CATÓLICA
FACULTY OF BIOTECHNOLOGY
PORTO

DIETARY INTAKE AND TAILORED FERMENTATION TOWARDS THE DEVELOPMENT OF FUNCTIONAL CEREAL FIBRE-RICH FOOD PRODUCTS: BRIDGE BETWEEN AFRICA AND EUROPE

Thesis submitted to Portuguese Catholic University to attain the degree of Ph.D. in Biotechnology, with specialization in Food Science and Engineering

Catarina Pereira de Melo Vila Real

May, 2021

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Catarina Pereira de Melo Vila Real

Supervisor: **Elisabete Cristina Bastos Pinto**

Co-supervisor: **Ana Maria Pereira Gomes**

May, 2021

Começa o desafio.
Vejo-te na primeira fila.
Olhos molhados de orgulho.
Coração inquieto de receio.
Ânimo e expectativa
Vestiam-me ao de leve.
A ti, esperança e inquietação.
Fui. Ficaste.
Aprendi. Sorriste-te.
Chorei. Levantaste-me.
Viveste de perto a distância que nos separava.
Voltei. Abracei-te e matámos saudades.
Continuaste na entrega diária aos meus problemas.
Perdida no novelo, começo a temer.
Ouço a voz de amparo.
Sinto o abraço de conforto.
A salva de palmas do sucesso.
O brilho nos olhos e orgulho eterno.
Inigualável, insubstituível.
Do nada, somos invadidas.
Um teste. A nós, à nossa relação, à nossa vida.
Levaram-te. Foste. Ficaste.
Termina o desafio.
Continuas na primeira fila.
Agora, não te vejo. Sinto-te.

A ti, minha mãe

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"*Number one in your life's blueprint should be a deep belief in your own dignity, your own worth and your own somebodiness. Don't allow anybody to make you feel that you are nobody. Always feel that you count. Always feel that you have worth, and always feel that your life has ultimate significance. (…)*

Secondly, in your life's blueprint you must have as a basic principle the determination to achieve excellence in your various fields of endeavor. You're going to be deciding as the days and the years unfold what you will do in life, what your life's work will be. And once you discover what it will be, set out to do it, and to do it well. (…) Be a bush if you can't be a tree. If you can't be a highway, just be a trail. If you can't be a sun, be a star. For it isn't by size that you win or fail. Be the best of whatever you are.

And finally, in your life's blueprint must be a commitment to the eternal principles of beauty, love and justice. (…) Well, life for none of us has been a crystal stair but we must keep moving, we must keep going. If you can't fly, then run. If you can't run, then walk. If you can't walk, then crawl, but whatever you do you have to keep moving."

Martin Luther King Jr., 1967

RESUMO

Uma alimentação rica em produtos açucarados e processados e pobre em alimentos de origem vegetal, tem-se demonstrado potenciadora do aparecimento e progressão de doenças não transmissíveis, enquanto que o consumo de produtos de origem vegetal, nomeadamente cereais integrais, tem-se revelado promissor na saúde do consumidor, sendo o alto teor em fibra um dos principais responsáveis por estes benefícios. Além da importância inegável que a avaliação da ingestão alimentar e nutricional tem no delineamento de estratégias para a promoção de hábitos alimentares mais equilibrados e estilos de vida saudáveis, a indústria alimentar é também um interveniente fundamental neste contexto. O desenvolvimento de produtos alimentares com propriedades benéficas à saúde é o primeiro passo para o seu consumo efetivo. Assim, é necessário investir esforços para o desenvolvimento de produtos diferenciadores, funcionais, com perfil nutricional de qualidade customizados às necessidades dos consumidores.

Neste sentido, esta tese de doutoramento vem dar resposta a estes dois eixos de investigação nas áreas das ciências da nutrição e alimentação, tendo especificamente os seguintes objetivos: estudar a ingestão alimentar e nutricional da população adulta residente em zonas urbanas do Quénia, e sua comparação com a realidade Portuguesa; e, desenvolver e caracterizar um produto alimentar inovador, fermentado, à base de cereais integrais Africanos isentos de glúten, com propriedades funcionais e perfil nutricional melhorado, que possa dar resposta a défices nutricionais das populações em estudo.

Para responder ao primeiro objectivo delineado neste trabalho de investigação, foi desenvolvido um questionário de frequência alimentar (QFA) semi-quantitativo para ser aplicado na população em estudo, o qual foi posteriormente validado (método comparativo: diários alimentares) e estudado em termos de reprodutibilidade (aplicação do QFA em dois momentos distintos). O QFA desenvolvido resultou numa ingestão nutricional mais elevada quando comparada com os diários alimentares (mediana para a energia total: 2998 kcal *vs*. 2032 kcal), mostrando concordância moderada na classificação em quartis de ingestão; não obstante, o QFA revelou-se uma ferramenta válida e reprodutível (mediana para a energia total nas duas aplicações: 2978 kcal *vs*. 2506 kcal) para classificar os adultos urbanos Quenianos de acordo com sua ingestão alimentar.

O QFA validado foi usado para estudar a ingestão alimentar e nutricional de uma amostra mais abrangente de adultos, residentes em zonas urbanas de Nairobi. A gama de ingestão dos macronutrientes revelou-se de acordo com as directrizes alimentares da OMS/FAO. Em termos de contributos alimentares, *cereais e derivados* (34,0 %), *snacks açucarados e salgados* (9,8 %), *fruta* (9,7 %) e *carnes e ovos* (8,8 %), foram os grupos alimentares que mais contribuíram para a energia total. Características individuais como sexo, idade e nível de escolaridade revelaram influência na escolha dos grupos alimentares. Quando comparada com a dieta Portuguesa, a dieta Queniana revelou-se semelhante em termos de contribuidores para a energia, mas com algumas diferenças em termos de fontes alimentares.

Para responder ao segundo objectivo, foram estudadas várias estirpes bacterianas (indígenas produtoras de exopolissacarídeos (EPS) e probióticos comerciais) em cultura simples ou combinada, na fermentação de três cereais integrais, Sorgo, *Pearl millet* e *Finger millet*, na expectativa de encontrar um consórcio bacteriano que revelasse capacidade fermentativa e, posteriormente, impacto positivo no perfil físico-químico, nutricional, sensorial e biológico, e com potencial na modulação da microbiota intestinal humana.

O consórcio bacteriano selecionado incluiu o probiótico comercial *Lactiplantibacillus plantarum* 299v e a bactéria indígena produtora de EPS *Weissella confusa* 2LABPT05, inoculados na razão 1:1 (v/v), numa suspensão de *finger millet*, em solução aquosa de sacarose a 10 %, cuja fermentação decorreu a 30 ºC, 200 rpm, durante 8 horas. O produto final obtido, doravante denominado de bebida fermentada tipo iogurte (*yoghurt-like beverage* em inglês - YLB), promoveu o crescimento significativo de ambas as bactérias (>10^8 unidades formadoras de colónias (UFC)/g produto fermentado), revelou maior viscosidade (35 *vs*. 12 mPa.s), maior teor em EPS (16 % *vs*. 0,3 %) contribuindo para a texturização da matriz e em alguns aminoácidos essenciais e não essenciais (treonina, arginina, GABA, e glutamina), maior digestibilidade da proteína (64 % *vs*. 25 %) e alto teor em fibra (4g/100g), comparativamente ao produto nativo antes da sua fermentação. Foi positivamente avaliado do ponto de vista sensorial, tanto *per si*, bem como quando incorporado numa matriz láctea, um iogurte natural não açucarado. Em termos de actividade biológica, a YLB demonstrou ter uma actividade antidiabética (21 % *vs*. 14 %) e nível de compostos fenólicos totais (244 *vs*. 181 mg equivalentes ao ácido gálico (EAG)/kg YLB) aumentados com a fermentação. A YLB submetida a simulação de digestão no tracto gastrointestinal contribuiu para o aumento, nas primeiras seis horas de fermentação fecal, do número de cópias do gene 16S rRNA do grupo bacteriano *Bifidobacterium*, com produção concomitante significativa de ácido láctico e dos ácidos gordos de cadeia curta acetato, butirato e propionato.

Este trabalho de investigação permitiu, por um lado criar ferramentas de epidemiologia nutricional úteis, passíveis de serem aplicadas em estudos posteriores levados a cabo no Quénia. Por outro lado, veio aumentar as alternativas alimentares na área das formulações fermentadas probióticas, concretamente com o desenvolvimento de um produto inovador simbiótico e fermentado à base de cereais integrais isentos de glúten. Assim, o trabalho desenvolvido nesta tese veio aumentar o conhecimento científico e trazer inovação pioneira em várias áreas de investigação.

Palavras-chave: Questionário de frequência alimentar; Ingestão alimentar; África; Cereais integrais; *Millet*; Sorgo; Fermentação; Probióticos; Modulação microbiota intestinal; Exopolissacarídeos; Propriedades biológicas; Alimento funcional.

Nota: Por opção da candidata, a redacção deste resumo não segue o novo acordo ortográfico.

ABSTRACT

Diets rich in high-sugar and -fat foods and poor in vegetable food products have been leading to the promotion or worsening of non-communicable diseases, whereas the consumption of vegetable products, namely whole grains, has been associated with beneficial effects on consumers' health, with high fibre content being one of the main factors involved. In addition to the undeniable importance that the assessment of the dietary intake has in the design of strategies for the promotion of more balanced eating habits, the food industry is also a key player in this context. The development of food products with beneficial health properties is the first step for an effective consumption. Therefore, it is necessary to invest research efforts towards the development of innovative, functional food products, with a high-quality nutritional profile and tailored to consumers' needs.

In this sense, this Ph.D. thesis responds to these two research areas in the fields of food science and nutrition, proposing a two-fold strategy based on the following objectives: to study the dietary intake of adult urban Kenyans, and further comparison with Portuguese data; and, to develop and characterize an innovative fermented gluten-free whole grain-based product, with functional properties and an improved nutritional profile, expecting a positive impact on the nutritional deficits of the populations under study.

Regarding the first objective outlined within this research work, a semi-quantitative food frequency questionnaire (FFQ) was firstly designed and developed, towards its further application in the target population. Once developed, this FFQ was validated (against three non-consecutive twenty-four hours recalls) and studied for its reproducibility (test-retest method). The developed FFQ presented higher nutrient intakes compared to the multiple recalls (median for total energy: 2998 kcal *vs*. 2032 kcal), thus showing moderate agreement between the classification of intake quartiles; nevertheless, it was shown to be a valid and reproducible (total energy median: 2978 kcal vs. 2506 kcal) tool for ranking Kenyan urban adults according to their dietary intake. This validated FFQ was then used for the study of the dietary intake of a wider heterogeneous and representative sample of urban adult Kenyans. Macronutrients' intake ranges were within the WHO/FAO dietary guidelines. *Cereals and grain products* (34.0 %)*, sugar, syrups, sweets, and snacks* (9.8 %)*, fruits* (9.7 %), and *meat and eggs* (8.8 %) were the major contributors to total energy. Individual characteristics such as gender, age and level of education seemed to have implications on the choice of food groups. When compared to the Portuguese diet, the Kenyan diet was similar in terms of contributors to energy, but with some differences regarding food sources.

Responding to the second objective, several bacterial strains (indigenous exopolysaccharides (EPS)-producing strains and commercial probiotics), in plain and combined cultures, were studied for fermentation of three different whole grain African flours, Sorghum, Pearl millet and Finger millet, expecting the selection of a bacterial consortium which would reveal fermentative capacity and after all show a positive impact on the physicochemical, nutritional, sensorial, biological profiles, and also on the human gut microbiota of the product under study.

The selected bacterial consortium included the *Lactiplantibacillus plantarum* 299v probiotic strain and the EPS-producing *Weissella confusa* 2LABPT05 indigenous strain, inoculated in a 1:1

ratio (v/v), in finger millet suspended in an aqueous sucrose-based solution at 10 %, fermented at 30 ºC, 200 rpm, for 8 h. The final product obtained, hereinafter referred to as fermented yoghurt-like beverage (YLB), was shown to promote a significant growth of both strains (>10^8 colony-forming unit (CFU)/mL), and to have higher viscosity (35 *vs*. 12 mPa.s), significant EPS content (16 % *vs*. 0.3 %) contributing to matrix texturization and essential and non-essential amino acids (threonine, arginine, GABA, and glutamine), increased protein digestibility (64 % *vs*. 25 %) and high fibre content (4 g/100 g) comparatively to the native cereal prior fermentation. It was organoleptically acceptable, both *per si* and combined with a dairy matrix, an unsweetened plain yogurt. In terms of biological activity, antidiabetic activity (21 % *vs*. 14 %) and total phenolics (244 *vs*. 181 mg of gallic acid equivalents (GAE)/kg YLB) increased with fermentation. The digested fermented YLB contributed to the increase, during the first 6 h of feacal fermentation, of the *Bifidobacterium*'s number of copies of the 16S rRNA gene, which results were supported by the acidification, concomitant with an expressive metabolic activity reflected in the significant production of lactic acid and the acetic, propionic and butyric short chain fatty acids.

In conclusion, this research work has created useful nutritional epidemiology tools that can be applied in future studies carried out in Kenya. In addition, it has increased food alternatives in the area of fermented probiotic products, specifically with the development of a novel synbiotic functional fermented African gluten-free cereal-based product. Thus, the work developed in this thesis brought increased scientific knowledge and pioneering novelty in several areas of research.

Keywords: Food frequency questionnaire; Dietary intake; Africa; Whole grains; Sorghum; Millets; Fermentation; Probiotics; Gut microbiota modulation; Exopolysaccharides; Biological properties; Functional food.

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LIST OF ABBREVIATIONS

16s rRNA – 16 Subunit of ribossomal Ribonucleic Acid
24hR – Twenty-four-hour Recall
2T-RFLP – Terminal Restriction Fragment Length Polymorphism
AAE – Ascorbic Acids Equivalents
ABTS – 2,2-Azinobis, 3-ethylbenzthiazoline-6-sulphonic acid
Ala – Alanine
AMPM – Automated Multiple-Pass Method
Arg – Arginine
Asn – Asparagine
Asp – Aspartic Acid
BMI – Body Mass Index
BPB – Bromophenol blue
C – Control
C-SIDE – Software for Intake Distribution Estimation
CI – Confidence Interval
Cq – Quantification Cycle
CVD – Cardiovascular Diseases
DF – Dietary Fibre
DF-YLB – Digested Fermented Yoghurt-Like Beverage
DGGE – Denaturing Gradient Gel Electrophoresis
DHS – Demographic and Health Survey
DNA – Deoxyribonucleic Acid
DPPH – 2,2-Diphenyl-1-picryl-hydrazyl
DRI – Dietary Reference Intake
EFSA – European Food Safety Authority
EI – Energy Intake
EPS – Exopolysaccharides
FAO – Food and Agriculture Organization
FCT – Food Composition Table
FDA – Food and Drug Administration
FFC – Functional Food Centre
FFQ – Food-Frequency Questionnaire
FISH – Fluorescent In Situ Hybridization
FOS – Fructooligosaccharide
F-YLB – Fermented Yoghurt-Like Beverage
GABA – Gamma-Aminobutyric Acid
GAE – Gallic Acid Equivalents
gDNA – Genomic DNA

GIT – Gastrointestinal Tract
Gln – Glutamine
Glu – Glutamic Acid
GOS – Galactooligosaccharide
GRAS – Generally Recognized As Safe
HDI – Human Development Index
HePS - Heteropolysaccharides
HIV – Human Immunodeficiency Virus
HoPS - Homopolysaccharides
HPLC – High-Performance Liquid Chromatography
IAN-AF – *Inquérito Alimentar Nacional e de Atividade Física* (National Food, Nutrition and Physical Activity Survey)
ICC – Intra-class Coefficients
Ile – Isoleucine
IQR – Interquartil Range
ISAPP – International Scientific Association for Probiotics and Prebiotics
KE – Kenya
LAB – Lactic Acid Bacteria
Leu – Leucine
LOD – Limit of Detection
LOQ – Limit of Quantification
Md – Maltodextrin
Met – Methionine
MRC – Medical Research Council
MRS – Man-Rogosa-Sharpe
MUFA – Monounsaturated Fatty Acids
N – Nitrogen
N. A. – Not Applicable
NCD – Noncommunicable Diseases
NDSR – Nutrition Data System for Research
N. I. – No Information
NO – Nitric Oxide
NSP – Non-Starch Polysaccharides
OD – Optical Density
OPA – Orthophthalaldehyde
OR – Odds Ratios
ORAC – Oxygen Radical Absorbance Capacity
P25 – 25th quartiles
P75 – 75th quartiles
PBS – Phosphate-Buffered Saline

PCA – Plate Count Agar
PD – Protein Digestibility
PDA – Potato Dextrose Agar
Phe – Phenylalanine
PPO – Polyphenol Oxidase
PSC – Pre-School Children
PT – Portugal
PUFA – Polyunsaturated Fatty Acids
QFFQ – Quantitative Food Frequency Questionnaire
QPS – Qualified Presumption of Safety
RDA – Recommended Dietary Allowance
RNI – Recommended Nutrient Intake
RT qPCR – Real Time quantitative Polymerase Chain Reaction
Sb – Sorbitol
SCFA – Short Chain Fatty Acids
SDG – Sustainable Development Goals
Ser – Serine
SFA – Saturated Fatty Acids
SGF – Simulated Gastric Fluid
SIF – Simulated Intestinal Fluid
SPSS – Statistical Package for the Social Sciences
SQFFQ – Semi-Quantitative Food Frequency Questionnaire
SSF – Simulated Salivary Fluid
TE – Trolox Equivalents
TEI – Total Energy Intake
TFA – Trans-Fatty Acids
Tg – Glass-Transition Temperature
TGGE – Temperature Gradient Gel Electrophoresis
Thr – Threonine
THUSA – Transition Health and Urbanization in South Africa
Tp – Product Temperature
TPC – Total Phenolics Content
Tr – Trehalose
Tyr – Tyrosine
UN – University of Nairobi
UL – Tolerable Upper Intake Level
UNDP – United Nations Development Programme
UNESCO – United Nations Educational, Scientific and Cultural Organization
USAID – United States Agency International Development
USDA – United States Department of Agriculture

Val – Valine

VRBGA – Violet Red Bile Glucose Agar

VTT – Technical Research Centre of Finland

WHO – World Health Organization

WR – Food Weighed Records

XOS – Xylooligosaccha

LIST OF EQUATIONS

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CHAPTER 1.

FRAMEWORK, OBJECTIVES AND THESIS OUTLINE

"You can't stop marching. (…) Because we know the rightness of our cause. (…) The future rewards those who press on. With patient and firm determination, I am going to press on. (…) I don't have time to feel sorry for myself. I don't have time to complain. I'm going to press on."

Barack Obama, 2011

1.1. FRAMEWORK

Non-communicable or chronic diseases are the major cause of death and disability worldwide (World Health Organization, 2018c). In Europe, cardiovascular diseases (CVD), cancer and other noncommunicable diseases (NCD) account for high mortality (World Health Organization, 2018b). Approximately 85 % of worldwide NCD premature deaths (between the ages of 30 and 69 years) occur in low- and middle-income countries and it is expected to rise in the coming years (World Health Organization, 2018c). Whole grains' consumption, mainly due to their high content of fibre, is associated with reduced risk of chronic diseases (Aune et al., 2016; Fujii et al., 2013; He et al., 2010). Despite the demonstrated benefits of fibre-rich products, there is fibre intake inadequacy in Portugal (Lopes et al., 2017) and, although data are scarce and currently there are no specific recommendations, it might also happen in African countries. Moreover, whole grain products have poor acceptability by the consumer, mainly due to their organoleptic properties.

Fermentation is an ancient process responsible for improving nutritional properties and enhancing the flavour of food products. Non-dairy fermented products are gaining great market interest due to consumers' willingness to try different concepts, 'free of' trend, as well as, due to its contribution to an increased offer with respect to required food products for people with gluten and lactose intolerance. During cereal fermentation microorganisms produce functional metabolites, including exopolysaccharides (EPS) and antimicrobial compounds. EPS improve texture and viscosity of fermented foods, enhancing sensory properties (Kalui et al., 2010), and may reveal biological activities (Prasanna et al., 2012). Antimicrobial compounds, such as organic acids, or bacteriocins, minimize product microbial load, allowing natural preservation of foods (Kalui et al., 2010). The consumption of fermented foods has demonstrated a positive impact on gut microbiota modulation (Bell et al., 2018; Dimidi et al., 2019; Marco et al., 2017; Mota de Carvalho et al., 2018). Adding probiotic strains in fermented products may enhance this effect, given their recognized competitive exclusion of pathogens, regulation of intestinal transit and immunomodulation (Hill et al., 2014).

Set within the framework of the pioneer ERAfrica strategy, in which European and African research centres jointly defined common societal challenges within health and food security, this thesis proposes a two-fold research Europe-Africa bridge strategy:

1) selection of Kenya among African countries to study the typical adult diet. Food consumption knowledge about Kenyan people, where comprehensive data are scarce (Vila-Real et al., 2016), will be increased and compared with that in the Portuguese adult population. Dietary intake assessment tools will be tailor-developed for further application in epidemiological studies to investigate associations of diet with risk of disease.

2) to use major African staple gluten-free whole grain sorghum and millet (Macauley, 2015; Shim et al., 2013; L. Zhang et al., 2014) for the development of innovative fermented products, capable of holding a nutritional fibre claim, that can contribute to overcome fibre inadequacy, in both

European and African markets. Probiotics strains and specific harboured lactic acid bacteria (LAB) in indigenous African cereal-based products will be selected as starter cultures, enabling, therefore, the achievement of *in situ* expression of desired functional properties.

Fermentation of dehulled grains has been characterized (Blandino et al., 2003), but less is known on the impact of probiotics on the fermentation of whole grain sorghum and millet food/beverage products. Besides, according to the recent literature few studies have focused on this subject, especially in terms of application of indigenous strains (Kohajdová & Karovičová, 2007), cereal fermentation impact thereby and stability and bioactivity assessment including impact on gut microbiota, of such products.

1.2. OBJECTIVES AND SCIENTIFIC PRODUCTION

Based on the above rationale, the main objectives of this thesis are, on the one hand, to assess the dietary intake in Kenya and, on the other hand, to develop affordable, acceptable and stable functional fermented cereal-based products for both African and European markets. Considering current European trends and the demonstrated need for increased fibre consumption, this study will be impactful on the health of African and European populations, thereby contributing to the strengthening of economic and research links between Africa and Europe. The scope of this thesis is multidisciplinary, branching different scientific areas from nutrition, microbiology, biochemistry and biotechnology, and consequently, these two objectives can be divided into the following specific objectives:

i. To assess the nutritional intake and the respective adequacy in Kenya, and further comparison with relative dietary intake data, previously collected in Portugal;
ii. To assess the contribution of main food groups for energy and nutrients intake, namely fibre, of an adult urban Kenyan population and comparison with Portuguese population data;
iii. To select the best bacterial combination in terms of fermentative capacity and of positive impact on the matrix texture of African cereals;
iv. To perform the nutritional, physicochemical, microbiological, sensory and biological characterization of the final cereal products (yoghurt-like and powdered product) and assess their stability assessment throughout storage.
v. To assess *in vitro* the impact of the high-fibre yoghurt-like cereal-based product on the modulation of the gut microbiota.

The aforementioned objectives culminated in the present Ph.D. thesis, where results were disseminated in the format of three papers published in peer-reviewed international journals in the Science Citation Index (SCI® Thomson ISI), and other communications (three oral communications at international conferences and eight posters at national and international meetings, two of which best poster awards, that can be found in the Ph.D. candidate *curriculum vitae*). The published scientific papers are the following:

1) How Dietary Intake Has Been Assessed In African Countries? A Systematic Review. *Crit Rev Food Sci Nutr*. 2016. DOI: 10.1080/10408398.2016.1236778;
2) Nutritional Value of African Indigenous Whole Grain Cereals Millet and Sorghum. *Nutr Food Sci Int J*. 2017. DOI: 10.19080/NFSIJ.2017.04.555628;
3) A culture-sensitive semi-quantitative FFQ for use among the adult population in Nairobi, Kenya: development, validity and reproducibility. *Public Health Nutrition.* 2020. DOI: 10.1017/S136898002000169X.

Two other scientific papers have been submitted to peer-reviewed journals, and are awaiting approval for publication, namely:

1) Novel synbiotic fermented finger-millet-based yoghurt-like beverage: nutritional, physicochemical, microbiological and sensory characterization; submitted to *Food Chemistry* (Impact Factor in 2019: 6.306, and classified with Q1 in all the scientific areas);
2) Nutritional intake and its food sources in an adult urban Kenyan population; submitted to *Journal of the Academy of Nutrition and Dietetics* (Impact Factor in 2019: 4.151, and classified with Q1 in all the scientific areas).

Finally, there are three more papers currently under revision to be submitted to peer-review journals:

1) Fermentation of African native whole grain red sorghum and finger millet flours from different milling processes by potential probiotic lactic acid bacteria;
2) Fermentation of African native whole grain red sorghum and pearl millet flours by indigenous *Weissella* strains;
3) *In vitro* fermentation assay for screening the gut microbiota effects of a novel synbiotic fermented finger millet-based yoghurt-like beverage and its biological potential.

1.3. THESIS OUTLINE

This thesis is composed of six chapters, of which the present chapter, Chapter 1, outlines the framework, the general and specific objectives of this thesis, as well as the scientific production resulting from this research work. Besides, chapter 1 describes how the thesis is organized, giving a brief description of the contents of each chapter. The following three chapters contents are divided into three sections corresponding to the three main areas covered in this thesis, namely:

Part I – Dietary intake assessment;

Part II – Fermentation of African whole grains;

Part III – Novel synbiotic fermented whole grain-based products.

Chapter 2 introduces the most important concepts and recent findings related to the three main topics covered in this thesis as discriminated above. It starts with a general overview, followed by a more descriptive approach for each specific topic, highlighting the most relevant publications

about each one, giving an up-to-date literature review and current perspectives on dietary intake assessment, whole grains fermentation and symbiotic cereal-based fermented products.

Chapter 3 gives an extensive detailed description of the methodology followed at every step, organized according to each of the three sections discriminated above.

Chapter 4 presents all the results obtained, duly analysed, and provides a comprehensive discussion thereof focusing on the main findings and contributions to science. As previously mentioned, this chapter is also organized into the previous three parts. The first part provides the main findings upon the dietary intake assessment survey, carried out in Kenya, from the development of the necessary epidemiological tools to their application towards the nutrient intake status of the Kenyan population. Also, it details the secondary analysis of the Portuguese dietary intake data, aiming a comparison of both countries' realities. The second part reports on the role that different commercial probiotic strains and indigenous *Weissella* strains, plain or in combined form, play in the fermentation of sorghum, pearl millet and finger millet both in terms of growth, acidification capacity and matrix texturisation. The third part describes the development of a new synbiotic fermented food based on whole grain finger millet with incorporated probiotic and EPS-producing *Weissella* strains (best bacterial consortium) and provides a detailed description of its biological and technological potential. Nutritional and biochemical characterization, sensorial evaluation, antioxidant, antidiabetic and *in vitro* gastrointestinal stability and gut microbiota modulation properties and storage stability of the developed yoghurt-like beverage (fresh and dried forms) are presented and discussed.

Chapter 5 outlines the concluding remarks for each of the experimental phases of this Ph.D. work, highlighting the main achievements.

Chapter 6 gives a brief overview of the future perspectives and suggestions for further research work.

The flowchart displayed in Figure 1.1 includes a schematic diagram of the experimental work divided into the three parts presented in this Ph.D. thesis.

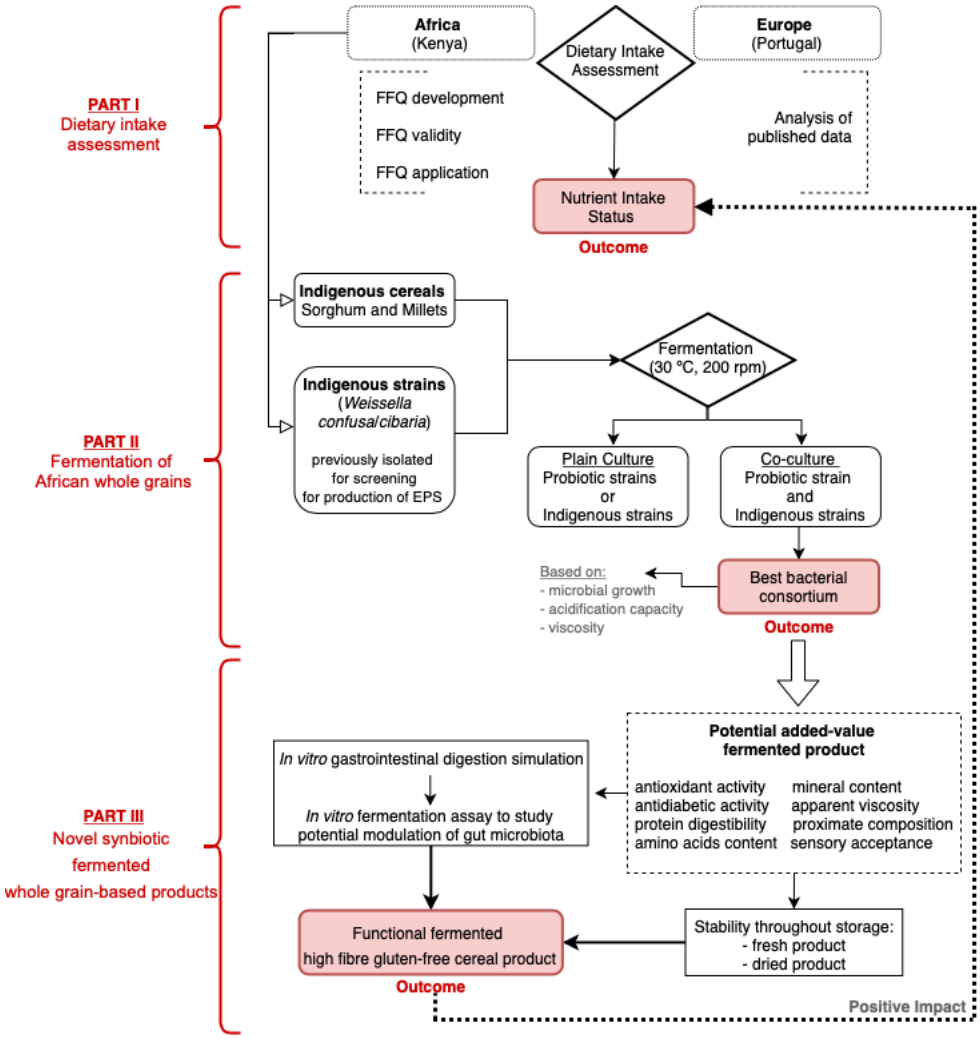


Figure 1.1 Flowchart of the outlined experimental work.

CHAPTER 2.

INTRODUCTION

"E*ducation (…) is not the learning of facts, but the training of the mind to think.*"

Albert Einstein, 1921

2.1. GENERAL OVERVIEW

According to the United Nations' studies on population growth and urbanization, it is expected that by 2050 the world population reaches 9 772 million people, from which 66 % are projected to be urban (United Nations Department of Economic and Social Affairs Population Division, 2014). At the year of 2014, 63 % of the Portuguese people were considered urban, while in Kenya the numbers were significantly lower (25 %) (United Nations Department of Economic and Social Affairs Population Division, 2014). This urbanization rate has grown and it is expected to grow in the coming years, almost doubling the percentage for the African countries and not being more than 20 % for Portugal (United Nations Department of Economic and Social Affairs Population Division, 2014). It is known that in urban areas people's food habits rely on more heterogeneous diets, which normally include more processed foods (consumed inside or outside home), more animal foods, more added sugar and fat, and often more alcohol when compared to traditional diets. This shift in food habits has been known as nutrition transition (Popkin et al., 2001). Besides diets modification, nowadays people tend to have a less active life, which combined with poor diets can lead to overweight and obesity. Moreover, there is evidence that these Westernized diets contribute to the degradation of the intestinal mucous membrane, leaving it more susceptible to harmful substances and compounds (Moschen et al., 2012). In consequence, we are watching another transition, a disease transition, from endemic deficiency and infectious diseases towards diet-related chronic diseases (Popkin et al., 2001).

Non-communicable or chronic diseases are the major cause of death and disability worldwide (World Health Organization, 2018b). In Portugal, CVD, cancer and other NCD account for high mortality (World Health Organization, 2018b). Developing countries, especially in Africa, have shown an increase in the prevalence of NCD, which contributes to almost 80 % of deaths in lower-middle-income countries and it is foreseen a switch of trends in 2030 (World Health Organization, 2014). Nevertheless, communicable diseases are still a major challenge, despite the success of vaccination programs (Boutayeb, 2006; Haregu et al., 2014; Islam et al., 2014; World Health Organization. Regional Office for Africa, 2014).

Dietary patterns are evidently related with prevention, development or worsening of NCD (Ben-Shlomo & Kuh, 2002; Popkin, 1999; Uusitalo et al., 2002). Therefore, it is of utmost importance not only to truly understand the influence that food choices have in health, and to quantify the impact of diet in this group of diseases, but also to deeply know the dietary habits of a population towards the establishment of the best nutritional strategies. Western Europe has nationally representative diet surveys, mainly collected after 2000 (Rippin et al., 2018), namely in Portugal, the last survey occurred in 2015-2016 (Lopes et al., 2017). In contrast, in lower-middle-income countries, this work is scarce or insufficiently documented, probably driven by a lack of resources and logistical constraints. The lack of reliable dietary assessment methodologies might be one of the major reasons that justify this absence of nutritional data (Pisa et al., 2014). This fact upholds the emergent

need for the development, validity, and standardization of tools for measuring and monitoring food intake in different countries enabling improved comparative methodologies (Pisa et al., 2014).

Cereals are a staple food for most of the world population, either in developed or developing countries. In particular, African native cereals such as millets and sorghum besides having advantages related to cereal production and stress adaptation, they also deserve attention due to their nutritional profile, and consequently, their potential impact on human health (Cardoso et al., 2017; He et al., 2010; Saleh et al., 2013; Shobana et al., 2013). Sorghum and millets are among the most significant cereal crops grown in arid and semi-arid developing nations, namely Africa and Asia (Food Security Department, 1999; Kajuna, 2001). In contrast to more developed places, such as America and Europe, where these cereals are mostly used for animal feed, in African countries they are mainly used as human food. When they are consumed as whole grains, they are a good source of carbohydrates, dietary fibre, proteins, minerals, and bioactive compounds. Particularly the high content in fibre, polyphenols, phosphorus, potassium, zinc, calcium, and iron and the protein and lipid profiles make these cereals a food option that potentially offers several health benefits to the consumer, as previously reviewed (Cardoso et al., 2017; Saleh et al., 2013; Vila-Real et al., 2017). Scientific evidence is consensual in what concerns the health-protective effect of whole grains consumption, specifically related to the reduced risk for chronic diseases such as type 2 diabetes (De Munter et al., 2007; Fujii et al., 2013), CVD (Fujii et al., 2013; He et al., 2010), metabolic syndrome (Sahyoun et al., 2006) and some types of cancers (Aune et al., 2011; Jacobs et al., 1998). Despite the demonstrated benefits of fibre-rich products, there is inadequate dietary fibre consumption in Portugal (Lopes et al., 2017) and it might also happen in African countries, however data is scarce and there are currently no existing specific recommendations. Moreover, whole grain products have poor acceptability by the consumer, mainly due to their organoleptic properties.

Fermentation is an ancient process responsible for improving nutritional properties and enhancing the sensory properties of food products. Many biochemical changes take place during fermentation, affecting the bioactivity, bioavailability, content, quality and digestibility of some nutrients (Kohajdová & Karovičová, 2007; Mallasy et al., 2010; Osman, 2011; Poutanen et al., 2009; Sindhu & Khetarpaul, 2001). Non-dairy fermented products are gaining great market interest due to consumers' willingness to try different concepts, aligned with sustainability and 'free of' trends as well as due to their contribution to an increased offer with respect to required food products for people with gluten and lactose intolerance (Kandylis et al., 2016; Kumar et al., 2015; Min et al., 2018). In Africa, spontaneous fermentation of cereals products is common. However, such naturally fermented products contain both functional and non-functional microorganisms, liable to affect both positively and negatively product attributes, eventually posing a hazard to human health. In response, lactic acid bacteria (LAB) have been used as starter cultures, successfully contributing to product quality and safety, increased shelf-life, texture, and sensory properties, adding value to the product. Lactobacilli comprise the most common LAB species used in fermented cereal probiotic foods (Tamang et al., 2016).

The consumption of fermented foods has demonstrated a positive impact on gut microbiota modulation, and the addition of probiotic strains into fermented products may enhance this effect

(Bell et al., 2018; Dimidi et al., 2019; Marco et al., 2017; Mota de Carvalho et al., 2018). Development of functional foods and beverages has been a trend over the last years, and the search and effective achievement of these products are increasing all over the world (Tripathi & Giri, 2014). Moreover, this is a tremendous opportunity to scientifically explore evidence-based strategies to develop, characterize and validate novel functional fermented cereal-based products, nutritionally and sensory improved, targeting different population groups from children to the elderly and that can be effectively introduced in the market in the near future.

PART I – DIETARY INTAKE ASSESSMENT

2.2. DIETARY INTAKE ASSESSMENT TOOLS

The assessment of dietary intake is imperative to know the population's food habits, including the inadequacy prevalence of different nutrients, as well as to study the relationships between dietary patterns and disease. Dietary assessment may be done at national, household and individual levels, when approaching food supply and production, food purchases or food consumption, respectively (Gibson, 2005; Thompson & Byers, 1994). At the individual level, several methodologies may be used, and these can be divided into two major groups: prospective and retrospective methods. Prospective methods include food weighed records (WR) (Gibson, 2005; Thompson & Byers, 1994), while retrospective methods comprise the twenty-four-hour recall (24hR) and the food-frequency questionnaire (FFQ).

The prospective method WR is a detailed list of foods and beverages consumed by an individual in a variable period, a single day or more than a day, that is constructed by the individual at the time the items are being consumed (Willet, 2013a). The quantification can be done by weighing or by determining volumes using household measures. The respondent should be trained aiming the attainment of accurate and complete records. One of the disadvantages of WR is related to the possibility of the respondent changing the meal content or servings. They must register all the information and by that, they might feel uncomfortable and be induced to make changes towards what they think is healthier and more balanced. Another limiting aspect is the requirement of literacy to conduct this register, which in some populations can be a problem. On the other hand, WR do not rely on memory, since the register is made at the same time people are eating, which figures an advantage.

Concerning retrospective methods, a 24hR implies asking the subjects to remember and report all foods and beverages consumed in the preceding 24 hours (h) or the previous day (Willet, 2013a). This register should be applied by a trained interviewer, capable of conducting quietly but effectively the interview, knowledgeable about foods' availability in the market, usual food habits and possible ethical issues of the population under study and able to probe for additional foods/food preparation methods. The interview can be stratified in five phases. Initially, the respondent reports all food/beverages consumed with no interruption from the interviewer, then the interviewer describes a list with the most common omitted foods in order to complete the previous food items list. After all the consumed items are reported, each one is associated with the time and occasion of consumption, and then the portion size of each is also asked. Finally, an overview is done in order to check all the obtained information and to assure that no details are omitted (Thompson & Subar, 2013). Typically, a 24hR requires from 20 to 30 minutes (min) to be completed and it can be a face-to-face interview or a telephone interview. In order to estimate the amounts consumed by the respondents, different tools can be used, such as food photography manuals, three-dimensional food models, food containers, real foods, or household typical recipients (Willet, 2013a). Notwithstanding, accurate

quantification of consumed foods is a critical component of data collection with this kind of dietary register. Another disadvantage of 24hR is the dependence on the subjects' short-term memory, which may lead to misreporting (Willet, 2013a). The other common method within this category of retrospective methods is the FFQ. The need of long-term frequency methods like this one was noticed when it was realized that the short-term methods were too expensive and not enough to describe the usual intake. The first method developed was a dietary history, which included a 24hR, a three-day diet record and a checklist of foods consumed over the preceding month (Willet, 2013b). Then, mainly because this method showed to be very time consuming, expensive and demanding in terms of highly skilled professionals, FFQs were created. These questionnaires are designed to measure the individual's usual food intake during a specific period. FFQs are comprised of a limited check-list of food and beverages with a frequency response section for subjects to report how often each item is consumed over a specific period (Willet, 2013b). FFQs can be differentiated in quantitative, semi-quantitative and qualitative. While in the first there is an exact quantification of the consumed portions, in the second the quantification is made by comparison, in which either a specific portion size is part of the question on frequency, or a medium portion is given to the respondent and one is asked to refer if one's portion is bigger or smaller than that the medium one (Willet, 2013b). Qualitative FFQs only include the frequency of consumption section, without mention to quantities. The major strength of frequency methods is that they are representative of the usual intake of the individuals, which does not happen with recalls and WR. FFQs are practical and easy to be completed by the respondents, what enables self-administration to be often used (Willet, 2013b). On the drawbacks' side, FFQs reliable results are dependent of long-term memory, and in case of semi-quantitative FFQs, the perception of the presented portion size is crucial (Willet, 2013b).

Ideally, these tools need to be reproducible and valid in order to assure the consistency and accuracy of measurements (Willet, 2013d). The choice of an appropriate method will depend on the aims of the study, the population under study as well as the research teams' experience.

2.2.1. Development of a Food Frequency Questionnaire

As mentioned before, the application of a FFQ to a specific population in order to study the dietary intake is more reliable, accurate and realistic if it has been developed taking into account specificities of the target population, towards the creation of a culture-adapted instrument. There are two possible situations while developing a FFQ, it is either based on a pre-existing questionnaire, or, in case of its absence, a new instrument should be designed. Several issues have to be considered during this plan, namely, the objective of the study, the questionnaire design, the food list, the way of grouping foods, the type of questions (open or closed), the frequency categories of response, the away of estimating the portions size and the method of administration (Cade et al., 2004; Willet, 2013b). All these characteristics are dependent on the population under study.

Regarding the food list, its length should be carefully planned. If a comprehensive total dietary intake is intended, a comprehensive food list should be created. According to Walter Willett (Willet, 2013b), people are willing to complete relatively long food lists, however, 130 food items may be approaching the limit. Otherwise, long lists will promote the respondents' fatigue and boredom.

The selection of the food items should be based on three important aspects: the items must be usual for a significant number of people, the items must be nutritive and, to be discriminating, their consumption must vary from person to person (Willet, 2013b). The compilation of the food list can be done using different approaches. In general, a comprehensive list is primarily developed, then items that are more infrequent between the population are removed from the list. Another approach is to compile a list with the foods that mainly contribute to substantial amounts of the nutrients of interest. Using the 24hR to gather information about the most consumed foods among a population is the third possible approach (Willet, 2013b). The way that foods are arranged within the questionnaire is also important. In their review, Cade and colleagues (2004) mentioned that one study (Boutron et al., 1989) revealed that people preferred to be asked about foods with the questionnaire organized by meals. Nevertheless, the most common format of food lists is food group-based. It is important to group foods with similar characteristics, for example, the nutrient profile, and more specific items should precede general items (Willet, 2013b).

The frequency response section can be displayed in two main formats: a closed scale of consumption or an open-ended format. In the study of Subar and collaborators (1995) it was found that the first approach leads to increased clarity and reduced errors compared to the latter approach. If a frequency response closed scale is chosen, it should be wide enough to avoid loss of information and to discriminate between less and more consumed foods or beverages (Willet, 2013b). The advisable number of categories by Willett is nine: never or less than once a month; 2–3 times per month; once per week; 2–4 times per week; 5–7 times per week; once a day; 2–3 times per day; 4–6 times per day; over 6 times per day (Willet, 2013b).

The respondents' estimation of the consumed portions can be addressed in different ways. They can be asked about the consumption of a determined food portion, for example, a slice of bread or a glass of milk, or they can be asked to place their usual consumed portion, as smaller, equal or bigger, in relation to a standard one that is detailed and described in the FFQ or by the interviewer. As previously mentioned, this type of questionnaire is known as semi-quantitative questionnaire. According to Cade *et al*. (2004) allowing the respondent to estimate their portion sizes is even more advantageous than using a standard portion. However, as the contribution of estimating food portions is not definitively clear, Willett argues that this topic requires further investigation (Willet, 2013b).

2.2.2. Reproducibility and Validity of Food-Frequency Questionnaires

According to Willett (2013d), the concept of reproducibility '*refers to the consistency of questionnaire measurements on more than one administration to the same person at different times, realizing that conditions are never identical on repeated administrations*'. To '*the degree to which the questionnaire actually measures the aspect of diet that it was designed to measure*' the author call validity. Reproducibility and validity are specific of the tools, which means that for every newly developed instrument or for every new population that the developed instrument is applied to, it is required a new process of assessment of reproducibility and validity.

The study of the reproducibility is useful to estimate the performance of the FFQ, *id est* it shows if the tool is consistently measuring the same parameters for the same subjects. This test is done at two different moments, and the period from which they differ is challenging to define. It should not be too close, in order to avoid respondents' ability to remember the answers, nor too apart, because diet can suffer major modifications inherent to dietary habits (Willet, 2013d). The comparison between both applications is evaluated in terms of correlation coefficients. Generally, in these types of studies, typical correlation coefficients are around 0.5 to 0.7 (Willet, 2013d). This range of values is understandable given the conditions under which these studies are carried out, among human beings, over a considerable period of months or years. Notwithstanding, low correlation coefficients are indicative that the FFQ is not suitable to measure medium- or long-term intakes. Nevertheless, obtaining high correlation coefficients does not mean that the FFQ is perfectly reproducible. They might result from correlated errors and systematic within-person errors (Willet, 2013d). Because of this, the reproducibility test does not exclude the need for assessing the validity of the instrument.

The validity of a FFQ implies the comparison with a method that is considered to be more accurate, not forgetting that regarding dietary intake there is no gold standard. Moreover, the ideal strategy is to compare methods with uncorrelated sources of error, aiming to improve correlations. According to Willett, WR are one of the few methods that have a different source of errors compared to the FFQ; they do not rely on memory, or estimation of portion sizes or interpretation of questions, what make them a suitable option to validate a FFQ (Willet, 2013d). However, when cooperation or literacy is limited within the population envisaged in the study, 24hR is likely to be a better option. Cade and colleagues (2004) concluded that both 24hR and WR are appropriate to be used as the reference method. Moreover, they advise the collection of data on multiple days. A source of error that might remain correlated is the food composition database, mainly because it is used for the conversion of foods into nutrients in all the methods. In terms of statistical assessment, a multiple approach should be followed. The sole use of correlation coefficients seems not to be enough, being more effective when in combination with the Bland–Altman method (Cade et al., 2002, 2004). These plots assess the agreement between the methods across the range of intakes (Bland & Altman, 1986).

2.2.3. Dietary intake assessment studies in Africa

The tools that are mostly used to assess the dietary intake in Africa are the 24hR and the FFQ, applied separately or together (Vila-Real et al., 2016). Most of the studies carried out in African countries, involving African native people, only used 24hR, single or multiple recalls, covering a range from two to eight recalls (Vila-Real et al., 2016). Some of them followed a validated method for collecting interviewer-administered 24hR, the so-called United States Department of Agriculture (USDA) Automated Multiple-Pass Method (AMPM), which is a computerized method that can be applied in person or by telephone, while others mentioned different validated methods. Table 2.1 presents the dietary information collected from the reviewed studies which used the 24hR as the reference tool to study the dietary intake of African populations.

Table 2.1 Studies (n=54) that assessed dietary intake of different African populations using 24-hour recall [from 2005 to 2014].

Author	Country	Study Design	Study Population	Number of recalls	Determination of Portion Sizes	Tools for Dietary Analysis	
				2014			
(Zeba et al., 2014)	Burkina Faso	Cross-sectional study	110 Adults (25-6 years-old)	2 non-consecutive	Local kitchen utensils	Malian FCT[1]; C-SIDE[2]	(Iowa State University, 1996)
(Kim et al., 2014)	Tanzania	Cross-sectional study	80 Pregnant and/or Lactating Women (>18 years-old)	2 non-consecutive	Standardized food models	Tanzanian FCT Harvard University School of Public Health	
(May et al., 2014)	South Africa	Case Control Study	128 Women (Mean age of 35 years-old)	Single	Photographs of local alcoholic beverage	Nutrition Data System for Research	(Nutrition Coordinating Center's University of Minnesota, n.d.)
(Changamire et al., 2014)	Tanzania	Cohort Study	8428 Pregnant and/or Lactating Women (>18 years-old)	3	N.A.[3]	Tanzanian FCT Harvard University School of Public Health	
(Oldewage-Theron et al., 2014)	South Africa	Cross-sectional study	722 Women (19-90 years-old)	≥3	Food models, household utensils	FoodFinder®	(Grant et al., 1992)
				2013			
(Powell et al., 2013)	Tanzania	Cross-sectional study	274 Women	2 non-consecutive	Local serving sizes aids	Programme CANDAT; Tanzanian FCT, FAO FTC, USDA Database and scientific literature[4]	(Godin, 2007; USDA Agriculture Research Service Nutrient Data Laboratory, 1996)

[1] FCT: Food Composition Table;
[2] C-SIDE: Software for Intake Distribution Estimation;
[3] N.A.: Information not available;
[4] Lukmanji et al. 2008; Wu Leung 1968;

Author	Country	Study Design	Study Population	Number of recalls	Determination of Portion Sizes	Tools for Dietary Analysis	
(Koethe et al., 2013)	Zambia	Prospective Cohort study	142 Adults	4 non-consecutive	Artificial food models and serving utensils	FCT published by the Zambian National Food and Nutrition Commission; Nutrition Data System for Research	(Nutrition Coordinating Center's University of Minnesota, n.d.)
(Kolahdooz et al., 2013)	South Africa	Cross-sectional study	137 Adults (>19 years-old)	2 non-consecutive	3D food models; local household utensils	Nutribase	(CyberSoft, 1986)
2012							
(Walton et al., 2012)	Kenya	Cross-sectional study	111 Women	Single	Placing dried beans into the individual bowl to represent the serving	WorldFood Dietary Assessment System and the Kenyan FCT; USDA database[5]	(Bunch & Murphy, 1997; Sehmi, 1993; USDA Agriculture Research Service Nutrient Data Laboratory, 1996)
(Termote et al., 2012)	Democratic Republic of Congo	Cross-sectional study	492 Women	2 non-consecutive	1) a booklet with photographs of different calibrated portion sizes; 2) a price-weight-conversion list covering all foods/ingredients reported during the 24hR; and 3) direct measurements of estimated leftovers	Lucille food analysis software	(UGent Research Group Food Chemistry and Human Nutrition & Nutrition and Child Health Unit of the Institute of Tropical Medicine, n.d.)
(Steyn et al., 2012)	Kenya	Cross-sectional study	1008 Women (15-60 years-old)	Single	Dietary assessment kit comprising life-size drawings and generic food models	FoodFinder®	(Grant et al., 1992)
(Pereko et al., 2012)	Ghana	Cross-sectional study	252 Adults (20-50)	3 non-consecutive	N.A.	ESHA Food Processor® and the Ghanaian FCT	(Davison & Mandible, 1994)

[5] US Department of Agriculture-Agriculture Research Service (2007) USDA National Nutrient Database for Standard Reference;

Author	Country	Study Design	Study Population	Number of recalls	Determination of Portion Sizes	Tools for Dietary Analysis	
(López et al., 2012)	Morocco	Cross-sectional study	327 Adolescents (15-20 years-old)	3 non-consecutive	N.A.	DIAL 1.0; FCT for use in Africa, from FAO	(Ortega et al., 2008)
(Mupere et al., 2012)	Uganda	Cross-sectional study	131 Adults (>18 years-old)	Single	Local food photographs, portion-size images, volumetric vessels	East African FCT and African FCT; USDA database; NutriSurvey Program	(Erhardt & Gross, 2007; USDA Agriculture Research Service Nutrient Data Laboratory, 1996)
(Papathakis & Pearson, 2012)	South Africa	Cohort Study	142 Pregnant and/or Lactating Women	4 non-consecutive	Volume of cups, bowls and plates	FoodFinder®	(Grant et al., 1992)
(Nyuar et al., 2012)	Sudan	N.A.	113 Women (18-42 years-old)	Single	Household measures	Foodbase Nutritional Program[6]	
(Boumaiza et al., 2012)	Tunisia	Cross-sectional study	329 Adults (Mean age of 44,9 years-old)	3 non-consecutive	Household measures	Dietetik® and Nutrilog®	(SAS, 2007)
2011							
(Steyn et al., 2011)	Kenya	Cross-sectional study	1050 Women (15-60 years-old)	Single	Photographs, life-size drawings food models	FoodFinder®	(Grant et al., 1992)
(Naude et al., 2011)	South Africa	Cross-sectional study	162 Adolescents (12-16 years-old)	3 non-consecutive	Household measures; Pictures from the Dietary Assessment and Educational Kit[7]; MRC Food Quantities Manual	FoodFinder®	(Grant et al., 1992)
(Gibson et al., 2011)	Malawi	Cross-sectional study	80 Pregnant Women (14-45 years-old)	3 non-consecutive	N.A.	Malawian FCT	

[6] version 4, Institute of Brain Chemistry and Human Nutrition, London Metropolitan University;
[7] Steyn and Senekal, 2002;

Author	Country	Study Design	Study Population	Number of recalls	Determination of Portion Sizes	Tools for Dietary Analysis	
(Hansen et al., 2011)	Kenya	Cross-sectional study	1163 Adults (18-68 years-old)	2	Real food items/Paper models, utensils from the local market	GIES[8]	
(Addo et al., 2011)	Ghana	Cross-sectional study	70 Pregnant and/or Lactating Women (18-42 years-old)	3 non-consecutive	Household measures, weigh portion made at home or bought	ESHA Food Processor® and published food composition information	(Davison & Mandible, 1994)
(Irvine et al., 2011)	Tanzania	Case Control Study	171 Adults (Mean age of 38 years-old)	Single	Real food items; Kitchen utensils; Serving dishes	ESHA Food Processor®	(Davison & Mandible, 1994)
(Luke et al., 2011)	Ghana, South Africa, Seychelles, Jamaica and United States	Longitudinal observational study	2500 Adults (25-45 years-old)	2	Photographs; usual portions of local foods	Nutrition Data System for Research	(Nutrition Coordinating Center's University of Minnesota, n.d.)
(Alemayehu et al., 2011)	Ethiopia	Cross-sectional study	68 Women (15-49 years-old)	Single	Weigh of the estimated portion consumed (using a spoon); households measures and actual food samples purchased in markets	Ethiopian FCT and USDA database	(USDA Agriculture Research Service Nutrient Data Laboratory, 1996)
(Rankin et al., 2011)	South Africa	Cross-sectional study	131 Adolescents	7 non-consecutive	Validated food portion photograph book (Venter et al., 2000)	FoodFinder®	(Grant et al., 1992)
				2010			
(Scarcella et al., 2011)	Mozambique	Cohort Study	106 Adults	Single	Food models and images of portion sizes	FAO FCT for Africa and Mozambique [9]	

[8] GIES: General Intake Estimation System Program, GIES; National Food Institute, Søborg, Denmark;
[9] *Repartição de Nutrição* (1991) *Tabela de Composição de Alimentos* Maputo:MISAU;

Author	Country	Study Design	Study Population	Number of recalls	Determination of Portion Sizes	Tools for Dietary Analysis	
(Becquey & Martin-Prevel, 2010)	Burkina Faso	Cross-sectional study	182 Women (19-49 years-old)	3	Household measures	C-SIDE Software; Malian FCT, INFOODS[10] database (Senegal), USDA Database	(Iowa State University, 1996; USDA Agriculture Research Service Nutrient Data Laboratory, 1996)
(Oldewage-Theron et al., 2010)	South Africa	Cross-sectional study	235 Elderly (≥60 years-old)	2 non-consecutive: 2nd 59 % of the sample	Food Models	FoodFinder® III	(Grant et al., 1992)
(Nago et al., 2010)	Benin	Cross-sectional study	656 Adolescents (≥13 years-old)	2 non-consecutive	Household utensils	Malian FCT; FCT for use in Africa; East African FCT	
(Dapi et al. 2010)	Cameroon	Cross-sectional study	227 Adolescents (12-16 years-old)	3 non-consecutive	Household measures, real food portions and information about the amount of money spent on some foods. Colour picture booklet	Becel Institution Nutrition Software	
(Heimburger et al., 2010)	Zambia	Cohort Study	874 Adults	4 non-consecutive	N.A.	Nutrition Data System for Research	(Nutrition Coordinating Center's University of Minnesota, n.d.)
2009							
(Lamri-Senhadji et al., 2009)	Algeria	Cross-sectional study	46 Adults (Mean age: 24 years-old)	5 weeks of measurements	Small, medium or large (graduated and household measures)	*La composition des aliments Tableaux des valeurs nutritives*	

[10] INFOODS: International Network of Food Data Systems;

Author	Country	Study Design	Study Population	Number of recalls	Determination of Portion Sizes	Tools for Dietary Analysis	
				2009			
(Sodjinou et al., 2009)	Benin	Cross-sectional study	200 Adults (25-60 years-old)	3	Local household measures	WorldFood Dietary Assessment System; FCT of neighboring countries; C-SIDE Software	(Bunch & Murphy, 1997; Iowa State University, 1996)
(Huybregts et al., 2009)	Burkina Faso	Cross-sectional study	394 Pregnant and/or Lactating Women (15-45 years-old)	Single	Validated booklet with food photographs	Malian FCT; ESHA Food Processor®	(Davison & Mandible, 1994)
(Alaofè et al., 2009)	Benin	Quasi experimental	68 Adolescents (12-17 years-old)	48hR (single) 24hR (3)	Food models, portion-size models, containers and photographs of foods	Nutrifiq® software, DANA-INFRE FCT used in Benin[11]	
(Wiesmann et al., 2009)	Mozambique	Cross-sectional study	409 Women (15-49 years-old)	Single and 2 non-consecutive	Direct weighing, volume containers, photographs	Specific FCT based on USDA Database	(USDA Agriculture Research Service Nutrient Data Laboratory, 1996)
(Kennedy et al., 2009)	Mali	Cross-sectional study	102 Women (15-49 years-old)	2 non consecutive	Household measures	VBS Food Calculation System	(KOMEET, VBS MANAGER, ORION and FOOD GROUPS)[12]
(Becquey et al., 2009)	Burkina Faso	Cross-sectional study	182 Women (17-49 years-old)	3 non consecutive	Household measures, portion sizes or price	Malian FCT; Worldfood FCT for Senegal; USDA Database	(USDA Agriculture Research Service Nutrient Data Laboratory, 1996)

[11] DANA-INFRE: *Direction de l'alimentation et de la nutrition–Institut national pour la formation et la recherche en education*;
[12] Bas Nutrition Software, Arnhem, The Netherlands, www.bware.nl;

Author	Country	Study Design	Study Population	Number of recalls	Determination of Portion Sizes	Tools for Dietary Analysis	
				2008			
(Oldewage-Theron, Samuel, & Venter, 2008)	South Africa	Cross-sectional descriptive study	170 Elderly (≥60 years-old)	2	N.A.	FoodFinder® III	(Grant et al., 1992)
(Sodjinou et al., 2008)	Benin	Cross-sectional study	200 Adults (25-60 years-old)	3 non-consecutive	Local household measures	WorldFood Dietary Assessment System; FCT of neighboring countries; C-SIDE Software	(Bunch & Murphy, 1997; Iowa State University, 1996)
(Ijarotimi & Keshinro, 2008)	Nigeria	Cross-sectional study	452 Adults (≥20 years-old)	Single	Household measures	Food analysis: AOAC[13] method	
(Maruapula & Chapman-Novakofski, 2008)	Botswana	Cross-sectional study	99 Elderly (60-95 years-old)	Single	N.A.	Nutritionist Five[14]	
(Gewa et al., 2008)	Kenya	Cross-sectional study	44 Women	Single	Food models, measuring cylinders, local household measures	WorldFood Dietary Assessment System	(Bunch & Murphy, 1997)
(Tesfaye et al., 2008)	Ethiopia	Cross-sectional study	619 Adults (18-64 years-old)	Single	Household measures, described as S, M, L., pictures of foods and utensils	FCT for Ethiopia	
(Oldewage-Theron et al. 2008)	South Africa	Cross-sectional study	138 Elderly (60-93 years-old)	2 non-consecutive	Food Models	FoodFinder®	(Grant et al., 1992)
(Oldewage-Theron, Salami, et al., 2008)	South Africa	Cross-sectional study	101 Elderly (60-110 years-old)	2 non-consecutive	Food Models	FoodFinder®	(Grant et al., 1992)

[13] AOAC: Association of Official Agricultural Chemists.

[14] Nutritionist Five, Version 2.3; First DataBank, San Bruno, CA 2000.

Author	Country	Study Design	Study Population	Number of recalls	Determination of Portion Sizes	Tools for Dietary Analysis	
				2007			
(Kamau-Mbuthia & Elmadfa, 2007)	Kenya	Cross-sectional study	716 Pregnant and/or Lactating Women (reproductive age)	Single	Household measures (cups, tea and tablespoons and bowls) and also preparation methods for the different foods	NutriSurvey Program	(Erhardt & Gross, 2007)
(Wiig & Smith, 2007)	Ghana	Cross-sectional study	50 Adults (18-65 years-old)	Single	Food models; Portions size images	ESHA Food Processor® and published food composition information	(Davison & Mandible, 1994)
(Mounir et al., 2007)	Egypt	Cross-sectional study	1606 Adolescents (Menarcheal age)	Single	N.A.	Egyptian FCT, National Nutritional Institute	
(O'Keefe et al., 2007)	South Africa	Cross-sectional study	52 Adults (50-60 years-old)	3-consecutive	N.A.	South African Food Composition Database of the MRC	
				2006			
(Steyn & Nel, 2006)	Kenya	Cross-sectional study	1008 Women (15-60 years-old)	Single	Household utensils, life-size drawings and food models	Kenyan FCT and FoodFinder®	(Grant et al., 1992)
				2005			
(Mostert et al., 2005)	South Africa	Cohort Study	46 Pregnant and/or Lactating Women (<40 years-old)	2	N.A.	South African Food Composition Database of the MRC	
(Charlton et al., 2005)	South Africa	Cross-sectional study	285 Elderly (>60 years-old)	Single	Standard household measuring utensils, rulers and validated food photographs	FoodFinder® III	(Grant et al., 1992)

In what concerns the application of the FFQ, some researchers chose the quantitative format and other the semi-quantitative FFQ. The Transition Health and Urbanization in South Africa (THUSA) study questionnaire, design by MacIntyre *et al*. (2001a, 2001b) and the Dietary Assessment and Education Kit questionnaire, developed by Steyn and Senekal (2005) and launched by the Medical Research Council (MRC) are two of the most commonly referred validated questionnaires, specifically designed for the South African population. The questionnaire of Sharma and colleagues (1996), which was developed specifically for Cameroonian people is also a reference. Among semi-quantitative FFQ, it is possible to highlight the ones of Decarli *et al*. (1996) and Franceschi *et al*. (1993), developed in Italy and designed for a population with cancer, another in Tunisia based on the FFQ used in the birth-to-twenty study (Richter et al., 2007) and another questionnaire also used among Tunisian people (El Ati et al., 2004). In some other studies, the authors developed their quantitative questionnaires for implementation in their own research works, and those FFQs were not applied in other populations, according to the carried out literature reviewed (Jackson et al., 2012; Jordan et al., 2013; Lukmanji et al., 2013; Merchant et al., 2005; Sheehy et al., 2013). For example, Sheehy *et al*. (2013) developed a specific FFQ for use among rural South Africans and Jackson *et al*. (2012a) developed, validated and tested for reproducibility a FFQ for use among adults in Botswana. Jordan *et al*. (2013) and Merchant *et al*. (2005) developed semi-quantitative FFQs to assess the dietary intake of Tanzanian women and of the Zimbabwean population, respectively. Lukmanji *et al*. (2013), authors of a Tanzanian study, also developed their own semi-quantitative FFQ but gave no information about a validation study. Table 2.2 summarizes the characteristics of all the studies which used a FFQ to measure the dietary intake. Considering these released studies, it is possible to observe that there is the need for developing new food frequency tools in the majority of African countries. Moreover, there is still a lack of validated tools for use in a specific population, and thus the need for updating the validated dietary assessment methods across African countries is warranted.

As mentioned before, it is rather common to use a combination of both methods, recalls and questionnaires. Table 2.3 synthetizes the studies which performed the assessment of dietary intake that way. Namugumya and Muyanga (2011) applied 24hR aiming to study meal patterns and to assess meal quality, whereas with the application of a FFQ they intended to collect information on food selection patterns and portion sizes. Korkalo *et al*. (2014) developed their own questionnaire and Mala and collaborators used a pre-existent FFQ to collect information about the frequency of food consumption while the 24hR was used to quantify the dietary intake (Mala et al., 2012). Amare *et al*. (2012), Mbochi *et al*. (2012), Faber and Kruger (2005) used a qualitative FFQ and 24hR to determine the nutritional intake, while Oldewage-Theron and Kruger (2011) used a quantitative FFQ to assess dietary intake and food consumption patterns and a 24hR to confirm food variety and dietary intake. Baroudi *et al*. (2010) assessed dietary intake using a quantitative FFQ and performed 24hR in order to obtain more qualitative information, related to food brand names and food preparation methods. Waudo *et al*. (2005) used 24hR to assess what mothers had eaten in the preceding 24h and then applied a FFQ in order to obtain information about the types of foods commonly consumed.

Besides these two methods, WR are also utilized in African countries, but in a much smaller number. The authors Haileslassie *et al*. (2013) and Gibson and colleagues (2008) used only a single day as a measuring unit, while Olayiwola *et al*. (2012) and Abebe *et al*. (2008) applied WR for three and two non-consecutive days, respectively. More detailed information about these studies is compiled in Table 2.4.

Table 2.2 Studies (n=30) that assessed dietary intake of different African populations using food frequency questionnaires [from 2005 to 2014].

Authors	Country	Study Design	Study Population	Dietary Assessment Method (Source)	Number of food items	Reference time frame	Validity/ Reproducibility	Determination of Portion Sizes	Tools for Dietary Data Analysis	
2014										
(Wrottesley et al., 2014)	South Africa	Cross-sectional study	247 Women (23-39 years-old)	QFFQ[1]	214	Preceding 7 days	N.A.[2]	Household measures,2D/3D life-size drawings of foods and utensils.	FoodFinder® III	(Grant et al., 1992)
(Baroudi et al., 2014)	Tunisia	Case Control Study	348 Adults (20-89 years-old)	SQFFQ[3] (Decarli et al., 1996; Franceschi et al., 1993)	77	Preceding year	Tested for reproducibility and validated against 7-day WR	N.A.	Binult logiciel[4]	
(Botha et al., 2014)	South Africa	Cross-sectional Study	1068 Adults (Mean age: 56.4 years-old)	QFFQ (MacIntyre et al., 2001b, 2001a)	145	N.A.	Tested for reproducibility and validated against 7-day WR and biomarkers	N.A.	FoodFinder®	(Grant et al., 1992)
2013										
(Sheehy et al., 2013)	South Africa	Cross-sectional study	81 Adults (19-79 years-old)	QFFQ	71	N.A.	Not tested for reproducibility or validated	Household units/3D models; weighed portions	Nutribase	(CyberSoft, 1986)
(Lukmanji et al., 2013)	Tanzania	Longitudinal clinical trial	1078 Pregnant and/or Lactating Women	SQFFQ	85	Preceding 3 months	Not tested for reproducibility or validated	Standard utensils and Food Models	Tanzanian FCT[5]	

[1] Quantitative FFQ;
[2] N.A.: Information not available;
[3] Semi-quantitative FFQ;
[4] Binult logiciel,2.01 version;
[5] FCT: Food Composition Table;

Authors	Country	Study Design	Study Population	Dietary Assessment Method (Source)	Number of food items	Reference time frame	Validity/ Reproducibility	Determination of Portion Sizes	Tools for Dietary Data Analysis	
(Jordan et al., 2013)	Tanzania	Case Control Study and a validity study	345 Women (26-85 years-old)	SQFFQ	65	N.A.	Validated against 2 non-consecutive 24hR	Household measures and solid foods in pieces or slices (in the validity study)	NutriSurvey Program	(Erhardt & Gross, 2007)
2012										
(Jackson et al., 2012)	Botswana	Validity study	79 Adults (18-75 years-old)	QFFQ	122	Preceding year	Validated against 4 non-consecutive 24hR	Food models; household utensils; measuring cups and measuring tape	FoodFinder®	(Grant et al., 1992)
(Pretorius et al., 2012)	South Africa	Nutritional Survey	50 Adults (Mean age: 47 ± 18 years-old)	QFFQ (MacIntyre et al., 2001b, 2001a)	139	Usual intake (daily, weekly and monthly basis)	Tested for reproducibility and validated against 7-day WR and biomarkers	Standardized Portions pictures and utensils (cups, teaspoons)	FoodFinder®	(Grant et al., 1992)
(Kruger et al., 2012)	South Africa	Cross-sectional study	1325 Adults (25-64 years-old)	QFFQ (MacIntyre et al., 2001b, 2001a)	145	N.A.	Tested for reproducibility and validated against 7-day WR and biomarkers	Food portion photograph book[6] Household measures	South African Food Composition Database _MRC[7]	
(Pisa et al., 2012)	South Africa	Cross-sectional study	2010 Adults (≥ 35 years-old)	QFFQ (MacIntyre et al., 2001b, 2001a)	145	N.A.	Tested for reproducibility and validated against 7-day WR and biomarkers	Food models and food photographs	FoodFinder®	(Grant et al., 1992)

[6] Venter CS, MacIntyre UE, Vorster HH. The development and testing of a food portion photograph book for use in an African population. J Hum Nutr Dietet. 2000; 13:205–18;
[7] Langenhoven, ML, Kruger, M, Gouws, E, Faber, M. MRC Food Composition Tables. 3rd edition. Parow Valley: Medical Research Council; 1991.; Kruger M, Sayed N, Langenhoven ML, Holing F. Composition of South African foods: vegetables and fruit. Supplement to the MRC Food Composition Tables 1991. Parow Valley: Medical Research Council; 1998; Sayed N, Frans Y, Schönfeldt HC. Composition of South African foods: milk and milk products, eggs, meat and meat products. Supplement to the MRC Food Composition Tables 1991. Parow Valley: Medical Research Council; 1999;

Authors	Country	Study Design	Study Population	Dietary Assessment Method (Source)	Number of food items	Reference time frame	Validity/ Reproducibility	Determination of Portion Sizes	Tools for Dietary Data Analysis	
(Joffe et al., 2012)	South Africa	Cross-sectional study	256 Adults (18-45 years-old)	QFFQ (De Villiers et al., 2006; Steyn & Senekal, 2005)	129	N.A.	Relative-validated	Food Photographs	FoodFinder®	(Grant et al., 1992)
2011										
(Wentzel-Viljoen et al., 2011)	South Africa	Cross-sectional study	175 Adults (35-70 years-old)	QFFQ (MacIntyre et al., 2001b, 2001a)	145	Preceding month	Tested for reproducibility and validated against 7-day WR and biomarkers	Food models, food pictures, real food, food dishes, utensils	South African Food Composition Database _MRC, USDA database	(USDA Agriculture Research Service Nutrient Data Laboratory, 1996)
(Anderson et al., 2011)	Cameroon	Cross-sectional study	1790 Adults (24-74 years-old)	QFFQ (Sangita Sharma et al., 1996)	76	Preceding year	N.A.	Wooden food models and cutlery	Several FCT[8] and Microdiet Software	(Fletcher, 1994)
(Joffe et al., 2011)	South Africa	Case Control Study	148 Women (18-45 years-old)	QFFQ (De Villiers et al., 2006; Steyn & Senekal, 2005)	129	N.A.	Relative-validated	Food photographs	FoodFinder® III	(Grant et al., 1992)
(Aounallah-Skhiri et al., 2011)	Tunisia	Cross-sectional study	1019 Adolescents (15-19 years-old)	SQFFQ (El Ati et al., 2004)	134	Preceding month	Tested for reproducibility and validated	Visual tools	Tunisian Food Composition Database; ESHA Food Processor®	(Davison & Mandible, 1994)

[8] Tan S, Wenlock R & Buss D (1985) Immigrant Foods: Second Supplement to McCance and Widdowson's The Composition of foods. London: HMSO.; Ngosom J & Abono A (1989) Les resources alimentaires du Cameroun: Re´partition Ecologique, classification et valeur nutritive (The Food Resources of Cameroon: Ecological Distribution, Classification and Nutritional Value). **Yaounde´: Institut de Recherche Me´dicinale et d'e´tudes de plantes me´dicinales.** Holland B, Welch A, Unwin I, et al. (1991) McCance and Widdowson's The Composition of Foods. London: The Royal Society of Chemistry;

Authors	Country	Study Design	Study Population	Dietary Assessment Method (Source)	Number of food items	Reference time frame	Validity/ Reproducibility	Determination of Portion Sizes	Tools for Dietary Data Analysis	
(Kruger et al., 2011)	South Africa	Cross-sectional descriptive study	330 Adults (> 30 years-old)	QFFQ (MacIntyre et al., 2001b, 2001a)	145	N.A.	Tested for reproducibility and validated against 7-day WR and biomarkers	Validated food portion photograph book[9] common utensils and containers	South African Food Composition Database _MRC USDA database	(USDA Agriculture Research Service Nutrient Data Laboratory, 1996)
(Delport et al., 2011)	South Africa	Cross-sectional study	318 Men (18-40 years-old)	SQFFQ (MacIntyre et al., 2001a)	15 food groups	N.A.	Tested for reproducibility and validated against four 24hR	N.A.	FoodFinder®	(Grant et al., 1992)
2010										
(Joffe et al., 2010)	South Africa	Case Control Study	233 Women (18-45 years-old)	QFFQ (De Villiers et al., 2006; Steyn & Senekal, 2005)	129	N.A.	Relative-validated	Food Photographs	FoodFinder® III	(Grant et al., 1992)
2009										
(Zingoni et al., 2009)	South Africa	Cohort Study	83 Adolescents	QFFQ (De Villiers et al., 2006; Steyn & Senekal, 2005)	N.A.	Usual intake (daily, weekly and monthly basis)	Not validated or tested for reproducibility	Food Photo Manual; Food flour models; Household utensils	FoodFinder®	(Grant et al., 1992)
(Goedecke et al., 2009)	South Africa	Cross-sectional study	57 Adults (18-45 years-old)	QFFQ (De Villiers et al., 2006; Steyn & Senekal, 2005)	129	N.A.	Relative-validated	Food Photographs	FoodFinder®	(Grant et al., 1992)

[9] Venter CS, MacIntyre UE, Vorster HH. The development and testing of a food portion photograph book for use in an African population. J Hum Nutr Dietet. 2000; 13:205–18;

Authors	Country	Study Design	Study Population	Dietary Assessment Method (Source)	Number of food items	Reference time frame	Validity/ Reproducibility	Determination of Portion Sizes	Tools for Dietary Data Analysis	
2008										
(Tessier et al., 2008)	Tunisia	Cross-sectional study	724 Adults	QFFQ (El Ati et al., 2004)	146	N.A.	Tested for reproducibility and validated	N.A.	ESHA Food Processor®	(Davison & Mandible, 1994)
(Hogenkamp et al., 2008)	South Africa	Cross-sectional study	1605 Adults (15-65 years-old)	QFFQ (95)	145	N.A.	Relative-validated	Validated photographs[10] Household measures; Food models	FoodFinder®	(Grant et al., 1992)
2007										
(Oguntibeju et al., 2007)	South Africa	Cross-sectional study	35 Adults (18-65 years-old)	QFFQ (MacIntyre et al., 2001b, 2001a)	145	Preceding 6 months	Tested for reproducibility and validated against 7-day WR and biomarkers	Food models; Household measures; MRC Food Quantities Manual	FoodFinder®	(Grant et al., 1992)
(MacKeown et al., 2007)	South Africa	Cohort Study	143 Adolescents (10 and 13 years-old)	SQFFQ (Richter et al., 2007)	145	N.A.	Tested for Reproducibility and validated	NRPNI[11]; Food quantities manual; Household measures	South African Food Composition Database MRC[12]; SAS software	
(Vorster et al., 2007)	South Africa	Cross-sectional study	1854 Adults (≥15 years-old)	QFFQ (MacIntyre et al., 2001b, 2001a)	145	N.A.	Tested for reproducibility and validated against 7-day WR and biomarkers	N.A.	FoodFinder®	(Grant et al., 1992)

[10] Venter CS, MacIntyre UE, Vorster HH. The development and testing of a food portion photograph book for use in an African population. J Hum Nutr Dietet. 2000; 13:205–18;
[11] National Research Programme for Nutritional Intervention;
[12] Langenhoven, ML, Kruger, M, Gouws, E, Faber, M. MRC Food Composition Tables. 3rd edition. Parow Valley: Medical Research Council; 1991.; Kruger M, Sayed N, Langenhoven ML, Holing F. Composition of South African foods: vegetables and fruit. Supplement to the MRC Food Composition Tables 1991. Parow Valley: Medical Research Council; 1998; Sayed N, Frans Y, Schönfeldt HC. Composition of South African foods: milk and milk products, eggs, meat and meat products. Supplement to the MRC Food Composition Tables 1991. Parow Valley: Medical Research Council; 1999;

Authors	Country	Study Design	Study Population	Dietary Assessment Method (Source)	Number of food items	Reference time frame	Validity/ Reproducibility	Determination of Portion Sizes	Tools for Dietary Data Analysis	
(Jackson et al., 2007)	Cameroon	Cross-sectional study	547 Adults (25-74 years-old)	QFFQ (Sangita Sharma et al., 1996)	37	Preceding year	N.A.	N.A.	N.A.	
2006										
(Belgnaoui & Belahsen, 2006)	Morocco	Cross-sectional study	172 Pregnant and/or Lactating Women (16-44 years-old)	QFFQ	N.A.	Usual intake (daily, weekly and monthly basis)	N.A.	N.A.	Bilnut Software[13]	
(Hattingh et al., 2006)	South Africa	Cross-sectional study	488 Women (25-44 years-old)	QFFQ	N.A.	N.A.	Validated	Food Quantities Manual	FoodFinder®	(Grant et al., 1992)
2005										
(Kesa & Oldewage-Theron, 2005)	South Africa	Cross-sectional study	431 Pregnant and/or Lactating Women (16-35 years-old)	QFFQ	N.A.	Habitual consumption	Validated	Food Models	Dietary Manager Program®	
(Merchant et al., 2005)	Zimbabwe	Cross-sectional study	100 Adults (34-93)	SQFFQ	30	Previous year	Validated against 24hR	Standard portion sizes	ESHA Food Processor®; Zimbabwe FCT; USDA database.	(Davison & Mandible, 1994)

[13] Bilnut: SCDA Nutrisoft, Cerelles, France.

Table.2.3 Studies (n=11) that assessed dietary intake of different African populations using food frequency questionnaires and 24-hour recall [from 2005 to 2014].

Author	Country	Study Design	Study Population	Number of food items in the FFQ	Reference time frame of the FFQ	Number of recalls	Determination of Portion Sizes		Tools for Dietary Data Analysis
2014									
(Korkalo et al., 2014)	Mozambique	Cross-sectional study	551 Adolescents (14-19 years-old)	37	Preceding 7 days	4 non-consecutive (1: 100 %; 2: 76 %; 3: 67 %; 4: 59 %)	Food Photographs (Validated Food photographs in portion size estimation), Common utensils	Mozambique FCT[1] for NutriSurvey Program; Collection and Analysis of foods.	(Erhardt & Gross, 2007)
2012									
(Mbochi et al., 2012)	Kenya	Cross-sectional study	365 Women (25-54 years-old)	26	Preceding 7 days	Single	FFQ: no used of portion sizes; 24hR: local household utensils; Real foods; SA Food Photo Manual	NutriSurvey Program	(Erhardt & Gross, 2007)
(Amare et al., 2012)	Ethiopia	Cross-sectional study	356 Adults (>18 years-old)	8 food categories	Preceding 7 days	1 (100 %) 3 (10 %)	Household measures	Ethiopian FCT; FCT for use in Africa	
(Mala et al., 2012)	Kenya	Cross-sectional study	107 Pregnant and/or Lactating Women (15-49 years-old)	N.A.	Usual intake	Single	Calibrated list of UNICEF; Household measures	NutriSurvey Program	(Erhardt & Gross, 2007)
2011									
(Oldewage-Theron & Kruger, 2011)	South Africa	N.A.	375 Women	>40	Preceding 7 days	Single	Food models	FoodFinder®	(Grant et al., 1992)

[1] FCT: Food Composition Table;

Author	Country	Study Design	Study Population	Number of food items in the FFQ	Reference time frame of the FFQ	Number of recalls	Determination of Portion Sizes	Tools for Dietary Data Analysis	
(Namugumya & Muyanja, 2011)	Uganda	Cross-sectional	225 Adults (21-50 years-old)	55	Preceding week	Single	Food cost; Household utensils	NutriSurvey Program; USDA database; FAO FCT for African foods.	(Erhardt & Gross, 2007; USDA Agriculture Research Service Nutrient Data Laboratory, 1996)
					2010				
(Baroudi et al., 2010)	Tunisia	Cross-sectional study	94 Adults (32-64 years-old)	168	Preceding month	Single	FFQ: household measures; 24hR: 3D food models; measurement aids; food specific units	Bilnut Software[2]	
					2006				
(Oldewage-Theron et al., 2006)	South Africa	Cross-sectional study	357 Women	N.A.	N.A.	1	Food Models	Dietary Manager Program®	
					2005				
(Waudo et al. 2005)	Kenya, Tanzania e Uganda	Cross-sectional study	612 Women	N.A.	Previous 7-day	1	Water and measuring cylinders	Food Meter UK 07	
(Faber & Kruger, 2005)	South Africa	Cross-sectional study	187 Women (25-55 years-old)	60	Preceding month	Single	24hR: Food models, household utensils, 3D sponge models dry oats utensils, and 3D sponge models	South African Food Composition Database of the MRC[3].	
(Oldewage-theron et al., 2005)	South Africa	Cross sectional study	409 Women	N.A.	N.A.	1	Food Models	N.A.	

[2] Bilnut: SCDA Nutrisoft, Cerelles, France;
[3] Medical Research Council;

Table 2.4 Studies (n=4) which assessed dietary intake of different African populations using weight records [from 2005 to 2014].

Author	Country	Study Design	Study Population	Number of days	Other collected information	Tools for Dietary Data Analysis	
				2013			
(Haileslassie et al., 2013)	Ethiopia	Cross-sectional study	60 Pregnant and/or Lactating Women (15-49 years-old)	1 day	Description of the foods and their cooking methods	ESHA Food Processor®; Ethiopian FCT[1]	(Davison & Mandible, 1994)
				2012			
(Olayiwola et al., 2012)	Nigeria	Cross-sectional study	240 Elderly (>60 years-old)	3 consecutive days	Description of what was eaten/drunk on the day before	FAO FCT; Total Dietary Assessment Software (NUTRIDATA)	(Pirrone et al., 1993)
				2008			
(Gibson et al., 2008)	Ethiopia	Cross-sectional study	99 Women (Mean age: 27,8 years-old)	1 day	N.A.	Ethiopian FCT	
(Abebe et al., 2008)	Ethiopia	Cross-sectional study	99 Women (Mean age: 27,8 years-old)	2 non-consecutive days	Detailed weighed recipe data for all the composite dishes	Development of a database based on the Ethiopian FCT	

[1] FCT: Food Composition Table.

2.3. METHODS FOR THE ANALYSIS OF FOOD INTAKE DATA

To convert the dietary intake into nutrient intake some components are needed, such as a food composition database, a coding system for matching listed foods with the entries in the food composition database and a software to calculate the nutritional composition (Thompson & Byers, 1994; Willet, 2013d). The correct choice of the nutrients database is very important because its parameters will affect the estimation of nutrient intake. Parameters such as the completeness regarding the included food items and evaluated nutrients are related to the constant updating of the database, so it is imperative to support the analysis on the most recently updated version available (Thompson & Byers, 1994). These nutrients databases are commonly included in computer software programs that process data and calculate individual nutritional intake. The choice of the software should be based on the level of specification and detail needed, on the type of foods that are usually consumed by the target population and on the hardware and software requirements.

2.3.1. Analysis of food intake data in Africa

Currently, many software tools for the analysis of dietary data are being used. According to Table 2.5, which compiles the nutritional software tools used in the previously mentioned studies carried out between 2005-2014, it can be observed that researchers preferably select food databases of the countries of the same African region. Several countries had to update these tools with their own typical foods or recipes of composite dishes when these were not available.

In Western African countries, the most used nutritional programs are *ESHA Food Processor*® (Food Processor Diet Analysis & Fitness Software) (Addo et al., 2011; Huybregts et al., 2009; Pereko et al., 2012; Wiig & Smith, 2007) and the *Software for Intake Distribution Estimation* (C-SIDE), developed by Iowa State University (Becquey & Martin-Prevel, 2010; Sodjinou et al., 2008, 2009; Zeba et al., 2014). Several authors used other software, such as *NutriData*, developed in California (Olayiwola et al., 2012), *Nutrition Data System for Research* (NDSR), developed by the University of Minnesota (Luke et al., 2011) and *Nutrifiq*, based on the Canadian Nutrient File (Alaofè et al., 2009). A very comprehensive software system, named VBS Food Calculation System, was chosen for the Women's Dietary Diversity Project (in Mali) (Kennedy et al., 2009). The *VBS Food Calculation System* is a set of three softwares, which include *Komeet* (for food intake analysis), *VBS Manager* (nutrient composition information), *Orion and Food Groups* (both for nutrient intake by food group analysis).

In East Africa, almost all the studies performed, and available in literature, used the *NutriSurvey Program*, which has seventeen different food databases (food composition tables from Tanzania, Kenya, Senegal, Mali and Germany among others) (Jordan et al., 2013; Kamau-Mbuthia & Elmadfa, 2007; Korkalo et al., 2014; Mala et al., 2012; Mbochi et al., 2012; Mupere et al., 2012; Namugumya & Muyanja, 2011). *ESHA Food Processor*® (Haileslassie et al., 2013; Irvine et al., 2011; Merchant et al., 2005), *FoodFinder*® (Steyn et al., 2011, 2012; Steyn & Nel, 2006), which includes

the latest version of the South African Food Composition Database, NDSR (Heimburger et al., 2010; Koethe et al., 2013), and World Food Dietary Assessment System (Gewa et al., 2008; Walton et al., 2012) were also used. Softwares such as the *Programme CANDAT* (Powell et al., 2013), Food Meter UK 07 (Waudo *et al*. 2005) and *General Intake Estimation System*, developed by The National Food Institute, in Denmark (Hansen et al., 2011), were also chosen for nutrient analysis.

Northern African countries based their nutrient analysis on *Bilnut Software* (Baroudi et al., 2014; Baroudi et al., 2010; Belgnaoui & Belahsen, 2006). However, other softwares such as *ESHA Food Processor*® (Aounallah-Skhiri et al., 2011), *DIAL Programme* (López et al., 2012), developed by several authors from Alce Ingenieria, *Tableaux des valeurs nutritives* (Lamri-Senhadji et al., 2009), built by Souci and colleagues (2000), *Dietetik*®, designed for Tunisian foods, *Nutrilog*®, a software with eleven different databases (Boumaiza et al., 2012) and *FoodBase Nutritional Program* (Nyuar et al., 2012) were also used in some studies.

Investigations carried out in Southern African countries mainly used *FoodFinder*® (Grant et al., 1992) software (Charlton et al., 2005; Faber & Kruger, 2005; Hattingh et al., 2006; Jackson et al., 2012; Joffe et al., 2010, 2011; Kruger et al., 2011, 2012; Mostert et al., 2005; Naude et al., 2011; O'Keefe et al., 2007; Oguntibeju et al., 2007; Oldewage-Theron et al., 2010, 2014; Oldewage-Theron, Salami, et al., 2008; Oldewage-Theron, Samuel, & Venter, 2008; Oldewage-Theron, Samuel, Grobler, et al., 2008; Oldewage-Theron & Kruger, 2011; Papathakis & Pearson, 2012; Pretorius et al., 2012; Rankin et al., 2011; Vorster et al., 2007; Wentzel-Viljoen et al., 2011; Wrottesley et al., 2014; Zingoni et al., 2009). Other software databases such as NDSR (May et al., 2014), *NutriBase* (Kolahdooz et al., 2013; Sheehy et al., 2013), developed by CyberSoft (both based on USDA National Nutrient Database for Standard Reference), *Dietary Manager* Program® (Kesa & Oldewage-Theron, 2005; Oldewage-Theron et al., 2006) managed by Oskar Scharf of Dietetic Services/Rand Software and Nutritionist Five (Maruapula & Chapman-Novakofski, 2008) were used as well.

In Central Africa, the used software tools in Cameroon were *Microdiet* (Anderson et al., 2011) and *Becel Institute Nutrition Software* (Dapi et al., 2010) and *Lucille food analysis software* (Termote et al., 2012) in the Democratic Republic of Congo.

Some studies do not mention the use of a specific software, only the use of food composition databases, as a source of information for the nutrient analysis. Both Microsoft® Office Excel and the International Business Machines Corporation Statistical Package for the Social Sciences (IBM SPSS) software are widely used to compute dietary data.

Generally, there are few African countries with their own food composition table (FCT). The majority of African countries has no specific FCT and, consequently, need to use either FCTs from neighbouring countries or from the Food and Agriculture Organization (FAO), which decreases the reliability of the results. This is one of the most cited limitations by the authors working in this research field. Several softwares have been mentioned, however, most of them are composed of the same FCTs, which makes imperative the need for creating updated tools. An example of an effort to improve this lack of country specific databases is the study of Becquey *et al*. (2009), who developed a FCT for Burkina Faso bringing together the information of three sources, namely the FCT for Mali, supplemented by the WorldFood FCT for Senegal and the USDA database. This FCT was complete

for energy, macronutrients, and eleven micronutrients. The variability within the same continent is huge, and different lifestyles and typical food patterns are found even within the same country, which makes finding the uniformity in the FCTs quite challenging, and eventually impossible. Based on these limitations, the countries find themselves obliged to create their own tools. In order to fight against the current lack of updating of these tools, it is necessary to join forces geared towards the development of both new and country specific FCTs or at least to complete the existing ones.

Table 2.5 Nutritional Tools used in dietary data analysis in the selected studies carried out between 2005-2014, organized per African Regions (Western, Eastern, Central, Northern, and Southern Africa).

WESTERN AFRICA	Benin	Burkina Faso	Ghana	Mali	Nigeria
Nutritional tools	• Nutrifiq (Alaofè et al., 2009) • C-SIDE[1] (Sodjinou et al., 2008, 2009) • FCT[2] for use in Africa (Nago et al., 2010)	• USDA[3] Database (Becquey et al., 2009; Becquey & Martin-Prevel, 2010) • ESHA Food Processor® (Huybregts et al., 2009) • C-SIDE (Becquey & Martin-Prevel, 2010; Zeba et al., 2014) • FCTs for Mali and the Worldfood FCT for Senegal (Becquey et al., 2009)	• ESHA Food Processor® (Addo et al., 2011; Pereko et al., 2012; Wiig & Smith, 2007) • Nutrition Data System for Research (Luke et al., 2011)	• VBS Food Calculation System (Kennedy et al., 2009)	• Nutridata (Olayiwola et al., 2012) • Food analysis methods (Ijarotimi & Keshinro, 2008)

EASTERN AFRICA	Ethiopia	Kenya	Tanzania	Uganda	Zambia	Zimbabwe	Mozambique
Nutritional tools	• USDA Database (Alemayehu et al., 2011) • ESHA Food Processor® (Haileslassie et al., 2013) • FCT for use in Africa and Ethiopian FCT (Abebe et al., 2008; Alemayehu et al., 2011; Amare et al., 2012; R. S. Gibson et al., 2008; Haileslassie et al., 2013; Tesfaye et al., 2008)	• WorldFood Dietary Assessment System (Gewa et al., 2008; Walton et al., 2012) • NutriSurvey Program (Kamau-Mbuthia & Elmadfa, 2007; Mala et al., 2012; Mbochi et al., 2012) • GIES[4] (Hansen et al., 2011) • FoodFinder® (Steyn et al., 2011, 2012; Steyn & Nel, 2006) • Food Meter UK 07 (Waudo et al. 2005)	• NutriSurvey Program (Jordan et al., 2013) • ESHA Food Processor® (Irvine et al., 2011) • Programme CANDAT (Powell et al., 2013) • Tanzanian FCT (Changamire et al., 2014; Kim et al., 2014; Lukmanji et al., 2013; Powell et al., 2013) • Food Meter UK 07 (Waudo et al. 2005)	• USDA database, NutriSurvey Program (Mupere et al., 2012; Namugumya & Muyanja, 2011) • Food Meter UK 07 (Waudo et al. 2005)	• Nutrition Data System for Research software (Heimburger et al., 2010; Koethe et al., 2013) • Zambian FCT (National Food and Nutrition Commission) (Koethe et al., 2013)	• USDA Database; ESHA Food Processor® (Merchant et al., 2005)	• NutriSurvey Program (Korkalo et al., 2014) • FCT for Africa and FCT for Mozambique (Scarcella et al., 2011)

[1] C-SIDE: Software for Intake Distribution Estimation.
[2] FCT: Food Composition Table
[3] USDA: United States Department of Agriculture
[4] GIES: GIES: General Intake Estimation System Program, GIES; National Food Institute, Søborg, Denmark

NORTHERN AFRICA	Algeria	Egypt	Morocco	Sudan	Tunisia
Nutritional tools	• *Tableaux des valeurs nutritives* (Lamri-Senhadji et al., 2009)	• Egyptian FCT_Nutrition Institute (Mounir et al., 2007)	• DIAL Programme[5] (López et al., 2012) • Bilnut Software (Nutrisoft) (Belgnaoui & Belahsen, 2006)	• Foodbase Nutritional Program (Nyuar et al., 2012)	• Dietetik® and Nutrilog® (Boumaiza et al., 2012) • Bilnut Software (Logiciel and Nutrisoft) (Baroudi et al., 2014; Baroudi et al., 2010) • Tunisian Food Composition database (Aounallah-Skhiri et al., 2011) • ESHA Food Processor® (Aounallah-Skhiri et al., 2011; Tessier et al., 2008)

SOUTHERN AFRICA	Botswana	South Africa
Nutritional tools	• Nutritionist Five (Maruapula & Chapman-Novakofski, 2008) • FoodFinder® (Jackson et al., 2012)	• FoodFinder® (Charlton et al., 2005; Faber & Kruger, 2005; Hattingh et al., 2006; Jackson et al., 2012; Joffe et al., 2010, 2011; Kruger et al., 2011, 2012; Mostert et al., 2005; Naude et al., 2011; O'Keefe et al., 2007; Oguntibeju et al., 2007; Oldewage-Theron et al., 2010, 2014; Oldewage-Theron, Salami, et al., 2008; Oldewage-Theron, Samuel, & Venter, 2008; Oldewage-Theron, Samuel, Grobler, et al., 2008; Oldewage-Theron & Kruger, 2011; Papathakis & Pearson, 2012; Pretorius et al., 2012; Rankin et al., 2011; Vorster et al., 2007; Wentzel-Viljoen et al., 2011; Wrottesley et al., 2014; Zingoni et al., 2009) • Nutribase (Kolahdooz et al., 2013; Sheehy et al., 2013) • Dietary Manager Program® (Kesa & Oldewage-Theron, 2005; Oldewage-Theron et al., 2006) • Nutrition Data System for Research (Luke et al., 2011; May et al., 2014) • South African Food Composition Database of the MRC (Faber & Kruger, 2005; Kruger et al., 2011, 2012; MacKeown et al., 2007; Mostert et al., 2005; Wentzel-Viljoen et al., 2011)

CENTRAL AFRICA	Cameroon	D. R. Congo
Nutritional tools	• Microdiet (Anderson et al., 2011) • Becel Software (Dapi et al., 2010)	• Lucille food analysis software (Termote et al., 2012)

[5] DIAL: Programa para evaluación de dietas y gestión de datos de alimentación.

2.4. KENYAN AND PORTUGUESE NUTRITION PROFILES

2.4.1. Nutrition Profile – Kenya

The Human Development Index (HDI) is a statistical parameter that ranks countries into different levels of human development, based on life expectancy, education, and *per capita* income indicators. Kenya's HDI is classified as medium and it is equal to 0.590, correspondent to the position of 147 out of 189 countries and territories in the Human Development Report 2019, from the United Nations Development Programme (UNDP) (United Nations Development Programme, 2019a).

Urban population in Kenya was reported at 27.5 % (14,461,522 people) in 2019 (The World Bank, 2019b). These numbers, inversely proportional to rural population percentage, have been increasing. In the capital, Nairobi, 100 % of the population is urban (4,397,073 people). People are moving from rural areas to Nairobi county in search of jobs. Consequently, the working-age population, aged between 15-64 years old, around 68.3 % in 2019, is very representative from which the majority is aged between 15-39 years old (Kenya National Bureau of Statistcs (KNBS), 2019). Despite the increasing proportion of urbanization, Kenya is still classified by the World Bank and FAO as a low-income food-deficit country given the existence of low income and food inadequacy (Food and Agriculture Organization of the United Nations, 2018; The World Bank, 2018). This means that the nutritional status of vulnerable populations is at risk, given food insecurity and susceptibility to domestic and external shocks. Even though, the Kenyan hunger index score has improved, food insecurity still exists. The major reasons for food insecurity are the co-existence of poverty, low food safety, low dietary diversity and protein quality, insufficient food supply and infrastructure and scarce public investments in agriculture, as well as corruption (Rampa & Dekeyser, 2020).

Besides the burden of undernutrition, Kenya also faces overnutrition. According to the Global Nutrition Report, Kenya experiences overweight, anaemia and chronic undernutrition (stunting) (Development Initiatives Poverty Research Ltd., 2018). Primarily reasons for malnutrition are on the one hand poor or inappropriate dietary intake, and on the other hand high disease burden. Among children under five years old, the prevalence of acute undernutrition (wasting), stunting, and overweight, are 4.2 % (in 2014), 26.2 % (in 2012), and 4.1 % (in 2012), respectively (Development Initiatives Poverty Research Ltd., 2018). At higher ages (5-19 years old), overweight (16.2 % *vs*. 6.2 %) and obesity (3.2 % *vs*. 1.2 %) tended to be more prevalent among girls than boys, prevalence dated to 2016. On the opposite, boys (31.6 %) are more underweight than girls (18.4 %) (Development Initiatives Poverty Research Ltd., 2018). These trends tend to continue to adulthood, 34.3 % and 11.1 % of women are overweight and obese, respectively, while for men the correspondent levels are 16.1 % and 2.8 %, respectively (Development Initiatives Poverty Research Ltd., 2018). Consequently, diabetes (5.8-6.2 %) and high blood pressure (26.5-26.6 %) are two comorbidities prevalent in the adult population (Development Initiatives Poverty Research Ltd., 2018). According to the Kenya National Micronutrient Survey (2011) (Harambee Ministry of Health, 2011), micronutrient deficiencies are also a health problem for Kenyans; iodine deficiency disorder, iron deficiency anaemia, and vitamin A and zinc deficiencies are the most worrying ones. Pre-school

children (PSC) are one of the age groups that has more prevalence of these micronutrient deficiencies, namely 9.2 %, 22.1 %, 39.1 % and 83.3 % of PSC suffer from vitamin A, iodine, iron, and zinc deficiencies (Harambee Ministry of Health, 2011). For iodine, besides deficiency is present in 22.1 % school age children, excess of iodine is also a health concern, affecting 30.4 % of school age children (Harambee Ministry of Health, 2011).

2.4.2. Nutrition Profile – Portugal

Portugal is classified as a high-income country by the World Bank (The World Bank, 2019a), which is closely related to its high HDI, of 0.847, being positioned in the 40th place in the country ranking system (United Nations Development Programme, 2019b). In 2018, 65.2 % (6,710,000.99 people) of the Portuguese population was considered urban and 34.8 % (3,580,000 people) rural (Food and Agriculture Organization of the United Nations, 2019). Regardless of this higher status of the country, unfortunately, Portugal also faces food insecurity challenges. A study developed during the first decade of XXI century, identified the presence of social disparities in the distribution of chronic diseases, with a higher prevalence in low-income population groups (Furtado & Pereira, 2010). It is a common conclusion among studies in this area, that women, unemployed people or employees in precarious conditions, single-parent families, and large families with low levels of education, are the most vulnerable groups in terms of food insecurity (Correia et al., 2018). Unhealthy and unbalanced diets, rich in high-energy and poor-micronutrient food products, coexisting with sedentary lifestyles seem to justify an evident association between food insecurity and chronic diseases, such as obesity, dyslipidaemia, type 2 diabetes mellitus and high blood pressure (Correia et al., 2018).

In 2015, the first National Health Survey with Physical Examination in Portugal was carried out, by the Department of Epidemiology of the Ricardo Jorge Institute, including 4911 participants, aged between 25-74 years-old, recruited over the whole country (mainland and islands) (Barreto et al., 2016). From those, 53.9 % were adult females and 46.1 % adult males. Obtained results showed that 38.9 % of the adult population (25-74 years-old) had overweight and that obesity affected 28.7 % of Portuguese adults. In terms of gender distribution, overweight was more common among men (45.4 % *vs*. 33.1 %), while obesity was more common among women (32.1 % *vs*. 24.9 %). Moreover, the socio-economic factor turned out to be determinant to the prevalence of these unhealthy conditions. These high levels of overweight and obesity and the low physical activity level lead to an increase of food-related health concerns such as type 2 diabetes, high blood pressure, high cholesterol, among others (WHO Consultation on Obesity, 2000). According to this national survey, type 2 diabetes was observed in 9.8 % of total participants, but higher prevalence, 23.8 %, was observed among older people (65-74 years-old) than in 35-44 years adults (0.9 %). Moreover, high school levels and permanent job have seemed to be a proxy for lower diabetes levels. The prevalence of high blood pressure among the adult population was significant in both gender (women – 32.7 % and men – 39.6 %), and, not surprisingly, older ages were more affected, 71.3 %. Similarly to what happened with diabetes, the highest prevalence was noticed in individuals without education, or with primary education (≤ 4 schooling years) (62.6 %) and in individuals without occupation

(64.5 %) (Barreto et al., 2016). More than half (52.3 %) of the Portuguese adult population involved in this survey had high cholesterol levels, and this number increased to 63.3 % when people already taking specific medication for dyslipidaemia were included in the group. Once again, older people were more affected by hypercholesterolemia (Barreto et al., 2016).

2.5. DIETARY HABITS IN KENYA AND PORTUGAL

2.5.1. Dietary habits in Kenya

Kenya is a country with traditional food habits, but more and more modern lifestyles are gaining dimension, and both co-exist (Mohajan, 2014). According to Kenya's Ministry of Health, unhealthy diets are fairly frequent among Kenyans (Ministry of Health, 2017). The World Health Organization (WHO) recommendation of eating five servings of fruits and/or vegetables a day is followed by 5.2 % of adults, the habit of adding salt to their own food is generally implemented (20 % of adults) and adding sugar to beverages is often done by 83.5 % of adults (Ministry of Health, 2017).

Even though there is no updated dietary intake data for Kenya, justified by sociocultural/economic barriers, there are some released studies, carried out on specific populations such as children, women or focused on specific foods or Kenyan rural areas (Vila-Real et al., 2016). Hansen *et al*. (2011) studied the food habits and intake of three ethnic groups of rural areas, and they reported that, not surprisingly, carbohydrates was the major consumed macronutrient (in percentage of total energy intake), varying between 55.7 %, for men and 74.2 %, for women, followed by fat contributing between 14.5 % (women) and 30.2 % (men) and protein, that ranged from 11.3 % (women) to 14.1 % (men). Energy intakes were quite different among the three ethnic groups: 1386 and 1720 kcal, 1601 and 2007 kcal, and 2055 and 2509 kcal, for women and men, respectively. The research of Nguu-Gutu *et al*. (2017), which involved 31 adolescents from Nairobi, stated that around 39 %, 42 %, and 23 % had inadequate (less than the Recommended Dietary Allowance) protein, fat and carbohydrates intakes, respectively, showing that protein and fat were the most critical nutrients. Similarly to the Hansen's study, the nutrient that contributed the most for the dietary intake was carbohydrates (61 % for women and 60 %, for men), followed by fat (28 %, for women and 30 %, for men) and protein (10 %, for women and 11 %, for men) (Nguu-Gutu et al., 2017). Mean energy daily intake was similar between genders, namely 2311 kcal for women and 2454 kcal for men (Nguu-Gutu et al., 2017). This work found that snacking is frequent among students, but it did not affect the adequacy for macronutrients (Nguu-Gutu et al., 2017). Nevertheless, it was concluded that snacks availability could be improved in terms of nutrient quality, given their high content in fat and sugar (Nguu-Gutu et al., 2017). Some examples of snacks are crisps, sweets, *chapatti*, *mandazi*, *masala* sticks, and carbonated soft drinks.

The study of Mbochi *et al*. (2012) was focused only on obese women, from different social-economic levels. Results showed that women of higher socioeconomic groups had higher intakes of energy, protein, fat, cholesterol and alcohol and lower intakes of fibre and carbohydrates in comparison with low-income women. This is one more evidence that urbanization and the nutrition transition are contributing to the increased intake of nutritionally poor diets. Moreover, the work of Steyn and colleagues had similar conclusions (Steyn et al., 2011).

Not only in African countries, but also worldwide, it is well-established that carbohydrates are the major contributors in terms of energy. Cereals, one of the main food-groups rich in carbohydrates, are a staple food for most of the world population. Not differently, in Kenya, cereals

are a very important energy source, regardless of the urbanization level. Among cereals, maize is the most produced and consumed crop. Since 2010, local maize production is increasing, with a drop between 2016 and 2017, reaching almost four million tonnes in 2019 (Food and Agriculture Organization of the United Nations (FAOSTAT), 2020). Wheat and rice consumption is increasing among urban areas (Mohajan, 2014) and sorghum and millet are among the five most produced crops in Kenya, being expectable a production increase for the next years (Kenya National Bureau of Statistics, 2019a). After cereals (45.4 %), caloric supply is provided by pulses (11.7 %), sugar and sweeteners (7.5 %), starchy roots (7.4 %) and milk products, excluding butter (7.4 %) (Kenya National Bureau of Statistics, 2019a). Most common dishes in Kenya include *Ugali* (thick porridge traditionally made of maize), *Githeri* (boiled maize and beans), *Mathoke* (mashed plantain), *Mukimo* (mashed potatoes with vegetables, maize, and beans), *Pilau* (cooked chicken and rice prepared with a typical seasoning) and *Irio* (boiled maize, beans, vegetables, and potatoes). Bread, milk products and vegetables are also a crucial part of the diet (Mohajan, 2014).

2.5.1.1. Dietary Guidelines for the Kenyan population

Until 2010, Kenya was devoid of any dietary guideline, except for some Nutritional Practices developed to support the management in Tuberculosis and Human Immunodeficiency Virus. In 2010, the Kenya National Clinical Nutrition and Dietetics Reference Manual was created, in which a food guide for regular diet presents recommendations in terms of food servings per day (Ministry of Medical Services, 2010). This guide includes not only the recommended servings per adult but also per child. Seven years later, a new national document was developed, The National Guidelines for Healthy Diets and Physical Activity, a publication that was the responsibility of the Ministry of Health (Ministry of Health, 2017). This document includes important information regarding healthy eating principles for families and highlights the need for a balanced, varied, complete and adequate daily diet. Moreover, it also includes messages related to healthy diets for different age groups over the life cycle. However, it does not include recommendations in terms of nutrient requirements. Consequently, in order to study the nutrient adequacy of Kenyan populations, it is necessary to use, international recommendations as guidelines, for instance the energy and nutrient guidelines developed by the WHO in collaboration with FAO (Food and Agriculture Organization Expert Consultation, 2008; Joint FAO/WHO Expert Consultation on Human Vitamin and Mineral Requirements, 1998; Joint WHO/FAO/UNU Expert Consultation, 2001, 2002; Mann et al., 2007).

2.5.2. Dietary habits in Portugal

In Portugal, food availability, food consumption and consequently, dietary intake, have changed over time (Bento et al., 2018). The last national food survey, National Food, Nutrition and Physical Activity Survey of the Portuguese General Population, (*Inquérito Alimentar Nacional e de Atividade Física*, IAN-AF), carried out in Portugal dated 2015-2016 (Lopes et al., 2017). Dietary data were obtained by the application of a food propensity questionnaire and two non-consecutive 24hRs (two non-consecutive WR, for children under 10 years old) and during the interviews, food

photographs, included in a photographic album, were used aiming to help respondents to estimate food portions. Besides the dietary intake assessment, this survey allowed collection on physical activity habits as well as anthropometric data. This survey included 6553 participants, of which 5819 answered to the two interviews, aged between three months and 84 years-old, recruited over the whole country (mainland and islands). From these, 29 % were adult females and 24 % adult males (18-64 years-old), 23 % were children under 10 years-old, 11 % were adolescents (10-17 years-old) and 13 % were elderly (≥ 65 years-old).

According to the results of this survey, considering the whole population, protein contribution was 19.9 % of energy intake, while carbohydrates and fat intakes corresponded to 46.6 % and 31.4 %, respectively (Lopes et al., 2017). In terms of food consumption (in percentage), the most expressive food group, considering the total amount of food and beverages, not only the foods included in the Food Wheel Guide, was '*cereals, cereal products and starchy tubers*', contributing with 20 %, followed by '*dairy products'* (17 %), '*meat, fish and eggs'* (12 %), '*vegetables'* (10 %), '*fruits'* (9 %), '*pulses'* (1 %) and '*fats and oils'* (1 %). Foods non-represented in the Portuguese food guide, such as alcoholic beverages (7 %), non-alcoholic beverages (excluding water) (16 %), sweets and cookies (4 %) and salty snacks and pizzas (1 %) contributed in total with 29 % of total consumption (Lopes et al., 2017). The most consumed foods included in cereals and cereal-based products were bread and toasts (100 g/day), followed by potatoes (86.9 g/day) and rice (64.3 g/day). The consumption of protein-rich foods, such as red meat was far above recommendations, namely, 22.5 % eats more than 100 grams per day. White meat (42.9 g/day) and fish (41.8 g/day) are the most consumed items after red meat (51.6 g/day). Among non-alcoholic beverages, at national level the beverage of choice was '*soft drinks'* (83 g/day), followed by '*coffee'* (58 g/day), '*nectars and fruit juices'* (25 g/day), and '*tea and infusions'* (20 g/day) (Lopes et al., 2017). However, the consumption of these beverages differs according to age-group. In what concerns '*soft drinks'* and '*nectars and fruit juices'*, adolescents were the main consumers ('*soft drinks*': 164 g/day; '*nectars and fruit juices*': 49 g/day), while for *coffee* and *tea and infusions*, adults ('*coffee*': 88 g/day; '*tea and infusions*': 30 g/day) and the elderly ('*coffee*': 72 g/day; '*tea and infusions*': 27 g/day) were the main consumers (Lopes et al., 2017). A deeper analysis of the contribution of sugars was carried out by Marinho and colleagues (Marinho et al., 2019). They concluded that the mean daily intake of total sugars, added sugars and free sugars were 84.3 g/day, 32.1 g/day and 35.3 g/day, contributing in 18.5 %, 6.8 % and 7.5 % for total energy, respectively. Thereby, on average the Portuguese population showed adherence to the 'strong recommendation' by WHO for free sugars intake (< 10 % of total energy); according to the results 24.3 % of participants revealed free sugars' intake above this threshold, while 34 % of the sample went beyond the WHO 'strong recommendation', reporting intakes below 5 % of total sugars, thereby aligned with the WHO 'conditional recommendation'. Fruits, soft drinks, yoghurts, sweets and sugar were the main contributors toward these forms of sugar intakes (Marinho et al., 2019).

2.5.2.1. Dietary Guidelines for the Portuguese population

In Portugal, there are well-established dietary guidelines. The principal illustrative food guide is the Food Wheel Guide, created in 1977 and revised in 2003 (Faculty of Food Sciences and Nutrition from the University of Porto (FCNAUP) & Portuguese Consumer's Institute, 2003). This guide with a wheel format, aiming to remind a plate of food, is divided into seven food groups, namely cereal and cereal-based products and tubers; vegetables; fruits; milk and dairy products; meat, fish and eggs; fats and oils; and pulses. The larger the size of the group, the greater should be the consumption of the included foods. Within each group, there are nutritionally equivalent foods, which must be regularly replaced in order to ensure food variety. In the centre of the wheel water is represented, to highlight the importance of a proper hydration balance. For each group, there is a specification of a portion range to be consumed by the different age groups. Lower portions are adequate to children aged between one and three years-old, while upper portions are addressed to physically active men and adolescent boys. Average portions are adequate for the rest of the population, corresponding to a dietary plan of 2200 kcal per day. The food wheel is the national official food guide for the Portuguese population, nonetheless, nutrient international recommendations, from the European Food Safety Authority (EFSA) are used as guidelines (Table 2.6 and Table 2.7). Moreover, some recommendations issued by WHO and FAO, are also an anchor for setting food consumption goals. Some examples are the consumption of at least 400 g of fruit and vegetables per day (excluding potatoes and other starchy tubers), which together with whole grains should provide the intake of at least 25 g per day of dietary fibre (> 20 g per day of non-starch polysaccharides), and lastly the reduction of free sugars consumption to less than 10 % of total energy intake (Joint WHO/FAO Expert Consultation, 2003; World Health Organization, 2015).

Table 2.6 Reference intake range for total carbohydrates, dietary fibre and total fat, according to EFSA recommendations (adapted from (EFSA, 2017)).

Age group (years)	Total carbohydrates (E %)[1]	Dietary Fibre (g/day)[2]	Total fat (E %)
7- 11 months			40
1	45-60	10	35-40
2-3	45-60	10	35-40
4-6	45-60	14	20-35
7-10	45-60	16	20-35
11-14	45-60	19	20-35
15-17	45-60	21	20-35
≥ 18	45-60	25	20-35

[1]: E %: Percentage of total energy, expressed as the reference intake range;

[2]: Quantities expressed as the adequate intake.

Similarly to other European countries, Portugal recognizes the Mediterranean Diet as a healthy and sustainable food pattern. Moreover, it was recognized, in 2013, by the United Nations Educational, Scientific and Cultural Organization (UNESCO) as intangible cultural heritage of humanity. Nevertheless, Portugal is still slightly far from the ideals encouraged by this diet. Generally, the Mediterranean diet is based on high consumption of whole grains, vegetables, fruits, pulses and seeds, dry and oleaginous fruits, regular consumption of fish and low consumption of dairy and red meats. It promotes the selection of olive oil as the source of fat for cooking and seasoning, and the moderate consumption of red wine and preferable drinking of water (Rosato et al., 2019). In terms of healthy habits, it highlights the importance of practicing regular physical exercise, having pleasant meals shared in a social context and selecting minimally processed, seasonally fresh and locally grown foods and simple preparations (Serra-Majem et al., 2004).

The Portuguese Ministry of Health has had a commendable role on the promotion of nutrition policies, and two examples of this are the implementation of a national law establishing a maximum limit to the content of salt in cooked bread (1.4 g salt / 100 g) and the application of a tax for sugary beverages (soft drinks, energy drinks, concentrates and flavoured waters) available in the market. Another small example of important steps that are being done toward healthy nutrition promotion, is the supply of diverse informative material about foods and nutrients recommendations that is available on the Portuguese Ministry of Health's website. This kind of information is clear and concise, aiming to ease the access of this information to unacquainted people.

Table 2.7 Average requirements (AR) and population reference intakes (PRI) of protein, according to EFSA recommendations (adapted from (EFSA, 2017)).

Age	AR		PRI	
	Male	Female	Male	Female
0.5 years (y)	1.12		1.31	
1 y	0.95		1.14	
1.5 y	0.85		1.03	
2 y	0.79		0.97	
3 y	0.73		0.90	
4 y	0.69		0.86	
5 y	0.69		0.85	
6 y	0.72		0.89	
7 y	0.74		0.91	
8 y	0.75		0.92	
9 y	0.75		0.92	
10 y	0.75		0.91	
11 y	0.75	0.73	0.91	0.90
12 y	0.74	0.72	0.90	0.89
13 y	0.73	0.71	0.90	0.88
14 y	0.72	0.70	0.89	0.87
15 y	0.71	0.69	0.88	0.85
16 y	0.71	0.68	0.87	0.84
17 y	0.70	0.67	0.86	0.83
≥ 18 y	0.66		0.83	

PART II – FERMENTATION OF AFRICAN WHOLE GRAINS

2.6. AFRICAN INDIGENOUS WHOLE GRAINS

2.6.1. Consumption of Millets and Sorghum

Globally, the consumption of maize, wheat, and rice has surpassed the consumption of traditional crops such as millet and sorghum (Food Security Department, 1999; Kajuna, 2001; Musyoka et al., 2014). Wheat, maize, and rice are cereals that do not growth specifically in Africa and are harvested in other parts of the world (Macauley, 2015), while red sorghum, pearl and finger millets, teff and African rice are traditional crops native to Africa and some of them to Asia. These cereals were gathered as wild grasses many centuries ago, domesticated and eventually produced by farmers in their fields in Africa, and currently they maintain the hardy, tolerant self-reliance of their wild ancestors. One reason that justifies the increased consumption of soft cereals, such as maize, rice, and wheat, is the reduction in the import tariffs for these three main kinds of cereal (Musyoka et al., 2014). However, these cereals do not reveal growth properties as beneficial as millets or sorghum, which are very resistant to drought, birds damage and insects attacks, do not require many resources to be harvested and are capable of growing in semi-arid and arid drylands of sub-Saharan Africa and Asia, meeting what is advocated to be sustainable food production in marginal lands (Bichard et al., 2005; Food Security Department, 1999; Kajuna, 2001). The replacement of indigenous cereals by soft cereals brings some risks associated with the increase of the human population and, consequently, the need for augmented production, yet using poor quality soils with low productive capacities for cultivation. Additionally, climate change scenarios and drought stress are harmful to crops like maize and wheat and yields are predicted to decline. Model predictions have shown that for an increase of 1 ºC in global mean temperature, wheat yields may decrease by 6 % (Porter & Semenov, 2005). For these reasons, and not neglecting projects that are trying to minimize this reality, the importance of sorghum and millet must not be undervalued (Food and Agriculture Organization of the United Nations Statistics, 1988).

2.6.2. Nutritional value of whole-grain Millets and Sorghum

Besides the advantages related to cereal production and stress adaptation, millets and sorghum also deserve attention due to their nutritional profile, and consequently, their potential impact on human health (Cardoso et al., 2017; He et al., 2010; Saleh et al., 2013; Shobana et al., 2013). As it is well established, cereals are generally comprised of three different parts: the outer pericarp, the germ, and the endosperm. Each part is rich in different nutrients. In general, the pericarp is rich in non-starch polysaccharides and phenolic compounds, the germ is composed mainly by lipids, vitamins (complex B and fat-soluble), minerals, few proteins and partially by non-starch polysaccharides and in the endosperm proteins, starch, water-soluble vitamins, and minerals are available (Food Security Department, 1999; Kajuna, 2001). Regarding millets and sorghum, there

are several varieties of each of these cereals, however, this chapter will be focused only on finger millet (*Eleusine coracana* (L.) *Gaertn*, pearl millet (*Pennisetum glaucum* (L.)) and red sorghum (*Sorghum bicolour* (L.) *Moench.*), originating from different regions, an issue that strongly influences nutritional composition, as it will be discussed below.

2.6.2.1 Carbohydrates

Millets, like any other cereal, have carbohydrates as their major component, from which it is possible to differentiate between starch (59-65.5 %), composed mainly by amylose and amylopectin, and non-starch polysaccharides, or dietary fibre (DF) (7-20 %) (Devi et al., 2014; Shobana et al., 2013). The content of amylopectin is much higher than amylose for these cereals, which is on average less than 25 %. Finger millet has a markedly higher amount of DF (11.5-19.1 %) when compared with pearl millet (7.0-11.3 %), and also with other common cereals such as maize (2.3-2.8 % crude fibre), wheat (2.0-2.9 % crude fibre) and rice (1.0-10.2 % crude fibre) (Devi et al., 2014; Saleh et al., 2013; Shobana et al., 2013) (Table 2.8). Regarding sorghum, the range of starch can vary between 32-72 %, and the content of DF is about 6-15 %, of which the percentage of insoluble fibre is much higher (75-90 %) than soluble fibre (10-25 %) (Cardoso et al., 2017).

The consumption of high fibre cereals has been associated with positive health effects, and various health claims are accepted by EFSA. Soluble fibre has demonstrated to lower blood cholesterol and to delay glucose absorption, reducing the risk of heart disease (He et al., 2010), diabetes (De Munter et al., 2007; Pereira et al., 2002) and colorectal cancer (Jacobs et al., 1998). On the other hand, insoluble fibre speeds up intestinal transit, reducing disease symptoms of constipation and improves bowel function, modifying the microbiota towards a more health-promoting profile (Devi et al., 2014).

2.6.2.2. Proteins

The protein content of cereals is relatively low. The protein content of pearl millet (averagely 13 %) is higher than that of finger millet (about 7 %), and the concentration in different sorghums' varieties can range between 7-15 % (Cardoso et al., 2017; Proietti et al., 2015; Shobana et al., 2013) (Table 2.8). Moreover, the content of essential amino acids is not the ideal, when compared with other foods, especially animal food sources. Globally, sorghum and finger millet contain a low proportion of lysine (lys), whereas pearl millet seems to have a higher content of this essential amino acid (Kulp & Ponte Jr., 2000). Nevertheless, finger millet contains more threonine (thr) and valine (val) than most of the other millets (Porter & Semenov, 2005).

In general, these three cereals have prolamins and other types of proteins such as albumins, globulins, and glutelins, in their protein profiles (Taylor & Taylor, 2017). Prolamins are protein types that can be harmful to gluten intolerants or celiac patients. The ones found in these cereals (finger millet's prolamins, pennisetins (pearl millet), and kafirins (sorghum)) are not injurious to this population group, unlike those present in wheat (gliadin), rye (secalin) and barley (hordein) (De

Mesa-Stonestreet et al., 2010; Devi et al., 2014). This particularity makes these cereals a safe food option for such population groups.

2.6.2.3. Lipids

Lipids are the macronutrient with less expression within the nutritional composition of cereals, varying between 1.3 %-5.1 % in different varieties of millet (Table 2.8) and 1.24 %-3.2 % in sorghum (Cardoso et al., 2017; Devi et al., 2014; Saleh et al., 2013; Shobana et al., 2013). The fatty acid profile found in these cereals is nutritionally balanced, offering a predominance of unsaturated fatty acids. Millets have a higher content of oleic and palmitic acids than sorghum, 46 %-62 % (Shobana et al., 2013) *vs*. 32.2 %-42.2 % (Cardoso et al., 2017) and 20 %-35 % (Shobana et al., 2013) *vs*. 12.4 %-16.0 % (Cardoso et al., 2017), respectively. In contrast, linoleic acid is present in higher amounts in sorghum, 45.6 %-51.1 % when compared to millets 8 %-27 % (Cardoso et al., 2017; Shobana et al., 2013).

Table 2.8 Nutritional composition of millets and sorghum (per 100 g of edible portion). Data compilation from Cardoso *et al*. (2017), Devi *et al*. (2014) Saleh *et al*. (2013) and Shobana *et al*. (2013).

	Finger millet	Pearl millet	Sorghum
Energy (kcal)	328 - 336	361 - 363	329
Protein (g)	7.3 - 7.7	11.6 - 14.5	7 - 15
Fat (g)	1.3 - 1.5	4.8 - 5.1	1.24 - 3.2
Carbohydrates (g)	72.0 - 72.6	67.0 - 67.5	70.7
Dietary Fibre (g)	11.5 - 19.1 (crude fibre: 3.6)	7.0 - 11.3 (crude fibre: 2.3)	6.0 - 15.0 (crude fibre: 2.0 -2.7)
Calcium (mg)	330 - 350	10 - 42	25 - 40
Potassium (mg)	408 - 430	307 - 440	289 - 380
Iron (mg)	3.9	1.0 – 8.0	5.4 - 6.1
Zinc (mg)	2.3	3.1	1.65-2.42
Thiamine (mg)	0.42 - 0.48	0.33 - 0.38	0.38 - 0.46
Riboflavin (mg)	0.12 - 0.19	0.21 - 0.25	0.15
Niacin (mg)	0.30 - 1.1	2.3 - 2.8	4.6 - 4. 84
Total phenol (mg)	102	51.4	13.6-47.94 (Phenolic acids)

2.6.2.4. Micronutrients

Both sorghum and millets are good sources of micronutrients, especially minerals, however, compositions are variable according to the production area and cultivation strategies (Cardoso et al., 2017; Martino et al., 2012). Sorghum is rich in phosphorus, potassium, and zinc, whereas finger millet is more recognized to be an important source of calcium, phosphorus, iron, and potassium, and pearl millet is rich in iron and zinc (Cardoso et al., 2017; Devi et al., 2014; Kulp & Ponte Jr., 2000; Shobana et al., 2013). Regarding vitamins, the contents of niacin (especially in sorghum and pearl

millet), riboflavin and thiamine are important (Cardoso et al., 2017; Devi et al., 2014). Although the solubility, bioaccessibility and subsequent bioavailability of some minerals are low, they can be increased by processing technologies, such as biofortification, fortification, genetic improvement, germination, malting, fermentation among others (Porter & Semenov, 2005).

2.6.2.5. Bioactive compounds

Finger millet, among millets, and red sorghum, predominantly in the cereal outer layers, are very rich in phenolic compounds, namely phenolic acids such as ferulic (sorghum: 120.5-173.5 μg/g; finger millet: 186 μg/g), protocatechuic (sorghum: 150.3-178.2 μg/g; finger millet: 450 μg/g) and caffeic acids (sorghum: 13.6-20.8 μg/g; finger millet: 16.4 μg/g), and tannins (sorghum: 0.2-48.0 mg/g; finger millet: 0.1-2.3.47 mg/g), but flavonoids, namely flavons (sorghum: 87 μg/g, on average), are also present in smaller quantities (Cardoso et al., 2017; Devi et al., 2014; Kumari et al., 2017; Ramachandra et al., 1977; Subba Rao & Muralikrishna, 2001; Vanamala et al., 2017). Red sorghum, followed by finger millet, have a higher content of tannins when compared to white sorghum and pearl millet, non-pigmented grains (Cardoso et al., 2017; Shobana et al., 2013). This can be due to the presence of anthocyanins, polymerized phenolics present in darker cultivars (Devi et al., 2014).

These compounds have very interesting properties such as antioxidant, anticarcinogenic, anti-inflammatory, antimicrobial, antifungal and antidiabetic activities, playing an important role in minimizing the incidence of some diseases and all-cause mortality (Cardoso et al., 2017; Devi et al., 2014; Saleh et al., 2013; Shobana et al., 2013; Viswanath et al., 2009). According to the antimicrobial activity, it is reported that the polyphenols are active against different microorganisms, namely *Bacillus cereus*, *Salmonella* sp., *Escherichia coli* and others (Viswanath et al., 2009). The antioxidant properties of polyphenols result from their capacity of donating hydrogen atoms via hydroxyl groups on benzene rings to electron-deficient free radicals (Devi et al., 2014), suppressing the excessive oxidation (Devi et al., 2014; Viswanath et al., 2009). Antidiabetic properties of phenolics are due to the inhibition of amylase and α-glucosidase, active enzymes in the hydrolysis of complex carbohydrates, which might delay the absorption of glucose and reduce postprandial hyperglycaemia (Shobana et al., 2009). Despite these positive properties, tannins and polyphenols may reduce the bioavailability of some nutrients, acting as antinutrients, along with phytates and oxalates, also present in cereal matrices (Kamath et al., 2004; Shobana et al., 2009; Viswanath et al., 2009). High levels of these compounds can compromise the regular absorption of some minerals, protein and starch digestibility (Cardoso et al., 2017; Devi et al., 2014). The main mechanisms of action involved include their action as reducing agents, metal chelators, oxygen scavengers, radical quenchers or enzyme inhibitors (De Mesa-Stonestreet et al., 2010; Devi et al., 2014; Gulati et al., 2017; Shobana et al., 2013; Tatham et al., 1996).

Despite this downside, such richness in naturally bioactive compounds may uphold the use of these whole grains, or derived fractions, in food product development with multifunctional purposes toward health promotion and chronic disease prevention. Nonetheless, biological validation thereof with animal models and human trials still need to be performed.

2.7. TRADITIONAL FERMENTED CEREAL-BASED AFRICAN PRODUCTS

Fermentation may be divided into two types of processes, natural or spontaneous and provoked or controlled fermentation. In the first one, the involved bacteria are indigenously presented in the substrate, while in the second process, specific microorganisms, usually called as starter cultures, with desirable characteristics, are added to the product. In this sub-chapter, only fermented products obtained by spontaneous fermentation will be referred to. Traditional preparation of fermented foods is rather common since the beginnings of human civilization. Fermented food is defined as a food product that was subjected to the biological activity of certain microorganisms, whose action caused changes in the initial product, by the consumption of a substrate, usually carbohydrates, towards the production of a wide range of metabolites (Prajapati & Nair, 2008). This process is one of the oldest methods used for food preservation, which is possible due to the bacteriostatic and antimicrobial properties that the product gains, caused by the decrease of pH and, consequently, elevated acidity.

The traditional African process, normally carried out by women in households, starts with the collection of the cereals, followed by sieving for stones', debris', and foreign materials' removal. The next two phases are cleaning and manually grinding with a stone over several cycles (Figure 2.1) until the obtainment of a paste. Afterwards, the paste is put in a wood container and it is let to ferment for some days (depending on the desired type of product).

Figure 2.1 Manual stone grinding of cereal grain, typically done in Kenyan villages to produce cereal-based fermented products.

There is a wide range of fermented foods produced around the world, especially in Asia and in Africa, namely porridges, beverages (alcoholic and non-alcoholic), breads, meat, fish, vegetables, dairy products and condiments (Marshall & Mejia, 2011). For this thesis, the main focus was placed into fermented products obtained from cereals. The reviews of Blandino *et al.* (2003), Nout (2009), Achi and Asamudo (2011), Aka *et al.* (2014), Galati *et al.*, (2014), Mokoena *et al*. (2016), Adebiyi *et al.* (2016) and Tsafrakidou *et al*. (2020) give an extensive list of the most common indigenous cereal-based fermented foods and beverages from Africa, which were suitably compiled in Table 2.9. According to these reviews, the cereals which are more commonly preferred by native African people are sorghum (*Sorghum bicolour* (L.) *Moench*), millets (pearl millet (*Pennisetum glaucum* (L.)) finger millet (*Eleusine coracana* (L.) *Gaertn*)) and maize (*Zea mays* (L.)).

Table 2.9 Common indigenous cereal-based fermented foods and beverages in African countries (Achi & Asamudo, 2019; Adebiyi et al., 2018; Aka et al., 2014; Blandino et al., 2003; Galati et al., 2014; Mokoena et al., 2016; Nout, 2009; Tsafrakidou et al., 2020).

Product	Substrates	Microorganisms[1]	Nature of use	Regions
Akasan	Maize, Sorghum, Millet	*Pediococccus acidolactic Lactobacillus plantarum*, *Lactobacillus acidophilus*, *Leuconostoc*, *Streptococcus*, *Bacillus*	Gel-like traditional fermented starchy food item	Benin
Amgba	Sorghum, Millet	LAB[2], yeasts	Alcoholic beverage	Cameroon, Chad
Banku	Maize, Cassava	LAB, moulds	Dough as staple	Ghana
Ben-salga	Pearl Millet	LAB	Non-alcoholic thin beverage (gruel) as breakfast (children and sick people)	Burkina Faso
Bogobe	Sorghum	*Mucor rouxianus*	Soft porridge staple	Botswana
Borde Shamita	Barley	*Lactobacillus* spp.	Beverage	Ethiopia
Bouza	Wheat	Yeasts and bacteria	Alcoholic beverage	Egypt
Burukutu	Sorghum	*Saccharomyces cerevisiae S. chavelieri*, *Leuconostoc mesenteroides*, *Candida*, *Acetobacter*	Alcoholic beverage of vinegar-like flavour	Nigeria, Benin, Ghana
Busa	Rice, millet	*Lactobacillus*, *Saccharomyces*	Liquid beverage	Egypt
Busaa	Maize	*Lactobacillus helveticus*, *L. salivarus*, *L. casei*,*L. brevis*, *L. plantarum*,*L. buchneri*, *S. cerevisiae*, *Penicillium damnosus*	Alcoholic beverage	Nigeria, Ghana
Bushera	Sorghum, Millet	LAB	Beverage	Uganda
Cheka	Sorghum, Maize	Unknown	Beverage	Ethiopia
Chibuku	Sorghum	*Lactobacillus* spp., *S. cerevisiae*	Alcoholic beverage	Zimbabwe
Chikokivana	Maize, millet	*Saccharoyces cerevisiae*	Alcoholic beverage	Zimbabwe
Dalaki	Millet	Unknown	Thick porridge	Nigeria
Doro	Finger millet malt	Yeasts and bacteria	Colloidal thick alcoholic beverage	Zimbabwe
Fura	Maize, Sorghum	*L. plantarum*, *Pedicoccus*, *Leuconostoc*, *Streptococus*, *Enterococcus*, *S. cerevisiae, Pichia anomala*, Candida	Beverage	Nigeria
Gari	Cassava	*Leuconostoc mesenteroide, L. plantarum, Bacillus subtilis, Candida krusei*	Side dish, snack	West Africa
Ikigage	Sorghum	*Lactobacilli*	Alcoholic beverage	Rwanda
Ilambazi lokubilisa	Maize	LAB, yeasts and moulds	Porridge as weaning food	Zimbabwe
Injera	Sorghum, tef, Maize or wheat	*Candida guilliermondii, Aspergillus* sp., *Pullaria* sp., *Penicillinium* sp., etc.	Bread-like staple	Ethiopia
Kachasu	Maize	Yeasts	Alcoholic beverage	Zimbabwe
Kachasu	Maize	Yeasts	Alcoholic beverage	Zimbabwe
Kaffir beer	*Kaffir* corn	Yeasts, LAB	Alcoholic beverage	South Africa

Product	Substrates	Microorganisms	Nature of use	Countries
Kenkey	Maize	*Lactobacillus fermentum*, *L. reuteri*, *Candida*, *Saccharomyces*, *Penicillium*, *Aspergillus and Fusarium*	Mush, steamed eaten with vegetables	Ghana
Kishk	Wheat and milk	*L. plantarum*, *L. brevis*, *L. casei*, *Bacillus subtilis* and yeasts	Solid, dried balls, dispersed rapidly in water; Soup	Egypt
Kisra	Sorghum	Unknown	Staple as bread	Sudan
Koko	Maize	*Enterobacter clocae*, *Acinetobacter.*, *L. plantarum*, *L. brevis*, *S. cerevisiae*, *Candida mycoderma*	Porridge as staple	Ghana
Kunu	Maize, millet, sorghum	*Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Pediococcus*, *Weissella*	Beverage	West Africa
Kwunu-zaki	Millet	LAB, yeasts	Paste used as breakfast dish	Nigeria
Mahewu	Maize	*Streptococcus lactis, L. debrueckii*, *L. bulgaricus*	Solid staple	South Africa, Togo
Mangisi	Millet	Unknown	Sweet-sour non-alcoholic drink	Zimbabwe
Mawe	Maize	LAB, yeast	Basis for preparation of many dishes	South Africa
Merissa	Sorghum and millet	*Saccharomyces*	Alcoholic drink	Sudan
Munkoyo	Kaffir corn, millet or maize plus roots of munkoyo	Unknown	Beverage	Zambia
Muramba	Sorghum	Unknown	Alcoholic drink	Uganda
Mutwiwa	Maize	LAB, other bacteria and moulds	Porridge	Zimbabwe
Ogi	Maize, sorghum or millet	*L. plantarum*, *Saccharomyces cerevisiae*, *Candida mycoderma*, *Corynebacterium*, *Aerobacter*, *Rhodotorula*, *Cephalosporium*, *Fusarium*, *Aspergillus* and *Penicillium*	Paste as staple. For breakfast or weaning food for babies	Nigeria, West Africa
Otika	Sorghum	Unknown	Alcoholic beverage	Nigeria
Pito	Maize, sorghum, or maize	*Geotrichum candidum*, *Lactobacillus*, *Candida*	Alcoholic dark brown drink	Nigeria, Ghana
Seketeh	Maize	*S. cerevisiae*, *S. chevalieri*, *S. elegans*, *L. plantarum*, *L. lactis*, *Bacillus subtilis*, *Aspergillus niger*, *A. flavus*, *Mucor rouxii*	Alcoholic beverage	Nigeria
Sorghum beer	Sorghum, maize	LAB, yeasts	Liquid drink, acidic, weakly alcoholic	South Africa
Talla	Sorghum	Unknown	Alcoholic drink	Ethiopia
Tobwa	Maize	LAB	Non-alcoholic	Zimbabwe
Togwa	Sorghum, millet, maize	*Lactobacillus*, *Streptococcus*	Bevereage	Tanzania
Tchoucoutou	Sorghum	LAB, yeasts	Alcoholic beverage (opaque beer)	Benin
Uji	Maize, sorghum, millet	*Leuconostoc mesenteriodes*, *L. plantarum*	Porridge as a staple	Kenya, Uganda, Tanzania
Umqombothi	Maize or sorghum	*Lactobacillus* spp., *S. cerevisiae*	Beverage	South Africa

[1] The *Lactobacillus* taxonomy presented in this table is written according to the nomenclature prior to the recent reclassification of the genus *Lactobacillus* (Zheng et al., 2020). [2] LAB: Lactic Acid Bacteria.

In this type of process, there is no control of environmental settings and, consequently, homemade products are prepared under unhygienic conditions. Therefore, naturally, besides the desirable bacteria that change positively the product, undesirable species of other bacteria, yeasts and fungi also grow (Holzapfel, 1997). In consequence, those microorganisms might affect negatively the product, either by contributing to spoilage or by creating a pathogenic environment capable of being harmful to the consumer. Nevertheless, functional and safe strains can be isolated from those cereal autochthonous microbiota and be, subsequently, used as starter cultures, providing improvements in safety and quality aspects of fermented cereal foods, which can be foreseen and reproducible (Ogunremi et al., 2017).

The abovementioned reviews also highlight the main microorganisms involved in these traditional processes, summarizing several works about the microbiological characterization of traditional fermented products. The microflora of fermented cereal-based products is characterized mainly by LAB and yeasts. Bacteria and yeasts isolated from traditional Sorghum and Millets based products are presented in Table 2.10.

Table 2.10 Bacteria and yeasts isolated from traditional Sorghum and Millets based products.

Kingdom	Genus	Species	Reference
Bacteria	*Lactobacillus*[1]	*L. fermentum*	(Lei & Jakobsen, 2004; Mugula et al., 2003; Mukisa et al., 2017; Mukisa, Porcellato, et al., 2012; Owusu-Kwarteng et al., 2015)
		L. plantarum	(Mugula et al., 2003; Mukisa et al., 2017; Mukisa, Porcellato, et al., 2012; Poornachandra Rao et al., 2015)
		L. paraplantarum *L. salivarius*	(Lei & Jakobsen, 2004)
		L. pentosus	(Poornachandra Rao et al., 2015)
		L. lactis *L. delbruecki*	(Mukisa, Porcellato, et al., 2012)
		L. brevis *L. cellobiosus*	(Mugula et al., 2003)
		L. bifermentans	(Thapa & Tamang, 2004)
	Weissella	*W. confusa*	(Lei & Jakobsen, 2004; Mugula et al., 2003; Y. Wang et al., 2019)
	Pediococcus	*P. pentosaceus*	(Lei & Jakobsen, 2004; Mugula et al., 2003; Mukisa, Porcellato, et al., 2012; S. Thapa & Tamang, 2004)
		P. acidilactici	(Lei & Jakobsen, 2004
	Leuconostoc	*Leuconostoc lactis*	(Mukisa et al., 2017; Mukisa, Porcellato, et al., 2012);
	Streptococcus *Enterococcus*	*S. infantarius* *Enterococcus* spp.	(Mukisa, Porcellato, et al., 2012)
Fungi	*Issatchenkia orientalis* *Saccharomyces cerevisiae*		(Mugula et al., 2003; Mukisa et al., 2017; Mukisa, Porcellato, et al., 2012)
	Candida tropicalis; Candida peliculosa		(Mugula et al., 2003)
	Cyberlindnera fabianii; *Clavispora lusitaniae*		(Mukisa, Porcellato, et al., 2012)

[1] The *Lactobacillus* taxonomy presented in this table is written according to the nomenclature prior to the recent reclassification of the genus *Lactobacillus* (Zheng et al., 2020).

2.7.1. Indigenous bacteria *Weissella confusa*/*cibaria*

The genus *Weissella* appeared for the first time in 1993. The works of Martinez-Murcia & Collins (1990) and Martinez-Murcia *et al*. (1993) revealed phylogenetic differences between *Leuconostoc paramesenteroides* and *Leuconostoc mesenteroides* and similarities between *L*. *paramesenteroides* and five heterofermentative lactobacilli (*Lactobacillus confusus, Lactobacillus halotolerans, Lactobacillus kandleri, Lactobacillus minor and Lactobacillus viridescens*, according to the former nomenclature*).* It was Collins and colleagues that grouped these leuconostoc-like organisms, which are only differentiable by using molecular taxonomical techniques, and came out with the genus *Weissella*, named after the German microbiologist Norbert Weiss (Collins et al., 1993). Currently, the genus comprises a total of 19 validated species (Fusco et al., 2015), that are grouped into five different group species, based on their 16S rRNA gene sequence phylogeny: 1st group: *W*. *soli*, *W*. *diestrammenae*, *W*. *koreensis*, *W*. *kandleri*, and *W*. *oryzae*; 2nd group: *W*. *cibaria* and *W*. *confusa*; 3rd group: *W*. *thailandensis*, *W*. *hellenica* and *W*. *paramesenteroides*; 4th group: *W*. *ceti*, *W*. *halotolerans*, *W*. *viridescens*, *W*. *minor* and *W*. *uvarum*; and 5th group: *W*. *beninensis*, *W*. *fabalis*, *W*. *fabaria*, and *W*. *ghanensis* (Fusco et al., 2015). In terms of scientific classification, the genus *Weissella* belongs to the family *Leuconostocaceae*, order Lactobacillales, class Bacilli and phylum Firmicutes (Collins et al., 1993). Species from this genus are known to be gram-positive, catalase-negative, non-endospore forming cells with coccoid or rod-shaped morphology (occurring singly or in pairs), non-motile (with the exception for *W*. *beninensis*) (Björkroth et al., 2002; Collins et al., 1993). They are facultative anaerobic, hetero-fermentative, fermenting glucose via the hexose-monophosphate and phosphoketolase pathways, with production of lactic acid (with some species producing only D (−) and others both D (−) and L (+) lactic acid enantiomers), gas (CO_2) and ethanol and/or acetate (Fusco et al., 2015).

Weissella strains are commonly associated with spontaneous fermentation, including fruits, cereal-based and vegetables-based foods, fermented meat and fish products (Fessard & Remize, 2017). Although *Weissella confusa* or *W*. *cibaria* are two of the most dominant species in this kind of foods, the exploration of their use as starters lacks deeper investigation. Nevertheless, these species have been gaining interest among the baking industry given its ability to produce EPS, mainly dextran (Fusco et al., 2015). This topic will be further discussed in sub-chapter 2.9. Besides the synthesis of EPS, other technological, sensory and nutritional characteristics have been positively associated with *Weissella confusa*/*cibaria* (Fessard & Remize, 2017). Namely, the rapid growth and the high acidification rate, which will create a safe environment, the resistance to bile salts and acidic conditions, the production of antimicrobial metabolites, such as specific organic acids and bacteriocins, the presence of heterofermentative metabolism and, also, the increase of antioxidant activity (Fessard & Remize, 2017). Regarding the production of bacteriocins, few studies have explored this potentiality of *Weissella* strains, nevertheless two bacteriocins were isolated and further characterized. The study of Malik *et al*. (2009) described a bacteriocin-encoding plasmid of *W*. *confusa* isolated from homemade soya. The study of Goh and Philip (2015) characterized a bacteriocin produced by a *W*. *confusa* strain previously isolated from fermented milk, and for gene encoding bacteriocin production, possible colicin V production protein was detected from the genome.

The former bacteriocin revealed antimicrobial activity against several gram negative and positive bacteria, showed to be stable to heat, acid pH and lysozyme, lipase, catalase and lyticase enzymes activities, and caused *Bacillus cereus*' membrane disruption (Goh & Philip, 2015). All of these positive properties associated with this species turns promissor its use as a starter.

In spite of the previous positive aspects, some *Weissella* strains have been associated to some potential constraints, which may be behind the reason why they have not yet been approved for commercial use as a starter culture for the production of fermented foods. Thereby, *Weissella* spp. are not generally recognized as safe (GRAS), by the Food and Drug Administration (FDA), neither as Qualified Presumption of Safety (QPS), by EFSA (Fessard & Remize, 2017). However, some species of *Weissella*, including *W*. *confusa* and *W*. *cibaria*, are included in the inventory of the International Dairy Federation, which is the inventory of microbial food cultures with safety demonstration in fermented food products, recently updated in 2018 (Bourdichon et al., 2018). Some of the constraints that might be hampering *Weissella* of being considered as a commercial starter are related with potential biogenic amine production, antibiotic resistance pattern or infection hazard (Fessard & Remize, 2017).

There are few studies that evaluated the production of biogenic amines by *Weissella* strains isolated from vegetable-based (*kimchi* and *tofu-misozuke*) and meat-based fermented products and, also, from fish and fish products (Barbieri et al., 2019; Fessard & Remize, 2017). Jeong and Lee, (2015) focused their investigation on *Leuconostoc* and *Weissella* strains (*W*. *confusa*, *W*. *cibaria* and *W*. *paramesenteroides*) isolated from *kimchi*, and concluded that there were few strains able to produce one or more types of biogenic amines in varying amounts. Also, Takebe *et al*. (2016) studied the biogenic amine production of several LAB strains isolated from *tofu-misozuke*, including *W*. *viridescens*, which were found to be tyramine-producing and non-tyramine producing (and also demonstrated the presence/absence of the tyrosine decarboxylase gene, respectively). These results show that the production of this amino acid derivative is strain-dependent and not species-dependent. Moreover, isolates close to *W*. *hellenica* and *Weissella* sp. were negative for both tests. Pereira *et al*. (2009) demonstrated that a *W*. *haloterant* strain isolated from traditionally fermented sausage combines an arginine deiminase pathway and an ornithine decarboxylation pathway, conducing to the production of the biogenic amine putrescine. On the contrary, no production of biogenic amines or detection of genes encoding amino acid decarboxylase for *W*. *confusa*, *W*. *cibaria* or *W*. *viridescens* was found in three other research works (Muñoz-Atienza et al., 2011; Thapa et al., 2006; Yüceer & Tuncer, 2015). Both the studies of Muñoz-Atienza *et al*. (2011) and Yüceer and Tuncer (2015) investigate the PCR detection of amino acid decarboxylase encoding genes.

Regarding antibiotic resistance, it is well established that several LAB possess antibiotic resistance genes, however the main safety problem is associated with the possibility of being transferable to other bacteria, specifically pathogenic bacteria. Therefore, such property deserves careful evaluation when amidst a selection process of starter cultures. There are some studies that explored the antibiotic resistance of *Weissella* spp., and *W*. *confusa* has shown contradictory results, which may be consequence of strain-specificity. *W*. *confusa* showed to be resistant according to some authors, but also sensitive according to others, to tetracycline, kanamycin, streptomycin,

gentamicin, penicillin and chloramphenicol (Houngbédji et al., 2021; Jeong & Lee, 2015; Ouoba et al., 2008; Patel et al., 2014; Jing Wang et al., 2018). Vancomycin was the only antibiotic that showed consistent results among studies (Ouoba et al., 2008; Patel et al., 2014; Jing Wang et al., 2018). Besides the phenotypic tests, some works have explored the PCR-based detection of antibiotic resistance genes, in order to understand if the resistance found is intrinsic or acquired. Houngbédji and colleagues (2021) detected phenotypic resistance to kanamycin, streptomycin and tetracycline, but only the streptomycin resistance gene *aadA* was positive with two strains of *W. confusa*. In the work developed by *Aka et al*. (2020), the authors concluded that by the comparison of the *W. confusa* AB3E41 isolated strain with other available strains, there are no hazardous features related to that one. Nevertheless, they point out that due to the lack of strains isolated from clinical samples, a parallel with those, which could be used to define potential factors involved in opportunistic pathogenicity, is not feasible. There is one more study that detected a resistance gene in *W. confusa* genome, namely tetracycline resistant gene *tetM*, however no certain about its possibility to be horizontal transfer was outlined (Jing Wang et al., 2018). However, it is important to note that this strain was isolated from meat-based product and not from vegetable, cereal-based fermented products. Lastly, the work of Jeong and Lee (2015) concluded that the phenotypic resistance showed by *Weissella* strains to streptomycin may be intrinsic feature due to a very low risk of transfer. Moreover, according to the authors, the observed minimum inhibitory concentration, which were higher than the breakpoints suggested by EFSA (which are not specific to *Weissella* genera, due to their absence) but much lower than the values exhibited from the *Lactobacillus* isolates, are unlikely to be the result of acquired resistance by transferable plasmids or transposons (Jeong & Lee, 2015). Based on the abovementioned literature, it is important to carefully analyse each LAB strain in what concerns antibiotic resistance, given the dependence of the strain.

In what concerns the infection hazard, there are some *Weissella* strains associated with cases of infection in animals and humans. The strains isolated in those cases are *W. ceti* (only isolated from animals), *W. paramesenteoides, W. confusa*, *W. cibaria* and *W. viridescens* (Fessard & Remize, 2017). Nevertheless, the last two are also associated with healthy human faeces and vaginal microbiota (Fessard & Remize, 2017). In respect to *W. confusa* it has been detecteded in cases of bacteraemia and infections, but almost all of the involved patients were immune-compromised or had been subjected to chemotherapy or organ transplant (Fessard & Remize, 2017). This clinical state may have posed a gateway for the microorganism in the body (Fessard & Remize, 2017).

Despite in this sub-chapter only *Weissella* spp., mainly *W. confusa,* studies have been highlighted, the above-cited potentially harmful aspects, such as biogenic amine production, antimicrobial resistance or infection cases, have also been reported for other LAB strains, including *Lactobacillus* or *Lactococcus*, which are already use as commercial starters and in some cases considered as probiotics (Fessard & Remize, 2017). This means that their potential pathogenicity is not considered an obstacle for their application in product development. All the same, the most responsible and safe approach is the evaluation of the potential pathogenicity for each case, being clear that safety aspects about *Weissella* need further investigations.

2.8. PROBIOTICS AND CEREAL-BASED PROBIOTIC PRODUCTS

As mentioned above, naturally fermented products contain both functional and non-functional microorganisms, liable to affect both positively and negatively product attributes eventually posing a hazard to human health (Holzapfel, 1997). In response, LAB have been used as starter cultures, contributing successfully to product quality and safety, increased shelf-life, texture, and sensory properties (Leroy & De Vuyst, 2004; Waters et al., 2015). In addition, well-controlled LAB-fermented food products are in many cases aligned with positive biological properties, in particular when probiotic organisms such as *Bifidobacterium* and/or certain *Lactobacillus* species are included (Ogunremi et al., 2017). Such functionality beyond the inherent nutritional value places these fermented foods in the functional food category. Although there is not yet a world-wide acceptable definition for functional foods, a definition that may become integrative is that of the Functional Food Centre (FFC). The FFC defines functional food as 'natural or processed foods that contain known or unknown biologically-active compounds which, in defined, effective, and non-toxic amounts, provide a clinically proven and documented health benefit for the prevention, management or treatment of a chronic disease' (Martirosyan & Singh, 2015).

The development of functional probiotic foods and beverages has shown a marked increase over the last years, and the consumer and market demands for these products are increasing all over the world (Grand View Research, 2019). In particular, non-dairy functional probiotic beverages have been gaining an interesting market positioning. Besides some nutritional drawbacks of dairy products, such as cholesterol or lactose contents, trends such as plant-based diets linking human health to environmental sustainability, the high prevalence of lactose intolerance/malabsorption and gluten intolerance around the world have opened an opportunity for these products (Enujiugha & Badejo, 2017; Kumar et al., 2015; Min et al., 2018; Soccol et al., 2012). In consequence, fruit-, legume-, cereal-based products have been developed (Liptáková et al., 2017; Min et al., 2018; Rai et al., 2018). To hold the classification of probiotic food is challenging because some requirements must be accomplished (Joint FAO/WHO Expert Consultation, 2001). One of them is the delivery of an adequate quantity of probiotic organisms at the time of consumption, which has been identified as a minimum of 10^7 CFU/g (Gomes et al., 2017). The current definition for a probiotic 'live microorganisms that, when administered in adequate amounts, confer a health benefit on the host' was developed by a consensus among FAO/WHO expert panel and other internationally recognized experts (Joint FAO/WHO Expert Consultation, 2001) and updated by the International Scientific Association for Probiotics and Prebiotics (ISAPP) who reinforced the initial definition with a slight grammatical correction.

As previously stated, *Lactobacillus* is one of the most common LAB genus used in fermented probiotic foods (Tamang et al., 2016). This genus comprised close to 260 species, until March 2020 (Zheng et al., 2020). However, scientists were detecting high disparities within the genus' species, as DNA techniques became more sophisticated (Sanders & Lebeer, 2020). Thereby, researchers resorted to whole genome sequences of all species and concluded that the included species were extremely diverse in terms of phenotypic, ecological and genotypic characteristics (Zheng et al.,

2020). In consequence, a recent reformulation of the *Lactobacillus* genus was proposed, and the species once included in the *Lactobacillus* genus are now reclassified into 25 genera (Zheng et al., 2020). The new names for important probiotics can be found at the ISAPP webpage (Sanders & Lebeer, 2020).

According to latest investigations, *Lactobacillus* spp. (considering the previous genus classification) have several advantageous characteristics such as their capacity to ferment many carbohydrates, to tolerate low pH (Nout & Ngoddy, 1997; Walter, 2008) and high concentrations of oxygen, to prevent growth of pathogenic microorganisms through diverse mechanisms (competition or production of antimicrobial compounds such as organic acids, ethanol, diacetyl, bacteriocins) and to reduce antinutrients due to enzymatic activity (Soro-Yao et al., 2014; Waters et al., 2015).

According to the previous taxonomy, the probiotic species used in the present work belong to the genus *Lactobacillus*, namely *Lactobacillus plantarum* 299v, *Lactobacillus rhamnosus* R-11 and *Lactobacillus acidophilus* Ki. Now, these three species should be referred to as *Lactiplantibacillus plantarum* 299v *(Lactobacillus plantarum* 299v), *Lacticaseibacillus rhamnosus* R-11 (*Lactobacillus rhamnosus* R-11) and *Lactobacillus acidophilus* Ki, which remains unchanged. In the present thesis, the new scientific names will be used, when referring to the level of the species and strain (for instance *Lacticaseibacillus rhamnosus* R-11), nonetheless when in reference to the *Lactobacillus* genus, it is important to note that for these cases it is intended that all species so far included in the genus are considered.

Former *Lactobacillus* genus included a high number of GRAS (Generally Recognized As Safe) species, comprising seven phylogenetic groups: *L. buchneri*, *L. casei* (it includes *L. rhamnosus*), *L. delbrueckii*, *L. plantarum*, *L. reuteri*, *L. sakei* and *L. salivarius* (De Angelis & Gobbetti, 2016). *Lactobacillus* belong to phylum Firmicutes, class Bacilli, order II Lactobacillales and family *Lactobacillaceae*. Members of this genus are gram-positive, catalase-negative, non-spore-forming and rod-shaped bacteria that have different carbohydrates fermentation pathways: obligate homofermentative, facultative/obligate heterofermentative (De Angelis & Gobbetti, 2016). The three species under study (*L. plantarum* 299v, *L. acidophilus* Ki and *L. rhamnosus* R-11) have revealed antimicrobial activity against several pathogenic bacteria (Alemka et al., 2010; Ducrotté et al., 2012; Gomes et al., 1998). In particular, *L. plantarum* 299v, has shown a noticeable ability to adhere to human mucosa cells (Johansson et al., 1993), to improve symptoms of irritable bowel syndrome (Ducrotté et al., 2012), and also to benefit immunomodulatory activity (Johansson et al., 1998; Mack et al., 2003). Some of these effects together with the improvement of lactose intolerance have been shown by *L. rhamnosus* R-11 (Fiander et al., 2005; Kocián, 1994; Sherman et al., 2005). *Lactobacillus acidophilus* Ki has demonstrated to be bile resistant and to have a positive impact in controlling serum cholesterol concentrations (Gomes et al., 1998). The mechanisms of interaction between microorganisms and the host are complex and remain unclear, however, it seems that three different microbial approaches are behind this interaction: through the bacterial surface (Fernandez et al., 2011; G. Wang et al., 2018), the bacterial DNA (Lammers et al., 2003) or by the synthesis of specific metabolites (Gilad et al., 2011; Sokol et al., 2008).

2.8.1. Probiotic viability during processing and storage

Probiotics stability and viability can be hindered during processing and storage, mainly because it is affected by factors such as temperature, water activity, oxygen and pH (Tripathi & Giri, 2014). An example of one of these processes is drying, which can be used to remove water and augment the products shelf-life. One of the most common drying processes used with food probiotics, and that has been showing interesting results in maintenance of high probiotics cell viability, is freeze-drying (Goderska, 2012). In the food industry, the application of this treatment is becoming more and more common (European Freeze Dry, n.d.), since it is a water removal process targeting the development of safe, long-term storage products, preserved in terms of nutritional and sensory properties.

Freeze-drying works by freezing the material, then reducing the pressure and adding heat to allow the frozen water in the material to change directly to a vapor (sublimate). It is a three-step process that involves freezing, followed by two phases of drying: primary drying (sublimation) and secondary drying (adsorption) (Carvalho et al., 2002). Freeze-drying's first phase is freezing performed under atmospheric conditions, in which the crystallization of the solvent and the concentration of the unfrozen fraction occurs, by cooling the material below its triple point (Carvalho et al., 2002). This is essential in order to guarantee that sublimation, instead of melting, occurs. During the drying process, firstly (second phase) the pressure is lowered and heat is added to the material in order for the frozen solvent to sublimate. During this phase about 95 % of the water in the material is removed. During the third and final phase (secondary drying), the additional non-frozen solvent (ionically-bound water) is removed via adsorption (Carvalho et al., 2002). At this stage, the sample reaches its final water content. Most materials can be dried to 1-5 % residual moisture. However, freeze-drying also has some drawbacks affecting cells' integrity, such as damage to lipidic membranes and denaturation of sensitive proteins (Leslie et al., 1995). Bacterial cell membranes are constituted by a bilayer of phospholipids with a hydrophilic head group and a hydrophobic acyl chain. Usually, water acts as a spacer between the head of phospholipids and gives fluidity to the membrane, by avoiding the proximity of the hydrophobic part (Aschenbrenner et al., 2015). During drying, when water is removed, a packing region on the phospholipid bilayer is created, caused by acyl chains grouping. This increases the occurrence of Van der walls interactions, resulting in the transition from a liquid crystalline into a gel phase, at a specific temperature, called temperature of transition (T_g) (Leslie et al., 1995). During rehydration the reverse reaction takes place and dry membranes undergo from the gel to the liquid crystal phase, in which process membranes might become leaky, due to the inhomogeneous transition. Removing more water leads to a higher packing density of the acyl chains and, consequently higher T_g (Aschenbrenner et al., 2015). When sugars, sucrose or trehalose, are added, they substitute the space between lipids, once occupied by water, and lower the T_g, preventing the transition phase and, consequently, the leakage of membranes after rehydration (Leslie et al., 1995). Figure 2.2 outlines the water replacement theory for freeze-drying of bacteria.

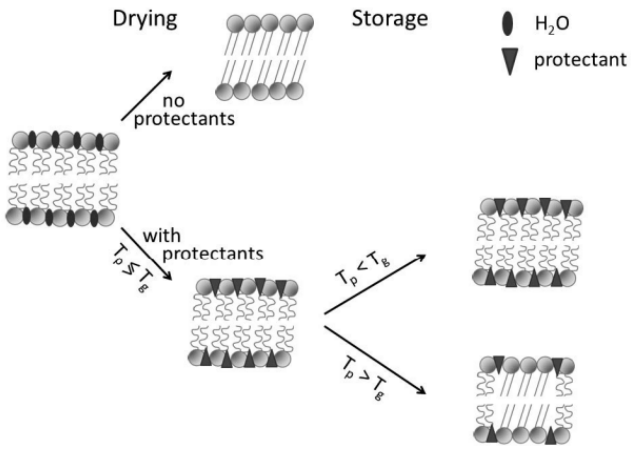


Figure 2.2 Diagram of the extended water replacement theory for freeze-drying of bacteria. Tp: Product temperature; Tg: Glass-transition temperature. From: Aschenbrenner *et al*. (2015)

In what concerns the second previously mentioned side effect of freeze-drying (namely denaturation of sensitive proteins) it can be prevented by the addition of specific sugars that enable the formation of hydrogen bonds between disaccharides and proteins in the absence of water (Leslie et al., 1995). In order to minimize loss of probiotic viability and functional properties, protective agents can be used prior to these processing steps or storage. By doing so, probiotic biomass is increased greatly and probiotic concentrations at the target site in the gastrointestinal tract are maximized. There are several protectants that may be used in drying processes, namely disaccharides (fructose, trehalose, sucrose, lactose), polymers (inulin or maltodextrin), sugar-alcohols or polyols (inositol, sorbitol, mannitol) and alcohols (glycerol, adonitol) or amino acids (betaine) (Goderska, 2012). Dimethyl sulfoxide is also a protective alternative substance, as well as gums (alginate, κ-carrageenan, and gellan/xanthan) that form a gel matrix that entrapped the bacteria, which process is called encapsulation (Goderska, 2012). Probiotic strains differ in their sensitivity to freeze-drying technology for long-term preservation purposes, thus requiring optimization of the relevant variables. One of the most common probiotic species (including different strains) used in these studies is *Lactiplantibacillus plantarum*. In this context, previous studies have concluded that the most favourable cryoprotectants to achieve higher viabilities of *L*. *plantarum* TISTR 2075 was the combination of protein (15 %) plus trehalose (5 %) (Savedboworn et al., 2017) and for *L*. *plantarum* and *L*. *rhamnosus* it was sorbitol (12.5 g/L) (Carvalho et al., 2002).

2.9. EXOPOLYSACCHARIDES PRODUCED BY LACTIC ACID BACTERIA

Microbial EPS are extracellular polymeric substances that can be divided into two categories, hetero (HePS) and homopolysaccharides (HoPS), and subdivided according to their structure (Sutherland, 1993; Zhou et al., 2019). Heteropolysaccharides are composed of different types of monosaccharides, such as D-glucose, D-galactose, and more rarely L-rhamnose, arabinose among others, while the homopolysaccharides, as the name implies, are composed of only one type of monosaccharides, such as D-glucose (α-D-glucans or β-D-glucans), D-fructose (fructans), or D-galactose (polygalactans) (Angelin & Kavitha, 2020; Maina et al., 2008). Within the glucans there are dextran, mutan, reuteran and alternan and among the fructans, levan and inulin-type (Angelin & Kavitha, 2020; Monsan et al., 2001; Zhou et al., 2019).

Among the bacteria that have demonstrated an over-production of EPS, because in fact most LAB can produce EPS, it is possible to point out *Lactococcus*, *Streptococcus* spp., *Lactobacillus*, *Bifidobacterium*, *Leuconostoc*, *Pediococcus, Weissella* and *Enterococcus* (Angelin & Kavitha, 2020; Korcz et al., 2018; Lynch, Zannini, et al., 2018). There are four biosynthesis pathways for these compounds, of which two are mainly considered as universal EPS biosynthesis pathways in LAB: the extracellular synthesis pathway and an intracellular pathway named the Wzx/Wzx-dependent pathway (Zhou et al., 2019). The first mentioned pathway is associated with the production of HoPS, mainly by the genera *Weissella*, *Leuconostoc*, *Pediococcus* and *Lactobacillus*, while the second targets the HePS production, synthesized by *Lactococcus*, *Lactobacillus* and *Streptococcus* (Zhou et al., 2019). Briefly, the extracellular synthesis pathway involves two steps, the first step related with the polymerization process, where sucrases (glucan or fructansucrases) act on the substrate (sucrose), lading to the monosaccharide transfer to the growing polysaccharide chain, followed by a second step, during which the release of the HoPS chains takes place (Angelin & Kavitha, 2020; Zhou et al., 2019). The Wzx/Wzx-dependent pathway is a more complex process, which involves firstly the transport, into the cell, and the phosphorylation of mono and disaccharides, followed by the formation of sugar nucleotides, and the synthesis of repeating units. These units are then translocated from intracellular to the extracellular surface, and here occurs the polymerization of the repeating units and the release of long chains (Angelin & Kavitha, 2020; Zhou et al., 2019). *Weissella* EPS is mainly dextran, a normally highly linear structure with few branches (Maina et al., 2008; Netsopa et al., 2018). *Weissella* strains have the ability to secrete the enzyme dextransucrase, which synthesizes dextran by transferring glucose residues from sucrose to the reducing tail of growing chains (Maina et al., 2008; Monsan et al., 2001). Such structural characteristic gives the dextran a high solubility and a high viscosity depending on its molecular weight. Consequently, dextrans from this bacterium are promissory food additives (Maina et al., 2008).

Microbial EPS have been shown to have an important technological and functional role. In what concerns their technological potential while applied in cereal-based products, mainly with sourdough technology (Lynch, Zannini, et al., 2018), EPS contribute notably to increasing specific volume, lowering crumb firmness and reducing staling (Lynch, Coffey, et al., 2018). In their study Katina and co-authors (2009) noticed an improvement of volume (up to 10 %) and crumb softness

(25–40 %) after fermentation of wheat sourdough by *Weissella confusa*. This resulted from the production of EPS, mainly dextran, from the added sucrose. Besides sourdough technology, EPS have other food applications such as in the dairy industry, namely in cheese and yoghurt (Barcelos et al., 2020; Duboc & Mollet, 2001; Lynch, Zannini, et al., 2018), and, a particular application, in the area of cereal-based beverages, with some works exploring quinoa, emmer flour and, also, barley malt wort-based beverages (Coda et al., 2011; Lorusso et al., 2018; Zannini et al., 2013). Regarding these beverages the main purpose of the EPS application was the increase of viscosity. In what concerns dairy products, EPS have revealed an important role in texture improvement and also in moisture retention, and thereby reducing syneresis. In recent studies, focused on Cheddar cheese making, results revealed that the presence of EPS-producing LAB (*Lactiplantibacillus plantarum* and *Lactococcus lacti*s ssp. *cremoris*) induced higher moisture and lower hardness and cohesiveness than the control cheese (Ji Wang et al., 2019), and, also, improved texture, higher moisture and cheese yield (Costa et al., 2010). More details about the use of EPS in the food industry can be found in the reviews of Lynch *et al*. and the other of Zannini *et al*. and (Lynch, Coffey, et al., 2018; Lynch, Zannini, et al., 2018; Zannini et al., 2016).

In what concerns the biological functionality of these compounds, these molecules have been claimed to exert specific beneficial health effects (Angelin & Kavitha, 2020; Ryan et al., 2015). They are potentially important in the modulation of the immune function (Laiño et al., 2016) and of beneficial commensal gut microbiota (Amaretti et al., 2020), of the cardiovascular function, due to their cholesterol-lowering effects (Korcz et al., 2018) and they also reveal antioxidant capacity (Liu et al., 2011). Furthermore, they may act as antitumoral agents, as pathogen antagonists (Korcz et al., 2018; Ruas-Madiedo et al., 2010), and they also have shown anti-inflammatory, anti-biofilm and anti-viral activities (Angelin & Kavitha, 2020).

The above-cited effects on functionality, whether physiological or technological, are dependent on the structure of the polysaccharide (Maina et al., 2008). EPS bring a two-fold advantage: not only are EPS a suitable natural alternative to chemical food additives or expensive ingredients, such as hydrocolloids, that are currently being used as texture improvers, stabilizing, emulsifying, gelling or water biding agents, in many food applications (Lynch, Coffey, et al., 2018; Rai et al., 2018), but they also add value through the abovementioned health benefits. Based on these rationale, it might be interesting and convenient to use EPS-producing strains as starter cultures for the enhancement of both gluten-containing and gluten-free cereal products.

PART III – NOVEL SYNBIOTIC FERMENTED WHOLE GRAIN-BASED PRODUCTS

2.10. RECENT DEVELOPMENTS ON FERMENTED CEREAL-BASED PRODUCTS

As stated before, in sub-chapter 2.7, fermented foods and beverages, in particular gluten-free-products, have been gaining attention among consumers and, in consequence, there is an ongoing development of novel products (Liptáková et al., 2017; Rai et al., 2018). According to Innova Market Insights, the plant-based claims for food and beverages launches have been increasing, with a compound annual growth rate of 57 % over the 2015 to 2019 period (Green, 2020). Furthermore, Mintel in their very recent 2020 report, affirmed that the plant-based products are being diversified into new categories such as yoghurt and ice cream, beyond the most representative of the segment, the plant-based drinks (Mintel, 2020). In terms of commercialized probiotic fermented foods or beverages made with cereals, oat is the privileged cereal, unique constituent of Yosa® and Proviva®. Yosa® is a oat bran-based pudding fermented by *Lactobacillus acidophilus* and *Bifidobacterium bifidum*, mostly consumed in Scandinavian countries (Blandino et al., 2003), whereas Proviva®, an oatmeal gruel fermented by *L. plantarum* 299v, is a product consumed in Sweden (Blandino et al., 2003; Coda et al., 2017; Prado et al., 2008).

Several researchers have explored this potential of plant-based probiotic fermented products and some recent developments may be found in the scientific literature, as reviewed by other authors (Enujiugha & Badejo, 2017; Kandylis et al., 2016; Kumar et al., 2015; Min et al., 2018; Peyer et al., 2016; Valero-Cases et al., 2020). For example some studies have focused their research on symbiotic functional drinks, made from the fermentation of oat-flour/flakes by *Lactiplantibacillus plantarum* (Angelov et al., 2006; M. Gupta & Bajaj, 2017; Luana et al., 2014; C. Wang et al., 2018) or by a combination of *Lactiplantibacillus*, *Lactobacillus* and *Lacticaseibacillus* strains (Gokavi et al., 2005). *Lactiplantibacillus plantarum* and *Limosilactobacillus fermentum* were strains studied for the development of fermented rice based-beverages (Ghosh et al., 2015; Giri et al., 2018). Quinoa was also investigated for the production of novel yoghurt-like beverages, where *L. plantarum, L. rhamnosus* and *Weissella confusa* were singly used as starter cultures (Lorusso et al., 2018), and combined cultures including *L. plantarum*, *L. casei* and *L. lactis* also showed interesting results (Ludena Urquizo et al., 2017). Finger millet, pearl millet and sorghum were also used for the formulation of fermented beverages using mainly *Lactobacillus* strains in single or combined cultures (Di Stefano et al., 2017; Fasreen et al., 2017; Mukisa, Byaruhanga, et al., 2012; Sanni et al., 2013). Matejčeková and colleagues, studied the fermentation of amaranth by a combination of different species, including *Lactococcus*, *Streptococcus* and *Lactobacillus*, and strains reached high levels of viable cell numbers (10^8 CFU/mL) upon eight hours fermentation (Matejčeková et al., 2016). Coda *et al*. (2011) used emmer flour to produce different fermented beverages prototypes with single (*Lactobacillus* strains) and combined cultures of different strains of *Lactobacillus*, together with *Weissella cibaria* and *Pediococcus pentosaceus* strains. Barley malt wort was also explored as a basis of a cereal-fermented beverage, in which *W. cibaria* was used as the starter culture (Zannini

et al., 2013). A multi-cereal mixture, supplemented with milk-based products, was the matrix to develop a fermented baby food, using a combined culture of *Lacticaseibacillus casei* and *Lactiplantibacillus plantarum* as the starter culture (Rasane et al., 2015). Considering these examples highlighted above, the multiplicity of attempts to develop cereal- or pseudo-cereal-based beverages is undoubtedly growing in this field of research.

2.11. CEREAL FERMENTATION AND IMPACT ON NUTRITIONAL AND BIOLOGICAL VALUES

Cereal fermentation reveals positive impact on several technological and functional properties of a food matrix. From the reduction of the bacterial load to the improvement of nutritional properties there are important roles that can be attributed to fermentation (Kohajdová & Karovičová, 2007; Steinkraus, 1994). From the nutritional perspective, fermentation contributes to the enrichment of foods through the increase of some important nutrients, such as essential amino acids and vitamins and the decrease of antinutrients, such as tannins, polyphenols or phytates, having as consequence an increased minerals' bioavailability (Kohajdová & Karovičová, 2007). Moreover, fermentation enriches a food product through the improvement of sensory properties, including flavours, aroma, texture, and overall appearance, due to the formation of volatile compounds or other texturizing agents (Blandino et al., 2003; Mukisa et al., 2017; Salmeron et al., 2009). Additionally, during fermentation, the production of an acidic environment protects the matrix against bacterial contamination, promoting preservation and detoxification of the food product (Kohajdová & Karovičová, 2007; Steinkraus, 1994).

2.11.1. Total phenolics and antioxidant activity

As mentioned before, the high levels of phenolic compounds in darker grains, such as sorghum and finger millets can be seen as a positive characteristic, however, it is a double-edged sword, because these compounds potentially reduce mineral absorption (Towo et al., 2006). Generally, sorghums' and millets' fermentation over at least 24 hours reduces phenolic compounds (Dlamini et al., 2007; Taylor & Duodu, 2015; Towo et al., 2006). However, considering other research works (Dordević et al., 2010; Ghosh et al., 2015; Nionelli et al., 2014; Rasane et al., 2015), conclusions are controversial. It seems that the effect of fermentation is dependent on the previous processing steps or the matrix as well. A combination of some mechanisms of action have been suggested to explain this reduction in polyphenols. One is related to the enzyme polyphenol oxidase (PPO), which origin may be from the cereal or from the fermenting microbiota itself (Dhankher & Chauhan, 1987; Taylor & Duodu, 2015). PPO uses phenolic compounds as substrates and its action will contribute to their oxidation and consequent removal. Another explanation is their reduced extractability caused by the interaction of these compounds with macromolecules, such as proteins, present in the acidic aqueous cereal matrix (Beta et al., 2000; Taylor & Duodu, 2015).

2.11.2. Antidiabetic activity

It has been reported that the consumption of millet, namely finger and pearl millets, has a blood glucose-lowering effect in rats (Elewechi et al., 2015; Sada et al., 2016; Shobana et al., 2010) and even in humans (Alyami et al., 2019; Kumari & Sumathi, 2002). In whole grain millets, the presence of fibre can both delay the absorption of carbohydrates, and also reduce its absolute

absorption, by slowing gastric emptying time and by forming unabsorbable complexes with available carbohydrates in the gut lumen (Kumari & Sumathi, 2002). Besides dietary fibre, there are other compounds in whole grains that may be involved in treatment and prevention of diabetes, such as polyphenols, anthocyanins, saponins, polysaccharides, and proteins (Gong et al., 2020). Polyphenols, more evidently present in sorghum and finger millet, can also be responsible for this lowering effect, due to the inhibition of glucose absorption in the gut or its uptake by peripheral tissues (Scalbert et al., 2005). The reduction of intestinal absorption of glucose might be explained by a mechanism that involves inhibition of intestinal glucosidases and glucose transporter (Matsui et al., 2001). The inhibitory compounds of this enzyme act as competitors of enzymes involved in carbohydrates hydrolysis and by that the transformation of oligo-, tri- and disaccharides to monosaccharides is compromised. Consequently, postprandial glucose absorption is delayed, decreasing postprandial increase of blood glucose level (Kwon et al., 2008; Puls et al., 1977). For instance, Shobana *et al*. (2009) observed that finger millet seed coat phenolics showed a lower required concentration to inhibit the activity of α-glucosidase in 50 % (IC_{50}=16.9 µl/mL) when compared to the value found by Pradeep and Sreerama (2018), which was slightly higher (IC_{50}, for soluble whole finger millet phenolics was around 20 µl/mL), nevertheless both of them were higher than the required concentration for acarbose, a recognised positive control. In the review of Gong *et al*. (2020) more examples with other cereals are highlighted, and in addition to the inhibitory effect of polyphenols on α-glucosidase, other bioactive compounds are also explored. Sada and colleagues also found that the content of minerals might be involved in the hypoglycemic action of pearl millet (Sada et al., 2016). The presence of magnesium and selenium stimulates insulin secretion by the pancreas and/or enhances insulin sensitivity (Sada et al., 2016).

The antidiabetic activity of fermented products, such as mung (Kwon et al., 2010; Yeap et al., 2012), soy (Keishing & Banu, 2015), beans and leaf tea (Park et al., 2013), has been studied and interesting results have been achieved, showing that fermented products ameliorate glucose metabolism. However, the mechanism of action behind these effects still needs further research. Nevertheless, the work of Yeap *et al*. (2012) reported that fermentation, by increasing the content of amino acids such as Gamma-Aminobutyric Acid (GABA) and lys, can enhance the regulation of blood sugar in mice (Yeap et al., 2012). Specifically, lys was found to be an enhancer of the insulin-receptor tyrosine kinase, contributing to the reduction of blood sugar levels (Sulochana et al., 2001). In the study of Aisoni *et al*. (2018) the authors compared the effect of the consumption of processed finger millet (roasted *vs*. fermented) with the consumption of a control food (food containing 50 g carbohydrate) as standard. They concluded that roasted millet showed higher capacity on reducing blood glucose when compared to fermented millet and the control, and that fermented millet showed to elevate blood glucose concentration more that the control; however, both processed millets revealed lower glycaemic index in comparison with the control. Some microorganisms are able to produce valuable bioactive compounds that can exert biological activities such as antidiabetic activity. The work of Yang *et al*. (2012) found that different fractions of EPS, extracted from the fermentation culture medium of *Coriolus versicolor* LH1, demonstrated high α-glucosidase inhibition. Furthermore, Huang *et al*. (2020) found that an EPS produced by a specific strain of *L*. *plantarum* isolated from

pickled cabbage revealed potential for the inhibition of α-amylase pancreatic activity, showing the potential of this *Lactiplantibacillus* for the prevention and alleviation of diabetes mellitus.

2.11.3. Protein digestibility

In order to be absorbed, proteins need to be digested into smaller peptides and amino acids, and so they must suffer a breakdown. Proteins in plants are more poorly digestible, compared to animal proteins (Bhutta & Sadiq, 2012). This low digestibility, a consequence of restrictively accessible substrates for their hydrolytic enzymes, may result from endogenous and exogenous factors (Joye, 2019). Naturally, proteins are structurally conformed according to their amino acids profile. These macromolecules may occur in different forms, such as supramolecular structures with dense networks formed by crosslinking forming protein bodies (Herman & Larkins, 1999; Pernollet, 1978). Moreover, low permeability of plant cell walls and relative insolubility can also justify lower digestibility (Becker & Yu, 2013). It is also known that proteins with high proline levels are typically less digestible (Joye, 2019). As exogenous factors, the presence of antinutrients or the physical entrapment in the cell can be highlighted (Bhattarai et al., 2017). Proteases inhibitors, tannins, and phytates are some examples of antinutrients found in different foods (Gilani et al., 2012; Joye, 2019; Kostekli & Karakaya, 2017). Proteases inhibitors such as trypsin and chymotrypsin inhibitors inactivate the gastrointestinal peptidases trypsin and chymotrypsin, respectively (Joye, 2019). Tannins are prone not only to associate with minerals, but also with proteins forming complexes that precipitate, reducing the availability for the protein to be hydrolysed (Joye, 2019). Phytates sequester minerals that are essential cofactors for peptidases activity. Dietary fibre also hinders protein digestibility by increasing viscosity in the gastrointestinal tract preventing enzymes to easily access their substrates (Joye, 2019).

Some of these mechanisms can be avoided by heat treatments and extrusion (trypsin inhibitors, and phytates) (Clemente et al., 2000; Nikmaram et al., 2017), dehulling, soaking or acid or alkaline treatments (tannins) (Osman, 2011), however, a balance between those treatments should be studied, mainly because they can have conflicting effects on protein digestibility (Nikmaram et al., 2017). Fermentation has been suggested as a way to increase cereals protein digestibility (Nkhata et al., 2018). The proposed mechanisms behind this improvement are, on the one hand, related to the reduction in pH, which promotes the breakdown of protein into smaller fractions, a consequence of augmented proteolytic enzymes activity of fermenting bacteria (Hesseltine, 1983; Ogodo et al., 2018; A. Singh et al., 2012); on the other hand, the decrease in undesirable substances that affect digestion, such as phytic acid or tannins seems to improve protein digestibility (Çabuk et al., 2018; Hesseltine, 1983; A. Singh et al., 2012). The study of Pranoto *et al.* (2013), in which the fermentation of sorghum by *L. plantarum* was studied, revealed an increase of protein digestibility after 36 hours from 41.81 % to 80.31 %. In the same study, authors also mentioned that the *L. plantarum* tannase activity might have been a justification for this increase, due to the liberation of previously complexed proteins to tannins. Singh and colleagues (2012), who evaluated the effect of fermentation on protein digestibility of sorghum, pearl millet, and maize, and El Hag *et al*. (2002), who studied the same effect on pearl millet, also found a significant increase in

protein digestibility. The effect of fermentation on other food products such as pea and soybean flour has been studied as well (Çabuk et al., 2018; Ogodo et al., 2018). The study of Çabuk and co-authors (2018) highlighted the decrease of phenolics and tannins as the main reason for the consequent reduction in protein cross linking, thereby making proteins more available for enzyme action. On the other hand, the research work of Ogodo *et al*. (2018) attributed to the pH downfall the main justification for the enhanced activity of proteolytic enzymes, which leads to the higher breakdown of proteins into smaller, easily accessible, polypeptides.

2.11.4. Mineral content

The positive impact of fermentation on the mineral content and bioavailability, is associated with the reduction of antinutrients (Singh et al., 2015). As already mentioned, antinutrients, such as phytic acid, are naturally present in cereals. This compound has the ability to bind itself to bivalent and trivalent cations, including minerals, forming complexes and consequently, hampering their absorption. For instance, 80-87 % of the total phosphorus present in sorghums' kernel is bound to phytate (Doherty et al., 1982). One way to reduce the amount of phytate in cereal products, and thus improve their quality, is resorting to microorganisms with phytase activity (Cizeikiene et al., 2015; Haros et al., 2001). According to Nuobariene *et al*. (2015), there are only a few LAB that revealed consistent extracellular activity, namely *Lactobacillus amylovorus* B4552 and *Lactiplantibacillus plantarum*. Fermentation by LAB has been reported to reduce the phytate content in plant-based food products (Reale et al., 2007). Reale and co-authors (2007), who studied the role of LAB in the degradation of phytate during lactic acid fermentation, concluded that the reduction of phytate after LAB fermentation was mainly due to intra-cellular phytase activity, which was triggered by the pH fall. During this process, at the ideal pH, phytases hydrolyse a complex form of phytate, myo-inositol hexaphosphate (or IP6) into lower myo-inositol esters, such as IP5, IP4 or IP3, liberating free inorganic phosphate (Ragon et al., 2008). These lower forms have inferior binding capacity to minerals (Gupta et al., 2013) and, consequently, its availability and solubility will be greater.

Moreover, bioavailability of minerals might be enhanced by the increase in organic acids concentrations, which can form soluble ligands with minerals and, in consequence the formation of insoluble complexes with present phytase is hindered (Hemalatha et al., 2007).

2.12. FOOD DIGESTION AND THE GASTROINTESTINAL TRACT

Foods provide energy and the nutrients essential for a healthy growth. However, the utilization of these molecules is dependent on the way they are physiologically and biochemically processed. After food intake, it is expected that the human body triggers the pathway leading to the metabolism of nutrients, enabling the occurrence of the digestion, absorption, transportation, and excretion processes.

The gastrointestinal tract (GIT) has three main functions, namely the extraction of macronutrients from the ingested foods and beverages, their absorption, as well as the absorption of micronutrients, and also it should serve as a physiological and immunological barrier to microorganisms and foreign molecules (Beyer, 2008). The digestion process begins in the mouth, where foods are chewed and by that reduced into smaller particles, allowing a greater exposure of the food matrix to salivary secretions. Here, the active enzyme is ptyalin (salivary amylase), which main substrate is starch (Beyer, 2008). After this first phase, the bolus reaches the stomach through the oesophagus. In this compartment, the bolus suffers action by gastric juices, containing pepsin and gastric lipase, the moment when lipid and protein digestion begins (Beyer, 2008). At this point, the food mixture is called chyme. Later, in the small intestine, is where the most intense activity occurs. The presence of food triggers the release of hormones that stimulate the activity of specific enzymes originating from the pancreas, from the brush border of intestinal mucosa, as well as from the bile and the gallbladder (Beyer, 2008).

Carbohydrates are exposed to a group of enzymes, α-amylase and other specific-substrate enzymes such as amyloglucosidase, sucrase, maltase, lactase, isomaltase, and simpler sugars are produced (Beyer, 2008). Exocrine secretions from the pancreas (with trypsin, chymotrypsin, carboxypeptidase) together with small intestine enzymes also act on proteins and complete their digestion by transforming larger peptides into small peptides and free amino acids (Beyer, 2008). Fats are converted into fatty acids and monoglycerides, mainly by the action of pancreatic lipase (Beyer, 2008). Absorbed nutrients can follow different destinations after they reach the liver, where they are stored or transformed into other nutrients or they are released into the blood stream. Most of the macronutrients, vitamins, minerals, trace elements are absorbed in the small intestine. Remaining nutrients and fluids are absorbed in the colon and rectum. In the colon, the residing microbiota ferments the remaining fibre, resistant starch, sugar, and amino acids, resulting in the production of short-chain fatty acids (SCFA) and gas (Beyer, 2008).

Ideally, when analysing diet-related questions, human nutritional studies should be implemented, however, in many cases such procedure is not feasible. In consequence, the only practicable solution is to mimic *in vitro* the *in vivo* physiological conditions of food digestion processes. Generally, *in vitro* methods are static and include the oral, gastric and small intestinal phases of digestion. More sophisticated laboratories work with dynamic models, that are more realistic because they can simulate aspects such as food transport, enzymes concentrations and pH variation over the GIT (Minekus et al., 2014). Due to the wide diversity of existent protocols for the *in vitro* simulation of the gastrointestinal digestion and, consequently, the impossibility in comparing results between

laboratories, a consensus protocol was proposed by a comprehensive research team – the INFOGEST 2.0 protocol (Brodkorb et al., 2019; Minekus et al., 2014). Even though this method is a very interesting achievement, and is being used for many dietary validations worldwide, the authors recognize that it is more suitable for the simulation of the digestion process of the adult population, mainly because other age-groups such as children and the elderly may differ in terms of biological conditions, for instance, enzyme concentrations (Minekus et al., 2014).

2.13. MODULATING THE GUT MICROBIOTA

2.13.1 Methods for studying the gut microbiota

As mentioned above, it is the colon residing microflora that is responsible for the ultimate fermentation of some remaining substrates, as well as the storage and excretion of waste materials (Gibson & Roberfroid, 1995). There are several methods for studying the gut microbiota, which can be divided in two main groups, the culture-dependent and the culture-independent methods (Gong & Yang, 2012). The first group of techniques is based on selective culturing of bacteria, and morphological, biochemical, and physiological assays, whereas the independent methods involve DNA techniques, and allow a wider characterization of the bacterial community (Gong & Yang, 2012). Within this last group, the most common used techniques are polymerase chain reaction (PCR)-based DNA profiling, quantitative PCR (qPCR), fluorescent *in situ* hybridization (FISH), flow cytometry, DNA sequencing, and DNA microarray (Gong & Yang, 2012).

In qPCR, also known as real-time PCR, there is an amplification of the DNA, followed by the quantification of targeted bacterial populations. This process involves a non-specific fluorescence molecule which binds to the double-stranded DNA and fluorescently labelled sequence-specific primers, resulting in measured fluorescence that reflects the amount of amplified product in each cycle (Fraher et al., 2012; Gong & Yang, 2012). In each cycle of qPCR a sequence of temperature dependent steps occurs: (i) denaturation, in which the double-stranded DNA 'melts' into single strands; (ii) annealing, during which step primers bind to the target sequence; and (iii) extension, in which DNA polymerase acts on primers extension (ThermoFisher, 2015). By plotting the level of fluorescence against the number of PCR cycles, the amount of DNA in a sample test can be quantified using, as reference, a standard curve derived from parallel amplification of known target copy numbers (Fraher et al., 2012). The target gene that is mostly used in these assays is the small subunit (16S) ribosomal ribonucleic Acid (16S rRNA) gene, named after its function, to code for the 16S rRNA (Clarridge, 2004). The choice of this as the target gene is based not only on the fact that the 16S subunit, which contains one molecule of RNA, is very well conserved in all eubacteria, but also because it varies in a manner that enables its differentiation between species. (Fraher et al., 2012). Quantitative PCR is widely used and is believed to be more sensitive than other techniques such as FISH or DNA microarray analysis. The main advantages include speediness in the process and post-PCR procedures, phylogenetic discrimination and also the possibility to design primers targeting specific species (Fraher et al., 2012; Gong & Yang, 2012). As drawbacks, it can be pointed out the lack of ability to perform a global assessment of bacterial communities, the identification of novel species, the limited number of species that can be measured per assay, and the possibility of bias during PCR amplifications (Fraher et al., 2012; Gong & Yang, 2012). Table 2.11 summarizes the main advantages and disadvantages associated with each method for the characterization of the gut microbiota.

Table 2.11 Culture-dependent and independent methods used for gut microbiota characterization. Adapted from Fraher *et al*. (2012) and Gong & Yang (2012).

Technique	Brief description	Advantages	Disadvantages
Culture	Isolation of bacteria on selective media	Cheap, semi-quantitative	Labour intensive, < 30 % of gut microbiota have been cultured to date
qPCR [1]	Amplification and quantification of 16S rRNA. Reaction mixture contains a compound that fluoresces when it binds to double-stranded DNA	Phylogenetic identification, quantitative, fast	PCR bias, unable to identify unknown species
DGGE/TGGE [2]	Gel separation of 16S rRNA amplicons using denaturant/ temperature	Fast, semi-quantitative, bands can be excised for further analysis	No phylogenetic identification, PCR bias
T-RFLP [3]	Fluorescently labelled primers are amplified and then restriction enzymes are used to digest the 16S rRNA amplicon. Digested fragments are separated by gel electrophoresis	Fast, semi-quantitative, cheap	No phylogenetic identification, PCR bias, low resolution
FISH [4]	Fluorescently labelled oligonucleotide probes hybridize complementary target 16S rRNA sequences. When hybridization occurs, fluorescence can be enumerated using flow cytometry	Phylogenetic identification, semi-quantitative, automated, no PCR bias	Dependent on probe sequences— unable to identify unknown species, relatively low sensitivity
DNA [5] **microarrays**	Fluorescently labelled oligonucleotide probes hybridize with complementary nucleotide sequences. Fluorescence detected with a laser	Phylogenetic identification, semi-quantitative, fast, high-throughput, cost-effectiveness	Cross hybridization, PCR bias, species present at low levels can be difficult to detect, unable to identify unknown species
Cloned 16S rRNA [6] **gene sequencing**	Cloning of full-length 16S rRNA amplicon, Sanger sequencing and capillary electrophoresis	Phylogenetic identification, quantitative	PCR bias, laborious, expensive, cloning bias
Direct sequencing of 16S rRNA amplicons	Massive parallel sequencing of partial 16S rRNA amplicons	Phylogenetic identification, quantitative, fast, identification of unknown bacteria	PCR bias, expensive, laborious
Microbiome shotgun sequencing	Massive parallel sequencing of the whole genome	Phylogenetic identification, quantitative, high sensitivity, no cloning bias, no PCR bias	Expensive data analysis, computationally intense

[1] qPCR: quantitative polymerase chain reaction;
[2] DGGE: denaturing gradient gel electrophoresis; TGGE: temperature gradient gel electrophoresis;
[3] T-RFLP, terminal restriction fragment length polymorphism;
[4] FISH: fluorescent in situ hybridization;
[5] DNA: Desoxyribonucleic Acid;
[6] 16S rRNA: small subunit of the ribosomal ribonucleic Acid.

2.13.2. Gut Microbiota and Health

It is estimated that the large intestinal microbiota counts with 1000 bacterial species (Gilbert et al., 2018). The phyla that are more predominant in the adult gut microbiota are essentially Firmicutes, Bacteroidetes, Verrucomicrobia, Proteobacteria, and Actinobacteria (Ruan et al., 2020; Wilson et al., 2020). Given this wide and complex microbial ecosystem, it is expected to find a multiplicity of nutritional and metabolic patterns, that will be reflected in beneficial or harmful effects on the host (Gibson & Roberfroid, 1995) (Figure 2.3).

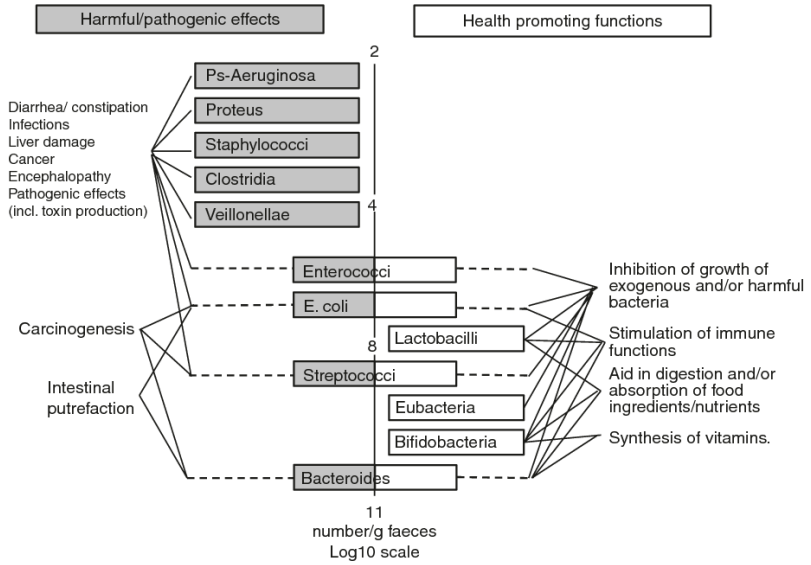


Figure 2.3. Generalized scheme of the composition and overall harmful or health effects of predominant human faecal bacteria. From: Gibson and Roberfroid (1995).

Among the positive effects on host health, regulation of hepatic lipid and glucose homeostasis, of the immune system and inflammatory response, maintenance of intestinal barrier integrity, *de novo* biosynthesis vitamins and amino acids and also appetite modulation must be highlighted (Morrison & Preston, 2016; Nagai et al., 2016; Thursby & Juge, 2017; Wilson et al., 2020). In contrast, the prevalence of specific bacterial groups has been associated with the presence of diseases such as colorectal cancer, inflammatory bowel disease, irritable bowel syndrome, and type 2 diabetes (Chong et al., 2019; Halfvarson et al., 2017; Marchesi et al., 2011; Ruan et al., 2020; Sapna Sharma & Tripathi, 2019).

Considering this duality of effects, there is an interest in manipulating the composition of the commensal population, the autochthonous microbial community, towards a potentially healthier community. This might be done by the usage of probiotics, which are proven to modulate microbiota positively, creating an allochthonous bacterial community, however, their colonization time is short and consequently, their beneficial effects are limited (Rinninella et al., 2019; Zhang et al., 2016). Some examples of probiotics, some of which were previously mentioned, are *Lactobacillus acidophilus*, *Lactiplantibacillus plantarum*, *Streptococcus thermophilus*, *Enterococcus faecium*,

Bifidobacterium adolescentis, *B*. *bifidum*, and *B*. *longum.* Their positive impact on health includes modulation of the immune system, production of antimicrobial substances, adhesion to the intestinal epithelial cells, and contribution to a healthier gut ecology, as slightly discussed above in sub-chapter 2.8.

It is also known that diet plays an important role in the modulation of microbiota. As previously described, the colonic microbiota uses as a source of energy any non-digested food that reaches the large intestine. The non-digestible food or food ingredients that positively stimulate growth or activity of specific resident bacteria, whose metabolism will improve host health, are called prebiotics (Gibson & Roberfroid, 1995). Recently, a group of experts from the ISAPP updated the concept of prebiotics, and they are now defined as 'a substrate that is selectively utilized by host microorganisms conferring a health benefit' (Gibson et al., 2017). Some examples of prebiotics are inulin and oligofructose (fructooligosaccharide (FOS)), galactooligosaccharides (GOS) and xylooligosaccharide (XOS). These substrates are fermented by the resident bacterial community resulting in the production of SCFA, namely butyrate, propionate, and acetate, positively influencing the lipid metabolism and strengthening the mucosal barrier and defence (Gibson & Roberfroid, 1995; Wilson et al., 2020). Considering the rationale of probiotics and prebiotics, their combination brought on the concept of synbiotic, updated by ISAPP, in 2019, and now defined as 'a mixture comprising live microorganisms and substrate(s) selectively utilized by host microorganisms that confers health benefit on the host' (Swanson et al., 2020). This symbiosis increases the potential for probiotic strains to enhance their activity and the ability of the prebiotic to increase beneficial microbial populations indigenous to the host GIT (Tuohy et al., 2005). Besides probiotics and prebiotics, dietary bioactive compounds, for example, carotenoids, flavonoids, proanthocyanidins, polyphenolic compounds, omega-3 fatty acids are also known to positively modulate gut microbiota (Charaslertrangsi, 2014). Some of them are absorbed in the small intestine, while others pass throughout the GIT until reaching the colon, where they are metabolized and absorbed into the systemic circulation (Charaslertrangsi, 2014).

The research studies of Connolly *et al*. (2010, 2012) demonstrated that in faecal batch fermentation of whole grain oat and wheat flakes resulted in increased numbers of *Bifidobacterium* (oat: 7.85 *vs*. 8.43, and wheat: 7.8 *vs*. 8.5, in log10 cells mL batch culture fluid), *Prevotella-Bacteroides* group (oat: 7.90 *vs*. 8.61, and wheat: 7.8 *vs*. 8.4, in log10 cells mL batch culture fluid), along with the production of acetate, butyrate, and propionate in significant amounts. These cereal-based products behaved similarly to the prebiotic oligofructose.

Despite this positive modulation of the gut microbiota, it is known that those alterations in the microbial community are transient and that the rate of change of the human microbiome composition is personalized (Flores et al., 2014). Very important findings were achieved in the last years (Ruan et al., 2020; Wilson et al., 2020), though more research is needed to better understand the complexity, composition, and function of this almost claimed human organ, that is the gut microbiota.

2.14. EXPECTED IMPACTS AND ALIGNMENT WITH THE SUSTAINABLE DEVELOPMENT GOALS

This research brings forward a win-win Europe-Africa bridge strategy. In Africa, specifically in Kenya, there are no recent dietary intake studies at individual level in adults, which allow the establishment of a solid and adapted food and nutritional plan of action. Therefore, the present research work is likely to fill this gap and take the research in this field one step forward. Knowing food and dietary habits of a population increases efficiency in tailored-food product development. Although the production and, consequently, the consumption of fermented cereal-based food products in Africa is very common, the traditional way in which they are being produced may be harmful to the consumers' health. In Europe consumption of fermented food or beverages is, generally, limited to bread, dairy-based products and beers. Controlled fermentation can reduce potential hazards, by the employment of starter cultures with well-known characteristics. The combination of probiotic *Lactobacillus* and indigenous *Weissella* might give the product a unique environment, improved in terms of physiological or technological functionality. Moreover, there is a clear opportunity for the cereal industry to be innovative. The development and characterization of functional fermented cereal-based products suitable for both the European and African markets, targeting more health-conscious and planet-caring consumers, aligned with the plant-based trend, has a sufficiently supportive scope to be impactful.

Taking all this into account, the present work is aligned with four, out of the 12 sustainable development goals (SDGs) established by the United Nations (Figure 2.4). In alignment with the SDG3, a healthy, nutritionally enriched, with biological activity potential cereal-based product, able to be consumed by different population groups, including, celiac or lactose intolerants is envisaged. Concerning the SDG9, the novelty in what concerns, on the one hand, the dietary assessment, nutritional tools development and nutritional data generation, and on the other hand, in terms of product development, ensure the fostering of innovation. The use of traditional crops, as sorghum and millets, with interesting harvesting properties contributes to a sustainable production, being aligned with the SDG15. Finally, and responding to the SDG17, the involved partnerships in the present work creates bridges between countries - Portugal, Finland, Kenya and Burkina Faso, and continents, Europe and Africa, promoting a global network.

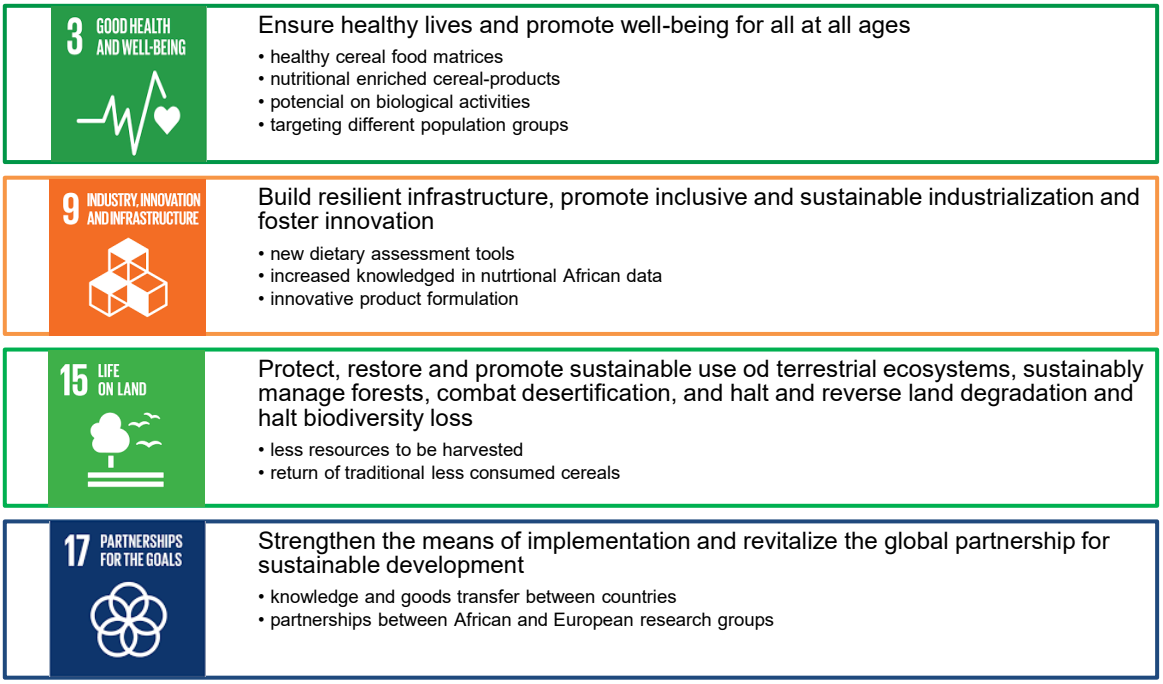


Figure 2.4 Alignment and expected impact of the work within this thesis with the sustainable development goals of the United Nations.

CHAPTER 3.

METHODOLOGY

“Take a method and try it: If it fails, admit it frankly and try another. But above all, try something.”

Franklin D. Roosevelt, 1932

PART I – DIETARY INTAKE ASSESSMENT

3.1. THE DIETARY SURVEY IN KENYA

This cross-sectional type study was carried out in Nairobi County, Kenya (in different constituencies), and performed in three phases:

Phase 1) development of a semi-quantitative FFQ;

Phase 2) test for validity and reproducibility of the developed FFQ;

Phase 3) study of dietary intake of the Kenyan population using the previous FFQ.

The methodological design for phases 1 and 2 is outlined in Figure 3.1 (a) and (b), respectively.

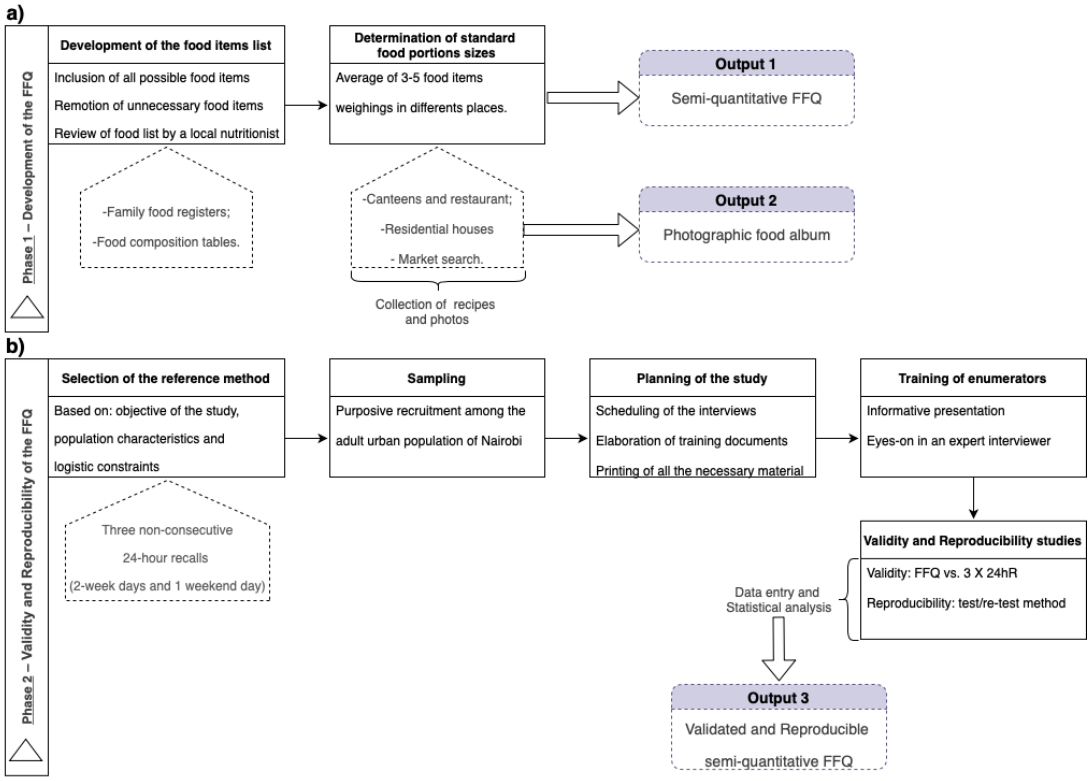


Figure 3.1 Methodological design for the (a) development and (b) study of validity and reproducibility of the culture-specific semi-quantitative food frequency questionnaire.

3.1.1. Phase 1 – Development of a semi-quantitative FFQ

3.1.1.1. FFQ structure and characteristics

A semi-quantitative FFQ, intended to report dietary intake during the previous month, was designed and constructed considering the food patterns of the urban Kenyan adult population. This FFQ was comprised of the food list, the frequency categories of response, and the area for specifying the consumed portion size, based on a three-portion option. The questionnaire, written in English, was designed to be applied in face-to-face interviews, conducted also in English, one of the official languages of the population under study, apart from Swahili. For this phase, the research team deeply discussed the most critical aspects of the FFQ development task.

3.1.1.2. Data collection

The food items used to compile the food list were obtained by accessing different sources and crossing these with information on generally consumed food, namely:

- different questionnaires applied in Kenyan and other African populations (Baroudi et al., 2014; Jackson et al., 2012; Jordan et al., 2013; Lukmanji et al., 2013; Mbochi et al., 2012; Merchant et al., 2005; Sheehy et al., 2013; Wrottesley et al., 2014);
- 30 food records from Kenyan people, obtained in a previous pilot study;
- the Kenyan national food composition tables.

For the composite dishes included in the FFQ, recipes from native people, local restaurants, and from the internet were collected and analysed in order to determine a standardized recipe. The compiled food list was evaluated by local nutritionists for further adaptation.

After the conclusion of the food list, standard portion sizes were established. For packed foods sold as single units, a market survey and personal observations were conducted, and the most commonly consumed quantity was used as the standard portion. For unpacked foods sold as single units (for instance fresh fruit), three samples of each (edible part) were weighed and the average weight was used as the standard portion. For composite dishes and cooked foods, three canteens, eleven residential houses, and one restaurant were accessed to obtain several weight measurements. Consequently, the average value from all the measurements was used as the standard portion. To calculate the smallest and the largest portion, the standard portion was multiplied by the following factors: 0.75 and 1.5, respectively. During all the visits, pictures of the portions were taken and were used for the construction of a photographic food album (Appendix A).

3.1.2. Phase 2 – Test for validity and reproducibility of the developed FFQ

3.1.2.1. Study design and recruitment of participants

To determine the relative validity of the developed FFQ, the results obtained by the application of the first FFQ were compared to the results from the applied three non-consecutive 24hR. To test reproducibility, the FFQ was applied on two different occasions, to the same group of people, by the same interviewer. Several Kenyan volunteers were purposively recruited. Each respondent was told the objectives of the study and a written informed consent (Appendix B), according to the Declaration of Helsinki of the World Medical Association, was obtained from those who agreed to participate in the study.

Five interviewers from the University of Nairobi (UN) Department of Food Science, Nutrition and Technology with a Nutrition background were recruited. All the interviewers were given training and guiding documents (Appendix C) in order to standardize the interviews, ensuring the quality of data to be obtained. The methodological design for this phase is outlined in Figure 3.1 (b).

A power calculation was performed for sample size determination. Statistical power was established as 90 %, two-sided significance level as 0.05, and the correlation of 0.4 was based on previous similar validity studies. The combination of these indicated a minimum sample of 60 respondents. Considering a drop-out rate of 20 %, researchers set the sample size of 75 individuals. Exclusion criteria included age under 18 and over 60 years-old. During data analysis, potential implausible energy intake reports were further excluded, considered as outliers the values under 1000 kcal and over 5000 kcal, based on the 95 % of the values for energy intake.

3.1.2.2. Data collection

The study protocol was composed of five interviews: in the first, the respondents answered to a general questionnaire (Appendix D) and the FFQ (Appendix E); in the next three interviews, the respondents were asked to recall dietary intake in the previous 24 hours (the interviews were separated from each other by two days minimum); in the fifth and last interview the respondents answered, once again, to the FFQ (the two FFQs were applied 21 days apart). All interviews for each participant were conducted by the same interviewer trying to minimize inter-rater bias. The general questionnaire included questions about demographic data, anthropometrics (weight and height), lifestyles, and health status. In the 24hR the respondents gave detailed information about everything they ate and drank in the previous 24 hours (Appendix F), from the time they woke up on the day before, until they woke up on the interview day. Every respondent was asked to recall two week days and one weekend day, aiming to be as comprehensive as possible in terms of inclusion of dietary intake information. During the 24hR, to facilitate the estimation of food portion sizes, the interviewers used the developed photographic album. Every time the respondent mentioned a composite dish, an estimation of the consumed

ingredients, or in its absence, the quantities used during dish preparation, was requested and registered whenever possible.

3.1.2.3. Data computation

In order to transform the food into nutrient intake, there was a need to resort to a food nutrient software, namely the Food Processor® software, version SQL 11.0.3 (ESHA Research, Salem, OR, USA), which includes the United States Department of Agriculture's Food and Nutrient Database for Dietary Studies, the Canadian Nutrient File, and the United Kingdom Foods database along with manufacturer and restaurant data. All the food items included in the FFQ and the ones mentioned during the recalls had to be identified in this software. In order to better choose the most adequate software code for each food within the range of existing items in Food Processor®, a comprehensive and detailed comparison between the nutritional compositions of the food items in Food Processor® and the foods consumed in Kenya (by assessing the Kenyan food composition tables) was performed by two nutritionists. When a food item was absent in the software or the nutritional composition of the Kenyan native food was different, data from the Kenyan food composition tables were used to supplement Food Processor®. Some recipes of composite dishes, obtained from native Kenyans, were also added to Food Processor® software. These codes were organized in a codification manual (an example of a part of it can be found in Appendix G) and used for the conversion of foods into nutrients. This manual was constructed to warrant the systematization of the codification of the recalls and the questionnaires. Consequently, all the food items of the FFQ and the 24hR were coded. The information from the FFQ was introduced in a Microsoft Access® database, for further crossing with Food Processor®, and the information of the 24hR was introduced into the Food Processor® software directly. Resultant nutrient information from both databases was exported to Microsoft Excel® databases for further statistical analyses. Regarding the food information obtained by the application of the FFQ, each food portion (weight in grams) was multiplied by the respective frequency of consumption and divided by the number of days correspondent to the frequency consumption, in order to estimate daily consumptions of each food item. Dietary data were analysed as energy, macro, and micronutrient intakes.

3.1.2.4. Statistical analysis

Descriptive data were calculated as frequencies (%) and presented as means and standard deviations, for normally distributed variables, and as median (25^{th} (P25) and 75^{th} quartiles (P75)) for non-normally distributed variables. The Kolmogorov-Smirnoff test was used to study the variables' distribution normality. The Wilcoxon test was used to compare medians of absolute nutrients' intakes estimated for both methods, in the validity study and for both applications of the FFQ, in the reproducibility study.

The correlation between the FFQ (FFQ1) and the mean of the three 24hR was studied by calculating unadjusted, energy-adjusted and de-attenuated Spearman correlation coefficients. The nutrients' energy-adjustment was conducted using the residual method (Willet, 2013c). The

de-attenuated correlation coefficients were used to adjust for random within-subject variability in the three 24hR (Willet, 2013c). Ranking agreement between the FFQ and the 24hR was quantified as the percentage of subjects who were categorized in the same (complete agreement) and/or adjacent and in the opposite (extreme disagreement) nutrient quartiles. The Bland–Altman plots (Bland & Altman, 1986) were also examined for each nutrient.

To evaluate the reproducibility of the FFQ, the correlation between the two applications was also studied by calculating unadjusted and energy-adjusted Spearman correlation coefficients. Moreover, intra-class correlations (ICC) between both FFQ were calculated. Statistical analyses were conducted using IBM® SPSS® Statistics, version 23 (SPSS Inc., Chicago, IL, USA).

3.1.3 Phase 3 – Study of dietary intake of the Kenyan population using the previous FFQ

3.1.3.1. Study design and recruitment of participants

To study the dietary intake of the urban Kenyan population, the previously developed and validated FFQ was applied to a tentatively representative sample. This cross-sectional study was carried out in April 2016, and several Kenyans (≥ 18 and < 60 years-old) were purposively recruited, in shopping malls, open-air markets, churches and urban informal settlements, such as slums, aiming to comprise different social backgrounds, with diverse incomes and lifestyles. All the required authorizations to access the sampled institutions were provided by the local manager. Respondents were randomly selected within each population. Each respondent was told the objectives of the study and a written informed consent (Appendix H), according to the Declaration of Helsinki of the World Medical Association, was obtained from those who agreed to participate in the study.

Eleven interviewers were recruited and given training by a nutritionist of the research team, along with all the necessary information to perform the interviews (Appendix I). In total, the team was composed of twelve trained interviewers. Daily meetings were crucial to the efficient and fruitful development of the plan. Major challenges were highlighted by the team and discussed between everyone, trying to obtain the best way to pursuit.

The sample size for a prevalence survey calculation (Glaziou, 2005) was carried out, considering the urban population of Nairobi (13,876,112 people) (The World Bank, 2019b), a precision of 5 %, a prevalence of 50 %, to obtain the highest sample size possible, and the confidence interval level of 95%. The combination of these criteria indicated a minimum sample of 385 respondents. Considering a misreporting of 25 %, researchers set a sample size of 500 individuals. Exclusion criteria included age under 18 and over 60 years-old or people who did not know their age and potential implausible energy intake reports, considered as outliers the values 1.5 X IQR above the third quartile (Q3 + 1.5 X IQR) or 1.5 X IQR below the first quartile (Q1 + 1.5 X IQR; 531 kcal) (Kipnis et al., 2003).

All procedures involving the research study participants, in both phases 1 and 2, were approved by the Institute for Bioethics of the *Universidade Católica Portuguesa* (Catholic University of Portugal).

3.1.3.2. Data collection

Dietary intake was assessed using the previously developed and validated semi-quantitative FFQ. Respondents were interviewed in a single moment. As in the validity study, the objectives of the study were explained to the participants and if they were interested in participating, a written statement of consent was given to them. During the interview, the participants, first answered a general questionnaire (Appendix J), including questions about demographics, anthropometrics (weight and height), lifestyles, and health status (presence of chronic diseases, specifically asked by the interviewer), followed by the FFQ.

3.1.3.3. Data computation

The methodology followed for the computation of data relative to the FFQ was the same as that in phase 2, described in the previous sub-chapter 3.1.2.3. Dietary data were analysed as energy, macro, and micronutrient intakes and contributions of each macronutrient was calculated and expressed as percentages of energy intake (EI). Food groups' contributions for energy or nutrient intake were also calculated and expressed as percentages of energy or nutrient intake. Nutrient adequacy was studied by the comparison of the actual intakes with the recommended specific nutrient requirements defined by the consortium WHO/FAO (FAO Expert Consultation, 2010; Fao & Consultation, 2001; Joint FAO/WHO Expert Consultation on Human Vitamin and Mineral Requirements, 1998; Joint WHO/FAO/UNU Expert Consultation, 2001, 2002).

3.1.3.4. Statistical analysis

Data were descriptively expressed as frequencies (%) and means and standard deviations for normally distributed variables and as median and 25^{th} (P25) and 75^{th} quartiles (P75) for non-normally distributed variables. The Kolmogorov-Smirnoff test was used to study the variables' distribution normality. Associations between food sources, *id est* the contribution of each food group to energy or nutrient intake, and participants' characteristics were studied calculating the odds ratios (OR) and 95 % confidence intervals, using logistic regression, Enter method, with binary and continuous predictors, depending on the variable. Consequently, the contribution of each food group (dependent variables) was divided into two categories, using as threshold the median for each variable. The models were adjusted to the predictor variables which had shown significance when studying the statistical model to apply: gender (0: female; 1: male), education level (0: none, primary and secondary; 1: tertiary), occupation (0: student, casual labourer and unemployed; 1: self or formally employed), marital status (0: single, widow and divorced; 1: married), all binary; total energy intake which was divided into quartiles (0: EI ≤ P25 (2352 kcal); 1: P25 < EI ≤ P50 (2892 kcal); 2: P50 < EI ≤ P75 (3509 kcal); 3: EI > P75), and age and BMI were considered as continuous predictors. In case of missing information on at least one

predictor, the respondent in question was excluded from the models' adjustment. Statistical analyses were conducted using IBM® SPSS® Statistics, version 24 (SPSS Inc., Chicago, IL, USA).

3.2. PORTUGUESE SECONDARY DATA ANALYSIS AND COMPARISON WITH KENYAN DATA

Data analysis of Portuguese dietary intake was done using the results obtained from the previously mentioned national survey – IAN-AF – carried out in 2015-2016 (Lopes et al., 2017). Even though the Portuguese survey has been applied to a wide range of age groups (from three months to 84 years-old) only the adult age group (18-64 years-old) was considered for this analysis. Twenty-four hours recalls (in two non-consecutive days) together with a food propensity questionnaire were used to assess the dietary intake of the adult Portuguese population (3445 participants at the first interview and 3104 participants in both previously 8moments of dietary assessment).

Macronutrients' intake ranges and main food sources were compared between Portuguese and Kenyan data. Nevertheless, nutrient adequacy was studied using different nutrient requirements' guidelines: for Portuguese data, the European EFSA recommendations (EFSA, 2017) were followed, while for Kenya, the guidelines developed by FAO/WHO were chosen (Joint FAO/WHO Expert Consultation on Human Vitamin and Mineral Requirements, 1998; Joint WHO/FAO/UNU Expert Consultation, 2001; Joint WHO/FAO Expert Consultation, 2003).

PART II – FERMENTATION OF AFRICAN WHOLE GRAINS

The methodology followed to study the fermentation of African whole grain flours can be divided into three phases:

Phase 1) African whole grain fermentation by probiotic strains (*Lacticaseibacillus, Lactiplantibacillus and Lactobacillus* strains - all former *Lactobacillus)*;

Phase 2) African whole grain fermentation by *Weissella* strains;

Phase 3) African whole grain co-fermentation by one of the abovementioned strains in phase 1 and the indigenous *Weissella,* in phase 2.

The methodological design describing these three phases is outlined in Figure 3.2.

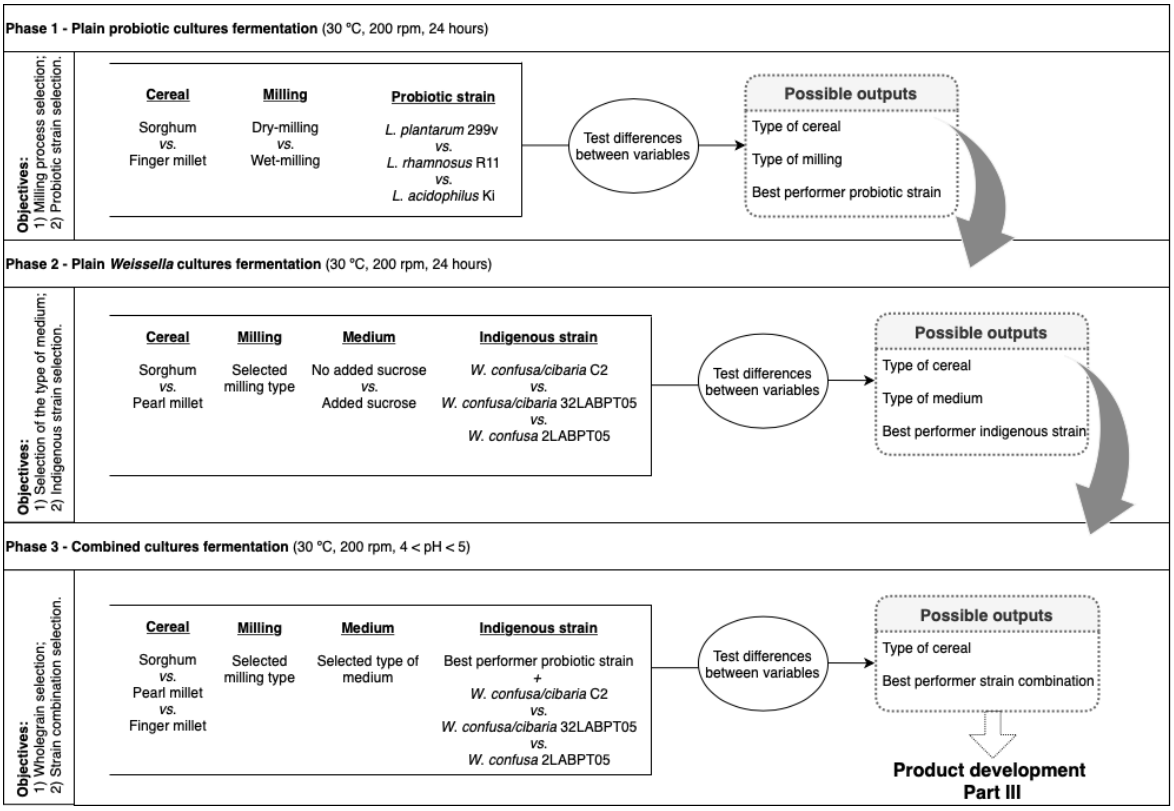


Figure 3.2 Methodological workflow design for the study of the fermentation of African whole grain flours by plain and combined cultures.

3.3. PHASE 1 – FERMENTATION OF AFRICAN WHOLE GRAINS BY PROBIOTIC STRAINS

3.3.1. Sample preparation

Cleaned finger millet (*Eleusine coracana* (L.) *Gaertn*) and red sorghum (*Sorghum bicolour* (L.) *Moench*) grains were obtained from a local producer in Nairobi, Kenya. Grains were milled using two different processes: dry milling and wet milling. For dry milling, for both cereals, a Thermomix (Type TM5-2, Vorwerk Elektrowerke, Germany) was used (2 cycles of 15 seconds each at maximum velocity). For sorghum wet milling: overnight soaking was followed by removal of water, milling with a hand blender, sieving (particles < 500 µm), and flour sterilization at 121 °C, for 15 minutes. For millet wet milling: grains were sterilized at 121 °C, for 15 minutes, followed by overnight soaking, removal of water and milling (soaking and milling were carried out in a flow chamber).

After milling, the cereal flours were suspended in deionized water (1:9 (v/v)), a suspension that will be referred to thereafter as slurry.

3.3.2. Microorganisms, growth conditions and inoculation

Commercial starter cultures *Lactiplantibacillus plantarum* 299v (LP299V®) (obtained from PROBI AB, Lund, Sweden), *Lactobacillus acidophilus* Ki (obtained from CSK, Ede, Netherlands) and *Lacticaseibacillus rhamnosus* R-11 (Rosell®-11) (obtained from Lallemand, Montréal, QC, Canada) were used. The microorganisms, previously kept at -80 ºC, were individually activated at 2 % (v/v) in Man-Rogosa-Sharpe (MRS) broth (Biokar Diagnostics, Beauvais, France), at 37 °C, overnight, and the inoculum was propagated (at least twice) at 1 % in MRS-Broth, every 24 h until the day of inoculation. Each bacterium was inoculated at 1 % (v/v) in each slurry (Table 3.1), totalizing a final number of 12 inoculated slurries, corresponding to all possible combinations of three probiotic strains x two types of milling process (wet- and dry-milling) x two cereals (sorghum and finger millet). Each slurry was prepared in duplicate.

Table 3.1 Description of the different cereal slurries inoculated with *Lactiplantibacillus plantarum* 299v, *Lactobacillus acidophilus* Ki, and *Lacticaseibacillus rhamnosus* R-11, in plain culture.

Cereal	Milling process	*Lactobacillus* species
Sorghum	Wet	*L. plantarum* 299v
		L. acidophilus Ki
		L. rhamnosus R-11
	Dry	*L. plantarum* 299v
		L. acidophilus Ki
		L. rhamnosus R-11
Finger millet	Wet	*L. plantarum* 299v
		L. acidophilus Ki
		L. rhamnosus R-11
	Dry	*L. plantarum* 299v
		L. acidophilus Ki
		L. rhamnosus R-11

3.3.3. Fermentation Process

Each slurry fermentation occurred in an orbital incubator (Wiggen Hauser, Germany) at 30 °C, for 24 h. Samples were collected at different time points (before inoculation and at 0, 2, 4, 6, 8 and 24 h of fermentation) for microbiological analyses (enumeration of viable cell numbers of lactobacilli and of contaminant bacteria as indicators of microbiological quality control) and for chemical analysis (pH, concentrations of organic acids and sugars, total phenolics content, antioxidant activity, and iron content).

3.3.4. Enumeration of microorganisms

Different culture media were prepared for the enumeration of viable cell numbers of the different abovementioned bacterial strains: MRS Agar (Biokar Diagnostics, Beauvais, France), for enumeration of lactobacilli, Potato Dextrose Agar (PDA) (Biokar Diagnostics, Beauvais, France), for the enumeration of yeasts and moulds, Plate Count Agar (PCA) (Biokar Diagnostics, Beauvais, France), for the total bacterial count and Violet Red Bile Glucose Agar (VRBGA), for enumeration of Enterobacteriaceae (BioMérieux France). MRS Agar and PCA were used in all sampling time points, whereas PDA and VRBGA were only used before strain inoculation of the strains to confirm the microbiological quality of the cereal slurries (absence of contaminants).

Before inoculation, an aliquot of 1 mL of each slurry was diluted (1:10 (v/v)), using an aqueous isotonic solution of 1 g/L peptone (Sigma–Aldrich, Munich, Germany) and 8.5 g/L sodium chloride (Panreac, Barcelona, Spain), and plated on PDA, VRBGA, MRS agar and PCA (in duplicate) using the spread-plate technique. At each sampling time, decimal dilutions were plated on MRS agar and PCA (in duplicate), using the Miles and Misra drop-plate technique (Miles & Misra, 1938). Microorganisms were enumerated according to Miles and Misra following appropriate incubation at 37 ºC for 72 h in the case of PCA plates, and 48 h for MRS and VRBGA, and at 30ºC for PDA for 48 h. The results were expressed in colony forming units (CFU) per millilitre (mL) (CFU/mL).

3.3.5. Chemical analysis

3.3.5.1. Fermentative capacity - acidification, sugars consumption, and organic acids production

The pH was measured, at every time point, at room temperature using a pH meter, equipped with a pH electrode (Crison micro pH 2002, Spain).

Concentrations of sugars and organic acids were measured simultaneously with refractive index and ultra-violet detection, respectively, using high-performance liquid chromatography (HPLC). HPLC analysis was performed according to Sousa *et al*. (2015), based on Zeppa *et al*. (2001) with some modifications: approximately 2 g of each sample (eight-hour fermented slurries) were diluted in 5 mL of sulphuric acid 13 mM (95–97 % (p.a.), Merck), homogenized with an Ultra-Turrax (T18 Basic; IKA Works, Inc., Wilmington, NC, USA) at 18.000 rpm (rotations per minute) for 3 min, then filtered with nº 1 filter paper (V. Reis, Lisbon, Portugal) and, immediately prior to injection, with 0.45 µm pore size filters (Chromafil® PET - 45/25, Macherey-Nagel, Germany).

The HPLC system consisted of an auto sampler Midas (Spark Holland), a K1001 isocratic pump (Knauer Germany), an ion-exchange Aminex HPX-87H Column (300 mm × 7.8 mm) (Bio-Rad), which was maintained at 65 °C (Gecko 2000), and two detectors in series, a K2300 refractive index detector (Knawer Germany) to analyse sugars and organic acids, and a K2501 UV/Vis spectrophotometry detector (Knawer Germany) to analyse organic acids (220 nm). The mobile phase used was 13 mM sulphuric acid, at a flow rate of 0.6 mL/min. The running time was 30 min, and the injection volume was 20 µL. Each sample was injected once, and data were collected and analysed by Clarity System Software (version 5.0.5.98) and quantified using the appropriate chromatographic standards' calibration curve.

3.3.5.2. Total phenolics content and antioxidant activity

Before these analyses, samples were prepared in order to extract the compounds to measure. Briefly, the samples (eight-hour fermented slurries) were freeze-dried (Christ-Alpha1-4 B.Braun, pump vacuum-Oerlikon Trivac Leybold, Barcelona, Spain), then diluted (1:50) in acidified methanol (1 % Hydrocloric acid (32 %) in methanol), and slowly stirred for 2 h in a roller stirrer. Samples were centrifuged (Hettich Centrifugen Universal 32R, Germany) at 4300 g, for 10 min. The supernatant was kept at -20 °C for further analyses.

Total phenolics content was estimated as the concentration of gallic acid equivalents (GAE) (mg GAE/kg of slurry), according to the Folin-Ciocalteu method (Mendes et al., 2016). Briefly, to 50 µL of sample, 50 µL of Folin-Ciocalteu reagent (Merck, Germany), 1 mL of 75 g/L sodium carbonate (Sigma-Aldrich) and 1.4 mL of deionized water were sequentially added. The samples were then mixed using a vortex, and kept away from light for 60 min. Finally, absorbances were read at 750 nm (UV-Vis spectrophotometer UVmini 1240, Shimadzu, Tokyo, Japan), in triplicate.

Antioxidant activity was determined using two methods, namely the ABTS (2,2-Azinobis (3-ethylbenzothiazoline-6-sulphonic acid)) and DPPH (2,2-Diphenyl-1-picryl-hydrazyl) methods.

Despite the fact that both methods are based on an electron transfer mechanism and involve oxidation-reduction reactions, DPPH, which is insoluble in water, is more efficient for the measurement of less polar compounds, while ABTS, soluble in both water and organic (alcoholic) solvents, is suitable for the measurement of both polar and apolar compounds, depending on the solvent used.

The ABTS procedure was based on the methodology proposed by Re *et al*. (1999) and, later, modified by Gião *et al*. (2007). The ABTS•+ solution was prepared by adding in the proportion of 1:1 (v/v), 7 mmol/L ABTS diammonium salt (Sigma-Aldrich) solution to 2.45 mmol/L potassium persulfate (Merk, Darmstadt, Germany) solution. Then, the reaction was allowed to occur, on a roller stirrer for 16 h, at room temperature, protected from the light. The final solution that was obtained was kept at 4 °C until further use. On the day of measurements, the aforementioned ABTS•+ solution was duly diluted in deionised water, in order to obtain an absorbance of 0.700 ± 0.020, at 734 nm (UV-Vis spectrophotometer UVmini 1240, Shimadzu, Tokyo, Japan). Briefly, 10 µL of each sample were added to 1 mL of ABTS•+ diluted solution, and the absorbance at 734 nm was measured after 6 min. Each sample was read in triplicate. A calibration curve was prepared with ascorbic acid as standard, which was used to express the final results as the equivalent concentration of ascorbic acid (g/L) Also, antioxidant capacity was calculated and expressed as the percentage of inhibition (PI), according to the equation:

$$PI = \frac{Abs_{ABTS^{\bullet+}} - Abs_{sample}}{Abs_{ABTS^{\bullet+}}} \times 100,$$

Equation 3.1

where $Abs_{ABTS^{\bullet+}}$ corresponds to the initial absorbance of diluted $ABTS^{\bullet+}$ and Abs_{sample} corresponds to the absorbance of the sample after 6 min of reaction.

The DPPH procedure followed the method proposed by Bondet *et al*. (1997), with few modifications. A DPPH• concentrated solution was prepared by dissolving DPPH (Sigma-Aldrich) in methanol (Honeywell, Riedel-de Haen™) at the concentration of 600 µM, and kept at -20 °C, away from the light, until further use. On the day of measurements, the aforementioned DPPH• concentrated solution was ten-fold diluted in order to obtain an absorbance of 0.600 ± 0.100, at 515 nm (UV-Vis spectrophotometer UVmini 1240, Shimadzu, Tokyo, Japan). Briefly, 250 µL of sample was added to 1.75 mL of methanolic DPPH• solution allowed to react for 30 min, after which the absorbance was read at 515 nm. Each sample was read in triplicate. A calibration curve was prepared with Trolox as standard, which was used to express the final results as the equivalent concentration of Trolox (g/L). Also, antioxidant capacity was calculated and expressed as the percentage of inhibition, according to the equation:

$$PI = \frac{Abs_{DPPH^{\bullet}} - Abs_{sample}}{Abs_{DPPH^{\bullet}}} \times 100,$$

Equation 3.2

where $Abs_{DPPH^{\bullet}}$ corresponds to the initial absorbance of $DPPH^{\bullet}$ solution and Abs_{sample} corresponds to the absorbance of the sample after 30 min of reaction.

3.3.5.3. Iron content

Prior to the analysis of the iron content, samples were prepared for a pre-step of digestion in Speedwave MWS-3 (Berghof, Germany) microwave system: approximately 2 g of each sample (eight-hour fermented slurries) were centrifuged (Boeco Hematocrit Centrifuge H-240, Germany) at 14.000 rpm for 10 min: supernatants were kept, and pellets were freeze-dried (Christ-Alpha1-4 B.Braun, pump vacuum-Oerlikon Trivac Leybold) for 24 h. A certified reference material (rice flour standard – IRMM 804) was used as a positive control for digestion. Briefly, 250 mg of the previously freeze-dried samples and control were mixed with 5 mL of nitric acid (65 %, Merck, Germany) and 1 mL of hydrogen peroxide (30 %, Merck, Germany) in a teflon vessel, and heated in Speedwave MWS-3 (Bergof, Germany) microwave system, in a specific digestion programme (Gião et al., 2007). The digestion was divided into five steps (temperature/time, °C/min): 130/10, 170/15, 200/16, 100/7, 100/3. The resulting solutions were diluted with deionised water and then, iron concentration was determined using Inductively Coupled Plasma Optical Emission Spectrometry (ICP-OES) model Optima™ 7000 DualView (PerkinElmer, USA) with a radial plasma configuration, according to Rodrigues *et al*. (2015). A calibration curve was prepared with a multi analyte custom grade solution of HNO_3 5 % (stock concentration of iron 150 mg /L), which was used to express the final results.

3.3.6. Statistical Analysis

One-way analyses of variance (One-way ANOVA) followed by the Tuckey HSD post hoc test were tested aiming to study the effect of cereal type (differences between the cereals) or bacterial strain (differences between the bacterial strains). The independent variables used were the absolute difference (△) between the beginning (0 hours), after eight hours, and the end of fermentation (24 hours), for each analysed parameter: $\triangle t_{24} - t_0$, $\triangle t_{24} - t_8$, and $\triangle t_8 - t_0$. Moreover, in order to study the variables behaviour over the fermentation time, pairwise comparisons were carried out, using paired-samples T-tests.

Normality and homoscedasticity of data were checked by Shapiro-Wilk and Levene tests, respectively. In cases in which normality and/or homoscedasticity of data were not verified, Kruskal-Wallis and Mann-Whitney non-parametric tests were used as alternatives to ANOVA and Tukey's test, respectively. Significance level was set at 5 % (p-values ≤ 0.05) for all tests performed. Statistical analyses were conducted using IBM® SPSS® Statistics, version 23 (SPSS Inc., Chicago, IL, USA).

3.4. PHASE 2 – FERMENTATION OF AFRICAN WHOLE GRAINS BY *WEISSELLA* STRAINS

3.4.1. Sample preparation

In this second phase, red sorghum, and pearl millet (*Pennisetum glaucum* (L.)) were studied. Red sorghum was obtained from Nairobi, Kenya as described in sub-chapter 3.3.1. and pearl millet flour (already cleaned and milled < 500 µm particles' size) from Ouagadougou, Burkina Faso. This cereal was only possible to obtain from local producers at this stage of the work. In terms of milling, and based on the results achieved in phase 1, only the dry-milled flour proceeded from this stage onwards. In this second phase, where *Weissella* strains were under scrutiny, besides studying their fermentative capacity, a further objective was to assess their ability to produce EPS. Hence, two types of slurries were prepared:

a) dry-milled cereal flours were suspended in deionized water (1:9 (v/v));

b) dry-milled cereal flours were suspended in an aqueous sucrose-based solution (10 % (w/v)) (1:9 (v/v)).

3.4.2. Microorganisms, growth conditions and inoculation

Three strains of *Weissella confusa/cibaria*, namely *W. confusa/cibaria* C2, *W. confusa/cibaria* 32LABPT05 and *W. confusa* 2LABPT05, were isolated from traditionally fermented cereal-based products from Kenya and Burkina Faso (Table 3.2). Another *Weissella* strain, *W. confusa* VTT E-90E392, from the culture collection of the Technical Research Centre of Finland (VTT) was used as a reference strain for EPS production. Microorganisms' activation and propagation were carried out in MRS broth according to the description provided in sub-chapter 3.3.2. Each bacterium was inoculated at 1 % (v/v) in each slurry (Table 3.3), totalizing a final number of 16 inoculated slurries, corresponding to all possible combinations of four *Weissella* strains x two types of media (water-based or sucrose-based solution) x two cereals (sorghum and pearl millet). Each slurry was prepared in duplicate.

Table 3.2 Details about the origin of the isolated *Weissella* strains from African traditionally fermented cereal-based products.

***Weissella* strains**	**Origin**	**Cereals in formulation**
W. confusa/cibaria C2	*Uji* - Traditionally fermented cereal-based Kenyan beverage	A mixture of millets, sorghum, and maize
W. confusa 2LABPT05 *W. confusa/cibaria* 32LABPT05	*Massa* - Traditionally fermented cereal-based Burkinabe fried pancake	Pearl Millet

Table 3.3 Description of the different cereal slurries inoculated with *W. confusa*/*cibaria* C2, *W. confusa*/*cibaria* 32LABPT05, *W. confusa* 2LABPT05, *W. confusa* VTT E-90E392, in plain culture.

Cereal	Milling process	Suspension solution	*Weissella* species
Sorghum	Dry	Deionised water	*W. confusa/cibaria* C2
			W. confusa/cibaria 32LABPT05
			W. confusa 2LABPT05
			W. confusa VTT E-90E392
		Sucrose-based solution (10 % (w/v))	*W. confusa/cibaria* C2
			W. confusa/cibaria 32LABPT05
			W. confusa 2LABPT05
			W. confusa VTT E-90E392
Pearl millet	Dry	Deionised water	*W. confusa/cibaria* C2
			W. confusa/cibaria 32LABPT05
			W. confusa 2LABPT05
			W. confusa VTT E-90E392
		Sucrose-based solution (10 % (w/v))	*W. confusa/cibaria* C2
			W. confusa/cibaria 32LABPT05
			W. confusa 2LABPT05
			W. confusa VTT E-90E392

3.4.3. Fermentation Process

Fermentation was performed under the same conditions as previously described in the sub-chapter 3.3.3. Samples were collected at different time points (before inoculation and at 0, 2, 4, 8, 12, and 24 hours of fermentation) for microbiological analyses (enumeration of viable cell numbers of *Weissella* strains and of contaminant bacteria as indicators of microbiological quality control) and chemical analyses (pH, concentrations of organic acids and sugars, total phenolics content and total amino acids content).

3.4.4. Enumeration of microorganisms

Media preparation, microbiological growth and enumeration were carried out according to the methodology described in sub-chapter 3.3.4. Different culture media were prepared for the enumeration of viable cell numbers of the abovementioned *Weissella* strains: MRS Agar, PDA, PCA and VRBGA. As before, MRS Agar and PCA were used in all sampling time points, whereas PDA and VRBGA were only used before strain inoculation of the strains to confirm the microbiological quality of the cereal slurries (absence of contaminants).

Serial dilutions and platting techniques followed the previously described methodologies in the abovementioned sub-chapter: each slurry was diluted (1:10 (v/v)) in an isotonic aqueous solution of peptone (0.1% (m/v)) and sodium chloride (0.85% (m/v)), and plated on PDA, VRBGA, MRS agar and PCA (in duplicate) using the spread-plate technique. At each sampling time, decimal dilutions were plated on MRS agar and PCA (in duplicate), using the Miles and Misra drop-plate technique (Miles & Misra, 1938), and microorganisms' enumeration was performed also according to Miles and Misra, following appropriate incubation.

3.4.5. Chemical analysis

3.4.5.1. Fermentative capacity - acidification, sugars consumption, and organic acids production

The pH and concentrations of sugars and organic acids were measured according to the methodologies described in sub-chapter 3.3.5.1, with the exception of the Aminex HPX-87H Column temperature operation, used for organic acids and sugars quantification, which was established at 40 °C.

3.4.5.2. Viscosity – qualitative analysis

A qualitative analysis, based on visual evaluation, was carried out to detect differences in the viscosity of the final product, a consequence of the EPS production. A scale of intensity was used to classify the samples: high, medium, and low production indicated as +++, ++, and +/-, respectively.

3.4.5.3. Total phenolics content

Samples (eight-hour fermented slurries) were centrifuged (Boeco Hematocrit Centrifuge H-240, Germany) at 14.000 rpm, for 10 min. The supernatant was kept at -20 °C until further analyses. Total phenolics content was estimated according to the methodology described in sub-chapter 3.3.5.2. Results are presented as the concentration of GAE (mg GAE/kg of slurry).

3.4.5.4. Total amino acids content

Total amino acids content was determined by pre-column derivatization using the orthophthalaldehyde (OPA) methodology, according to the procedure of Pripi-Nicolau *et al*. (2000), based on Soufleros *et al*. (1998). Prior to the determination of the total amino acids content by HPLC, freeze-dried samples were subjected to acidic hydrolysis, based on the procedure described by Campos *et al*. (2017). Briefly, 10 mg of dried sample were hydrolysed with 3 mL de HCl 6 M, at 115 °C, for 20 h, in sealed glass vials that had undergone previous nitrogen injection to remove oxygen. The pH of the hydrolysed samples was adjusted to 9.5 and these were diluted to 10 mL with borate buffer. The HPLC system consisted of a 126 System Pump Beckman a Chromolith® Performance RP18 (4.6 mm x 100 mm) column (Merck), a fluorescence detector (Waters 474 FLD Detector, Milford. MA, USA) and a Spark Midas auto-sampler. The mobile phase consisted of two different solvents:

(A) 20 g sodium phosphate dibasic dihydrate (Sigma-Aldrich), 7.4 g of propionic acid (Sigma), 40 mL of dimethyl sulfoxide (Sigma), and 130 mL of acetonitrile (VWR). The pH was adjusted to 6.65 and the solution was made up to 2 L with Mili-Q water;

(B) 330 mL of methanol (Sigma-Aldrich), 70 mL of dimethyl sulphoxide, 400 mL of acetonitrile and 200 mL of Mili-Q water.

Three different reagents were sequentially added to the sample:

- reagent A (composed of homoserine (Merck) and norvaline (Merck) internal standards, mercaptoethanol (Sigma-Aldrich), sodium tetraphenylborate (Sigma-Aldrich) and borate (Boric Acid, Merck) buffer);
- reagent B (composed of iodoacetic acid (Sigma-Aldrich) and borate buffer, pH 9.5);
- reagent C (composed of OPA (Merck), methanol, borate buffer and mercaptoethanol).

The derivatization reaction was performed by the auto-sampler: to 100 µL of sample, 250 µL of reagent A and 250 µL of reagent B were added and mixed and left to react for 3 min. Then, 250 µL of reagent C were added and mixed and left to react for 3.5 min, followed by mixing and injection. Separation and quantification were performed by injecting 10 µL of the derivate using the HPLC system, with a linear multistep solvent gradient (100 % A initially, 10 % B at 30 min, 20 % B at 40 min, 25 % B at 45 min, 40 % B at 80 min, 85 % B at 110 min, 50 % B at 120 min, 5 % B at 123 min, ending at 125 min.) and were detected by fluorimetric detector ($\lambda_{excitation}$=356 nm, $\lambda_{emission=}$445 nm). Samples were analysed in duplicate, by 32 Karat Software (version 8.0, Beckman Coulter) and quantified using amino acids pure standards' calibration curve (Sigma-Aldrich).

3.4.6. Statistical Analysis

Statistical analyses were carried out according to the methodology described in the previous sub-chapter 3.3.6, using one-way ANOVA followed by the Tuckey HSD post hoc test. The only difference was that the variables analysed were the following: $\triangle t_{24} - t_0$, $\triangle t_{12} - t_{,0}$ and $\triangle t_8 - t_0$. As previously described, all statistical analyses were conducted using IBM® SPSS® Statistics, version 23 (SPSS Inc., Chicago, IL, USA).

3.5. PHASE 3 – CO-FERMENTATION OF AFRICAN WHOLE GRAINS BY *LACTIPLANTIBACILLUS* AND *WEISSELLA* STRAINS

3.5.1. Sample preparation

In this third phase, the three different cereals were studied, namely: red sorghum, finger millet, and pearl millet. From this stage onwards, the dry-milled flours were suspended in an aqueous sucrose-based solution (10 % (w/v) (1:9 (w/w)).

3.5.2. Microorganisms, growth conditions and inoculation

In this third phase, both plain and combined cultures were used. In the case of the plain cultures, the indigenous *Weissella* strains that were also used in phase 2, were inoculated alone. In the case of combined cultures, the inocula consisted of *Lactiplantibacillus plantarum* 299v combined with each *Weissella* strain). Each strain was individually cultured and propagated (at least twice) as indicated previously in sub-chapter 3.3.2. Both inocula were standardised to the same initial number of viable cells (10^6 CFU/mL or 10^8 CFU/mL, which value was indigenous strain-dependent) immediately mixed in a 1:1 ratio and inoculated at 1 % (v/v) in each slurry (Table 3.4), totalizing a final number of 18 inoculated slurries, corresponding to all possible combinations of three *Weissella* strains x two types of culture (plain or combined) x three cereals (sorghum, finger millet and pearl millet). Each slurry was prepared in duplicate.

Table 3.4 Description of the experimental conditions for each cereal slurry inoculated either with plain *Weissella* cultures (*W. confusa*/*cibaria* C2, *W. confusa*/*cibaria* 32LABPT05 or *W. confusa* 2LABPT05) or with co-cultures of *Lactiplantibacillus plantarum* 299v combined with each of the abovementioned *Weissella* strains.

Cereal	Type of culture	Probiotic strain	Indigenous *Weissella* strain
Sorghum	Plain	-	*W. confusa*/*cibaria* C2
	Combined	*L. plantarum* 299v	*W. confusa*/*cibaria* C2
Finger millet	Plain	-	*W. confusa*/*cibaria* C2
	Combined	*L. plantarum* 299v	*W. confusa*/*cibaria* C2
Pearl millet	Plain	-	*W. confusa*/*cibaria* C2
	Combined	*L. plantarum* 299v	*W. confusa*/*cibaria* C2
Sorghum	Plain	-	*W. confusa*/*cibaria* 32LABPT05
	Combined	*L. plantarum* 299v	*W. confusa*/*cibaria* 32LABPT05
Finger millet	Plain	-	*W. confusa*/*cibaria* 32LABPT05
	Combined	*L. plantarum* 299v	*W. confusa*/*cibaria* 32LABPT05
Pearl millet	Plain	-	*W. confusa*/*cibaria* 32LABPT05
	Combined	*L. plantarum* 299v	*W. confusa*/*cibaria* 32LABPT05
Sorghum	Plain	-	*W. confusa* 2LABPT05
	Combined	*L. plantarum* 299v	*W. confusa* 2LABPT05
Finger millet	Plain	-	*W. confusa* 2LABPT05
	Combined	*L. plantarum* 299v	*W. confusa* 2LABPT05
Pearl millet	Plain	-	*W. confusa* 2LABPT05
	Combined	*L. plantarum* 299v	*W. confusa* 2LABPT05

3.5.3. Fermentation Process

Each slurry fermentation was performed as previously described in the sub-chapter 3.3.3, except for the time of fermentation that was shortened, corresponding to the time required for a final pH around 4-5 to be reached (~ 8 hours). Samples were collected at different time points (before inoculation and at 0, 4, 8 h of fermentation) for microbiological analyses (enumeration of viable cell numbers of lactobacilli and *Weissella* strains and of contaminant bacteria as indicators of microbiological quality control), and for chemical analysis (pH, concentrations of organic acids and sugars, and total phenolics content).

3.5.4. Enumeration of *Lactiplantibacillus* and *Weissella* colonies

As bacteria were inoculated in a combined culture, a differential medium was used for individual enumeration. The preparation of this medium was based on Lee and Lee (2008). Briefly, to previously prepared MRS agar medium, bromophenol blue (BPB) was added at 0.002 % (final concentration), then the medium was autoclaved and L-cysteine×HCl (final concentration of 0.05 %, Sigma Aldrich, St Louis, MO, USA) was filtered (0.22 µm Minisart® High Flow syringe filters, Sartorius AG, Germany) and added. Enumeration of the two types of bacteria was based on specific morphology. Both produced colonies with a dark blue circle in the centre, however *Weissella* strains were much darker than the *Lactiplantibacillus* strain and, consequently, easily distinguishable

(Figure 3.3). All the other media were prepared, plated and incubated as previously described in sub-chapter 3.3.4.

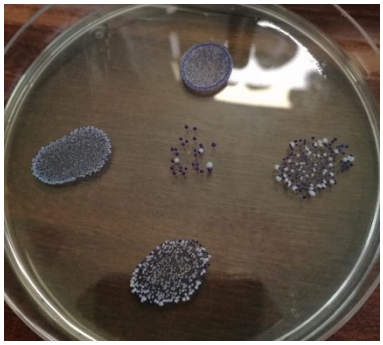


Figure 3.3 Colonies of *Lactiplantibacillus plantarum* 299v (lighter blue) and *Weissella confusa* 2LABPT05 (darker blue), as they appeared on MRS containing BPB.

3.5.5. Chemical analysis

3.5.5.1. Fermentative capacity - acidification, sugars consumption, and organic acids production

The pH of each slurry was measured, and the concentrations of organic acids and sugars were determined, at each sampling point, by using the same methodologies previously described in sub-chapter 3.4.5.1.

3.5.5.2. Viscosity – qualitative analysis

The viscosity of each slurry was analysed in order to follow the fermentation properties of the specific strains involved. A qualitative analysis was carried out following the same methodology previously described in sub-chapter 3.4.5.2.

3.5.5.3. Total phenolics content

In order to assess the impact of fermentation on the total phenolics content of each slurry, the determination was carried out using the same methodology previously described in sub-chapter 3.4.5.3.

3.5.6. Statistical Analysis

Statistical analyses were carried out according to the methodology described in the previous sub-chapter 3.3.6, using one-way ANOVA followed by the Tuckey HSD post hoc test. The only difference being that the variable analysed was $\triangle t_8 - t_0$. Moreover, at this phase in order to study differences between the type of cultures the independent T-student test was used. All statistical analyses were conducted using IBM® SPSS® Statistics, version 24 (SPSS Inc., Chicago, IL, USA).

PART III – NOVEL SYNBIOTIC FERMENTED WHOLE GRAIN-BASED PRODUCTS

After the selection of the best combination match between bacterial consortium and cereal matrix, a new fermentation run, under the same operational conditions, was carried out, aiming the production of a new yoghurt-like fermented beverage. Dry-milled finger millet flour was suspended in an aqueous sucrose-based solution (10 % (w/v)) (1:9 (w/w)), and the resulting slurry was inoculated with the co-culture including *L. plantarum* 299v and *W. confusa* 2LABPT05 (each slurry was prepared in triplicate). Microorganisms' activation and propagation were carried out according to the previous description in sub-chapter 3.3.2. In this fermentation process, strains were inoculated at the same percentage (1 % (v/v)) but not standardised to the same initial number of viable cells (in a ratio of 1:100, *Weissella*:*Lactiplantibacillus*). Preliminary trials showed that such protocol step allowed the probiotic strain to achieve higher viable cell numbers than when inoculated on a similar viable cell number basis. Non-inoculated slurries were similarly prepared and followed during the fermentation process, working as a control. Slurries were incubated for 8 h at 30 ºC, 200 rpm. Samples were collected at different time points of fermentation process (before inoculation and at 0, 4, 6, 8 hours of fermentation). The final fermented product that will be referred to hereafter as yoghurt-like beverage (YLB), was characterized in terms of microbiological, physicochemical and sensory profiles.

3.6. VISCOUS FERMENTED FINGER MILLET-BASED PRODUCT: FRESH FERMENTED YOGHURT-LIKE BEVERAGE

3.6.1. Fermentative capacity - acidification, sugars consumption, and organic acids production

The pH of the product was measured, and the concentrations of organic acids and sugars were determined by using the same methodologies previously described in sub-chapter 3.4.5.1.

3.6.2. Biological activity

3.6.2.1. Total phenolics content and antioxidant activity

Prior to these analyses, the EPS produced during fermentation were precipitated with cold ethanol (1:1 (v/v)) overnight, followed by centrifugation at 4.000 rpm for 20 mi at 4 °C (Pintado et al., 2006). The supernatant was recovered and used for further analyses. Unfermented and fermented samples were studied. Total phenolics content and antioxidant activity were determined as described in the sub-chapter 3.3.5.2.

3.6.2.2. Antidiabetic activity –α-glucosidase inhibitory activity

Samples used for this analysis were the same as those used for organic acids and sugars determination. Unfermented and fermented samples were studied. The α-glucosidase inhibitory activity was determined in 96 well plates according to the method described by Kwon *et al*. (2008). The supernatant (50 µL) at the concentration of approximately 30 mg cereal flour/mL, was mixed with 100 µL of 0.1 M phosphate buffer (pH 6.9) containing α-glucosidase solution (1.0 U/mL) and pre-incubated at 25 ºC for 10 min. Then, 50 µL of 5 mM p-nitrophenyl-α-D-glucopyranoside solution in 0.1 M phosphate buffer (pH 6.9) was added to each well at 5 seconds intervals. The reaction mixtures were incubated at 25ºC for 5 min, and the absorbance readings were recorded at 405 nm by a multiscan microplate fluorimeter (FLUOstar optima, BMG Labtech, Offeuburg, Germany) and compared to a control which had 50 µL of H_2SO_4 13 mM in the place of the sample. Acarbose (Sigma) was used as a positive control at the concentration of 10 mg/mL. The α-glucosidase inhibitory activity was expressed as percentage of inhibition and was calculated as follows:

$$PI = \frac{\Delta Abs_{control} - \Delta Abs_{sample}}{\Delta Abs_{control}} \times 100,$$

Equation 3.3

where $\Delta Abs_{control}$ is the variation of absorbance of the control sample and ΔAbs_{sample} is the variation of absorbance of the samples, between the beginning and after the 5 min of reaction.

3.6.3. Nutritional properties

3.6.3.1. Mineral content

The mineral content of the samples was determined according to the methodology described in sub-chapter 3.3.5.3, with some modifications. Briefly, 400 mg of samples (unfermented and fermented samples) previously freeze-dried were mixed with 5 mL of nitric acid (65 %, Merck, Germany) and 2 mL of hydrogen peroxide (30 %, Merck, Germany) in a teflon vessel and heated in the microwave system. The digestion was divided into five steps (temperature, °C/time, min): 130/5, 170/5, 190/15, 100/2, 100/2. The following steps were carried out according to the methodology described in the abovementioned sub-chapter.

3.6.3.2. Free-amino acids content

The objective was to determine the free amino acids content, hence acidic hydrolysis was not performed before the analysis. The sample used was the supernatant, obtained after precipitation of EPS, as previously described above in sub-chapter 3.6.2.1. The free amino acids content was determined by pre-column derivatization with OPA methodology, according to the methodology described in sub-chapter 3.4.5.4.

3.6.3.3. Protein digestibility

The *in vitro* protein digestibility (IVPD) of the samples was determined by the combination of the methods of Arte *et al*. (2015) and Elmaki *et al*. (1999) with some modifications. Prior to this analysis, unfermented and fermented samples were freeze-dried. Samples (500 mg) were incubated with 1.5 mg of pepsin in 15 mL of 0.1 M HCl at 37 °C, 150 rpm for 3 h. After neutralization with 2 M NaOH, 4 mg of pancreatin in 7.5 mL of phosphate buffer (pH 8.0) were added, followed by 1 mL of toluene to prevent microbial growth, and the solution was incubated at 37 °C, 150 rpm, for 24 h. After 24 h, the enzyme was inactivated by the addition of 10 mL of trichloroacetic acid (10 %, w/v), and the undigested protein was precipitated. Samples were centrifuged at 5000 *g* for 20 min, at room temperature. Nitrogen (N) in the supernatant was determined by the Kjeldahl method (AOAC, 1965). A negative control (following the same protocol, but without sample) was included in the run together with the samples. Protein digestibility was calculated as follows:

$$\text{IVPD (\%)} = \frac{\text{N}_{\text{supernatant}}}{\text{N}_{\text{original sample}}} \times 100,$$

Equation 3.4

where the original sample is the freeze-dried sample before digestion.

3.6.3.4. Proximate composition

The obtained fermented whole grain finger millet-based YLB was nutritionally characterized. Protein (Kjeldahl method in which assessed nitrogen content was multiplied by 6.25 to estimate protein content), total fat (Soxhlet method), total sugars (Munson and Walker technique), fibre (AOAC

991.43, and AOAC 985.29), sodium (Flame Atomic absorption spectroscopy), and moisture (drying at atmospheric pressure at 102 °C) were analysed. These analyses were outsourced, carried out by the CINATE laboratories, an autonomous certificated structure of the Faculty of Biotechnology.

3.6.3.5. Apparent viscosity – quantitative analysis

The apparent viscosity of the YLB was determined using the rotating springless viscometry B-ONE PLUS (Lamy Rheology, Champagne au Mont d'Or, France), with the MS ASTM measuring system, and a R-2 mobile disc. Apparent viscosity was measured at 20 ± 2 ºC and 8 ± 2 ºC, at constant shear rate (60 s^{-1}), after 10 seconds, in triplicate. This combination of parameters intended to simulate the texture of the YLB perceived by the consumer at the moment of tasting (Moufle et al., 2018). Moreover, viscosity (η (γ)) was also measured at different shear rates in order to study the type of fluid in question. Viscosity values were given by the viscometer, which calculation is based on the following equation:

$$\eta = \tau\gamma = KTAU \times MKD \times \omega,$$

Equation 3.5

where η is the apparent viscosity (Pa.s), τ is the shear stress (Pa), γ is the shear rate (s^{-1}), KTAU and KD are constants related to the measuring system (KTAU = 55.65 and KD = 1), M is the torque (mN.m) and ω is the rotational speed (rpm).

3.6.3.6. Exopolysaccharides' characterization and quantification

EPS structural characterization - NMR analysis

Prior to structural analysis, EPS were extracted from the cell mass of *W. confusa* 2LABPT05, grown on MRS agar, according to Maina *et al*. (2008). EPS structural analysis was carried out using nuclear magnetic resonance (NMR) spectroscopy, according to Maina *et al*. (2008). Briefly, samples (10 mg of the extracted EPS) were exchanged three times with D_2O (1 mL), by dissolving and freeze-drying. The samples were then dissolved in 700 µL D_2O, filtered and placed in NMR tubes (Wilmad NMR tubes, 5 mm, ultra-imperial grade, Aldrich, Milwaukee, WI, USA). NMR data were recorded on a Bruker Avance III NMR spectrometer (Bruker BioSpin, Rheinstetten, Germany) working at 600 MHz with a QCI cryoprobe. The measurements were performed at 50 °C, and the chemical shifts were referenced to acetone (^{1}H=2.225 ppm).

The NMR system was composed by a Varian Unity 500 spectrometer (Varian NMR Systems, Palo Alto, CA, USA) operating at 500 MHz for ^{1}H and using 5 mm triple-resonance pulsed-field gradient (PFG) probes. The one-dimensional (1D) ^{1}H spectrum was acquired with a spectral width of 8000 Hz, pulse width of 9 µs, 4 s acquisition time, and 64 scans.

Dextran quantification - enzyme-assisted method

EPS (dextran) quantification was accomplished using an enzyme-assisted method, using the unfermented and fermented freeze-dried samples according to the methodology of Katina *et al*. (2009). To the freeze-dried samples (100 mg), 3 mL of aqueous ethanol (50 % v/v) was added. Then, the mixture was placed in a boiling water bath over 5 min, vortexed and another 3 mL aqueous ethanol were added. Samples were vortexed, centrifuged (10000 *g*, 10 min) and the supernatant discarded. The pellet was re-suspended in 5 mL of aqueous ethanol, followed by another centrifugation and supernatant disposal. This step of sample washing with ethanol was included in order to remove free sugars and short oligosaccharides that interfered with the assay by increasing the level of background glucose. For hydrolysis, the resultant pellet was re-suspended in 4.5 mL of sodium citrate buffer (pH = 5.5) and kept in a boiling water bath for 2 min. Then, samples were vigorously vortexed and placed back in the water bath for 3 min more. After cooling, 10 000 nkat/g dextranase (originated from *Chaetomium erraticum*, Sigma–Aldrich, Germany) and 1000 nkat/g α-glucosidase (from *Aspergillus niger*, Megazyme, Ireland), were added to the solutions and the final volume adjusted to 5 mL. Enzyme hydrolysis was then carried out for 48 h at 30 °C, after which the solutions were placed in a boiling water bath for 10 min, to inactivate enzymes. Subsequently, samples were centrifuged, and the supernatants recovered for glucose quantification, using high performance anion exchange chromatography. For correction of background glucose, a control blank sample was treated as the other samples but only hydrolysed with the α-glucosidase. The amount of dextran was determined as the sum of anhydro-glucose, which calculation was based on the following equation:

$$\text{Dextran content (\%)} = \frac{\frac{(Glc\ in\ B - Glc\ in\ A)\ mg \times 5\ mL \times 0.9 \times 1.6}{mL}}{100\ mg} \times 100,$$

Equation 3.6

where Glc is the glucose content in mg/mL, B refers to the tested samples (hydrolysed by dextranase and glucosidase), A is relative to the control sample (hydrolysed only by α-glucosidase), 5 mL is the final volume, 0.9 is the correction of anhydro-glucose and 1.6 is the correction factor determined for dextrans in the finger millet matrix.

3.6.4. Sensory Analysis

The fermented finger millet-based YLB was subjected to sensory analysis by a non-trained consumer tasting panel (n= 30), using a questionnaire with open and closed questions (Appendix K), developed by the authors together with an expert in sensory evaluation. Five different cereal YLB samples were tasted in two subsequent phases (Table 3.5):

1) the objective of the first phase was to evaluate the original YLB (with no added aroma) in terms of appearance, colour, consistency, and texture using a 9-point hedonic scale ranging from dislike extremely (1) to like extremely (9);

2) the second phase (first step) intended to evaluate several parameters, namely, global acceptability, sweetness, acidity, quality of the aroma and flavour of four samples flavoured with different aromas; understanding that the fermented cereal beverage may stand on its own but may also be used as an ingredient to be incorporated in a dairy or non-dairy food matrix. This second phase included a second step in which participants were also asked to taste the flavoured cereal beverage together with a non-sweetened natural yoghurt and to evaluate it in terms of global acceptability using the same 9-point hedonic scale.

All the aromas (coffee, chocolate, hazelnut and blueberry) were kindly provided by FRULACT S.A (Maia, Portugal). Final questions related to the hypothetical purchase of the product, regardless of the aroma, were also asked. The incorporation of the YLB in another food, namely a yoghurt, was planned to target an additional market positioning, eventually easier to be achieved, since the combination of both products – fermented finger-millet-based product and yoghurt – is likely to be more easily accepted and successful in terms of consumer appreciation.

Table 3.5 Cereal yoghurt-like beverages (YLB) subjected to sensory analysis by a non-trained consumer tasting panel (n = 30).

Tasting phases	Description
1	Cereal YLB tasting without aroma (1 sample))
2	**2.1.** Cereal YLB tasting with aroma[1] without yoghurt (4 samples)
	2.2. Cereal YLB tasting with aroma[1] with yoghurt (4 samples)

[1] Coffee; Chocolate; Hazelnut; Blueberry.

3.6.5. Stability throughout storage – fresh fermented finger millet yoghurt-like beverage

Upon fermentation, the resulting fresh fermented YLB (F-YLB) was stored, in triplicate, at 4 ºC, and acidification and microbiological stability were assessed at three different moments: at 7, 14, and 21 days of storage. Enumeration of microorganisms, pH measurement and quantification of sugars and organic acids were carried out according to the previously described methodologies in sub-chapter 3.4.5.1.

3.6.6. Statistical Analysis

Paired-sample T-tests were used to study differences between each parameter before and after fermentation, and also during storage. Shapiro-wilk was used for checking normality of data. In cases in which normality of data was not verified, the Wilcoxon non-parametric test was the alternative test used.

Sensory data were descriptively expressed by the median, minimum, and maximum. The non-parametric Wilcoxon test was applied to study differences between the global appreciation of the YLBs with and without yoghurt. The chi-square (χ^2) test of independence or Fischer test was used to study associations between texture and global appreciation/certainty of purchase. The Kruskal-Wallis test was used to study differences in classifications between the four aromas.

Significance level was set at 5 % (p-values ≤ 0.05) for all tests performed. Statistical analyses were conducted using IBM® SPSS® Statistics, version 24 (SPSS Inc., Chicago, IL, USA).

3.7. POWDERED FERMENTED FINGER MILLET-BASED PRODUCT: DRIED FERMENTED YOGHURT-LIKE BEVERAGE

3.7.1. Preliminary test – Spray-drying, Tray-drying and Freeze-drying

In order to obtain a powdered fermented finger-millet-based product, different drying methods were tested, namely spray-drying, tray drying and freeze-drying. Drying yield was assessed for each drying process.

3.7.1.1. Spray-drying

A trial was carried out with 100 mL of sample, in a laboratory-scale mini-spray dryer model B-191 (Büchi Laboratoriums-Technik, Flawil, Switzerland), with a two-fluid nozzle atomizer with a 1 mm inside diameter, a concurrent drying chamber of 10.5 cm, and a single cyclone air separator system. The drying conditions were established as follows: drying air flow rate at 86 %, feeding solution flow rate at 8 %, inlet and outlet air temperatures of 130 °C, resulting in an outlet temperature of 65 °C. In this equipment, it is not possible to regulate the outlet temperature, since it depends on the inlet air temperature, the feed rate, the drying gas flow rate and the solids content of the feed. More trials were carried out to test the effect of sample dilution (1:50 and 1:10), and also feeding solution flow rate (8%,12%, and 15%).

3.7.1.2. Tray-drying

A first trial was done in order to select the most appropriate material to pour the sample to be dried. Three different materials were tested: aluminium foil, tracing paper, and plastic film. For this phase, only 5 g of sample were dried and the factor evaluated was the easiness with which the sample unstuck from the respective material, in a laboratory-scale dryer (Tray Dryer Armfield Ltd, England), with coupled scale and thermometer. Drying conditions were established as follows: air velocity at 6 m/s and drying temperature at 45 ± 1°C. During the whole process, air outlet velocity, temperature, and weight were controlled. For air outlet velocity monitoring an anemometer (Airflow instrumentation LCA 6000) was used. After the selection of the best supporting material for the fermented liquid sample, 25 g of sample were poured into it and dehydrated until no variation of weight was measurable.

3.7.1.3. Freeze-drying

Fifty grams of each sample were transferred into appropriate flasks under aseptic conditions and frozen at -80 °C overnight. The freeze-drier (Telstar lyoquest, Barcelona, Spain) was operating at 0.004 mbar and -60 °C for 48 h. Cell viability after freeze-drying was assessed. Microbiological analyses were carried out according to the methodology described in sub-chapter 3.5.4.

After selection of the best drying process (freeze-drying), different protective agents were tested: a polysaccharide and a polyalcohol, maltodextrin (Md) and sorbitol (Sb), respectively, both at 1 % and 2.5 % (w/w) and a non-reducing disaccharide, trehalose (Tr) at 1 % (w/w) (Table 3.6).

Table 3.6 Cryoprotectants (maltodextrin, sorbitol, and trehalose) concentration used in freeze-dried samples (in duplicate).

	Cryoprotectant concentration (%)		
Sample	**Sorbitol**	**Maltodextrin**	**Trehalose**
1	0	0	0
2	1	0	0
3	0	1	0
4	0	0	1
5	2.5	0	0
6	0	2.5	0

Once the effect of the cryoprotectant was tested and the most favourable cryoprotectant selected, another experiment was carried out with such protectant in order to assess the protective effect on strains' survival. Fifty grams of the fresh F-YLB were transferred into each of six flasks under aseptic conditions and frozen at -80 °C overnight. Prior to freezing, to a set of three flasks the cryoprotectant sorbitol was added at 1 %, and to the other set of three flasks no cryoprotectant was added, acting as a control. Similarly, microbiological analyses were carried out according to the methodology described in sub-chapter 3.5.4.

3.7.2. Stability throughout storage – freeze-dried fermented finger millet yoghurt-like beverage

After the freeze-drying process, the resultant fermented powders (in the presence and absence of cryoprotectant) were stored in a desiccator, in triplicate, at room temperature and away from light. During storage microbiological stability, acidification, and concentrations of sugars, organic acids and free amino acids were analysed at four different moments: at three, six, nine and 12 weeks. Enumeration of microorganisms, pH measurement and quantification of sugars and organic acids were followed according to the methodologies described in sub-chapter 3.4.5.1, whereas the quantification of amino acids followed the described methodology in sub-chapter 3.6.3.2.

3.7.3. Statistical Analysis

Paired-sample T-tests were used to study differences between each parameter before and after the drying process and also during storage. One-way ANOVA followed by Tukey HSD post-hoc test were performed to study the impact of cryoprotectants on microbial viability and differences of viable cell numbers after freeze-drying and at each time during storage, for the different cryoprotectants.

Normality and homoscedasticity of data were checked by Shapiro-Wilk and Levene tests, respectively. In cases in which normality and/or homoscedasticity of data were not verified, the Wilcoxon, Kruskal-Wallis and Mann-Whitney non-parametric tests were the used alternatives to paired-T tests, ANOVA and Tukey's test, respectively. Significance level was set at 5 % (p values ≤ 0.05) for all tests performed. Statistical analyses were conducted using IBM® SPSS® Statistics, version 24 (SPSS Inc., Chicago, IL, USA).

3.8. *IN VITRO* GI DIGESTION SIMULATION AND GUT MICROBIOTA IMPACT

The fermented finger-millet-YLB was submitted to a two-phase digestion, where the first step was the *in vitro* GI digestion simulation and the second step the faecal culture fermentations. Afterwards, beneficial and potentially harmful bacteria were enumerated using real-time quantitative-PCR (RT qPCR).

3.8.1. *In vitro* gastrointestinal digestion simulation

3.8.1.1. Simulated digestion fluids

In order to simulate the digestion of the fermented product, the standardized static *in vitro* digestion method, advanced by Minekus *et al*. (2014), specifically the protocol developed from it (Brodkorb et al., 2019), was followed. All the simulated digestion fluids (Simulated Salivary Fluid (SSF), Simulated Gastric Fluid (SGF), and Simulated Intestinal Fluid (SIF)), $CaCl_2$ (Honneywell Fluka) and water were previously prepared. Enzymatic solutions were prepared on the day of the experiment:

- salivary α-amylase (α-amylase from human saliva Type XIII-A, lyophilized powder, 300-1500 units/mg protein, Sigma) solution at 75 U/mL in the final mixture, was made up in SSF electrolyte stock solution;

- porcine pepsin (pepsin from porcine gastric mucosa, powder, ≥ 250 units/mg solid, Sigma) at 2000 U/mL in the final mixture, was made up in SGF electrolyte stock solution;

- pancreatin (pancreatin from porcine pancreas, powder solution), at 100 U/mL and bile salts (Oxoid Limited, United Kingdom) at 12 g/L, were made up in SIF electrolyte stock solution.

3.8.1.2. Oral, gastric and intestinal phases

After the eight-hour fermentation process, the obtained liquid fermented product (the so-called fermented YLB) was freeze-dried, without cryoprotectants. To simulate the oral phase, water was added to this freeze-dried fermented product (1:2 (w/w)), followed by the addition of the SSF electrolyte stock solution (800 µL/ml sample), $CaCl_2$ (5 µL/ml sample), salivary α-amylase solution (195 µL/ml sample) and water (final ratio of food to SSF of 50:50). The mixture was thoroughly mixed and incubated for 2 min at 37 °C, at 200 rpm in an orbital incubator. To simulate the gastric phase, to the previous oral bolus, SGF electrolyte stock solution (800 µL/mL oral bolus), $CaCl_2$ (0.5 µL/mL oral bolus), pepsin solution (50 µL/mL oral bolus), HCl (to reach pH 3.0) and water were sequentially added (final ratio of oral bolus to SGF of 50:50). The mixture was thoroughly mixed and incubated for 2 h at 37 °C, at 200 rpm in an orbital incubator. After gastric digestion simulation, the intestinal phase was mimicked. To the gastric chyme, SIF electrolyte stock solution (425 µL/mL gastric chyme), $CaCl_2$ (2 µL/mL gastric chyme), pancreatin solution (250 µL/mL gastric chyme), bile solution (125 µL/mL gastric chyme), NaOH (to reach pH 7.0) and water were sequentially added (final ratio of oral

bolus to SIF of 50:50). The mixture was thoroughly mixed and incubated for 3 h at 37 °C, at 200 rpm in an orbital shaker.

Afterwards, the absorption in the small intestine was simulated by including a step of dialysis. The digested fermented YLB (DF-YLB) was transferred to 1 kDa molecular weight cut-off regenerated cellulose dialysis tubing (Spectra/Por 6 dialysis tubing, 1 kDa, 45 mm flat-width, 10 meters/roll, Spectrum Europe, Netherlands) and dialysed against NaCl 0.01 M, to remove low molecular mass digestion products, at room temperature, at 500 rpm in a magnetic plate stirrer. After 15 h, the fluid was changed and dialysis continued for two more hours, after which the content was transferred to freeze-drying recipients and DF-YLB was freeze-dried, aiming to produce a powder to be used for the next phase, the faecal culture fermentations.

Cell viability was evaluated after the digestion process simulation. Suitable dilutions of the DF-YLB suspension were plated on MRS agar supplemented with BPB and PCA, by the Miles technique, as previously described in sub-chapter 3.3.4. Also, free *L. plantarum* 299v and *W. confusa* 2LABPT05 suspensions were followed in order to study the impact of the cereal matrix carrier on strains' viability.

3.8.2. Faecal fermentations for studying gut microbiota impact

3.8.2.1. Recruitment of participants and collection of samples

Healthy adult volunteer donors (n=5) were purposively recruited in the Faculty of Biotechnology, Catholic University of Portugal. Eligibility criteria included being a healthy adult between 18 and 40 years-old, to have an omnivorous diet, without any food intolerances/allergies, and no consumption of prebiotic or probiotic supplements (including 'yoghurts with bifidus') or antibiotics in the prior six months. An informed consent form (Appendix L) was signed by the volunteers who agreed to participate in the study. Plus, each participant was given a document with the information about the study, and also with all the necessary instructions about on how to collect faecal samples together with the required collection material (Appendix M). Collection of samples was done by the participant, into clean containers, which were immediately closed after the placement of an anaerobic sachet inside the container together with the sample.

3.8.2.2. Faecal fermentation conditions and procedure

A basal nutrient medium, pH 7.0 (peptone water 2 g/L, yeast extract 2 g/L, NaCl 0.1 g/ L, K_2HPO_4 0.04 g/L, KH_2PO_4 0.04 g/L, $MgSO_4 \cdot 7H2O$ 0.01 g/L, $CaCl_2 \cdot 6H_2O$ 0.01 g/L, $NaHCO_3$ 2 g/L, Tween 80 2 mL/L, hemin 0.05 g/ L, vitamin K 10 µL/L, L-cysteine HCl 0.5 g/L, bile salts 0.5 g/L and resazurin 4 mg/L) was previously prepared according to Mota de Carvalho *et al*. (2019). Three independent fermentations, with independent tubes for each sampling time, were run in parallel:

1) the digested fermented YLB;

2) a positive control (C+), FOS from chicory root, purity:>95 %, degree of polymerization between 2 to 8 (Megazyme, Bray, Ireland);

3) a negative control (C-), which had no source of carbon added (instead of sample, the basal medium was added).

The DF- YLB and the C+ were suspended at 2 % (w/v) in the basal nutrient media and then dispensed to the tubes (9.8 mL), in an anaerobic workstation (Don Whitley Scientific, West Yorkshire, UK), maintained at 37 °C, under anaerobic atmosphere (10 % CO_2, 5 % H_2 and 85 % N_2). For the C-, the medium was directly dispensed to the tubes (9.8 mL). Faecal samples were used within 30 min of collection. Five independents faecal inocula were prepared in phosphate-buffered saline (PBS) solution (10 %, w/v), in the above-cited anaerobic atmospheric conditions. Faecal inoculum was added at 2 % to each tube containing the basal medium.

Samples were collected at different time points (0, 6, 12 and 24 h after inoculation) for pH control during fermentation. Moreover, these sampling aliquots were used for the determination of organic acids and sugars concentration and extraction of genomic DNA (gDNA).

3.8.2.3. Acidification and organic acids production

The pH of independent tubes (C+, C- and DF-YLB) was measured, at every time point, at room temperature using a pH meter, equipped with a pH electrode (Crison micro pH 2002, Spain). Samples were then stored at -20 °C, until further analyses. Previously frozen samples, were defrosted and aliquots of 2 mL were centrifuged (Boeco Centrifuge U-320R, Germany) at 4000 *g*, for 10 min. The supernatant was used for the evaluation of organic acids production, according to the operational conditions described in the sub-chapter 3.4.5.1. The pellet was further used for gDNA extraction.

3.8.3. Bacterial enumeration using Real-Time quantitative-PCR

3.8.3.1. DNA extraction and quantification

DNA was extracted using the NZY Tissue gDNA Isolation kit (NZYTech, Lisbon, Portugal) and some adaptions were made to the standard manufacturers' protocol (Annex I). To the previously mentioned pellet, an aliquot of 2 mL of sample was added and another centrifugation was carried out, at 4000 *g*, for 10 min. The supernatant was discarded and then the pellet washed with 1 mL of TE buffer (10 mM Tris/HCl; 0.1 mM EDTA, pH 8.0, previously prepared), vortexed, and centrifuged at 4000 *g*, for 10 min, which process was repeated until obtention of a colourless supernatant. To promote the pre-lysis of the sample, 300 µL of buffer NT1 was used to re-suspend the sample, dissolving the pellet by an up and down movement with the pipette. From this, 200 µL were transferred to a new eppendorff, which was vortexed and incubated at 95 °C, for 10 min. After this incubation time, 25 µL of Proteinase K were added and the samples were vortexed and incubated at 56 °C, for 1 h 45 min, vortexing after half of the period. In order to lyse the sample, samples were vortexed and 200 µL of buffer NL was added, followed by vortex agitation for 10 seconds and centrifugation at 11500 *g*, for 10 min. The obtained supernatant was transferred to a new eppendorff and the pellet discarded. The following steps, namely the addition of ethanol, the DNA binding, the washing and drying of silica membrane, and, finally the elution of the DNA were carried out exactly

according to the abovementioned protocol. Once extracted, the concentration and purity (through the analysis of Abs_{260nm}/Abs_{230nm} and Abs_{260nm}/Abs_{280nm} ratios) of DNA were determined using a Thermo Scientific™ µDrop™ Plate coupled with a Thermo Scientific™ Multiskan™ FC Microplate Photometer (Thermo Fisher Scientific, Waltham, USA). The obtained concentration of gDNA was diluted and equilibrated to 20 ng/µL. The stock and diluted genomic DNA samples were stored at – 20 °C until further analysis.

3.8.3.2. Bacterial enumeration using Real-Time quantitative-PCR

Standard Calibration curves with gDNA

Standard calibration curves were created according to the instructions provided by Applied Biosystems, in which the following steps are described:

1) identification of the genome size of the organism of interest;

2) identification of the number of copies of the gene of interest (16S rRNA gene) per genome of the organism of interest;

3) calculation of the mass of DNA per genome and per copy of 16S rRNA gene;

4) calculation of the mass of gDNA needed to contain the number of copies of interest;

5) calculation of the concentration of gDNA needed to achieve the number of copies of interest;

6) preparation of serial dilutions of the gDNA.

Four bacterial genomic DNA standards (*Bifidobacterium longum* subsp. *Infantis*, *Clostridium leptum, Roseburia hominis, Phocaeicola vulgatus*) were obtained from DSMZ (DSMZ, Braunschweig, Germany), and the other two bacterial strains (*Lactocaseibacillus rhamnosus* Lcr35, obtained from *Laboratórios Azevedos – Indústria Farmacêutica*, *S.A*. and *Faecalibacterium prausnitzii* A2-165 (DSM 17677), obtained from DSMZ), stored in the internal collection of the Faculty of Biotechnology, were cultured and genomic DNA was extracted from the bacterial cells.

Information about the genome size and the number of copies of the 16S rRNA gene was obtained in public databases, namely the GenomeNet database (Kyoto University Bioinformatics Center), the ribosomal RNA database (Center for Microbial Systems, University of Michigan) or the NCBI (National Center for Biotechnology Information) genome database. Standard calibration curves were constructed by plotting the log of the number of copies of the 16S rRNA gene of each bacterial strain against the quantification cycle (Cq).

Real-Time quantitative-PCR

A CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad Laboratories, Inc., Hercules, USA) was used to perform qPCR. The cycling program of qPCR was made up by the following steps:

1) initial denaturation/enzyme activation, at 95 ºC, for 5 min;

2) denaturation, at 95 ºC, for 10 seconds;

3) annealing, for 1 min, at primer specific temperature;

4) extension, at 72 ºC, for 30 seconds;

5) melt curve generation, at 60-97 ºC, with an increment of 0.5 ºC, for 5 seconds.

Steps 2, 3, and 4 comprised the amplification reaction and they were repeated 45 times (45 cycles). The PCR reaction volume was 10 µL of the following mixture: 5 µL of 2x iQTM SYBR® Green Supermix (Bio-Rad Laboratories, Inc., Hercules, USA), 2 µL of ultrapure water, 1 µL of sample diluted DNA (20 ng/µL) and 1 µL of forward and reverse primers (100 nM) targeting the 16S rRNA gene. All assays were performed in quadruplicate and in every run, a calibration curve was constructed.

The annealing temperature of each primer (STABvida, Lisbon, Portugal) was optimized. The optimal annealing temperature was determined by testing a range of annealing temperatures above and below the calculated T_m of the primers of each bacterial strain. The optimal annealing temperature was then established for the PCR runs. Table 3.7 presents the primers sequence, and respective optimal annealing temperature, the genome size and the number of copies of the 16S rRNA gene, for each used bacterial strain.

3.8.4. Statistical Analysis

For the GI simulation, independent-T test was used to detect differences in the number of viable cells between samples (free-cells or inoculated in the cereal slurries).

For the fermentation assay, one-way ANOVA coupled with Tukey's post-hoc test were carried out to detect significant differences on the log number of 16S rRNA gene copies obtained for between samples (C-, C+ and DF-YLB) at distinct times (0 h, 6 h, 12 h or 24 h).

Normality and homoscedasticity of data were determined by Shapiro-Wilk's and Levene's tests, respectively. In cases in which normality and/or homoscedasticity of data were not verified, Kruskal-Wallis and Mann-Whitney non-parametric tests were the used alternatives to ANOVA and Tukey's test/independent T-test, respectively. Significance level was set at 5 % (p-values ≤ 0.05) for all tests performed. Statistical analyses were conducted using IBM® SPSS® Statistics, version 26 (SPSS Inc., Chicago, IL, USA).

Table 3.7 Primer sequences targeting bacterial groups and respective optimal annealing temperature and genomic DNA standards and respective genome size (base pairs) and the number of copies of the 16S RNA gene.

Target group	Primers sequence (5'-3') (F: Forward; R: Reverse)	Optimal Annealing temperature (°C)	Microorganism	Strain reference	Genome size (base pairs)	Copies of 16S RNA gene
***Bifidobacterium* spp.**	F: CGC GTC YGG TGT GAA AG R: CCC CAC ATC CAG CAT CCA	62	*Bifidobacterium longum* subsp. *infantis*	DSM 20088 (S12)	2832748	4
***Lactobacillus* spp.**	F: CAC CGC TAC ACA TGG AG R: AGC AGT AGG GAA TCT TCC A	59	*Lacticaseibacillus rhamnosus*	Lcr35	2937400	5
***Clostridium leptum* subgroup**	F: GCA CAA GCA GTG GAG T R: CTT CCT CCG TTT TGT CAA	57.5	*Clostridium leptum*	DSM 753 (VPI T7-24-1)	3270109	2
***Roseburia* spp.**	F: TAC TGC ATT GGA AAC TGT CG R: CGG CAC CGA AGA GCA AT	60	*Roseburia hominis*	DSM 16839 (A2-183)	3592125	4
Faecalibacterium prausnitzii	F: GGA GGA AGA AGG TCT TCG G R: AAT TCC GCC TAC CTC TGC ACT	60	*Faecalibacterium prausnitzii*	DSM 17677 (A2-165)	3214418[1]	6[1]
***Bacteroides* spp.**	F: ATA GCC TTT CGA AAG RAA GAT R: CCA GTA TCA ACT GCA ATT TTA	54.5	*Phocaeicola vulgatus* (former *Bacteroides vulgatus*)	DSM 1447	5163189	7

[1] These parameters refer to *Faecalibacterium prausnitzii* SL3/3, given the lack of this information on the strain used.

CHAPTER 4.

RESULTS AND DISCUSSION

"*Give us the tools, and we will finish the job.*"

Winston Churchill, 1941

PART I – DIETARY INTAKE ASSESSMENT

4.1. THE DIETARY SURVEY IN KENYA

4.1.1. Phase 1 – Development of a semi-quantitative FFQ

The final food list of the FFQ had 123 food items distributed over eleven food groups (Table 4.1). This well-established food list did not cause boredom on participants; and it was comprehensive enough to include the most common items eaten in Nairobi. The selection of the food items was based on three important aspects: the items had to be usual for a significant number of people; the items had to be nutritive; and to be discriminating, their consumption had to vary from person to person (Willet, 2013b).

Table 4.1 Design characteristics of the developed FFQ.

Food Groups (number of items)	Frequency options		Consumed amount	
Cereals and grain products (22) Vegetables (21) Legumes, pulses, seeds and nuts (5)	Never 1-3 1	per month	Smaller than Equal to Bigger than	Standard portion
Fruits (15) Meat, fish and eggs (18)	2-4 5-6	per week		
Dairy products (7) Fats and oils (6) Sauces, seasonings and flavourings (7) Sugar, syrups and sweets (9) Beverages (10) Composite dishes (3)	1 2-3 4-6 + 6	per day		

The consumption frequency was recorded in nine pre-specified categories from ‘never or less than once per month’ to ‘six or more times per day’, as suggested by Willet (2013b), in order to discriminate between highly consumed and less consumed items and to avoid loss of information.

In what concerns food portion information, three portion size options were established – the standard portion, a smaller and a bigger portion – using the standard portion size as the comparison term. The definition of a standard portion for each item tried to facilitate the process of describing quantities by participants. The food weighting study allowed the establishment of portion sizes in a way that subjects were able to conceptualise the unit and relate it to their own habits. This procedure was successfully achieved for most items; however, some challenges emerged and will be discussed below. Although the layout of the questionnaire was explicit by

itself, and though liable to be self-administrated, the population under study had revealed some difficulties in visualising clearly the portion sizes and bearing in mind the period that was being covered in terms of consumption. Consequently, the research team found it to be more reliable, feasible and ascertained to do face-to-face interviews.

4.1.2. Phase 2 – Test for validity and reproducibility of the developed FFQ

4.1.2.1. Subjects

A total of 75 volunteers took part in the current study: 35 from the UN Kabete Campus, 10 from a residential area in Uthiru (Dagoreti South constituency) and 30 from the UN Main Campus (Starehe constituency). For the validity study, it is not imperative to obtain a representative sample, however, it is advisable that it should be heterogeneous in order to represent different food consumption patterns (Willet, 2013d). Consequently, young, middle-aged and older women and men were included.

Out of the 75 participants enrolled in the current study, a total of 71 (94.7 %) completed the three recalls, three respondents (n=3, 4 %) only completed two recalls and one FFQ, and one (n=1, 1.3 %) did not complete any recall. These latter respondents were therefore excluded from the final sample. Furthermore, respondents who had a daily dietary intake above 5000 kcal (n=3, 4.2 %) were considered outliers and, consequently, were also excluded. For the validity study, the first applied FFQ was used to be tested against the three recalls. The final sample considered for the validity study was composed of 68 (90.7 %) respondents. In the case of the reproducibility study, data from the first and last interviews (first and second application of the FFQ) were considered. In addition to the individuals previously excluded based on energy intake, another four who did not reply to the second FFQ were, consequently, also excluded. The final sample for the reproducibility study included 68 (90.7 %) participants.

The sociodemographic profile of participants (n=69), in both validity and reproducibility studies, is presented in Table 4.2 (67 participants were common to both studies; in what concerns the other two from the 69, one only participated in the validity study and the other only in the reproducibility study). The median age of the sample was 26 years-old (P25-P75, 23-31 years-old), with 48 % being women (n=33) and 52 % being men (n=36). At the time of interview, 46 % (n=32) of respondents had a job (self-employed or employee), while the other 54 % (n=37) were students or casual labourers. More than half of participants (n=43, 62 %) had no children under their care at home. According to lifestyle habits, almost the whole sample (96 %, n=66) reported to be non-smokers and 30 % claimed to be a regular consumer of alcoholic beverages (18 % (n=6) of women and 42 % (n=15) of men). More than half of respondents (n=46, 67 %) ate three meals a day, namely breakfast, lunch and dinner.

Table 4.2 Characterization of participants in both studies (validity and reproducibility) (n=69), by the application of the general questionnaire (Absolute (n) and relative frequency (%)).

	n (%)		n (%)
Age (years)		**Number of children living at home**	
≤25	33 (47.8)	0	43 (62.3)
26-29	13 (18.8)	1-2	23 (33.3)
30-34	14 (20.3)	3-4	3 (4.3)
35-39	5 (7.2)		
≥ 40	4 (5.8)		
Gender		**Number of meals per day**	
Female	33 (47.8)	1-2	74 (15.8)
Male	36 (52.2)	3	242 (51.6)
		4-6	153 (32.6)
Education Level		**Smoking habits**	
None	1 (1.5)	Yes	3 (4.3)
Secondary	45 (65.2)	No	66 (95.7)
Tertiary	23 (33.3)		
Occupation		**Alcohol consumption**	
Casual Labourer	8 (11.6)	Yes	21 (30.4)
Formally employed	27 (39.1)	No	48 (69.6)
Self-Employed	5 (7.3)		
Student	29 (42.0)		
Marital Status		**Number of meals per day**	
Married	25 (36.2)	2	6 (8.7)
Single	44 (63.8)	3	46 (66.7)
		4-5	17 (24.6)

4.1.2.2. Validity study

The developed FFQ presented higher nutrient intakes compared to the multiple recalls, thus showing moderate agreement. Table 4.3 shows a comparison between absolute daily nutrient intakes estimated by the first semi-quantitative FFQ and by the three non-consecutive 24hR. Intakes are expressed in medians, P25 and P75. For almost all nutrients, the FFQ resulted in higher values compared with the mean value of the three recalls. Values were similar for total fat, PUFA, trans-fatty acids (TFA) and vitamin D. According to Willet, such difference observed between these two methods is common (Willet, 2013d). Similar results were obtained by other investigators (Jackson et al., 2012; Mouratidou et al., 2005; Segovia-Siapco et al., 2007; Silva-Jaramillo et al., 2015; Zack et al., 2018), in the assessment of dietary intake in adults. The main sources of errors of a FFQ, and consequently, the causes for overestimation are: fixed list of foods, memory (medium-term memory, in this case), perception of portion sizes, and interpretation of questions (Willet, 2013b). In this study, the medium-term memory and perception of portion sizes were the main factors that affected results. To clarify the portion sizes to the respondents, it could have been possible to show them the developed photographic food album used for the recalls. However, there is no clear evidence that using photographs during the application of a FFQ improves correlations significantly (Paiva et al., 2004; Willet, 2013d). Besides, the time spent in an interview using the photographic album would also have been longer, which could have been critical for this study, considering our limited schedule and resources. It has been

observed that the more recalls are made, the less the difference between intakes from the questionnaire and recalls (Willet, 2013d). Based on this fact, it would be clear that increasing the number of recalls would improve the correlation between both methods. However, the study of Papazian and colleagues, in which one recall was obtained to validate a FFQ (Papazian et al., 2016), revealed correlation coefficients not so different compared to those obtained by other studies in which more recalls were conducted.

Spearman correlation coefficients (unadjusted, energy-adjusted and/or deattenuated) between both methods are also shown in Table 4.3. For all macronutrients, the unadjusted correlations were above 0.38. Vitamin B_{12}, vitamin D, TFA, carbohydrates and total fibre were the nutrients with the highest crude correlation coefficients (0.52, 0.52, 0.52, 0.50 and 0.49, respectively). The range of unadjusted Spearman correlations of the present study was similar to that obtained in other studies with analogously applied methodology (Athanasiadou et al., 2016; Jackson et al., 2012; Mouratidou et al., 2011; Silva-Jaramillo et al., 2015). However, other researchers reported better (Cheng et al., 2008; Papazian et al., 2016) and worse (Dehghan et al., 2012; Gunes et al., 2015) results when compared to those of the present study. Among these, only the study of Jackson and collaborators was conducted in Africa, specifically in Botswana, which confirms the region's poorness in these types of studies (Jackson et al., 2012).

Table 4.3 Absolute daily nutrient intakes estimated by semi-quantitative food frequency questionnaire (FFQ) and three non-consecutive 24-hour recalls (24hR), Spearman correlations coefficients (for unadjusted and energy-adjusted values and de-attenuated) and level of agreement and disagreement between both methods (FFQ and 24hR) (n=68).

Nutrient	FFQ			24hR			Spearman correlation coefficients				Agreement in the same quartile (%)	Agreement in the same or adjacent quartile (%)	Extreme disagreement (%)
	Median	P25[1]	P75[1]	Median	P25	P75	Unadjusted	Energy-adjusted	De-attenuated	Energy-adjusted and de-attenuated			
Energy (kcal)	2997.5**	2417.0	3586.7	2031.6	1744.7	2359.4	0.444	-	0.547	-	39.7	77.9	2.94
Protein (g)	95.5**	84.0	127.9	59.5	52.5	77.9	0.390	0.383	0.479	0.470	27.9	76.5	7.35
Total Fat (g)	80.6**	66.9	107.0	71.2	58.2	89.8	0.381	0.171	0.468	0.210	32.4	76.5	5.88
SFA[2] (g)	25.1**	20.3	32.5	20.8	14.6	26.5	0.314	0.236	0.394	0.296	32.4	70.6	8.82
MUFA[3] (g)	26.5**	20.8	35.5	20.9	16.8	27.4	0.414	0.251	0.507	0.308	24.0	75.0	4.41
PUFA[4] (g)	20.4	15.8	26.2	20.4	17.4	24.3	0.296	-0.145	0.360	-0.176	45.6	72.1	7.36
TFA[5] (g)	0.7	0.4	0.8	0.5	0.3	0.8	0.516	0.526	0.529	0.540	35.3	85.3	4.41
Cholesterol (g)	220.1**	145.1	301.9	148.5	79.8	212.9	0.337	0.345	0.405	0.415	36.8	76.5	4.41
Carbohydrates (g)	474.1**	370.7	568.2	297.8	254.9	345.7	0.495	0.371	0.648	0.486	41.2	80.9	4.41
Total Fibre (g)	52.4**	39.6	64.8	25.9	19.6	31.9	0.491	0.377	0.593	0.455	41.2	83.8	4.41
Vitamin B_1 (mg)	2.5**	2.3	3.3	1.8	1.4	2.1	0.336	0.324	0.436	0.420	35.3	73.5	5.88
Vitamin B_2 (mg)	2.3**	1.7	2.7	1.4	1.1	1.7	0.445	0.454	0.501	0.511	38.2	75.0	4.41
Vitamin B_3 (mg)	27.4**	21.0	35.9	17.6	15.0	21.5	0.335	0.356	0.421	0.447	32.4	70.1	5.88
Vitamin B_{12} (µg)	4.6**	1.8	9.1	2.0	1.3	3.5	0.517	0.354	-	-	44.1	70.1	2.94
Vitamin D (µg)	3.4*	2.1	5.9	2.9	1.5	4.3	0.519	0.452	0.656	0.572	42.6	77.9	2.94
Vitamin E (mg)	8.9**	7.0	11.5	6.8	5.9	8.2	0.370	0.339	0.434	0.398	36.8	76.5	5.88
Folate (µg)	744.6**	569.8	967.6	418.8	304.2	497.5	0.389	0.113	0.496	0.144	38.2	79.4	8.82
Calcium (mg)	290.8**	961.0	1658.2	731.7	498.7	936.7	0.420	0.498	0.517	0.614	41.2	75.0	7.35
Iron (mg)	29.1**	23.7	36.9	18.5	14.2	22.9	0.371	0.174	0.477	0.224	35.3	69.1	2.94
Magnesium (mg)	653.5**	477.3	842.7	347.3	293.9	469.2	0.436	0.406	0.533	0.497	29.4	80.9	4.41
Zinc (mg)	11.6*	9.4	15.4	8.2	6.6	11.9	0.340	0.126	0.351	0.130	39.7	70.6	4.41

Median values were significantly different from those estimated from repeat recalls: *p≤0.05,**p≤0.001; *p*-values were calculated using the non-parametric Wilcoxon Signed Ranks test.

[1] P25: Percentile 25th; P75: Percentile 75th; [2] SFA: Saturated fatty acids; [3] MUFA: Monounsaturated fatty acids; [4] PUFA: Polyunsaturated fatty acids. [5] TFA: Trans fatty acids.

When energy-adjusted, these correlations tended to decrease. However, for some nutrients the correlation factor slightly increased, namely for TFA (from 0.52 to 0.53), cholesterol (from 0.34 to 0.35), vitamin B_3 (from 0.34 to 0.36) and calcium (Ca) (from 0.42 to 0.50). Some studies also experienced improvements (when energy-adjusted) in protein (Jackson et al., 2012; Mouratidou et al., 2011), fibre (Jackson et al., 2012) and in micronutrients correlations (Cheng et al., 2008; Jackson et al., 2012; Mouratidou et al., 2005; Papazian et al., 2016; Silva-Jaramillo et al., 2015), especially for Ca, iron (Fe) and vitamin E. The adjustment for random within-person day-to-day intake variation in recalls made the coefficients increase (unadjusted *vs*. deattenuated and energy-adjusted *vs*. energy-adjusted and deattenuated). Energy-adjusted and deattenuated coefficients ranged between 0.13 (zinc, Zn) and 0.61 (Ca). For macronutrients, these values were higher, with the exception of fat. De-attenuation adjustment showed a general improvement of coefficients (unadjusted or energy-adjusted), meaning that the contribution of the within-person error was considerable. This might be justified by the non-monotonous diet of urban Kenyans, not necessarily in terms of the type of foods, but especially in quantities and in the number of meals per day.

Although correlations are the most commonly used method in validity studies, they have some limitations since they do not study the agreement of both methods, but only if they are associated. Thus, other statistical approaches were followed. On the one hand, the levels of agreement in the same quartile, adjacent quartiles and extreme disagreement were also deeply studied and are expressed in Table 4.3. According to the results for the envisaged nutrients, on average, 36.7 % and 75.9 % of participants were classified into the same quartile (the values ranged from 24.0 % for monounsaturated fatty acids (MUFA) to 45.6 % for polyunsaturated fatty acids (PUFA)) and into the same or adjacent quartiles (85.3 % for TFA and 69.1 % for Fe). Only 5.2 % were classified in opposite quartiles (2.94 % for energy, vitamins B_{12}, vitamin D and Fe, and 8.8 % for folate and saturated fatty acids (SFA)). On the other hand, the Bland-Altman method was also followed in order to study the agreement between both methods. These plots assess the agreement between the methods across the range of intakes (Bland & Altman, 1986; Cade et al., 2002). The analysis of Bland–Altman plots enabled recognising that the agreement for energy [Figure 4.1(a)], carbohydrates [Figure 4.1(b)], total fibre [Figure 4.1(c)], total fat [Figure 4.1(d)] and PUFA [Figure 4.1(e)] was moderate. By the analysis of the plots, it was possible to conclude that for lower intakes the difference between both methods was smaller when compared with higher intakes, where the difference seemed larger. The overall mean difference corroborated the first conclusions since it was clear that there was an overestimation of the FFQ against the recalls. However, for total fat, especially for the PUFA fraction, despite the fact that the Bland-Altman diagram had shown a good agreement between both instruments, the correlation coefficient for energy-adjustment was very weak. Moreover, for this nutrient, the agreement into the same quartile was also high, as previously mentioned. These results show the importance of a multi-level evaluation of the correlation and association of different methods.

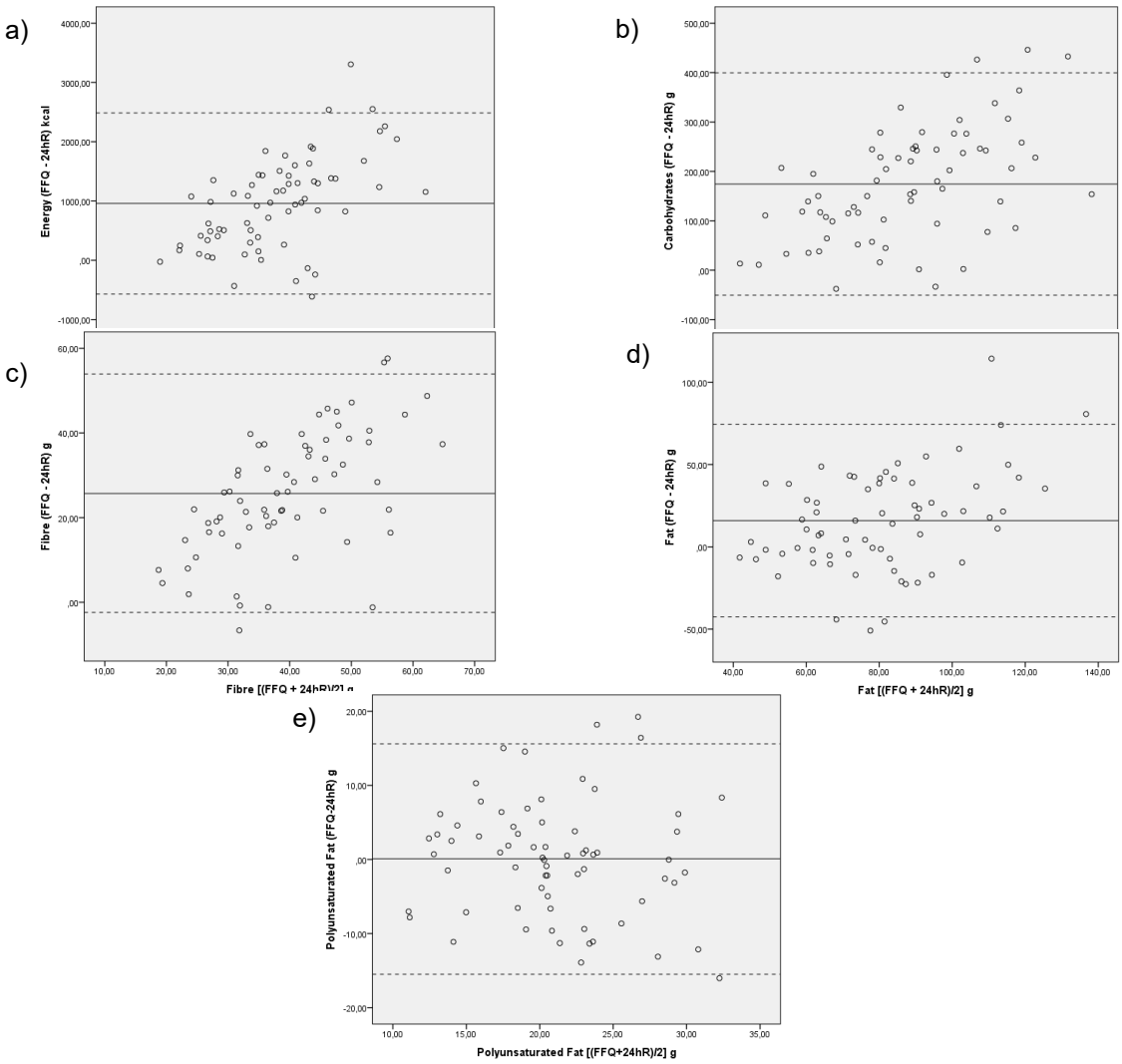


Figure 4.1 Bland–Altman plot between the food frequency questionnaire and the three non-consecutive 24-hour recalls estimating total energy intake (a – total energy, b – carbohydrates, c – total fibre, d – total fat, e – polyunsaturated fat). The solid line represents the mean difference in absolute intake between the two methods and the dashed lines represent the limits of agreement (mean ± 1.96 × standard deviation of the difference).

4.1.2.3. Reproducibility study

In order to assess reproducibility, FFQs were applied twice, within a period of 21 days. This FFQ captured the consumption of the previous month, so ideally the second questionnaire should have been applied, at maximum, 30 days apart. This interval was maximized as possible, in order to avoid answers' memorization between the first and the second application. Nevertheless, memorization was likely difficult due to the high quantity of food items in this FFQ.

In general, the participants tended to report higher intakes in the first application of the FFQ. Other studies experienced the same, i.e., in the first FFQ administration respondents gave answers which resulted in higher intakes (Dehghan et al., 2012; Jackson et al., 2012; Mouratidou et al., 2011; Silva-Jaramillo et al., 2015). This might be explained by the acquisition of the ability to answer the questions over time. According to Willet, the processing of diet recalling might

modify consciousness of food intake and, consequently, improve accuracy in completing the questionnaire (Willet, 2013d). Table 4.4 shows a comparison between absolute daily nutrient intakes estimated by the first application and the second application of the semi-quantitative FFQ.

In terms of the studied nutrients, crude Spearman's correlations were high, reporting an average value of 0.62 (ranging between 0.37 for vitamin D and 0.70 for Ca). Adjustments for total energy tended to decrease correlations, ranging between 0.15 for PUFA and 0.65 for niacin (vitamin B_3). Intra-class coefficients (ICC) ranged between 0.17 for vitamin B_{12} and 0.70 for magnesium (Mg). The correlations observed for macronutrients were all above 0.6. Jackson *et al*. (2012) and Mouratidou *et al*. (2011) obtained results similar to those of the present study, except for vitamin D. In contrast, other studies revealed poorer correlations (Cheng et al., 2008; Gunes et al., 2015; Mouratidou et al., 2005). The studies of Papazian *et al*. (2016) and Silva-Jaramillo *et al*. (2015) also resorted to ICC to evaluate the reproducibility of the FFQ, however correlations of both research works were higher than the ones observed in the present study. Nevertheless, ICC coefficients above 0.6 showed that within-person variation was not very high, showing moderate reliability (Koo & Li, 2016).

The mentioned studies and others that also tested the validity of a FFQ among adults were explored and organized, and the results of this exploitation are presented in Table 4.5.

Table 4.4 Absolute daily nutrient intakes estimated by the first (FFQ1) and second (FFQ2) applications of the semi-quantitative food frequency questionnaire. Spearman correlation coefficients (for unadjusted and energy-adjusted values) and intra-class correlation coefficients (FFQ1 and FFQ2).

Nutrient	FFQ1			FFQ2			Spearman correlation coefficients		Intra-class correlation coefficients
	Median	P25[1]	P75[1]	Median	P25	P75	Unadjusted	Energy-adjusted	
Energy (kcal)	2977.6**	2333.9	3586.7	2505.8	1834.4	2935.7	0.684	-	0.659
Protein (g)	95.5**	82.4	127.8	81.6	66.30	103.75	0.676	0.613	0.670
Total Fat (g)	80.6**	65.7	106.97	70.9	50.65	87.43	0.686	0.319	0.645
SFA[2] (g)	25.1**	19.9	32.53	21.3	15.92	27.52	0.632	0.263	0.575
MUFA[3] (g)	26.5**	20.7	35.49	21.8	14.94	29.24	0.651	0.434	0.619
PUFA[4] (g)	20.4**	15.2	26.19	15.7	9.74	19.53	0.617	0.150	0.611
TFA[5] (g)	0.62	0.36	0.82	0.54	0.34	0.69	0.575	0.495	0.575
Cholesterol (g)	220.1*	137.9	301.93	177.9	105.74	279.43	0.632	0.427	0.526
Carbohydrates (g)	474.1**	367.0	568.21	376.1	286.95	462.66	0.660	0.280	0.613
Total Fibre (g)	52.0**	38.4	64.80	37.6	30.58	50.58	0.634	0.479	0.630
Vitamin B_1 (mg)	2.6**	2.2	3.28	2.1	1.68	2.57	0.676	0.443	0.624
Vitamin B_2 (mg)	2.2**	1.7	2.74	1.8	1.48	2.30	0.552	0.470	0.612
Vitamin B_3 (mg)	27.4**	20.9	35.91	22.5	17.71	27.35	0.625	0.651	0.614
Vitamin B_{12} (µg)	4.4	1.7	9.09	2.8	1.63	6.59	0.562	0.503	0.166
Vitamin D (µg)	3.3	2.1	5.83	3.4	2.12	5.55	0.365	0.309	0.334
Vitamin E (mg)	8.9**	6.6	11.54	7.0	4.75	8.52	0.465	0.404	0.393
Folate (µg)	744.6**	565.0	967.62	555.7	443.46	725.76	0.593	0.400	0.623
Calcium (mg)	1290.8**	902.7	1658.17	1035.3	843.36	1341.27	0.696	0.597	0.638
Iron (mg)	28.8**	23.5	36.89	23.0	16.73	28.61	0.658	0.467	0.687
Magnesium (mg)	653.5**	477.3	842.69	496.0	367.47	664.73	0.673	0.622	0.704
Zinc (mg)	11.6**	9.2	15.41	9.8	7.92	12.15	0.623	0.387	0.560

Median values were significantly different from those estimated from FFQ2: *P≤0.05,**P≤0.001; *P*-values were calculated using the non-parametric Wilcoxon Signed Ranks test.

[1] P25: Percentile 25th; P75: Percentile 75th;

[2] SFA: Saturated fatty acids;

[3] MUFA: Monounsaturated fatty acids;

[4] PUFA: Polyunsaturated fatty acids.

[5] TFA: Trans fatty acids.

Some limitations should be considered when interpreting the present results. Regarding the reproducibility study, the period between interviews may have been short. It would have been more adequate to spread the interviews slightly more, however, this was not achievable within the time frame and resources available for the study. Concerning the validity of the FFQ, three non-consecutive recalls were used to validate the questionnaire, however other methods could have been hypothesised, for example, food records or even biomarkers. According to other investigations, researchers observed better agreements with FFQ (MacIntyre et al., 2001b). All methods have their own limitations and sources of errors. Besides, in some circumstances, the study population or the study site does not allow the implementation of the ideal method for comparison, which leads to the choice of a potentially less effective or appropriate method. In the present case, participants' illiteracy and poor motivation made the multiple 24hR the only reasonable choice. Additionally, the research team recognises the likely source of correlated errors by the usage of the same food software for the analysis of nutrient intakes. Considering the application of both tools, interviewers found it difficult for respondents to estimate the consumed amount of certain kinds of foods. *Vegetables* was the food group that offered more challenges, mainly because Kenyans do not eat a single vegetable at a time, but a mix of several types. Moreover, the estimation of meat (mainly beef and pork) was difficult in some cases, mainly because meat is not commonly eaten in large pieces such as beef, but in small pieces, in mixed preparations. Consequently, this hindered the portion size estimation. Nevertheless, meat is not a major component of the main course, and so, this might not have influenced that much the dietary intake estimation. Another specificity in Kenya is that in some places, even in urban areas, people share a big plate, or a bowl, which makes the estimation of portions sizes a challenge.

Considering the scarcity of tools to assess dietary intake in African populations, this work is a valuable contribution to future studies. The tool comprises a wide list of foods and it is possible to be completed in a 25-30 minute-interview, avoiding respondents' lack of patience and, consequently, inaccuracy in the dietary assessment. This validated tool may be used to rank individuals according to their nutrient or food intake, in order to further associate with disease development or prevalence.

Table 4.5 Literature review on the validity of food-frequency questionnaires (FFQ) among adults.

Author/Year	Country	Reference method	Sample	FFQ characteristics			Validity Study		Reproducibility Study
				Type of FFQ	Number of food items	Reference time period	Range of Correlation (energy-adjusted)	Level of Agreement (unadjusted)	Range of Correlation
(Zack et al., 2018)	Tanzania	Two 24hR	317 adults	semi-quantitative	179	1 month	-0.02 – 0.26	-0.04 – 0.26	N.A. [1]
(Athanasiadou et al., 2016)	Greece	Two 24hR	179 pregnant women	semi-quantitative	221	1 month	Crude: 0.35 (cholesterol) – 0.77 (SFA) [3]	0.15 (vit. B_{6}) – 0.42 (magnesium) [4]	N.A. [1]
(Papazian et al., 2016)	Lebanon	One 24hR	128 (validity) 38 (reproducibility) pregnant women	semi-quantitative	157	N.I. †	0.312 (cholesterol) – 0.563 (carbohydrates) [3]	62 % (folic acid) - 88 % (carbohydrates) [6]	0.935 (calcium) – 0.984 (vit. D) [7]
(Gunes et al., 2015)	Turkey	Four 24hR	120 adults	semi-quantitative	229	1 year	0.017 (vit. C and A) – 0.539 (zinc) [3]	21.7 % (vit. C) – 49.2 % (fat) [6]	N.A. [1]
(Silva-Jaramillo et al., 2015)	Ecuador	Three 24hR	345 adults	semi-quantitative	111	1 year	0.20 (total fat) – 0.61 (calcium) [3]	Energy-adjusted: 27.4 % (total fat) – 52.7 % (calcium) [6]	0.77 (vit. A) – 0.88 (calcium) [6]
(Dehghan et al., 2012)	Argentina	Three/Four 24hR	256 adults (urban/rural)	semi-quantitative	96	1 year	Crude: Urban: 0.20 (retinol) –0.47 (carbohydrate); Rural: 0.11 (PUFA) – 0.47 (Folate) [3]	Urban: 46.1 (energy) – 24.7 (fibre) Rural: 16.4 (phosphorus and PUFA) 49.3 (fibre) [6]	Urban setting: 0.30–0.56 Rural setting: 0.32–0.60 3
(Jackson et al., 2012)	Botswana	Four 24hR	79 adults	quantitative	122	1 year	0.23 (iron)- 0.49 (protein) [3]	27 % (retinol) to 72 % (alcohol) [6]	0.39 (retinol) – 0.66 (vit. E) [3]
(Mouratidou et al., 2011)	United Kingdom	Four 24hR	101 postpartum women	semi-quantitative	N.I. [2]	1 month	0.01 (vit. B12) – 0.57 (iron) [3]	Energy-adjusted: 54 % (starch, vit. B_{12}) – 84 % (manganese) [5]	-0.21 (total fat) – 0.67 (zinc) [3]

[1] N.A.: Not applicable;
[2] N. I.: No Information;
[3] Spearman/Pearson correlations coefficients; [4] Cohen's kappa; [5] Bland-Altman mean and 95 % CI; [6] Agreement in the same (or same and adjacent) percentile; [7] Intra-class correlations coefficients.

Author/Year	Country	Reference method	Sample	FFQ characteristics			Validity Study		Reproducibility Study
				Type of FFQ	Number of food items	Reference time period	Range of Correlation (energy-adjusted)	Level of Agreement (unadjusted)	Range of Correlation
(Cheng et al., 2008)	China	Six 24hR	12 pregnant women	semi-quantitative	68	3 months	0.22 (protein) – 0.60 (calcium, retinol) [3]	28 % (phosphorus) – 40 % (calcium) [6]	0.08 (zinc) to 0.50 (manganese) [3]
(Segovia-Siapco et al., 2007)	Southern California	Six 24hR	87 adults	semi-quantitative	171	6 months	De-attenuated: 0.33 (95 % CI -0.36-0.67) (protein) – 0.96 (95 % CI 0.40-1.00) (vit. C) [3]	N.I. [2]	N.A. [1]
(Mouratidou et al., 2005)	United Kingdom	Two 24hR	123 pregnant women	semi-quantitative	17	1 month	Crude: -0.15 (potassium) to 0.47 (Englyst fibre) [3]	48.0 % (alcohol) – 70.7 % (Englyst fibre) [6]	N.A. [1]
NO ASSESSMENT OF ABSOLUTE INTAKES/ OTHER REFERENCE METHOD									
(Landais et al., 2014)	Morocco	Three 24hR	100 women	quantitative	Fruit and vegetable intakes: 8	1 week	0.48 (vegetables) – 0.67 (fruits) [3]	N.I. [2]	0.48 (vegetables) – 0.54 (fruits) [3]
(Morita et al., 2015)	Papua New Guinea	Biomarkers and three-day Weighed Food Records	135 adults	semi-quantitative	Protein intake: 32	1 month	Median protein intake did not differ significantly in total protein intake (P=0.56) or animal protein intake (P=0.33)	FFQ vs. biomarkers: 27.2 % (total protein) – 34.8 % (animal protein) [6]	N.A. [1]
(Jayawardena et al., 2016)	Sri Lanka	7-day weighed-intake dietary records	77 adults	semi-quantitative	85	1 month	0.09 (vit. E) – 0.47 (carbohydrates) [3]	Mean: -96.20 (energy) – Mean: -40.92 (carbohydrates) [5]	N.A. [1]
(Macedo-Ojeda et al., 2013)	Mexico	Three weighed dietary records	97 adults	semi-quantitative	162	1 year	0.09 (vit. E) – 0.62 (calcium) [7] 0.16 (riboflavin) – 0.77 (cholesterol) [7]	56.7 % (vit. B_{12}) – 76.3 % (thiamine) [‖]	0.20 (vit. E) – 0.82 (energy) [7] 0.34 (vit. E) – 0.82 (ethanol) [7]

Author/Year	Country	Reference method	Sample	FFQ characteristics			Validity Study		Reproducibility Study
				Type of FFQ	Number of food items	Reference time period	Range of Correlation (energy-adjusted)	Level of Agreement (unadjusted)	Range of Correlation
NO ASSESSMENT OF ABSOLUTE INTAKES/ OTHER REFERENCE METHOD									
(Barrett & Gibson, 2010)	Australia	Four 7-day food diaries	100 adults	semi-quantitative	297	1 year	Crude: 0.239 (starch) – 0.810 (alcohol) [3]	Energy-adjusted: 43 % (riboflavin) – 67 % (alcohol) [6]	0.928 (galactans) – 0.352 (mannitol) [7]
(Bountziouka et al., 2012)	Greece	Three dietary register	500 adults	semi-quantitative	76	1 month	Crude: -0.02 (carbohydrates) – -0.15 (protein) [3]	-1.1 (mean-2SD: -64, mean+2SD: 62) (protein) – 27 (mean-2SD: -56, mean+2SD: 119) (fat) [5]	Crude: -0.08 (protein – -0.01 (fat, carbohydrates) [3]
(Pinto et al., 2010)	Portugal	Three food diaries	71 (reproducibility study)	semi-quantitative	86	1 month	0.20 (for protein) to 0.58 (for riboflavin) [4]	22.8 % (vit. C) – 36.6 % (folate) [6]	0.19 (vit. E and thiamine) –0.62 (riboflavin) [3]
(Moreira et al., 2003)	Portugal	Four-day dietary records	246 adults	semi-quantitative	89	1 month	0.20 (selenium) – 0.75 (iodine) [3]	Lowest two percentiles: 52 % – 84 %; Highest two percentiles: 51 % – 88 %	N.A. [1]
(MacIntyre et al., 2001b)	South Africa	7-day weighted records and biomarkers	144 adults	quantitative	145	N.I. †	Crude: 0.14 (fibre) and 0.59 (vit. C) [3]	31 % (protein) – 79 % (alcohol) [5]	Crude: 0.14 (calcium) – 0.75 (alcohol) [3]
(Decarli et al., 1996)	Italy	Two 7-day dietary record	395 cancer patients (adults)	semi-quantitative	77	1 year	0.19 (vegetable fat) – 0.64 (sugar) [3]	Lowest two percentiles: 59 % (vegetable fat, vit. E) – 96 % (alcohol); Highest two percentiles: 44 % (vegetable fat) – 94 % (alcohol)	N.A. [1]

[1]N.A.: Not applicable;
[2]N. I.: No Information;
[3] Spearman/Pearson correlations coefficients; [4] Cohen's kappa; [5] Bland-Altman mean and 95 % CI; [6] Agreement in the same (or same and adjacent) percentile; [7] Intra-class correlations coefficients.

4.1.3. Phase 3 – Study of dietary intake of the Kenyan population using the previous FFQ

4.1.3.1. Subjects

A total of 524 people were interviewed from different constituencies namely Langata, Dagoretti South, Westlands, Embakasi East, Starehe, Kibera and Ruaraka. Different clusters were selected based on the local knowledge about the demographic profile of each specific area, tempting to achieve a representative sample. Although no proxy about income level (regardless of education level) was included in the general questionnaire, lower, medium and medium-high social classes would be well represented, due to the broad selection of the residential areas.

From the 524 people, a total of 486 were included in the final sample. Twenty-two (4.2 %) did not know their age and 16 (3.1 %) were classified as having given implausible energy intake reports. Logistic regression models included 459 individuals (94.4%) because 27 respondents had missing information in at least one predictor.

More than half of the sample was aged below 30 years-old, had completed the secondary and tertiary level of education, and was self- or formally employed (Table 4.6). Despite the young median age of the sample, almost half of the respondents were married, and more than half had at least one child. In developing countries having large families was fairly common years ago, and there still exist families (even single parents) with five to seven children. This fact may be justified by cultural and/or social-linked reasons, which may include the early marriage, limited access to contraception, religion, lack of education, among others (Noakes, 2019).

In terms of meal patterns, half of the sample only consumed between one and three meals, and the other half more than three. Residents in lower-income settings, such as slums (n=52, 10 %), counted with a higher average number of meals, when compared to the other groups, which might indicate that in these poorer areas a snacking pattern is more common rather than having a complete-meal pattern. Interestingly, Hansen and collaborators (2011) concluded that rural ethnic groups with less frequency of main meal consumption had a higher consumption of snacks.

In what concerns lifestyle habits, smoking was very infrequent, and the practice of exercise very common, being the most common activities walking, running, dancing, playing football, and skipping rope. Nevertheless, walking was also included as physical exercise, since it fits in the concept determined by the WHO (World Health Organization, 2020) and it was averagely done on a daily basis for more than 50 min. In Nairobi, it is a common practice to walk to the job.

Table 4.6 Characterization of the participants (n=486), regarding socioeconomic characteristics and lifestyles (Absolute (n) and relative frequency (%)).

	n (%)		n (%)
Age (years)		**Number of children living at home**	
≤25	173 (35.6)	0	211 (44.1)
26-29	108 (22.2)	1-2	209 (43.6)
30-34	80 (16.5)	3-4	51 (10.6)
35-39	56 (11.5)	5-7	8 (1.7)
≥ 40	69 (14.2)		
Gender		**Number of meals per day**	
Female	243 (50.0)	1-2	74 (15.8)
Male	243 (50.0)	3	242 (51.6)
		4-6	153 (32.6)
Education Level		**Smoking habits**	
None/ Primary	54 (11.2)	Yes	18 (3.7)
Secondary	203 (41.9)	No	467 (96.3)
Tertiary	227 (46.9)		
Occupation		**Alcohol consumption**	
Unemployed	25 (5.2)	Yes	132 (27.3)
Student	36 (7.4)	No	351 (72.7)
Casual Labourer	41 (8.5)		
Self-Employed	199 (41.1)		
Formally employed	183 (37.8)		
Marital Status		**Practice of physical exercise**	
Single	246 (51.0)	Yes	440 (90.5)
Married	227 (47.1)	No	46 (9.5)
Divorced/widow	9 (1.9)		

The low prevalence of smoking and the high level of activity of the present sample can be related not only to the low prevalence of chronic diseases (Table 4.7), but also to the young age of the sample. The more prevalent chronic diseases among the Kenyan sample were hypertension and respiratory diseases, such as asthma. Some respondents were not sure about whether or not they had the chronic diseases listed in the general questionnaire, which might have underestimated the prevalence of these diseases. Moreover, it is known that half of adult hospital admissions and 55 % of all deaths are due to NCD (World Health Organization, 2018a). Also, according to the Demographic and Health Survey (DHS) (2014), 9.4 % of women and 3 % of men have hypertension, whereas for both types of diabetes the percentages are lower, namely 1.3 % for women and 0.8 % for men (Kenya National Bureau of Statistics, 2015).

In accordance with the DHS, the prevalence of over-weight and obese women totalized 32.8 %, values in line with the present results. Mbochi *et al.* (2012) and Steyn *et al*. (2012) also concluded that excess weight and obesity were highly prevalent among urban women. Even though the present results are consistent with other studies, the reliability of both weight and height obtained in the current study might be questioned because these are self-reported measures. Kenyans do not have the habit to take periodic body weightings mainly because they neither have a personal scale at home nor is it easy to find public scales where they can monitor their bodyweight. Understandably, this fact should be taken into account when taking conclusions regarding the BMI of the present sample.

Table 4.7 Description and comparison of participants' characteristics (BMI and prevalence of chronic diseases), according to gender.

	Total (n=486)	Female (n=243)	Male (n=243)
	n (%)	n (%)	n (%)
BMI[1] classification (kg/m^2)			
Under weight (< 18.5)	23 (4.9)	13 (5.5)	10 (4.3)
Normal weight (≥ 18.5–24.9)	297 (63.7)	137 (58.3)	160 (69.2)
Pre-obesity (≥ 25.0–29.9)	108 (23.2)	61 (26.0)	47 (20.3)
Obesity (≥ 30.0)	28 (6.0)	24 (10.2)	14 (6.1)
Chronic disease			
Diabetes	3 (0.7)	1 (0.4)	2 (0.8)
Hypertension	21 (4.5)	16 (6.7)	5 (2.1)
Dyslipidaemia	6 (1.4)	4 (1.9)	2 (0.8)
Respiratory diseases	15 (3.1)	9 (3.8)	6 (2.5)
Cancer and others (such as arthritis)	5 (1.0)	4 (1.7)	1 (0.4)
Medical appointment (previous year)			
Yes	180 (37.0)	95 (39.1)	85 (35.0)
No	306 (63.0)	148 (60.9)	158 (65.0)

[1] BMI: Body Mass Index.

4.1.3.2. Energy and nutrient intakes and recommendations

Carbohydrates (60.3 %, P25-P75, 57.2-63.8 %) were the nutrient class that mostly contributed to total energy intake (TEI). The respondents had a high total fat intake, representing almost 30 % (P25–P75, 24.6–30.4 %) of the TEI, where the MUFA and the SFA were the most contributory fats. The third most relevant nutrient contributing to TEI was total sugars (19.8 %). A summary of all nutrients' contributions is presented in Table 4.8.

All the median macronutrient intake ranges were in accordance with WHO/FAO range recommendations, although nutrient inadequacy was prevalent in part of the sample. Protein intake higher than the maximum level (15 % of TEI), by more than half of the sample, and fat intake above the upper recommended level, by 27.4 % of participants, might be associated to the ongoing rapid urbanization in Kenya (Steyn et al., 2012). Concerning fat, TFA and SFA were the most critical, due to inadequacy by excess of 81.1 % and 100 % of the sample, respectively. The lower limit of 55 % of TEI for carbohydrates was not reached by 15.2 % of respondents. Half of the sample had a salt intake up to 10.1 g/day (P25–P75, 7.9–12.7 g/day). Although the free sugars intake was not explored in this study, and no global recommendation exists regarding total sugars intake, the percentage of total sugars (19.8 % of TEI) was high. The only reference intake for total sugars was established by the United Kingdom National Health Service and corresponds to 90 g/d, equivalent to 18 % of TEI, considering the reference value of 2000 kcal per day (National Health Service (NHS), 2017). In the study of Mwenda *et al*. (2018) the reported total sugar intake corresponded to 13.7 % (95 % confidence interval (CI), 11.7–15.8 %), coming from foods such as carbonated drinks, biscuits, wafers, cakes, candy, sweets, chocolate and alike, all good contributors to free sugars. Regarding dietary fibre, the observed intake is in agreement

with the WHO/FAO guidelines, nevertheless the authors recognize that this value might be overestimated, since FFQs are prone to overestimate dietary intake (Willet, 2013d). Nevertheless, calculating the ratio of fibre per 1000 kcal, the median consumption is about 17 g of fibre, which is closer to the Eurodiet (3 g/MJ or 12.5 g/1000 kcal) (EFSA Panel on Dietetic Products, 2010; Eurodiet, 2001) and the American recommendations (3.4 g/MJ or 14 g/1000 kcal) (EFSA Panel on Dietetic Products, Nutrition, 2010; U.S. Department of Health and Human Services & U.S. Department of Agriculture., 2015). Still, this might be indicative that Kenyans have a reasonable fibre intake. Lower inadequacy prevalence were found for Fe, iodine and Zn, micronutrients typically problematic in developing countries (Harambee Ministry of Health, 2011), probably due to overestimation of dietary intake. Calcium might be in deficit in this sample, due to its proximity to the recommended values. Also, Steyn *et al*. (2012) concluded that Ca adequacy ratio was 62.2 %. Vitamin D intake can also be a concern among Kenyans, since their privileged food sources, such as salmon, mushrooms or fortified foods, are not common in the Kenyan diet.

There are few studies, carried out in Nairobi, evaluating the dietary intake of the urban adult population, including women and men. It is more common to analyse the nutritional intake of low-income or rural areas (Beatrice, 2009; Dominguez-Salas et al., 2016; Hansen et al., 2011; Jayne, 2011; Long et al., 2011; Van't Riet et al., 2002; Wanjihia et al., 2009) and in population groups such as children and women (Gegios et al., 2010; Gewa et al., 2007, 2009, 2012; Kamau-Mbuthia & Elmadfa, 2007; Long et al., 2011; Mala et al., 2012; Mwaniki & Makokha, 2013; Stephenson et al., 2010; Steyn et al., 2011, 2012; Steyn & Nel, 2006; Walingo & Musamali, 2008; Walton et al., 2012; Waudo et al., 2005), normally targeted because they might be at a disadvantaged in household food distribution. Hence, it is difficult to conclude on the trends highlighted in this study. Nonetheless, the slight deviations from other studies performed in more rural areas reveal that urbanisation has the potential to modify the well-being of the population.

Table 4.8 Comparison between energy and nutrients' intake of the total sample (n=486), and according to gender, and the WHO/FAO dietary recommendations.

		Total (n=486)			WHO/FAO	Female (n=243)			Male (n=243)		
	Unit	Median	P25[1]	P75[1]	Guidelines[2]	Median	P25	P75	Median	P25	P75
Energy (E)	kcal	2892	2352	3512	F[3]: 1800-2400[4]; M[3]: 2200-3200[4].	2647	2220	3347	3111	2532	3636
Protein	% E	14.8	13.6	16.0	10 – 15	14.6	13.4	16.0	15.0	13.8	16.0
Total Fat	% E	27.5	24.6	30.4	15 – 30	27.6	24.3	30.2	27.5	24.9	30.7
SFA[5]	% E	8.4	7.4	9.6	< 10	8.5	7.3	9.7	8.3	7.4	9.6
MUFA[6]	% E	8.7	7.4	10.2	by difference	8.6	7.2	10.2	8.8	7.6	10.2
PUFA[7]	% E	5.9	5.2	6.7	6 – 10	5.8	5.3	6.6	6.1	5.2	6.7
n-3 [8] PUFA	% E	0.45	0.40	0.52	1 – 2	0.46	0.40	0.53	0.45	0.39	0.52
n-6 [9] PUFA	% E	5.1	4.4	5.8	5 – 8	5.0	4.4	5.8	5.2	4.4	5.9
TFA[10]	% E	0.20	0.16	0.26	< 1	0.20	0.15	0.27	0.21	0.16	0.26
Cholesterol	mg	258	168	365	< 300	252	157	360	266	177	371
Carbohydrates	% E	60.3	57.2	63.8	55 – 75	60.6	57.6	64.3	60.0	56.9	62.8
Total Fibre	g	50	40	65	> 25	48	38	61	51	41	68
Total Sugars	% E	19.8	17.4	22.6	< 10	20.2	18.2	22.9	19.3	16.6	22.2
Vitamin B_{12}	µg	5.9	3.1	10.8	2.4	5.8	2.9	10.7	5.9	3.1	10.8
Folate	µg	658	515	866	400 (1000)	629	497	851	677	545	891
Vitamin C	mg	285	215	370	45 (1000)	288	219	377	283	208	359
Vitamin D	µg	4.4	2.7	6.2	19-50 y: 5; 51-65 y: 10	4.2	2.6	5.7	4.5	3.0	6.4
Calcium	mg	1406	1112	1729	1000 (3000)	1387	1078	1748	1421	1126	1708
Iron	mg	30	24	40	F: 19.6; M: 9.1 (45)	28	23	38	31.6	24.0	41.0
Magnesium	mg	636	510	824	F: 220; M: 260	599	478	799	650	541	847
Zinc	mg	13.3	10.2	16.6	F: 4.9; M: 7.0	11.9	9.4	15.8	14.5	11.5	17.3
Salt	g	7.9	10.1	12.7	< 5	9.7	7.8	12.1	10.7	8.0	13.3

[1] P25: Percentile 25th; P75: Percentile 75th;

[2] Macronutrients: ranges of population nutrient goals; Micronutrients: recommended nutrient intake (RNI), stratified by gender in specific cases, followed by upper limit in brackets;

[3] F: Female; M: Male;

[4] Estimated calorie needs per day, by sex, regardless of the physical activity level, for adults (18-59 years-old), according to the Dietary Guidelines 2015-2020, defined by USDHHS and USDA (U.S. Department of Health and Human Services & U.S. Department of Agriculture., 2015).

[5] SFA: Saturated fatty acids; [6] MUFA: Monounsaturated fatty acids; [7] PUFA: Polyunsaturated fatty acids. [8] n-3: Omega-3; [9] n-6: Omega-6; [10] TFA: Trans fatty acids.

4.1.3.3. Contribution of food groups to energy and nutrient intakes

Amidst the food groups analysed in this study, the major contributors to TEI were *cereals and grain products* (34.0 %), followed by *sugar, syrups, sweets, and snacks* (9.8 %), *fruits* (9.7 %), and *meat and eggs* (8.8 %; Table 4.9). *Cereals and grain products, fruits,* and *vegetables* are the groups that contain the most consumed foods on a daily basis by Kenyans (Mohajan, 2014). These results are in agreement with the Kenyan food balance sheet, which refers that vegetable products contribute between 86 % and 88 % to total energy, being one of the most important parts of daily *per capita* supply of calories (Kenya National Bureau of Statistics, 2019b). In consequence, the contribution of foods from animal origin to energy is low. The food group *meat, fish, and eggs* was divided for this analysis (fish was considered an individual group), as it was observed that the consumption of fish was low, and this way, it is not taken into account and more realistic results are being considered. Interestingly, other researchers found that a specific rural ethnic group had a higher contribution from fish to TEI (7 %), showing that dietary intake and habits vary according to the region and most important to the type of population (Hansen et al., 2011).

Cereals and grain products contributed with 42.5 % to carbohydrates intake, and expectably *fruits* (12.4 %) and *sugar, syrups, sweets, and snacks* (10.6 %) were also noteworthy. The contribution to carbohydrates from the first food group of the FFQ was mainly given by maize products, namely *ugali* and porridge, rice, potatoes, wheat products such as *chapati* and bread, as also found by other researchers (Steyn et al., 2012; Waudo et al., 2005). *Ugali* and *chapati* are very popular in the Kenyan diet, being eaten as a main course, while bread is mostly consumed at breakfast time. *Fruits* are very popular in Kenya and avocado, bananas, watermelon, oranges, and mangoes were the most consumed in terms of the average daily amount. The high consumption of fruits and their contribution to energy and carbohydrates might be due to their availability in street markets, where they are usually sold as individual doses (even watermelon and pineapple) making their consumption easier, even at the moment of buying. *Cereals and grain products* (26.7 %) occupied the first place in what concerns sources of dietary fibre, followed by similar contributions of *vegetables* and *legumes, pulses, seeds and nuts*, 17.2 %, and 19.8 %, respectively.

The most important sources of protein were *cereals and grain products* (23.3 %) and *meat and eggs* (22.0 %), followed by *dairy* (11.7 %) and *legumes, pulses, seeds and nuts* (10.7 %). Half of the sample consumed more protein coming from plant (46 %) compared to animal sources (36 %). Among cereals, white *chapati*, *ugali*, and white bread were the most relevant protein contributors. The consumption of fish was uncommon in this sample, mainly Tilapia was consumed in the previous month period, and fish such as *Omena* (*Silver cyprinid*), Nile perch, canned fish or other type were not consumed at all by more than 63 % of the sample. In Nairobi centre, the fish offer is not great, and it is more expensive than meat, which might be the reason that justifies its low consumption and reduced contribution to protein's intake. Dairy products assumed an important contribution to protein, mainly due to the consumption of milk, which is

commonly mixed with tea, and consumed as a snack. However, other types of dairy products did not have much popularity among the studied sample. During the previous month, 95 %, 59 %, and 38 % of the sample had not consumed any type of cheese, soured milk (known as Mala), or yoghurt. Even though the recruited sample lives in an urban setting, products such as yoghurts and cheese lose popularity due to their higher price, compared with staple foods.

In what concerns total fats, *cereals and grain products* (19.7 %) and *meat and eggs* (18.7 %) were the main contributors, followed by *dairy* (13.7 %), which also showed a considerable contribution. At a first glance it might be unexpected that *cereals and grain products* are one of the main contributors to total fat, however, it is easily understood since this group includes cooked food, and, so, added fats and salt are taken into account. Also, for SFA and MUFA, the previous food groups were the main sources, however, *fruits* group was the third most relevant source (11.2 %) of MUFA. Given the contribution of *meat and eggs* to the three types of analysed fats (more SFA and MUFA and less PUFA), it might indicate that the sample had a higher consumption of red meats rather than white meats. It would be desired that fish would have been a considerable part of fats' contribution given the well-recognized benefits from fish's fats, specifically the omega-3 PUFA (Shahidi & Ambigaipalan, 2018). The contribution from *dairy* to fats would be reduced if the consumption of low-fat dairy options were common among Kenyans, which does not happen. Only 28 respondents (5.8 %) mentioned the consumption of skimmed milk. *Legumes, pulses, seeds and nuts* provided 10.3 % of total PUFA, being surpassed only by *cereals and grain products* (38.8 %). Among *fats and oils*' food items, butter and margarine were fairly the most cited ones, while olive oil and other oils were not consumed by 94 % of the sample in the previous four weeks, considering adding fats, not those used for cooking. Eating salads is not common in Nairobi, and thereby using olive oil, for example, as dressing, as it happens in European countries is not usual. A rebalance of these fats would also invert the contribution of SFA and PUFA for the diet.

Fruits and *sugar, syrups, sweets, and snacks* counted for 23.2 % and 20.8 % of total sugar, respectively, while *cereals and grain products* (32.7 %), *vegetables* (14.9 %) and *legumes, pulses, seeds and nuts* (13.2 %) were the main sources of salt. In what concerns sugary products, cookies, biscuits, cakes, and ice-cream were the main providers, besides the sugar itself that was used on a daily basis given the high consumption of tea (with or without milk). This indicates that this sample had high consumption of sugary products, and consequently, the intake of free sugars (World Health Organization, 2015) might have been high. Regarding salt consumption, besides the salt used for cooking, nuts and groundnuts might have been one of the most contributors to salt, since they are sold as a salty snack in Nairobi, a trend which was also found by other researchers (Steyn et al., 2012). Mwenda and colleagues concluded that despite possessing a general awareness (more than 88 % of the sample) about the health risks derived from the consumption of dietary sugar and salt, almost 50 % of the respondents were not implementing any strategies to reduce the intake of both sugar and salt (Mwenda et al., 2018). The current results could work as an incentive to national authorities to implement nutritional strategies aiming at the reduction of sugar, salt, and relative products consumption. Some examples could include

the creation of legislation identifying maximum levels of sugar/salt in specific foods towards the development of healthier products, as well as the promotion of awareness actions concerning the health risks associated with the consumption of such food products. In this sense, and according to the national Nutrition Action Plan 2018-2022, efforts are being taken by the Ministry of Public Health and Sanitation (Ministry of Public Health and Sanitation, 2018). Three examples of the envisaged strategies are: to promote consumption and marketing of healthy foods, as well as to establish collaboration with stakeholders and sensitize them to promote good nutrition for older children and adolescents; to strengthen behaviour change communication on the consumption of healthy diets among the populations; and to develop or to review relevant policies and guidelines to include nutrition of adults.

The last food group in this FFQ comprised the *composite dishes*, including *Pilau* (cooked rice with aromatic spices and meat), *Guitheri* (boiled maize, beans, and potatoes) and *Matoke* (mashed plantain), which also contributed to energy and macronutrients, namely by 4 %, 4 %, 4 % and 2 % to total energy, protein, fats, and carbohydrates intake, respectively.

Concerning micronutrients, the main providing sources of folate were *cereals and grain products*, contributing to almost a quarter of total intake (23.0 %), and *legumes, pulses, seeds and nuts* (16.4 %), followed by *fruits* (12.1 %) and *vegetables* (11.6 %; Table 4.10) groups. The contributors to Fe were the same groups with the exception of *fruits*. More than half of Zn was supplied by *cereals and grain products* and *meat and eggs.* The *dairy* group included the more relevant providers of iodine (77.5 %) and Ca (33.4 %), with the *vegetables* group also contributing to the latter (28.5 %). Within green-leaf vegetables, kales (*Sukuma wiki*), cabbages and spinach, were the most consumed ones, either in frequency or in quantity. The contribution of vegetables to Ca intake was also demonstrated by other authors (Jayne, 2011; Steyn et al., 2012; Waudo et al., 2005). Specifically, kales and spinach are a good source of Ca, while more traditional vegetables such as spider weed (*Sargiet*) and cowpeas leaves (*Kunde*) are excellent sources of Fe and Ca. One of the food groups that has a high contribution to total energy, around 10 %, but does not provide any interesting vitamin or mineral, is *sugar, syrups, sweets, and snacks*, which is also another reason to join efforts to reduce the consumption of these high-energy-dense and nutritionally-poor foods.

Table 4.9 Contribution of food groups to energy and macronutrient intakes (n=486).

	Nutrients									
	Energy	Protein	Total Fat	SFA[2]	MUFA[3]	PUFA[4]	Chol[5]	Carbohydrates	Dietary Fibre	Sugars
Food Group	Median (P25-P75) [1]	Median (P25-P75)	Median (P25-P75)	Median (P25-P75)	Median (P25-P75)	Median (P25-P75)	Median (P25-P75)	Median (P25-P75)	Median (P25-P75)	Median (P25-P75)
Cereals and grain products	34.0 (27.6-40.8)	23.3 (18.4-29.8)	19.7 (14.3-26.3)	11.8 (8.3-16.7)	15.4 (10.6-21.1)	38.8 (28.9-49.7)	0.05 (0.00-0.16)	42.5 (35.1-49.2)	26.7 (19.8-33.7)	14.7 (10.4-20.4)
Vegetables	5.2 (4.0-6.6)	7.8 (6.0-9.8)	1.1 (0.8-1.6)	0.32 (0.20-0.46)	0.18 (0.13-0.26)	1.1 (0.7-1.6)	0.0 (0.0-0.0)	7.3 (5.6-9.5)	17.2 (13.0-22.9)	8.0 (5.5-10.8)
Legumes, pulses, seeds and nuts	8.0 (4.7-12.1)	10.7 (6.2-17.2)	6.9 (3.0-11.5)	3.1 (1.4-5.6)	6.6 (3.0-12.3)	10.3 (5.2-17.6)	0.0 (0.0-0.0)	7.7 (4.1-11.7)	19.8 (11.2-28.4)	1.4 (0.8-2.3)
Fruits	9.7 (6.2-14.0)	4.0 (2.4-6.1)	7.2 (1.9-15-40)	4.4 (0.9-10.0)	11.2 (0.9-24.3)	6.1 (1.9-12.6)	0.0 (0.0-0.0)	12.4 (8.3-17.6)	21.7 (12.7-29.0)	23.2 (15.5-31.8)
Meat and eggs	8.8 (5.9-12.7)	22.0 (12.2-29.4)	18.7 (12.7-25.6)	20.4 (13.8-27.7)	23.7 (15.8-32.1)	9.8 (6.5-13.8)	74.0 (61.6-82.0)	0.39 (0.17-0.85)	0.13 (0.0-0.33)	0.22 (0.11-0.34)
Fish	0.7 (0.2-1.7)	2.4 (0.8-5.4)	1.0 (0.1-2.4)	0.3 (0.0-0.6)	0.0 (0.0-0.0)	0.0 (0.0-0.0)	0.4 (0.0-1.0)	0.18 (0.0-0.43)	0.07 (0.0-0.1)	0.0 (0.0-0.0)
Dairy	8.3 (5.3-11.6)	11.7 (7.9-16.4)	13.6 (9.01-19.18)	10.4 (4.57-19.9)	10.8 (7.12-15.7)	3.05 (1.89-4.70)	16.2 (10.3-25.3)	1.79 (0.7-3.7)	0.0 (0.0-0.0)	13.3 (8.6-18.3)
Fats and oils	0.44 (0.0-0.99)	0.0 (0.0-0.0)	1.6 (0.0-3.6)	2.5 (0.0-5.7)	1.8 (0.0-4.3)	0.69 (0.0-1.8)	0.16 (0.0-0.41)	0.0 (0.0-0.0)	0.0 (0.0-0.0)	0.0 (0.0-0.0)
Sauces and seasonings	0.36 (0.12-0.88)	0.07 (0.02-0.25)	0.04 (0.0-0.64)	0.002 (0.0-0.37)	0.015 (0.0-0.84)	0.003 (0.0-0.93)	0.0 (0.0-0.0)	0.45 (0.13-1.0)	0.034 (0.003-0.12)	0.43 (0.08-1.5)
Sugar, syrups, sweets and snacks	9.8 (6.0-13.7)	2.4 (1.3.4.2)	11.0 (5.6-16.7)	15.2 (8.6-23.7)	10.5 (5.1-16.1)	7.4 (3.6-11.6)	4.8 (1.8-10.0)	10.8 (6.9-14.8)	1.6 (0.8-3.0)	20.8 (13.6-29.3)
Soft and carbonated beverages	1.0 (0.4-2.5)	0.0 (0.0-0.09)	0.0 (0.0-0.0)	0.0 (0.0-0.0)	0.0 (0.0-0.0)	0.0 (0.0-0.0)	0.0 (0.0-0.0)	1.9 (0.7-4.4)	0.0 (0.0-0.36)	5.8 (2.3-13.0)

[1] P25: Percentile 25th; P75: Percentile 75th;

[2] SFA: Saturated fatty acids; [3] MUFA: Monounsaturated fatty acids; [4] PUFA: Polyunsaturated fatty acids. [5]Chol: Cholesterol.

Table 4.10 Contribution of food groups to salt and micronutrient intakes (n=486).

	Nutrients					
	Salt	Folate	Iron	Calcium	Iodine	Zinc
Food Group	Median (P25-P75)[1]	Median (P25-P75)	Median (P25-P75)	Median (P25-P75)	Median (P25-P75)	Median (P25-P75)
Cereals and starchy foods	32.7 (26.0-41.2)	23.0 (14.1-32.5)	27.5 (21.6-37.0)	12.1 (7.4-17.4)	2.2 (0.4-4.2)	26.1 (19.0-33.4)
Vegetables	14.9 (10.4-19.2)	11.6 (7.5-16.0)	19.6 (12.5-31.3)	28.5 (20.0-40.7)	0.0 (0.0-0.02)	4.8 (3.3-6.8)
Legumes, Pulses and Nuts	13.2 (7.4-21.2)	16.4 (8.7-27.2)	15.4 (8.13-23.9)	4.7 (2.5-8.8)	0.18 (0.48-1.3)	9.8 (5.6-15.4)
Fruits	0.04 (0.0-0.15)	12.1 (7.5-18.0)	3.6 (2.1-6.0)	3.7 (2.1-5.9)	1.8 (3.5-5.9)	4.4 (2.4-7.0)
Meat and Eggs	5.20 (2.9-8.4)	4.4 (2.2-6.8)	8.0 (5.0-12.0)	2.2 (1.4-3.2)	12.1 (5.13-20.5)	26.1 (16.6-35.1)
Fish	0.50 (0.0-1.0)	0.0 (0.0-0.0)	0.0 (0.0-0.8)	0.0 (0.0-0.01)	0.0 (0.0-0.0)	0.0 (0.0-0.0)
Dairy	4.2 (2.7-6.2)	3.4 (2.1-5.2)	0.90 (0.58-1.41)	33.4 (22.2-43.7)	77.5 (66.3-85.8)	13.3 (8.5-18.2)
Fats and Oils	0.12 (0.0-0.30)	0.0 (0.0-0.0)	0.0 (0.0-0.0)	0.0 (0.0-0.0)	0.0 (0.0-0.0)	0.0 (0.0-0.0)
Sauces and Seasonings	0.8 (0.02-6.78)	0.003 (0.030-0.13)	0.03 (0.004-0.11)	0.021 (0.001-0.070)	0.0 (0.0-0.0)	0.033 (0.003-0.17)
Sweets and pastry	4.4 (2.3-6.9)	5.5 (2.6-10.0)	3.7 (1.8-5.8)	3.4 (1.7-5.5)	0.0 (0.0-2.34)	1.9 (0.9-2.9)
Soft and carbonated beverages	0.2 (0.08-0.54)	0.0 (0.0-0.06)	0.0 (0.0-0.15)	0.0 (0.0-0.11)	0.0 (0.0-0.0)	0.0 (0.0-0.03)

[1]P25: Percentile 25th; P75: Percentile 75th.

4.1.3.4. Food sources of nutrients and individual characteristics

A deep analysis of food sources and their association with individual characteristics was carried out. Concerning the main abovementioned food sources of energy, interesting results were found. Diets of impoverished and uneducated people seemed to be nutritionally poorer compared with those of the respective reference groups. Being male, living in poorer settlements, such as slums, and having no education (n=5, 1 %) was significantly associated with higher contributions from *cereals and grain products* ($p \leq 0.05$) to TEI. The average contributions were 36 % for men (33 % for women), 40 % for residents in slums (34 % for other types of living conditions), and 53 % for no educated individuals (primary level: 35 %; secondary level 36 %; and tertiary level: 32 %). Women and people with no education had significantly higher contributions from *sugar, syrups, sweets and snacks* to TEI compared with men and people highly educated. In what concerns the contribution from *meat and eggs* to TEI, people living in slums had a significantly lower contribution, 7 %, compared with 10 % observed in the rest of the sample. For the *fruits* group the only characteristic that showed to be significant on having a higher contribution to TEI was being a woman. In residential informal settings, such as slums, the availability of quality food is rare and staple foods such as flour, rice or potatoes are cheaper than meat products for example, which might justify the contributions of these food groups to TEI. Regarding the education level, it seems that going to the university is a proxy for having healthier food habits. Higher levels of education might be indicative of not only belonging to a higher social class, and consequently having an increased purchase capacity, but also of possessing a greater knowledge and awareness about the food-health relationship. An example of this was the contribution of meat products to fats, provided by highly educated people that showed their diet diversity, not only rich in red meat.

Table 4.11 shows the association between food sources (only for those that provided at least 10 % of the TEI of each nutrient) and individual characteristics. According to these results, men are more likely to have a higher contribution from *cereals and grain products* to carbohydrates, total fibre, SFA, MUFA, sugars, salt, and to the micronutrients Mg, Zn and Ca. Higher contributions from *vegetables* to fibre, folate, Fe, Mg, and salt were more frequent in women. Women are more likely to consume *fruits* and *vegetables* as a source of fibre, while men choose *cereals and grain products*. In addition, men have a lower contribution from energy-dense foods, both to carbohydrates and to fat compared to women.

People with higher education levels were associated with having higher contributions from *meat and eggs* to protein, MUFA, omega-6 PUFA, and SFA. It is noteworthy that this group of people also use *dairy* foods as a source of protein and fat, compared to the reference group. People with lower levels of education had a significantly higher contribution to protein from *cereals and grain products*, and although not significant, it seems that the group of pulses was also a source of protein. The *vegetables* group was the only food source in which contribution to dietary fibre was significantly different among people with different educational levels.

Those belonging to the third quartile of EI, were inversely associated with having higher contributions from *cereals and grain products* for the macronutrients, total fibre, total sugars, and some micronutrients when compared to the respondents from the first quartile. Moreover, *legumes, pulses, seeds and nuts* revealed to be significantly important sources of protein, PUFA, and Fe. For people belonging to the first quartile of EI, *vegetables* were an important source of fibre, Ca, Mg and Fe, compared with those with higher EI. The *dairy* food group was shown to be an important source of fat for people belonging to lower EI quartiles, rather than fat from the group of sugary and salty snacks, as happened for people who have higher EI.

Older people are less likely to consume *cereals and grain products* as a source of the majority of the nutrients, except for carbohydrates and total sugars. The intake of fibre and PUFA from *legumes, pulses, seeds and nuts* were positively associated with being older. Moreover, as age increases it is more likely to have higher contributions to sugars from *fruits*, rather than sweetened products. These results showed that youngsters seemed to have unhealthy options in what concerns food sources of carbohydrates, sugar, and fat, which the apparent main contributor was the group of *sugar, syrups, sweets, and snacks*. *Mandazi*, a traditional deep-fried bread very typical in Nairobi, was frequently consumed among the respondents (75 % of respondents (n=382) mentioned its consumption during the previous month). This snack, which is normally consumed together with tea, is sold in canteens and in the streets at a low price, and for that reason, it is more available to students and people with less purchasing capacity.

Having a stable job did not seem to influence food sources unless regarding the contribution of *sugar, syrups, sweets and snacks* to total fat, in which students, unemployed and casual employers were 66 % more likely to have it. Higher contribution from *dairy* foods to total fats and cholesterol were associated with having higher BMI. No other associations between food sources and BMI or marital status were found.

Table 4.11 Adjusted odds ratio (OR) of having a higher or a lower contribution of different food groups to daily energy and nutrient intakes, according to participants' characteristics (n=459).

Predictor	Gender [1] (male)		Education level [2] (tertiary)		Occupation [3] (Self/Formally employed)		Marital Status [4] (married)		Daily Energy Intake (DEI) [5] (P25 < DEI < P50)		Daily Energy Intake [5] (P50 < DEI < P75)		Daily Energy Intake [5] (DEI > P75)		Age		BMI	
Food Group	Adjusted OR[6]	95 % CI	Adjusted OR	95 % CI	Adjusted OR	95 % CI	Adjusted OR	95 % CI	Adjusted OR	95 % CI	Adjusted OR	95 % CI	Adjusted OR	95 % CI	Adjusted OR	95 % CI	Adjusted OR	95 % CI
Cereals and starchy foods																		
Energy	1.37	0.94-1.99	0.73	0.50-1.07	0.75	0.46-1.23	0.99	0.65-1.51	-	-	-	-	-	-	**0.97***	0.95-1.00	0.97	0.93-1.02
Protein	1.31	0.89-1.95	**0.66***	0.45-0.97	0.84	0.51-1.38	0.93	0.61-1.43	**0.54***	0.32-0.93	0.66	0.38-1.16	**0.34****	0.19-0.59	**0.97***	0.95-1.00	0.96	0.92-1.01
Total Fat	1.48	0.99-2.21	0.66	0.44-0.97	0.69	0.41-1.14	0.71	0.46-1.10	**0.64***	0.37-1.10	**0.50***	0.28-0.88	**0.32****	0.18-0.56	**0.98***	0.95-1.00	0.96	0.92-1.01
SFA	**1.62***	1.09-2.40	**0.51***	0.34-0.75	0.88	0.53-1.45	0.90	0.59-1.39	0.86	0.50-1.48	0.74	0.42-1.29	0.66	0.38-1.15	**0.97***	0.94-1.00	**0.95***	0.90-0.99
MUFA	**1.51***	1.01-2.26	**0.63***	0.42-0.93	0.78	0.47-1.29	0.79	0.52-1.23	0.64	0.37-1.10	**0.48***	0.27-0.84	**0.28****	0.16-0.50	**0.97***	0.94-1.00	0.97	0.92-1.02
PUFA	1.33	0.89-1.98	0.81	0.55-1.19	**0.60***	0.36-1.00	0.76	0.50-1.18	**0.58***	0.34-1.00	**0.43***	0.24-0.75	**0.32****	0.18-0.55	**0.97***	0.94-1.00	1.00	0.95-1.04
Carbohydrates	**1.74***	1.17-2-6	0.79	0.54-1.16	0.68	0.41-1.11	0.89	0.58-1.37	**0.55***	0.32-0.94	0.60	0.34-1.04	**0.40***	0.23-0.69	0.99	0.96-1.01	0.97	0.93-1.02
Total Fibre	**2.26****	1.51-3.36	0.90	0.61-1.32	0.86	0.52-1.41	0.77	0.50-1.18	0.73	0.43-1.25	0.67	0.38-1.17	**0.39***	0.22-0.69	**0.98***	0.95-1.00	1.01	0.96-1.06
Sugars	**1.84***	1.24-2.73	**0.61***	0.41-0.89	0.76	0.46-1.25	0.98	0.64-1.51	**0.47***	0.27-0.81	0.61	0.35-1.07	**0.35****	0.20-0.61	1.00	0.97-1.02	0.98	0-93-1.02
Salt	**1.67***	1.11-2.49	**0.63***	0.42-0.93	1.24	0.75-2.05	0.78	0.50-1.21	0.93	0.54-1.60	0.85	0.48-1.49	**0.39***	0.22-0.69	**0.95****	0.92-0.98	0.98	0.93-1.03
Folate	1.24	0.83-1.84	0.84	0.57-1.24	0.82	0.50-1.35	0.85	0.55-1.3	0.59	0.34-1.01	0.58	0.33-1.01	**0.32****	0.18-0.56	**0.97***	0.94-1.00	0.96	0.92-1.01
Iron	1.39	0.94-2.05	0.88	0.60-1.29	0.99	0.61-1.63	0.68	0.44-1.03	1.00	0.58-1.71	0.93	0.53-1.62	0.60	0.35-1.04	**0.97***	0.94-1.00	0.96	0.91-1.01
Calcium	**1.55***	1.04-2.31	**0.61***	0.41-0.90	0.78	0.47-1.29	1.27	0.82-1.97	0.96	0.56-1.66	1.33	0.76-2.34	0.92	0.53-1.59	**0.95***	0.93-0.98	**0.95***	0.90-1.00
Zinc	**1.94***	1.29-2.92	**0.45***	0.30-0.67	0.78	0.47-1.30	1.12	0.72-1.75	0.67	0.39-1.17	**0.50***	0.28-0.89	**0.32****	0.18-0.57	**0.97***	0.94-1.00	0.96	0.92-1.01
Magnesium	**2.25****	1.51-3.37	0.73	0.49-1.08	0.94	0.57-1.54	0.76	0.49-1.17	0.78	0.45-1.34	0.77	0.44-1.35	**0.41****	0.23-0.73	**0.96***	0.94-0.99	0.97	0.93-1.02
Vegetables																		
Total Fibre	**0.55***	0.36-0.83	**1.62***	1.07-2.44	0.75	0.44-1.27	0.82	0.52-1.30	**0.31****	0.17-0.55	**0.32****	0.18-0.57	**0.10****	0.05-0.19	1.00	0.98-1.03	1.02	0.97-1.07
Salt	**0.50***	0.33-0.74	1.23	0.82-1.85	0.94	0.56-1.57	1.04	0.67-1.64	**0.39***	0.22-0.70	**0.26****	0.14-0.47	0.19	0.10-0.34	**1.04***	1.01-1.07	1.01	0.96-1.06
Folate	**0.44****	0.30-0.66	0.97	0.65-1.45	1.12	0.67-1.86	1.05	0.68-1.64	**0.30****	0.17-0.53	**0.32****	0.18-0.57	0.28	0.16-0.50	1.02	0.99-1.05	1.04	0.99-1.09
Iron	**0.60***	0.40-0.90	0.88	0.59-1.31	0.92	0.56-1.53	**2.09***	1.34-3.24	0.60	0.34-1.04	**0.36***	0.20-0.64	**0.30****	0.17-0.52	1.02	0.99-1.04	1.01	0.96-1.06
Calcium	0.72	0.48-1.07	0.72	0.48-1.06	0.75	0.46-1.24	1.46	0.95-2.26	**0.41***	0.24-0.71	**0.32****	0.18-0.57	**0.28****	0.16-0.50	1.02	0.99-1.05	1.02	0.97-1.07
Magnesium	**0.44****	0.30-0.66	1.01	0.68-1.50	1.13	0.68-1.86	1.22	0.78-1.88	**0.40****	0.23-0.69	**0.54***	0.30-0.95	**0.29****	0.16-0.51	1.01	0.98-1.04	1.03	0.98-1.08
Legumes, Pulses and Nuts																		
Protein	0.96	0.64-1.45	0.87	0.58-1.30	0.95	0.57-1.58	1.04	0.67-1.64	1.48	0.86-2.55	1.69	0.96-2.98	**1.98***	1.13-3.49	1.03	1.00-1.06	0.98	0.94-1.03
PUFA	0.97	0.66-1.43	1.35	0.92-1.98	1.29	0.79-2.11	0.72	0.47-1.10	1.50	0.88-2.56	**1.89***	1.09-3.28	**2.01***	1.17-3.46	**1.03***	1.99-1.06	1.03	0.98-1.08
Total Fibre	0.92	0.62-1.36	0.82	0.56-1.20	1.02	0.63-1.67	0.93	0.61-1.43	**1.93***	1.13-3.29	**2.39***	1.37-4.17	**2.16***	1.25-3.72	**1.04***	1.01-1.07	0.98	0.94.1.03
Salt	1.02	0.69-1.51	1.08	0.74-1.59	1.32	0.81-2.16	0.92	0.60-1.40	1.54	0.91-2.60	1.50	0.87-2.59	**2.70****	1.57-4.65	1.02	0.99-1.05	0.99	0.95-1.04
Folate	0.90	0.61-1.32	0.96	0.66-1.40	0.82	0.51-1.34	1.15	0.75-1.75	1.52	0.89-2.58	**1.82***	1.05-3.16	1.69	0.99-2.91	**1.04***	1.01-1.07	0.99	0.95-1.04
Iron	1.07	0.72-1.58	0.89	0.60-1.31	0.95	0.58-1.56	0.81	0.52-1.25	**1.94***	1.13-3.34	**2.09***	1.20-3.64	**3.73****	2.13-6.52	1.04*	1.01-1.07	0.98	0.94-1.03
Fruits																		
Carbohydrates	**0.66***	0.44-0.98	1.34	0.91-1.97	**1.66***	1.01-2.74	1.15	0.75-1.77	1.01	0.60-1.72	0.84	0.49-1.46	**1.89***	1.09-3.26	1.01	0.98-1.08	1.03	0.98-1.08
Total Fibre	**0.68***	0.46-1.00	1.22	0.83-1.78	1.25	0.77-2.03	1.30	0.85-2.00	1.10	0.65-1.85	1.16	0.68-2.00	**1.81***	1.06-3.11	**0.97***	0.94-1.00	1.02	0.97-1.07
Sugars	0.64	0.43-0.94	1.36	0.92-2.01	1.52	0.92-2.50	1.29	0.78-1.84	0.88	0.52-1.51	0.64	0.37-1.13	1.51	0.87-2.60	**1.03***	1.00-1.05	1.04	0.99-1.09
MUFA	0.86	0.58-1.27	**0.64***	0.43-0.94	1.39	0.85-2.28	1.48	0.96-2.28	1.14	0.70-1.92	1.27	0.74-2.20	2.62	1.51-4.53	0.99	0.97-1.02	0.96	0.92-1.01
Vitamin C	0.98	0.66-1.45	1.00	0.68-1.47	1.06	0.65-1.74	1.37	0.90-2.10	1.44	0.85-2.46	1.30	0.75-2.25	**2.63***	1.52-4.55	1.02	0.99-1.04	**1.05***	1.00-1.10
Folate	0.87	0.59-1.28	**1.49***	1.02-2.18	1.13	0.70-1.83	1.33	0.87-2.04	1.20	0.71-2.02	0.97	0.56-1.67	**1.74***	1.02-2.97	0.99	0.97-1.02	1.03	0.98-1.08
Magnesium	0.68	0.46-1.00	1.21	0.83-1.77	1.27	0.78-2.07	0.98	0.64-1.50	1.11	0.66-1.87	1.08	0.63-1.85	1.49	0.87-2.54	1.00	0.97-1.02	1.04	0.99-1.09
Meat and Eggs																		
Protein	1.02	0.69-1.51	**1.83***	1.25-2.68	0.94	0.57-1.53	0.84	0.55-1.29	1.39	0.82-2.36	1.63	0.94-2.82	1.44	0.84-2.47	**0.97***	0.94-1.00	1.03	0.98-1.08
Total Fat	0.91	0.62-1.33	**1.93***	1.32-2.83	1.10	0.68-1.79	0.88	0.58-1.35	0.96	0.57-1.62	1.11	0.64-1.91	1.01	0.59-1.72	1.00	0.97-1.02	1.04	0.99-1.09
SFA	0.98	0.67-1.45	**1.60***	1.09-2.33	1.03	0.63-1.67	0.74	0.49-1.13	1.44	0.85-2.43	1.32	0.77-2.28	1.32	0.77-2.24	1.00	0.97-1.02	1.02	0.97-1.07
MUFA	1.11	0.76-1.63	**1.68***	1.15-2.46	1.06	0.65-1.71	0.78	0.51-1.18	1.10	0.65-1.86	1.28	0.75-2.21	0.93	0.55-1.58	1.01	0.99-1.04	1.02	0.98-1.07
Cholesterol	1.36	0.93-2.00	1.08	0.74-1.57	0.99	0.61-1.60	0.92	0.60-1.40	1.43	0.85-2.42	0.93	0.54-1.60	1.17	0.69-1.98	1.02	0.99-1.04	0.96	0.92-1.00
Zinc	1.14	0.77-1.68	1.36	0.93-2.00	1.03	0.63-1.68	0.69	0.45-1.05	1.59	0.93-2.70	**1.82***	1.05-3.16	1.47	0.86-2.52	0.97	0.95-1.00	1.03	0.98-1.08
Omega-6	0.99	0.68-1.46	**1.72***	1.18-2.52	1.06	0.65-1.72	1.02	0.67-1.55	1.04	0.62-1.76	1.37	0.80-2.35	1.16	0.68-1.98	1.00	0.98-1.03	1.00	0.96-1.05

Predictor	Gender [1] (male)		Education level [2] (tertiary)		Occupation [3] (Self/Formally employed)		Marital Status [4] (married)		Daily Energy Intake (DEI) [5] (P25 < DEI < P50)		Daily Energy Intake [5] (P50 < DEI < P75)		Daily Energy Intake [5] (DEI > P75)		Age		BMI	
Food Group	Adjusted OR[6]	95 % CI	Adjusted OR	95 % CI	Adjusted OR	95 % CI	Adjusted OR	95 % CI	Adjusted OR	95 % CI	Adjusted OR	95 % CI	Adjusted OR	95 % CI	Adjusted OR	95 % CI	Adjusted OR	95 % CI
Dairy																		
Protein	1.02	0.70-1.50	**1.54***	1.06-2.25	1.00	0.62-1.62	0.88	0.58-1.35	0.84	0.50-1.42	0.77	0.45-1.32	0.73	0.43-1.25	0.99	0.96-1.02	1.04	1.00-1.09
Sugars	1.02	0.70-1.50	1.20	0.99-1.05	0.96	0.60-1.56	0.75	0.49-1.14	0.77	0.46-1.30	1.05	0.62-1.80	0.83	0.49-1.41	1.02	0.99-1.05	1.01	0.96-1.05
Fat	1.06	0.72-1.57	1.04	0.94-2.02	1.02	0.63-1.67	0.98	0.65-1.50	0.72	0.43-1.23	0.73	0.43-1.26	**0.40***	0.23-0.69	1.01	0.98-1.04	**1.04***	1.00-1.10
SFA	0.84	0.57-1.24	**2.10****	1.43-3.08	1.01	0.62-1.64	1.25	0.82-1.92	0.89	0.52-1.51	1.14	0.66-1.97	1.28	0.75-2.20	1.00	0.98-1.03	1.02	0-97-1.06
MUFA	1.06	0.72-1.55	1.23	0.84-1.80	1.17	0.71-1.90	0.91	0.59-1.38	0.77	0.45-1.30	0.77	0.45-1.32	**0.46***	0.27-0.78	1.02	0.99-1.05	1.03	0.98-1.08
Cholesterol	0.95	0.65-1.40	0.74	0.51-1.09	1.01	0.62-1.64	1.16	0.76-1.77	0.70	0.41-1.18	0.93	0.54-1.59	0.55	0.32-0.94	1.00	0.97-1.02	**1.05***	1.00-1.10
Calcium	1.04	0.71-1.53	1.41	0.97-1.06	1.08	0.67-1.76	0.76	0.50-1.16	1.22	0.72-2.06	**1.99***	1.15-3.44	1.01	0.59-1.72	1.00	0.97-1.02	1.02	0.98-1.07
iodine	1.13	0.77-1.65	0.98	0.67-1.43	1.14	0.70-1.85	1.34	0.88-2.04	0.86	0.51-1.44	1.03	0.60-1.76	0.98	0.57-1.66	1.00	0.98-1.03	**1.05***	1.00-1.10
Zinc	0.85	0.58-1.25	1.59	1.09-2.32	1.12	0.69-1.81	0.85	0.56-1.30	0.74	0.44-1.24	0.75	0.43-1.28	0.79	0.46-1.35	1.00	0.97-1.02	1.03	0.98-1.08
Sweets and savoury snacks																		
Carbohydrates	**0.64***	0.43-0.94	1.43	0.97-2.11	0.68	0.41-1.13	1.12	0.73-1.73	1.55	0.90-2.66	1.49	0.85-2.60	1.20	0.70-2.01	**0.95****	0.92-0.97	1.00	0.95-1.04
Total Sugars	0.80	0.54-1.18	1.05	0.72-1.53	0.96	0.59-1.57	1.14	0.75-1.74	1.44	0.85-2.44	1.24	0.72-2.13	0.74	0.43-1.27	**0.97***	0.95-1.00	0.97	0.97-1.02
Fat	**0.53***	0.36-0.79	1.40	0.94-2.07	**0.51***	0.31-0.85	1.35	0.87-2.09	1.33	0.78-2.29	1.28	0.73-2.25	1.87	1.07-3.25	**0.95***	0.93-0.98	0.99	0.95-1.04
SFA	0.77	0.52-1.14	1.22	0.83-1.79	**0.55***	0.34-0.91	0.90	0.59-1.37	1.10	0.65-1.86	1.31	0.76-2.25	**2.00***	1.16-3.43	0.98	0.96-1.01	1.01	0.96-1.06
MUFA	0.94	0.64-1.40	1.18	0.80-1.74	**0.54***	0.33-0.90	1.07	0.70-1.64	1.22	0.72-2.07	**1.84***	1.06-3.19	2.48*	1.43-4.29	**0.97***	0.94-1.00	1.00	0.95-1.04

* OR and 95 % confidence interval (CI) are statistically significant ($p < 0.05$). ** OR and 95 % confidence interval are statistically significant ($p < 0.001$).

[1] Reference category: female;

[2] Reference category: none, primary and secondary grouped;

[3] Reference category: Unemployed, student and casual laborer grouped;

[4] Reference category: single, widow, divorced grouped;

[5] Reference category: DEI < P25;

[6] OR adjusted for gender, education level, occupation, marital status, total energy intake, age and BMI.

These results brought forward a dietary assessment never performed in Kenya and, certainly, it will be revealed very useful for dietary intervention in this population. The results obtained not only provide an improved understanding for the potential impact of food products reformulation, an important current recommendation, but also encourage the establishment of targeting nutritional education interventions in different population groups. Still, this part of the research work had some limitations. It would be desirable to have obtained the anthropometric profile with actual measurements, instead of the reported values, mainly because Kenyans do not monitor their body weight which renders the values less reliable. Moreover, the application of a questionnaire in order to obtain more reliable information regarding physical activity would be interesting in order to explore associations between diet and physical activity levels. Similarly to what happened in the validity study, and inclusively discussed at the end of the sub-chapter 4.1.2.3, interviewers noticed that some respondents had difficulties in the estimation of the consumed amount of certain types of foods, namely vegetables and meat.

4.2. PORTUGUESE SECONDARY DATA ANALYSIS AND COMPARISON WITH KENYAN DATA

4.2.1. Nutrient adequacy and food sources

In both countries, men had a higher daily intake than women, approximately of about 500 kcal, considering the median values (Table 4.12). The major contributors to energy were the same, however carbohydrates had a higher expression in Kenyan diet (60 % *vs*. 46 %) whereas protein (15 % *vs*. 19 %) and total fat contributed less (28 % *vs*. 32 %) (Table 4.12). In Table 4.13 and Figure 4.2 it is possible to observe the percentage of nutrient inadequacy of the adult populations of both countries.

Within carbohydrates, food groups that showed higher contributions in each country are different, while in Kenya vegetables, fruits and legumes together with cereals and starchy foods were the main contributors, in Portugal the last group assumed a primordial role (Figure 4.3 (g)). Kenyans' traditional meal is composed by a generous part of a mix of several vegetables, being present on a daily basis, as previously described in sub-chapter 4.1.3. Also, the consumption of fruits throughout the day is very frequent among Kenyans. In Portugal, it is more common to eat vegetables in a form of a soup, rather than as a side dish, for which mix salad (lettuce, tomato and onion) is chosen more often. Moreover, the consumption of fruits is generally reserved for dessert, after a main meal, not being eaten that often as a snack. According to the IAN-AF survey the consumption of fruits among the adult population is about 129 g/day, which corresponds to less than a regular portion of fresh fruit (~160 g).

In what concerns the food groups that mostly contribute to protein intake, it is easily perceived that the Kenyan diet is richer in plant proteins, while Portuguese diet in animal proteins (Figure 4.3 (b)). In Portugal, every main dish either includes a piece of meat or a piece of fish (normally > 100 g), which is accompanied by a source of carbohydrates, more commonly rice, potatoes or pasta. The consumption of legumes is not that well implemented in Portugal, being on average for the adult population of 19 g/day, nonetheless the Portuguese Nutrition Association (APN, *Associação Portuguesa de Nutrição*, in Portuguese) is making an effort to change this reality. For instance, in 2016, during the international year of legumes and pulses, APN distributed close to 100 kg of dried legumes, in bags of 25 g throughout the country, appealing to the daily consumption of this food, and carried out more than 50 awareness-raising actions.

The fat profile is slightly different, with SFA and MUFA contributing more to energy in the Portuguese diet, and the PUFA having similar contributions. Considering the use of fats and oils, either for cooking or to be added as seasoning, Portuguese diet is very rich in olive oil, mainly composed by MUFA, while in Kenya margarine is the fat of choice, mainly used for cooking. Looking at Figure 4.3 (c), it is possible to observe that the more relevant sources of total fat for Kenyans were the food groups '*meat, fish and eggs*', '*vegetables, fruits and legumes*' and '*cereals and starchy foods*', whereas for Portuguese were '*meat, fish and eggs*' and '*fats and oils*'. For the Kenyan study, the '*fats and oils*' group might have had less expression because for this group

only added fats were considered, since cooking fats were considered within the recipe for each food item, such as rice, beans, or other. In what concerns cholesterol, Portuguese men showed to have diets with higher levels than women, a trend which was not observed in Kenya.

Table 4.12 Daily intake of macronutrients (% of daily energy intake) and micronutrients (absolute values) of the adult populations from Kenya (n=486) and Portugal (n=3104).

		Kenyan Population									Portuguese population								
		Total (n=486)			Female (n=243)			Male (n=243)			Total (n=3104)			Female (n=1675)			Male (n=1429)		
	Unit	**Median**	**P25[1]**	**P75[1]**	**Median**	**P25**	**P75**	**Median**	**P25**	**P75**	**Median**	**P25**	**P75**	**Median**	**P25**	**P75**	**Median**	**P25**	**P75**
Energy (E)	kcal	2892	2352	3512	2647	2220	3347	3111	2532	3636	1904	1568	2292	1606	1349	1891	2186	1812	2601
Protein	% E	14.8	13.6	16.0	14.6	13.4	16.0	15.0	13.8	16.0	19.9	18.1	22.1	19.7	17.8	21.9	17.7	15.8	19.9
Total Fat	% E	27.5	24.6	30.4	27.6	24.3	30.2	27.5	24.9	30.7	31.7	28.3	35.2	31.8	28.3	35.3	29.9	26.5	33.3
SFA[2]	% E	8.4	7.4	9.6	8.5	7.3	9.7	8.3	7.4	9.6	10.3	8.6	12.3	10.4	8.6	12.4	9.7	8.0	11.7
MUFA[3]	% E	8.7	7.4	10.2	8.6	7.2	10.2	8.8	7.6	10.2	12.7	10.9	14.6	12.6	10.8	14.5	12.3	10.6	14.2
PUFA[4]	% E	5.9	5.2	6.7	5.8	5.3	6.6	6.1	5.2	6.7	5.2	4.4	6.1	5.2	4.4	6.1	4.6	3.9	5.4
n-3 [5] PUFA	% E	0.45	0.40	0.52	0.46	0.40	0.53	0.45	0.39	0.52	-	-	-	-	-	-	-	-	-
n-6 [6] PUFA	% E	5.1	4.4	5.8	5.0	4.4	5.8	5.2	4.4	5.2	11	8	15	9	7	13	12	9	17
TFA[7]	% E	0.20	0.16	0.26	0.20	0.15	0.27	0.21	0.16	0.26	0.4	0.3	0.5	0.4	0.3	0.5	0.5	0.3	0.5
Cholesterol	mg	258	168	365	252	157	360	266	177	371	274	205	359	233	175	250	327	250	418
Carbohydrates	% E	60.3	57.2	63.8	60.6	57.6	64.3	60.0	56.9	62.8	45.9	41.5	50.1	48.1	43.9	52.1	45.1	40.6	49.7
Total Fibre	g	50	40	65	48	38	61	51	41	68	17.5	14.1	21.5	15.9	12.9	19.2	18.9	15.0	23.2
Total Sugars	% E	19.8	17.4	22.6	20.2	18.2	22.9	19.3	16.6	22.2	17.5	14.3	21.1	19.5	16.3	23.0	16.4	13.0	20.3
Vitamin B_{12}	µg	5.9	3.1	10.8	5.8	2.9	10.7	5.9	3.1	10.8	4.1	2.7	6.0	3.4	2.2	5.2	4.9	3.4	6.9
Folate	µg	658	515	866	629	497	851	676.7	544.6	890.9	219	169	284	205	156	269	238	188	301
Vitamin C	mg	285	215	370	288	219	377	283.4	208.3	359.5	100	66	148	90	59	136	99	66	146
Vitamin D	µg	4.4	2.7	6.2	4.2	2.6	5.7	4.5	3.09	6.4	3.7	2.0	6.8	3.4	1.8	6.7	3.6	2.3	5.8
Vitamin E	mg	8.8	6.7	11.8	8.4	6.5	11.6	9.0	6.9	11.9	9.0	6.7	12.1	8.4	6.2	11.5	9.7	7.3	12.9
Calcium	mg	1406	1112	1729	1387	1078	1748	1421	1126	1708	733	572	927	692	539	879	783	616	981
Iron	mg	30	24	40	29	23	38	31.6	24.0	41.0	11.4	8.9	14.7	9.9	7.7	12.7	13.3	10.6	16.6
Magnesium	mg	636	510	824	599	478	799	651	542	847	279	228	340	245	201	298	320	268	380
Zinc	mg	13.3	10.2	16.6	11.9	9.4	7.8	14.5	11.5	17.3	10.2	8.1	12.7	8.8	7.1	11.0	11.9	9.7	14.4
Salt	g	10.1	7.9	12.7	9.7	7.7	12.1	10.7	8.0	13.3	7.5	6.1	9.2	6.3	5.2	7.6	8.8	7.3	10.6

[1] P25: Percentile 25th P75: Percentile 75th; [2] SFA: Saturated fatty acids; [3] MUFA: Monounsaturated fatty acids; [4] PUFA: Polyunsaturated fatty acids; [5] n-3: Omega−3; [6] n-6: Omega−6 PUFA; [7] TFA: Trans fatty acids.

Table 4.13 Percentage of nutrient inadequacy of the adult populations from Kenya (n=486) and Portugal (n=3104), according to the respective nutrient recommendations (WHO/FAO: World Health Organization/Food and Agriculture Organization; EFSA: European Food Safety Authority).

WHO/FAO guidelines	Inadequacy (%)– Kenyan adults	EFSA guidelines	Inadequacy (%)– Portuguese adults
Protein (% E[1])		Protein (g/ kg body weight)	
< 10	0.6	< 1	31.7
> 15	45.9	> 2	5.3
Carbohydrates (% E)		Carbohydrates (% E)	
< 55	15.2	< 45	44.7
> 75	0.4	> 60	1
Free sugars (% E)		Free sugars (% E)	
> 10	N.A.[2]	> 10	24.1
Total fat (% E)		Total fat (% E)	
< 15	0.0	< 20	0.7
> 30	27.4	> 30	26.3
SFA (% E)		SFA (% E)	
> 10	81.1	> 10	54.4
PUFA (% E)		PUFA (% E)	
< 2	6.0	-	-
> 8	26.7	-	-
TFA (% E)		TFA (% E)	
> 1	100	> 1	0.2
Salt (g)		Salt (g) [3]	
> 5	97.3	> 5,75	79.7

[1] E: Energy;

[2] N.A.: not applicable;

[3] Percentage obtained from the comparison with the Tolerable Upper Intake Level (UL) for sodium (2300 mg/day), based on the Dietary Reference Intakes (DRIs) developed and published by the Institute of Medicine (IOM), due to the absence of a reference value recommended by EFSA. The calculation took in consideration that 1 g of salt provides 400 mg of sodium.

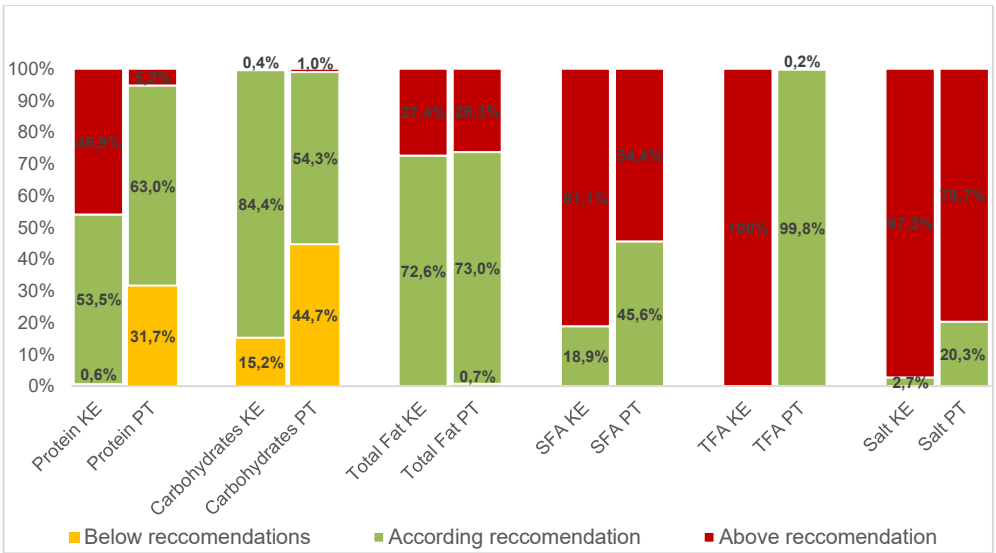


Figure 4.2 Percentage of nutrient inadequacy of the adult populations from Kenya (KE) (n=486) and Portugal (PT) (n=3104), calculated according to the respective nutritional recommendations.

The quantity of consumed fibre per 1000 kcal was around 17 g per day in Kenya, while in Portugal this value was almost half, specifically 9 g per day. Analysing more deeply the sources of fibre in each population, Portuguese get higher quantity of fibre from cereals, while Kenyans resort more to vegetables, fruits and legumes (Figure 4.3 (h)). As stated before, Kenyans eat large quantities of vegetables and fruits, using these as dietary fibre source. In Portugal, the consumption of white bread is much more expressive than in Kenya, being consumed not only at breakfast, but also at main meals and as a snack, which may support these results. In what concerns rice or potatoes, both countries have the habit to use this food item as a meal side dish. Nonetheless, Portugal is one of the European countries that has higher whole grains consumption, being surpassed by Nordic countries and Malta (Joint Research Centre - European Commission, 2017).

Total sugars intake revealed to be very significant in both diets, contributing in 20 % to TEI in half of the Portuguese population and in 18 % in Kenyans, with females being the gender with the higher consumption. In the Portuguese study, the inadequacy in free sugars (above 10 % of EI) was assessed, being around 76 % in the adult population. Analysing the sources of sugars in both countries, although the group with '*vegetables, fruits and legumes*' is the main source, it has more expression in Kenya, with only fruits contributing in 25 % to sugars intake. Furthermore, sweets, sweetened snacks, and soft drinks, were shown to be a common source of sugars, contributing in 26.6% in the Kenyan diet and 30.7% in the Portuguese diet. The consumption of these kinds of products, which are mainly composed of free sugars (World Health Organization, 2015), is recognized to be linked to weight gain and CVD (Evans, 2017). Salt intake was also high in both diets, however in this case men were the gender that revealed higher consumption. According to the results in Portugal, the major contributor to sodium levels is the added salt (29.2 %), followed by '*Bread and toasts*' (18.5 %), '*Soup*' (8.2 %) and '*Charcuterie and processed meats*' (6.9 %). This was similar to what was observed for the Kenyan reality, in which salt added for cooking cereal-based foods, vegetables and legumes was the main provider, with '*Sauces and seasonings*' and '*sweets and savoury snacks*' contributing by 4.4 % and 5.3 %, respectively. These results, relative to sugary and salty products consumption reinforce the need to design strategies towards the reduction of their consumption, and an effort is being done in Portugal as stated preciously in sub-chapter 2.5.2. In what concerns other micronutrients, absolute intakes of vitamins D and E and Zn were close, while for vitamins B_{12} and C and minerals such as Fe, Mg and Ca, concentrations were higher for the Kenyan population, which may be justified by the consumption of traditional vegetables, rich sources in these minerals, as previously discussed.

Figure 4.3 Average contribution (%) of the main food groups to daily intake of energy and nutrients.

Independently of the observed differences, it is important to highlight that the methods used to assess the dietary intake were different; while in Kenya a FFQ was used, in Portugal, 24hR (in two non-consecutive days) together with a food propensity questionnaire were the selected tools, hence, the comparison of both methodologies is compromised. Also, it would have been interesting to do a deeper comparison of the results, supported by a detailed statistical approach. However, the raw data of the Portuguese study were not available, which limited the exploration of the results in such a manner, making possible only the comparison of average values. Moreover, the contribution of food groups to energy and nutrients within the Portuguese diet was studied considering the whole population, not only the adult age group, because those results are not available yet.

PART II – FERMENTATION OF AFRICAN WHOLE GRAINS

4.3. PHASE 1 – FERMENTATION OF AFRICAN WHOLE GRAINS BY PROBIOTIC STRAINS

In this first phase, the performance of three probiotic lactobacilli in different whole grains, sorghum and millet, sourced from Africa was studied. To this end, twelve different fermentations using all combinations of both dry- and wet-milled sorghum and millet flours driven by the three bacterial species were performed. Specific behavioural differences were observed among the twelve fermentation experiments, and in some cases, fermentation was detrimental leading to significant loss of cell viability that might be justified by the production of inhibitory metabolites that prevent bacterial growth (data not shown). Hence, based on these observations, only the cases in which fermentation was effective are presented and discussed herein; a total of six fermentations were targeted for further analytical and statistical analysis (Table 4.14).

Table 4.14 Selected cereal slurries that underwent fermentation over 24 h at 30 °C and 200 rpm, in an orbital incubator.

Cereal	Milling process	Probiotic species
Sorghum	**Wet**	*L. plantarum* 299v
	Dry	*L. plantarum* 299v
Finger millet	**Wet**	*L. plantarum* 299v
	Dry	*L. plantarum* 299v *L. acidophilus* Ki *L. rhamnosus* R-11

4.3.1. Fermentative capacity - microbial growth and acidification rates

Analysis of Figure 4.4. reveals that *Lactiplantibacillus plantarum* 299v was the most efficient bacterium, being able to ferment cereal slurries from both milling treatments (dry and wet) of both cereals (sorghum and finger millet). On the other hand, *L. rhamnosus* R-11 and L. acidophilus Ki were unable to survive fermentation in sorghum cereal independently of the milling treatment. Sorghum cereal has a high tannins content, which are known to inhibit the growth of several microorganisms being resistant to their attack (Chung et al., 1998); such properties may justify the strains' lack of growth capacity. *Lactiplantibacillus plantarum* 299v's ability to ferment this type of matrices might be related to its possession of tannase activity (Osawa et al., 2000) and, consequent ability of metabolising these compounds (Barthelmebs et al., 2001), preventing them from hindering associated growth.

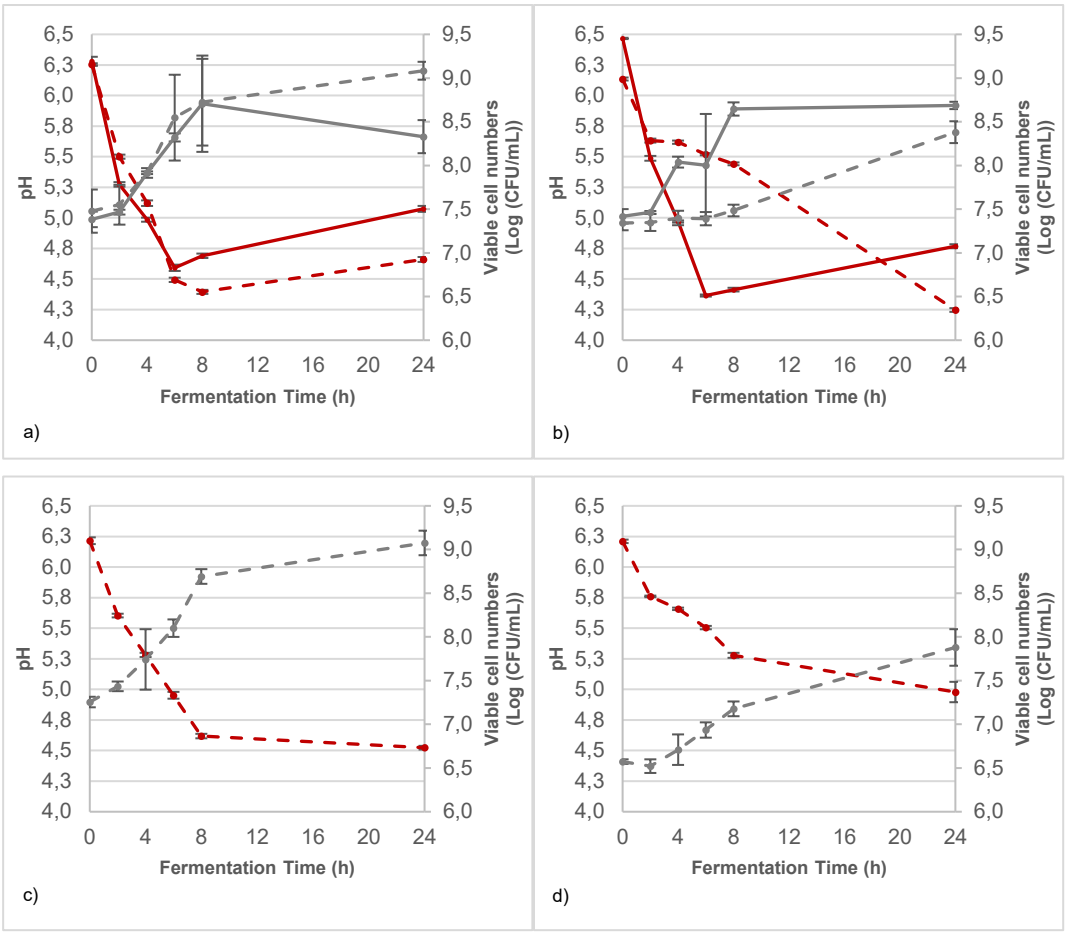


Figure 4.4 Evolution of viable cell numbers (grey lines) and of pH (red lines) of *L. plantarum* 299v (a, b), *L. rhamnosus* R-11 (c) and *L. acidophilus* Ki (d) inoculated in dry milled (dashed lines) or in wet-milled (full lines) of finger millet (a, c, d) and sorghum (b) slurries over 24 h of fermentation at 30 ºC and 200 rpm in an orbital incubator. Error bars represent standard deviation of independent replicate experiments.

By the analysis of the fermentation performance of *L. plantarum* 299v the four different cereal slurries (Figure 4.4 a and b) it is possible to observe that the type of cereal (sorghum *vs*. finger millet) and the cereal-processing operations (dry *vs*. wet) affected the acidification rate ($p < 0.05$) and the variation of the bacterial viable cells ($p < 0.05$) over the fermentation time.

The difference in pH values before and after fermentation (delta pH) was used as an indicator of strains' acidification performance in each cereal slurry. The delta pH was 0.4 and 0.2 units higher for the dry-milled cereal millet and sorghum slurries, respectively, than for the wet-milled counterparts (1.6 and 1.9 units in dry-milled millet and sorghum slurries *vs*. 1.2 and 1.7 units in wet-milled millet and sorghum slurries) revealing an important impact of the cereal-processing conditions ($p < 0.05$). Microbial growth was higher in dry-milled millet fermentations than in the other cereal slurries, showing an increase in cell density of nearly 2 log cycles (21 %) ($p < 0.05$). Wet-millet and dry-sorghum slurries were those in which lower cell populations were reached (12 % and 15 %, respectively) over fermentation, *id est* provided the lowest bacterial

growth. Analysis of Figure 4.4 also shows that the main fermentation activity occurred during the first 8 h, with exception for dry sorghum. Considering wet-sorghum, wet-millet and dry-millet slurries, the acidification rates, over the first 8-h period are quite different from those registered over the subsequent 16-h period up to the total 24 h fermentation: 0.26, 0.20 and 0.23 pH units per hour, respectively, and 0.02 units of pH per hour, for the three matrices, respectively. This rate difference is supported by the deltas in both intervals, namely $\triangle_{t8-t0}$ and $\triangle_{t24-t8}$ ($p < 0.05$). The bacterial viable cell numbers followed a similar trend, as expected, *id est*, the growth rate for wet-sorghum, wet-millet and dry-millet in the first 8 h of fermentation (0.15, 0.17 and 0.16 log (CFU/mL) h^{-1}, respectively) was greater than that observed in the subsequent 16 h of fermentation (0.003, 0.02 and 0.02 log (CFU/mL) h^{-1}, respectively). Consequently, the variation in viable cell numbers in the first (-h incubation, $\triangle_{t8-t0,}$ was significantly higher than in the subsequent 16-h period, $\triangle_{t24-t8}$, for the three lactobacilli ($p<0.05$). These results are in accordance to those reported by other researchers (Coda et al., 2012; Magala et al., 2015; Pranoto et al., 2013), who demonstrated the coupling effect between the most effective fermentative activity and exponential growth phase.

Analysis of bacterial growth performance in dry millet (Figure 4.4 a, c and d) shows that *L. rhamnosus* R-11, *L. plantarum* 299v*,* and *L. acidophilus* Ki were all able to increase viable cell numbers by approximately 1.8 log cycles, 1.6 log cycles, and 1.3 log cycles, respectively. No statistically significant differences in viable cell numbers were observed for either strain: *L. rhamnosus* R-11 and *L. plantarum* 299v, even though *L. plantarum* 299v revealed higher mean viable cell numbers over time, inclusively by 24-h fermentation. In contrast, the poor acidification and lower increment in viable cell numbers of *L. acidophilus* Ki was significant over the fermentation period ($p < 0.05$). The improved growth achieved by *L. plantarum* and *L. rhamnosus* may be justified by their acid tolerance, that seems to be superior to the ability of *L. acidophilus* to survive at low pH to the organic acids that are duly produced (Anukam & Koyama, 2007; Corcoran et al., 2005; Pan et al., 2009). In the studies of Pranoto *et al*. (2013) and Mukisa *et al*. (2012)*,* in which sorghum flour was analysed, *L. plantarum* viable cell numbers increased approximately 2.5 log cycles, and around 2 log cycles, respectively. During rice and millet fermentations, LAB grew no more than 1.5 log cycles during 24 h or 10 h, respectively (Di Stefano et al., 2017; Magala et al., 2015).

4.3.2. Fermentative capacity - Sugars consumption and organic acids production

The decreasing evolution of pH throughout the 24 h of fermentation was reflected in the production of organic acids, consequence of fermentation of cereal sugars. For *L. plantarum* 299v fermentation in the different cereal slurries, the most abundant organic acid produced during the first 8 h of fermentation was lactic acid, although increases in acetic acid were also detected, whereas the main sugars consumed were glucose and fructose (Table 4.15), similarly to that observed in other studies with these cereals (Di Stefano et al., 2017; Mukisa, Byaruhanga, et al., 2012). Mukisa *et al*. (2012) reported higher lactic acid concentrations by 24-h fermentation and

there was no reduction at the end of fermentation, however, the highest rate of lactic acid production also happened during the first 12 h. In their study, using hulled pearl millet, Di Stefano *et al*. (2017) reported similar final lactic acid concentrations (at 12 h sampling time), yet here it is not possible to compare the evolution since only two time-points were followed (0 h and 12 h). Rathore *et al*. (2012), in their study with mixed cereals, also found a reduction in lactic acid concentrations, however such reduction was registered only after 24 h of fermentation. Good negative correlations, above 0.70, were observed between lactic acid production and glucose and fructose depletion for dry-milled cereals (-0.95 and - 0.96 for sorghum and -0,78 and -0.95 for millet, respectively) in comparison to wet-milled counterparts (-0.51 and -0.66 for sorghum and -0.34 and -0.99 for millet, respectively). Indeed, although not statistically significant ($p > 0.05$), the highest absolute increase in lactic acid concentration and subsequent glucose/fructose depletion was observed during fermentation of dry-milled millet slurry, in particular during the first 8-h incubation period, which is in agreement with the acidification and microbial growth results. Concentrations of citric acid and maltose were also analysed, but no major concentrations (< 0.1 g/kg) or variations were observed during fermentation of all the cereal matrices fermented by *L. plantarum* 299v.

Table 4.15 Evolution of sugars consumption, namely of fructose and glucose (g/kg of slurry), and organic acids production, namely of acetic and lactic acids (g/kg of slurry) in finger millet wet or dry-milled sorghum and slurries fermented by *L. plantarum* 299v, over 24 h of incubation at 30 ºC and 200 rpm, in an orbital incubator.

				Lactic Acid (g/kg)	Acetic Acid (g/kg)	Glucose (g/kg)	Fructose (g/kg)
***L. plantarum* 299v**	**Sorghum**	Dry-milled	0	0.10 ± 0.02	0.09 ± 0.05	0.26 ± 0.03	0.23 ± 0.01
			8	0.27 ± 0.07	0.37 ± 0.08	0.109 ± 0.002	0.104 ± 0.002
			24	0.36 ± 0.03	0.42 ± 0.05	0.100 ± 0.003	0.091 ± 0.003
		Wet-milled	0	< LOD[1]	0.0560 ± 0.0008	0.18 ± 0.01	0.149 ± 0.008
			8	0.6 ± 0.4	0.17 ± 0.08	0.09 ± 0.05	0.10 ± 0.03
			24	0.07 ± 0.01	0.2 ± 0.1	0.08 ± 0.03	0.08 ± 0.02
	Millet	Dry-milled	0	< LOD	0.12± 0.02	0.44 ± 0.04	0.23 ± 0.03
			8	1.0 ± 0.1	0.33 ± 0.07	0.23 ± 0.04	0.102 ± 0.007
			24	0.7 ± 0.1	0.48 ± 0.04	0.108 ± 0.004	0.103 ± 0.004
		Wet-milled	0	< LOD	0.057 ± 0.002	0.18 ± 0.02	0.13 ± 0.02
			8	0.3 ± 0.2	0.12 ± 0.06	0.12 ± 0.04	0.08 ± 0.01
			24	0.10 ± 0.02	0.4 ± 0.1	0.11 ± 0.03	0.11 ± 0.03

[1]LOD: Limit of Detection (Lactic acid: 0.05 g/L).

Values are expressed as the mean ± standard deviation of independent replicate experiments.

Final concentrations of glucose and fructose were very low, almost totally consumed by 24-h fermentation as in other studies (Di Stefano et al., 2017; Mukisa, Byaruhanga, et al., 2012); maltose was also detected but in small quantities (Mukisa, Byaruhanga, et al., 2012) and it was almost not consumed. Similar trends of sugars consumption and organic acids production were observed in rice fermentation studies, even though the concentrations' magnitude was different (Magala et al., 2015). Regarding the bacterial growth and metabolic activity, the cereal matrix in which more impactful achievements were observed during fermentation by the three bacteria was dry millet.

Dry-milled millet was also the most favourable cereal slurry for *L. rhamnosus* R-11 and *L. acidophilus* Ki acidification performance. In fact, *L. rhamnosus* R-11, followed by *L. plantarum* 299v, was the species that produced the highest amount of lactic acid at a higher rate in the first 8 h of fermentation, but no statistically significant differences were observed ($p > 0.05$). After 24 h of fermentation, *L. rhamnosus* R-11, *L. plantarum* 299v and *L. acidophilus* Ki had produced 1.3 ± 0.2 g/kg, 0.74 ± 0.09 g/kg and 0.40 ± 0.06 g/kg of lactic acid, respectively. In terms of acetic acid concentrations over fermentation no differences were observed, although *L. acidophilus* Ki was, once again, the strain that produced the lowest concentration. At the end of fermentation, the concentrations of acetic acid were 0.47 ± 0.04 g/kg, 0.42 ± 0.08 g/kg and 0.17 ± 0.06 g/kg for *L. plantarum* 299v, *L. rhamnosus* R-11 and *L. acidophilus* Ki, respectively. Glucose was the main consumed sugar with initial concentrations of 0.44 ± 0.04 g/kg, 0.3 ± 0.1 g/kg and 0.167 ± 0.007 g/kg and final concentrations of 0.108 ± 0.004 g/kg, 0.073 ± 0.005 g/kg and 0.05 ± 0.00 g/kg, for *L. plantarum* 299v, *L. rhamnosus* R-11 and *L. acidophilus* Ki, respectively.

4.3.3. Total phenolics content and antioxidant capacity

It is important to know the antioxidant content and associated efficacy in foods, since not only do they play a preservation role but also provide protection against oxidative damage, preventing loss of nutritional and biological values. Total phenolic compounds are commonly associated with antioxidant capacity. A potential mechanism by which they may confer such antioxidant capacity involves their donation of an electron/transfer hydrogen atom to free radicals, leading to the activation of endogenous antioxidant mechanisms, increasing antioxidant enzymes levels, and acting as chelators of trace metals involved in free radical protection. Observing data in Table 4.16, it is clear that sorghum, either in dry- or wet-milled form and either fermented or non-fermented, showed higher amounts of total phenolics when compared with finger millet counterparts ($p < 0.05$). In terms of cereal processing conditions, average total phenolics content (TPC) was higher in dry-milled cereal slurries than in wet-milled counterparts, especially for sorghum ($p < 0.05$). In what concerns dry-milled millet slurries fermented by each of the three lactobacilli, while for *L. rhamnosus* R-11 and *L. acidophilus* Ki an increase in TPC was observed over the 24-h fermentation period, in the fermentation by *L. plantarum* 299v, lower levels of TPC were obtained after 24 h, which might be justified by its possession of the tannase enzyme, as previously observed in sub-chapter 4.3.1.

Focusing on *L*. *plantarum* 299v fermentation, in particular, higher values of Trolox equivalents (TE) and Ascorbic acid equivalents (AAE) were obtained for sorghum when compared with millet slurries ($p < 0.05$), and in consequence the percentage of inhibition of DPPH and ABTS radicals was much higher for sorghum, showing its higher antioxidant potential, in comparison with millet slurry. A 100-fold diluted 20 mg of sorghum extract/mL of methanol is capable of inhibiting around 20-23 % of free DPPH radicals while a 10-fold diluted 20 mg of millet extract/mL of methanol inhibits Results show that antioxidant activity was not apparently affected by 24 h of fermentation. Regarding the scavenging of free ABTS radicals, the potential of sorghum slurries is also higher than millet counterparts, since for the same concentration of 20 mg of extract /mL of methanol, sorghum slurry extracts inhibit about 50-62 % of the ABTS radicals and millet slurry extracts about 10 %. Higher values for DPPH relative to ABTS, in sorghum slurries, might be associated with the presence of larger amounts of non-polar compounds (higher content of fat) in the cereal. Comparing the activity of the three lactobacilli during the fermentation of dry-milled millet slurry, DPPH antioxidant capacity was highest in slurries fermented by *L*. *acidophilus* Ki whereas a higher ABTS antioxidant capacity was observed in cereal slurries fermented by *L*. *rhamnosus* R-11 and *L*. *plantarum* 299v, especially for the former strain over the last 16 h of fermentation.

Table 4.16 Evolution of total phenolics content (TPC) (mg GAE[1]/g of lyophilized extract) and antioxidant activities (DPPH, mg TE[2]/ g of lyophilized extract; ABTS mg AAE[3]/ g of lyophilized extract) in wet- or dry-milled sorghum and millet slurries fermented by *L*. *plantarum* 299v, dry-milled finger millet fermented by *L*. *rhamnosus* R-11 and *L*. *acidophilus* Ki, over 24 h of incubation at 30 °C, and 200 rpm, in an orbital incubator.

	***L. plantarum* 299v**				***L. rhamnosus* R- 11**	***L. acidophilus* Ki**
	Sorghum		**Millet**		**Millet**	**Millet**
	Wet-milled	Dry-milled	Wet-milled	Dry-milled	Dry-milled	Dry-milled
TPC (mg GAE[1]/g of lyophilized extract)						
0	16 ± 1	21 ± 8	3.1 ± 1.4	4.5 ± 0.9	2.8 ± 0.4	2.8 ± 0.5
8	14.9 ± 0.5	19 ± 7	3.2 ± 1.1	4 ± 1	3.9 ± 0.4	3.2 ± 0.5
24	15.4 ± 0.9	17.6 ± 0.9	2.97 ± 0.08	3.1 ± 0.9	3.8 ± 0.4	3.1 ± 0.5
DPPH (mg TE[2]/ g of lyophilized extract)						
0	36 ± 1	42 ± 2	7.6 ± 0.2	9.6 ± 0.7	8.9 ± 0.2	17.7 ± 0.6
8	34.2 ± 0.7	28 ± 5	7.9 ± 0.1	9.2 ± 0.7	8.8 ± 0.1	18 ± 1
24	34 ± 4	36 ± 6	7.74 ± 0.05	8.4± 1.1	8.2 ± 0.1	18 ± 1
ABTS (mg AAE[3]/ g of lyophilized extract)						
0	10.7 ± 0.6	12.5 ± 0.3	3.2 ± 0.3	5 ± 1	3.7 ± 0.1	3.2 ± 0.3
8	9.9 ± 0.1	11.4 ± 0.3	3.7 ± 0.3	4.0 ± 0.2	3.7 ± 0.2	2.9 ± 0.2
24	9.6 ± 0.2	10.9 ± 0.1	3.4 ± 0.3	3.8 ± 0.1	6.30 ± 0.04	3.2 ± 0.3

[1] GAE: Gallic Acid Equivalents; [2] TE: Trolox Equivalents; [3] AAE: Ascorbic Acid Equivalents.

Values are expressed as the mean ± standard deviation of independent replicate experiments.

The general decrease in TPC observed within 24 h of fermentation is consistent with results reported in some recent studies, as reviewed by Taylor and Duodu (2015). However, the effect of fermentation on these compounds is not very clear, as in the literature controversial results are found. The results presented herein revealed that for some cases, concentrations seemed to vary slightly, within 8 h of fermentation. Unfortunately, the monitorization during the process is usually not followed and only an analysis before and after fermentation is assessed, which hampers the comparison of the evolution during fermentation with other studies. Nevertheless, Towo *et al*. (2006) also found a reduction in total phenolics after fermentation of sorghum and concentration values before and after fermentation were similar to those of the present study. Svensson *et al*. (2010) studied the effect of fermentation by combined cultures of *Lacticaseibacillus, Lactiplantibacillus, Limosilactobacillus fermentum* and *Limosilactobacillus reuteri* on sorghum and they concluded that fermentation reduced concentrations of phenolic acids with some exceptions. Focusing on millet fermentation, Gabaza *et al*. (2016) also observed a general decrease in bound and total phenolics, but an increase in soluble phenolics. Venkateswaran and Vijayalakshmi (2010) studied the effect of fermentation by *Monascus purpureus* on the tannins' concentration in finger millet, which was reduced in 13.2 % after processing. The mini review of Taylor and Duodu (2015) has highlighted that fermentation decreases the levels of phenolic compounds in both cereals, unlike other processes such as malting for example. Dlamini *et al*. (2007) evaluated the effect of fermentation on antioxidant activity (using the ABTS and DPPH assays) in sorghum and sorghum products and reported that either ABTS or DPPH activities were strongly reduced after fermentation.

A combination of mechanisms has been suggested to explain this reduction. A first possibility is related with the enzyme polyphenol oxidase (PPO), which source can be the cereal or the fermenting microbiota themselves (Dhankher & Chauhan, 1987; Taylor & Duodu, 2015). PPO uses phenolic compounds as substrates and its action might lead to their removal/depletion. Additional microbial enzymes (decarboxylases or hydrolases) may play similar roles and be responsible for phenolic compound metabolism. Another explanation is their reduced extractability caused by the interaction of these compounds with macromolecules, such as proteins, present in the acidic aqueous cereal matrix (Adebo & Medina-Meza, 2020; Beta et al., 2000; Taylor & Duodu, 2015).

In contrast, the increase in TPC and antioxidant activity after cereal fermentation is also reported. Rasane *et al*. (2015) analysed different cereal blends with pearl millet, oat and sorghum, and for those blends containing a high percentage of sorghum, fermentation led to increased levels of total phenolics and DPPH activity as well, contradicting the present results. However, it might be noted that cereals have been roasted and germinated before fermentation, which could have had some influence on the latter effect of fermentation. Considering other cereals matrices, Dordević *et al*. (2010) studied unfermented and fermented buckwheat, barley, wheat and rye, Ghosh *et al*. (2015) explored a rice-based fermented beverage, and Katina *et al*. (2007) focused their research on wholemeal rye flour, and all concluded that after fermentation higher values were obtained. Coda *et al*. (2012) determined the TPC of several yoghurt-like beverages made

from rice and other cereals, concluding that fermented products had more 10.8–18.0 % of TPC when compared to the control. These results seem to show that the matrix might have an influence on the effect of fermentation; it is believed that the substrate matrix may influence the metabolic pathway by influencing the expression of enzymes, *id est*, enzymatic activities can shift from decarboxylase action to reductase to glucosidase activity. Hence this relationship between different enzymes of the fermenting organisms and the grain types, may lead to bound phenolics release (Adebo and Medina-Meza, 2020).

4.3.4 Iron content

Evolution of iron content in wet- or dry-milled sorghum and millet slurries fermented by *Lactiplantibacillus plantarum* 299v, and in dry-milled millet slurry fermented by *Lacticaseibacillus rhamnosus* R-11 and *Lactobacillus acidophilus* Ki, throughout fermentation is shown in Figure 4.5.

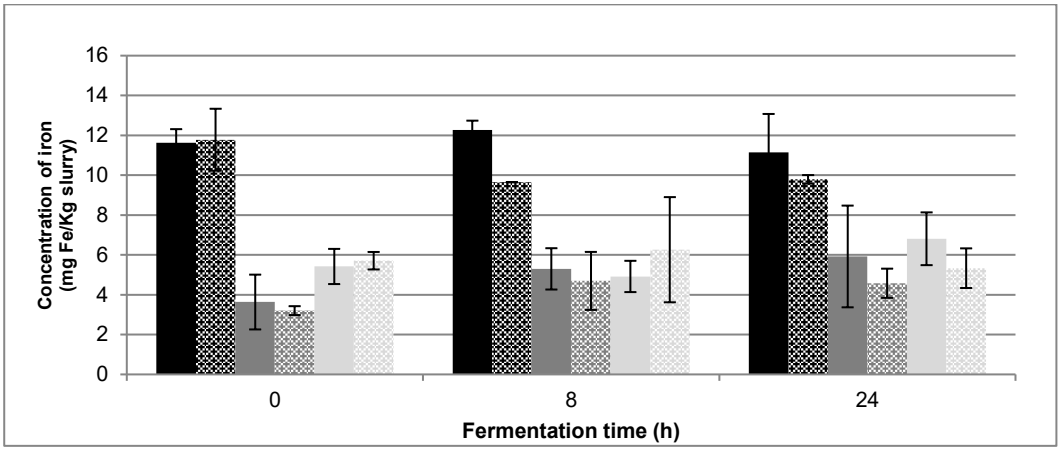


Figure 4.5 Evolution of iron content (mg Fe/kg of slurry) in wet- (pattern columns) or dry-milled (plain colour columns) sorghum (first and second columns) and finger millet (third and fourth columns) slurries fermented by *L. plantarum* 299v, and dry-milled millet slurry fermented by *L. rhamnosus* R-11 (fifth column) and by *L. acidophilus* Ki (sixth column), over 24 h of incubation at 30 ºC and 200 rpm, in an orbital incubator. Error bars represent standard deviation of independent replicate experiments.

All sorghum slurries either in dry- or wet-milled form and either fermented by *L. plantarum* 299v or non-fermented, had higher amounts of iron in comparison to the millet slurry counterparts ($p < 0.05$), which is in agreement with literature (Saleh et al., 2013; Vila-Real et al., 2017). Averagely, iron content is higher in dry-milled millet slurries at the end of fermentation by *L. plantarum* 299v and *L. rhamnosus* R-11, than those fermented by *L. acidophilus* Ki, however, no statistically significant differences were observed ($p > 0.05$). No differences are observed neither between bacteria nor over time. Towo *et al*. (2006) concluded that iron content was not affected by fermentation, however *in vitro* accessible iron was higher after treatment. The study of Ghosh *et al*. (2015) revealed that one day of fermentation was enough to increase the iron content in rice. In addition, iron content was also higher after germination and fermentation of the optimized

multi-cereal baby food formulation studied and developed by Rasane *et al*. (2015). In the study of Hemalatha *et al*. (2007), in which spontaneous fermentations were assessed, bioavailable iron content did not alter after fermentation, whereas Adebiyi *et al*. (2017) found higher iron content in fermented pearl millet flour. The increase in iron content might be justified by the decreased activity of antinutrients, namely phytate and polyphenolic compounds, in consequence of processing such as germination and fermentation (Vaughn & Duke, 1981). Although it was not an objective of this research work, it would be interesting to assess the effect of the presence of phytate on this mineral, given this known adverse effect on iron's availability.

Overall, it is possible to conclude that the most favourable milling treatment that enabled the growth of the three strains was dry milling. Among the probiotic strains, *L*. *plantarum* 299v revealed itself to be more versatile regarding the cereal matrix to ferment, and no significant differences were observed between *L*. *rhamnosus* R-11 and *L*. *plantarum* 299v in terms of growth and impact on iron contents. Nevertheless, *L*. *rhamnosus*' fermentation seemed to result in higher TPC and antioxidant activity (only considering the ABTS assay). Although an increase in the total phenolics' level after fermentation might hamper the minerals' bioavailability and specific biological activities, having higher antioxidant activity is desirable. Taking all this into consideration, and also that in the subsequent phases combined cultures and fermentation of another cereal are studied, *L*. *plantarum* 299v was the selected strain, and dry-milling the milling treatment to proceed for further studies.

4.4. PHASE 2 – FERMENTATION OF AFRICAN WHOLE GRAINS BY *WEISSELLA* STRAINS

4.4.1. Fermentative capacity - microbial growth and acidification rates

Four *Weissella* strains were tested for their fermentative capacity of dry-milled sorghum and pearl millet slurries. Analysis of Figures 4.6 and 4.7, representing pH and viable cell numbers evolution over 24-h fermentation, highlight the fact that millet's fermentation resulted in higher acidification and growth than sorghum fermentations ($p < 0.05$), for all four strains.

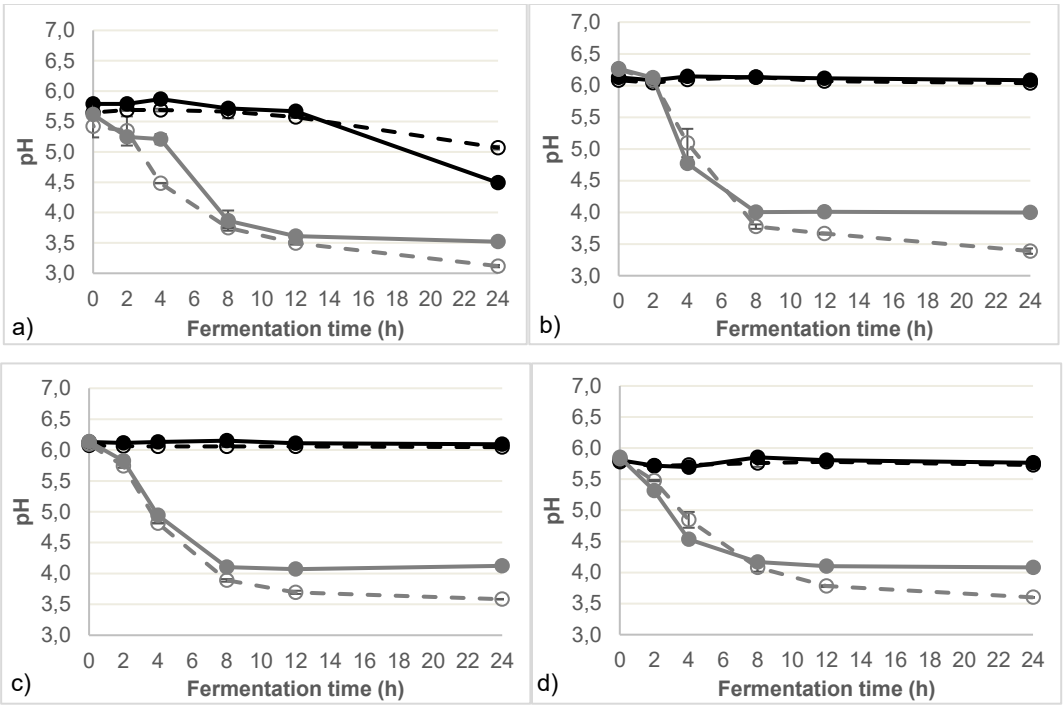


Figure 4.6 Evolution of pH of *W. confusa*/*cibaria* C2 (a), *W. confusa*/*cibaria* 32LABPT05 (b), *W. confusa* 2LABPT05 (c) and *W. confusa* VTT E-90E392 (d) inoculated in dry-milled pearl millet (grey lines) and sorghum (black lines) slurries in the presence (dashed lines) or absence (full lines) of sucrose, over 24 h fermentation at 30 °C and 200 rpm in an orbital incubator. Error bars represent the standard deviation of independent replicate experiments.

e) *W*eissella *confusa/cibaria* C2 was the only the strain capable of acidification of sorghum slurries during fermentation ($p < 0.05$) and also the only one in which the viable cell numbers increased steadily during sorghum fermentation ($p < 0.05$) (Figure 4.7 a)). During millet fermentation (in the presence or absence of sucrose), strains revealed a growth increase of more than 2 log cycles and final viable cell numbers around 10^9 CFU/mL were reached. There was no difference between the acidification and growth behaviour of the four strains over time ($p > 0.05$). Nevertheless, according to the results, it seems that the acidification rate is higher during the

fermentation of millet slurries with added sucrose than without sucrose. But, only the strain *W. confusa* 2LABPT05 lead to significantly higher acidification when fermenting millet in the presence of sucrose ($p < 0.05$). In terms of growth, no differences were observed between the fermentation over time of the two media (pearl millet and pearl millet with sucrose), for all strains.

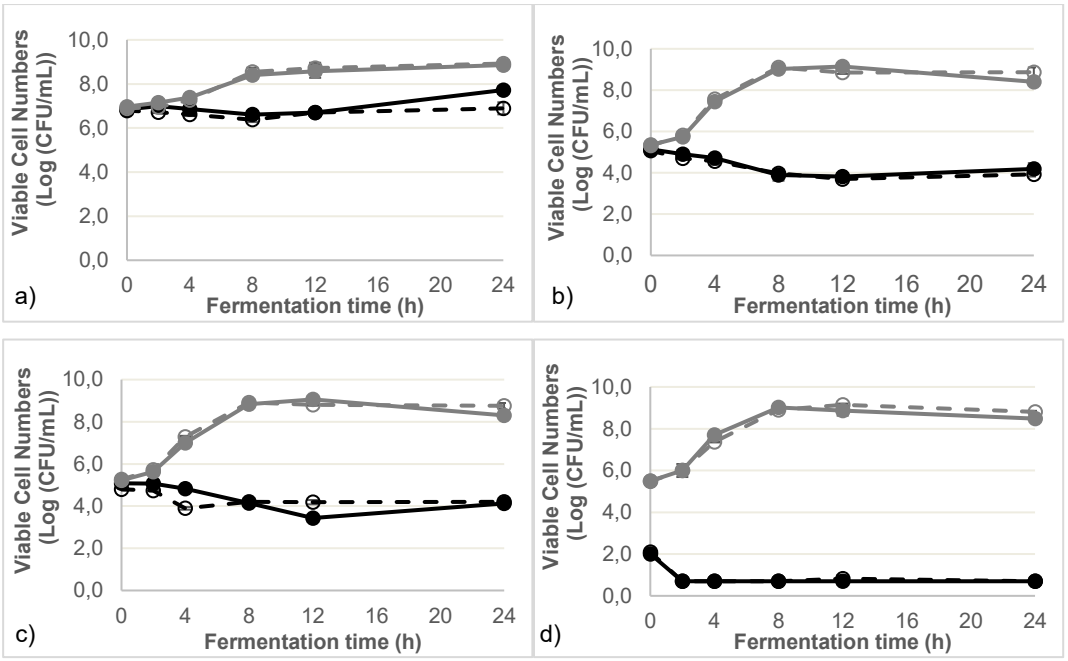


Figure 4.7 Evolution of the viable cell numbers of *W. confusa*/*cibaria* C2 (a), *W. confusa*/*cibaria* 32LABPT05 (b), *W. confusa* 2LABPT05 (c) and *W. confusa* VTT E-90E392 (d) inoculated in dry milled pearl millet (grey lines) and sorghum (black lines) slurries in the presence (dashed lines) or absence (full lines) of sucrose, over 24 h fermentation at 30 °C and 200 rpm, in an orbital incubator. Error bars represent the standard deviation of independent replicate experiments.

e) Based on the previous results, the discussion below only includes the results relative to the successful fermentations, namely pearl millet fermentations, which were targeted for further analytical and statistical analysis.

4.4.2. Fermentative capacity - Sugars consumption and organic acids production

Lactic and acetic acids were produced by all the *Weissella* strains (Table 4.17). After 8 h of fermentation, an increase in lactic acid concentration was observed, which is in agreement with the pH drop within the same time. Except for *W. confusa*/*cibaria* C2, the remaining fermentation time, after the 8 h, does not seem to have influenced organic acids concentrations. During the first 8 h of fermentation of millet in the absence of sucrose, lactic acid production rate varied between 0.18 g/kg h^{-1} (*W. confusa*/*cibaria* C2) and 0.22 g/kg h^{-1} (*W. confusa* 2LABPT05) while after this period the rate dropped intensively for every strain (0.003 g/kg h^{-1}), with the exception for *W. confusa*/*cibaria* C2 (0.13 g/kg h^{-1}), showing that it is during the first 8-h fermentation period

that of the major cell metabolism activity occurs. Nevertheless, at the confidence level of 95 %, no statistically significant differences were observed between the studied bacteria during the fermentation of pearl millet in the presence of sucrose, at the several evaluated time intervals: $\triangle t_{24} - t_0$, $\triangle t_{12} - t_0$ and $\triangle t_8 - t_0$. In accordance with the observed acidification, a more acidic environment was achieved during fermentation of pearl millet in the presence of sucrose, for all strains, showing that the presence of sucrose has an impact on the fermentation activity. However, no statistically significant differences, for the several absolute differences evaluated: $\triangle t_{24} - t_0$, $\triangle t_{12} - t_0$ and $\triangle t_8 - t_0$, were observed for the same strain between the two slurries. The increment of lactic acid at the end of fermentation ($\triangle t_{24} - t_0$) was highest for *W. confusa*/*cibaria* C2 (+ 6.7 g/kg), followed by *W. confusa*/*cibaria* 32LABPT05 (+ 3.64 g/kg) and *W. confusa* 2LABPT05 (+ 3.53 g/kg), however these differences were not statistically different were observed. Di Stefano *et al*. (2017), who studied the fermentation of pearl millet in a water-based formulation by *L. rhamnosus* and *S. thermophilus*, also observed that the main organic acids produced were lactic and acetic acids, however concentrations achieved after 12 h were lower than the ones of the present study. Rathore *et al*. (2012) have obtained similar conclusions regarding the organic acids produced when studying controlled fermentation of different cereals by other LAB.

Sugars levels, namely glucose and sucrose, decreased during fermentation, serving as substrates for the microorganisms. In the study of Osman, despite being focused on spontaneous fermentation, it was also observed a decrease of soluble sugars after 24 h of fermentation (Osman, 2011). Specifically, the strains *W. confusa* and *W. cibaria* are known for producing organic acids, and also EPS, from sucrose (Fusco et al., 2015). Sucrose was both metabolized as a carbon source for microbial growth, and more evidently, given the observed higher viscosity, after six hours of fermentation, it was used for EPS production. This decrease of sucrose, due to the activity of the glycansucrases (glucansucrases or fructansucrases) characteristic of *Weissella* strains (Amari et al., 2013; Van Hijum et al., 2006), leads to the formation of glucans, liberating fructose into the medium (Xu, Wang, et al., 2017), an observable trend within the present results, given the existence of fructose at the end of fermentation. Similar results were also observed by Wang *et al*. (2019). Table 4.17 shows the evolution of the lactic and acetic acids concentrations, and also of glucose, fructose and sucrose over the 24-h fermentation period of pearl millet in the presence or absence of sucrose.

Table 4.17 Evolution of glucose and fructose consumption (g/kg of slurry) and lactic and acetic acids production (g/kg slurry) by *W. confusa*/*cibaria* C2, *W. confusa*/*cibaria* 32LABPT05, *W. confusa* 2LABPT05 and *W. confusa* VTT E-90E392 inoculated in dry-milled pearl millet slurries in the presence or absence of sucrose, over 24 h fermentation at 30 °C and 200 rpm, in an orbital incubator.

	W. confusa/cibaria															
	C2				**2LABPT05**				**32LABPT05**				**VTT E-90E392**			
	Dry-milled Millet in water suspension															
(g/kg)	0	8	12	24	0	8	12	24	0	8	12	24	0	8	12	24
Lactic Acid	0.27	1.72	3	3.8	0.16	1.88	1.90	1.90	0.18	1.79	2.06	1.9	0.4	1.1	1.3	1.3
	±0.00	±0.02	±1	±0.4	±0.02	±0.09	±0.09	±0.08	±0.00	±0.00	±0.06	±0.2	±0.1	±0.4	±0.5	±0.5
Acetic Acid	0.14	0.145	0.17	0.25	0.16	0.27	0.28	0.285	0.14	0.245	0.335	0.3	0.14	0.22	0.20	0.25
	±0.02	±0.007	±0.06	±0.03	±0.01	±0.01	±0.04	±0.007	±0.00	±0.007	±0.007	±0.03	±0.04	±0.01	±0.02	±0.01
Glucose	0.72	0.57	0.30	0.3	0.72	0.195	0.4	0.185	0.74	0.22	0.3	0.23	0.34	0.15	0.15	0.20
	±0.01	±0.04	±0.08	±0.1	±0.06	±0.007	±0.3	±0.007	±0.06	±0.05	±0.1	±0.04	±0.04	±0.00	±0.05	±0.02
Fructose	0.61	0.56	0.4	0.21	0.65	0.175	0.3	0.15	0.70	0.20	0.24	0.165	0.63	0.16	0.16	0.075
	±0.08	±0.06	±0.2	±0.09	±0.01	±0.007	±0.2	±0.00	±0.05	±0.05	±0.08	±0.007	±0.01	±0.01	±0.01	±0.007
	W. confusa/cibaria															
	C2				**2LABPT05**				**32LABPT05**				**VTT E-90E392**			
	Dry-milled Millet in sucrose suspension															
(g/kg)	0	8	12	24	0	8	12	24	0	8	12	24	0	8	12	24
Lactic Acid	0.3	1.71	3.68	7.00	0.21	1.81	2.61	3.74	0.16	1.7	2.5	3.795	0.56	2.3	3.0	3.96
	±0.1	±0.06	±0.08	±0.07	±0.00	±0.06	±0.04	±0.08	±0.02	±0.1	±0.2	±0.007	±0.02	±0.1	±0.2	±0.04
Acetic Acid	0.145	0.11	0.17	0.25	0.16	0.245	0.27	0.29	0.14	0.24	0.34	0.365	0.15	0.22	0.28	0.29
	±0.007	±0.01	±0.01	±0.03	±0.02	±0.007	±0.00	±0.00	±0.00	±0.02	±0.06	±0.007	±0.00	±0.00	±0.00	±0.06
Sucrose	6.22	4.9	3.60	1.87	7.0	0.87	1.2	1.1	7.9	1.37	1.7	0.71	2.24	3.0	2.8	2.4
	±0.04	±0.3	±0.05	±0.04	±0.6	±0.06	±0.4	±0.6	±0.6	±0.04	±0.1	±0.01	±0.06	±0.2	±0.2	±0.1
Glucose	22.4	25.76	25.8	25.8	22	5.9	11	11.5	22.1	5.8	14.1	13.9	26	13.2	22.1	22.2
	±0.7	±0.05	±0.1	±0.1	±1	±0.2	±2	±0.3	±0.4	±0.2	±0.2	±0.3	±2	±0.5	±0.8	±0.2
Fructose	15.1	19.0	20.7	23.8	12.1	25.14	23	23.6	11.8	23.2	21.7	23.9	18.3	24.9	24	25.9
	±0.2	±0.4	±0.2	±0.4	±0.2	±0.02	±2	±0.3	±0.1	±0.5	±0.2	±0.1	±0.5	±0.2	±1	±0.3

Values are expressed as the mean ± standard deviation of independent replicate experiments.

4.4.3. Viscosity – qualitative analysis

Texture of the slurries varied over the 24-h fermentation period apparently due to the production of EPS. Table 4.18 shows the intensity of the observed viscosity, which intensity was classified in high, medium and low, indicated as +++, ++ and +/- respectively.

Despite the qualitative analysis of the viscosity, it was possible to observe clear differences between the strains' behaviour and consequent texture changes. The strains native from Burkina Faso (*W*. *confusa*/*cibaria* 32LABPT05 and *W*. *confusa* 2LABPT05) were the ones that revealed a more noticeable viscosity, observable by the more viscous texture of the slurry (Figure 4.8). The increment in viscosity was visible between 6 h and 8 h of fermentation, coinciding with the maximum viscosity of the products, which was continuously observed during the remaining fermentation period.

Although in this phase of the present research work EPS were neither characterized nor quantified, according to the literature, the EPS produced by *W*. *confusa*/*cibaria* are mainly homopolysaccharides, specifically dextran (Galle et al., 2010; Lynch, Coffey, et al., 2018). In the field of cereal-based products, EPS applications are more common in sourdough and baking products (Katina et al., 2009; Lynch, Zannini, et al., 2018; Zannini et al., 2016), rather than cereal-based beverages, as described in the introduction of this thesis, in sub-chapter 2.9. Wang *et al*. (2019) observed that the dextran produced by *Weissella* strains has contributed to improve bread characteristics, such as increased bread specific volume, decreased crumb firmness, moisture loss, and staling rate, compared to the control millet bread. Similar results were obtained by Wolter and colleagues and, also, by Katina and collaborators (Katina et al., 2009; Wolter et al., 2014). Nevertheless, among the studies which explored the application of EPS-producing *Weissella* strains in the production of cereal-based beverages (Coda et al., 2011; Lorusso et al., 2018; Zannini et al., 2013), all observed that viscosity increased during fermentation, a consequence of EPS synthesis by *W*. *confusa/cibaria,* giving good textural characteristics to the fermented products contributing to their more appreciable acceptance.

Table 4.18 Intensity of the viscosity, consequence of exopolysaccharides produced by *W*. *confusa*/*cibaria* C2, *W*. *confusa*/*cibaria* 32LABPT05, *W*. *confusa* 2LABPT05 and *W*. *confusa* VTT E-90E392 after 24 h of fermentation of dry-milled pearl millet slurries in two different media (water-based solution and aqueous sucrose-based solution), at 30 °C and 200 rpm, in an orbital incubator.

	Intensity of viscosity	
Strain/Code	Dry-milled millet in water suspension	Dry-milled millet in sucrose suspension
32LAB PT05	-	+++
2LAB PT05	-	+++
C2	-	++
VTT E-90E392	-	++

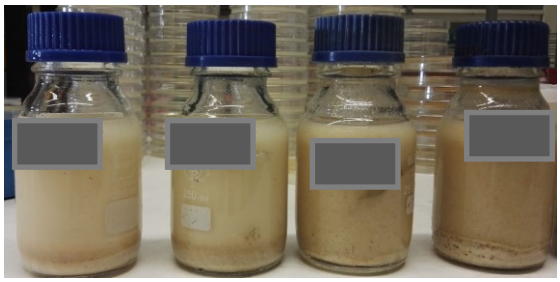
a)  b) 

Figure 4.8 Viscous appearance of dry-milled pearl millet slurries (two types of suspensions: in the absence of sucrose- first and second schott flasks; and in the presence of sucrose- third and fourth schott flasks), resulting from exopolysaccharides production by *W. confusa* 2LABPT05 after a 24-h fermentation period, at 30 °C and 200 rpm, in an orbital incubator (a). Slimy texture of the pearl millet slurry with added sucrose at 8 h of fermentation (b).

4.4.4. Total phenolics content

At this point the objective was to study the impact of fermentation on the content of total phenolics. Moreover, in this phase, no extracts were prepared since the main objective was to evaluate the free phenolics, the ones released into the medium, after 24 h of fermentation. Similarly to the results obtained in the first phase, sorghum slurries were shown to be three-fold richer in total phenolics than pearl millet slurries ($p < 0.05$), independently of the presence of sucrose or the fermentative strain. Generally, during fermentation of pearl millet over the 24 h, the concentration of total phenolics gradually increased. Table 4.19 presents the concentrations of total phenolics at the beginning, after 12 h and at the end of fermentation (24 h). The effect of the presence of sucrose in millet slurries was only noticed during the fermentation by *W. confusa*/*cibaria* 32LABPT05 ($p < 0.05$). The $\triangle t_{24} - t_0$ for TPC was higher when compared to the other bacteria ($p < 0.05$) and to the slurry without sucrose counterpart ($p < 0.05$). This same *Weissella* strain seems to be more effective in phenolic compounds metabolism than the previously tested strains (considering the water-based suspension - increment of 48 % in TPC by 24 h *vs*. -34 %, +36 % and +8 %, for *L. plantarum* 299v, *L. rhamnosus* R-11 and *L. acidophilus* Ki, respectively) (section 4.3.3). Both *W. confusa*/*cibaria* C2 (9 % *vs*. -34%) and *W. confusa* 2LABPT05 (7 % *vs*. -34%, increment in TPC by 24 h) reported better phenolics metabolism, by 24 h, than the *L. plantarum* 299v. Nevertheless, it must be highlighted that in this case pearl millet slurries were used instead of finger millet counterparts (for lactobacilli). Cereal seed coats may differ in composition and structure which may naturally influence the extractability of phenolic compounds by fermentation microorganisms.

Table 4.19 Evolution of the total phenolics content (TPC) (mg GAE[1]/kg of slurry) in dry-milled pearl millet slurries in the presence or absence of sucrose, fermented by *W. confusa*/*cibaria* C2, *W. confusa*/*cibaria* 32LABPT05, *W. confusa* 2LABPT05, over 24 h, at 30 ºC and 200 rpm, in an orbital incubator.

		Concentration of Gallic Acid Equivalents (mg GAE/kg)					
		Dry-milled millet in water suspension			Dry-milled millet in sucrose suspension		
		0	**12**	**24**	**0**	**12**	**24**
W. confusa/*cibaria*	C2	92 ± 15 ^{a}	77 ± 7 $^{a, b}$	100 ± 4 ^{a}	96 ± 4 ^{a}	131 ± 27 ^{b}	125 ± 5 ^{b}
	2LABPT05	84 ± 4 ^{a}	103 ± 10 ^{b}	90 ± 5 ^{a}	95 ± 7 ^{a}	121 ± 23 $^{a, b}$	136 ± 8 ^{b}
	32LABPT05	79 ± 6 ^{a}	98 ± 8 ^{b}	117 ± 8 ^{c}	98 ± 9 ^{a}	159 ± 7 ^{b}	189 ± 30 ^{b}

[1]: GAE-Gallic Acid Equivalents.

Values are expressed as the mean ± standard deviation of independent replicate experiments.

Different letters within each line and suspension type are significantly different ($p < 0.05$), using paired-samples T-tests.

Recall, that the fermentation process can lead to different fates of the phenolic compounds in wholegrain cereals either by changing inherent levels and/or by generation of subsequent monomers or polymers. Overall, and as previously stated the impact of fermentation on total phenolics is somehow controversial. Most of the discussion on this topic can be found in sub-chapter 4.3.3. Additionaly, an argument that was not previously mentioned is now explored. Gabaza *et al*. (2016) and Hur *et al*. (2014) highlighted that some microorganisms, namely LAB, might decrease the generation of reactive oxygen species due to the possession of enzymatic and non-enzymatic antioxidative mechanisms. In the case of *Weissella confusa*, it has been suggested that its produced EPS may have antioxidant activity (Adebayo-Tayo et al., 2018; Sharma et al., 2018). Specifically, in the work of Adebayo-Tayo *et al*. (2018), in which a wild type of *Weissella confusa* was studied, the authors concluded that the antioxidant activity increased in a dose-dependent manner. Although the antioxidant activity was not evaluated at this phase, the increase in total phenolics, consequence of specific enzymatic activity that may spur their release, might be related with this fact.

4.4.5. Total amino acids content

Fermentation of pearl millet slurries resulted in either a decrease, or, in some cases, in an increase of the amino acids content. Generally, in pearl millet suspended in water, without sucrose, the amino acids content after 24-h fermentation tended to be lower, with some exceptions for aspartic and glutamic acids, and valine when fermented by *W. confusa*/*cibaria* 32LABPT05. When sucrose was added, fermented pearl millet showed a higher content of amino acids. The more prevalent amino acids were aspartic and glutamic acids, alanine and leucine. Some bacterial strains had a higher impact on the increase of the threonine (essential amino acid), arginine (conditionally essential amino acid) and tyrosine (non-essential amino acid) contents, in the fermented pearl millet slurries. Table 4.20 shows the concentrations of several amino acids (mg of amino acid/ g of lyophilized slurry) before and after 24 h of fermentation. Sanni and colleagues also observed increased (lysine, valine, threonine, methionine, leucine, phenylalanine,

tyrosine, serine, and arginine) and decreased (aspartic acid, alanine, proline, asparagine, and glutamic acid) concentrations of total amino acids in cereal blends after 24 h of fermentation by *Saccharomyces cerevisiae* and *Lactiplantibacillus plantarum* (Sanni et al., 1999). Jannathulla and collaborators also found similar results when studying the effect of fermentation of several plant-based ingredients, by a microbial combination of LAB and yeasts (Jannathulla et al., 2017). Also, Ai and colleagues observed that the content of free amino acids of a mixed cereal substrate was reduced after 12 h of fermentation by *Lactobacillus helveticus*, cultured alone or in combination with *Saccharomyces cerevisiae* (Ai et al., 2015). The reviews of Singh *et al*. (2015) and Gänzle (2014) generally reported that fermentation tends to increase the content of amino acids, while Elkhalifa *et al*. (2007) reviewed that the effect of fermentation on the amino acids' content of foods is variable, although the evidence for the improvement, especially, of lysine content is substantial.

The mechanism that might be behind the decrease in amino acids concentrations involves their utilization by the fermenter microorganism, as a nitrogen source for growth or the production of enzymes and organic compounds (Imelda et al., 2008). Moreover, in the pioneering work of Garvie (1967), amino acids' requirements by different strains of *Leuconostoc* were explored, and the authors concluded that glutamic acid and valine were required by all strains, methionine was required or stimulatory for the majority of strains and that no requirement was observed for alanine for any strain. On the other hand, the increasing of amino acids content in the fermented slurries can result from a combination of circumstances. It can be related to the type of inoculum itself. In the case of the microorganisms belonging to the genus *Weissella,* it is known that their peptidoglycan cell wall is composed by several amino acids such as lysine, alanine, serine, glutamic acid, and glycine (Björkroth et al., 2002; Fusco et al., 2015). During fermentation, microorganisms can convert carbohydrates into microbial proteins and also produce enzymes, which are proteinaceous in nature (Imelda et al., 2008), and consequently, levels of amino acids might increase. In addition, peptide hydrolysis carried out by LAB intracellular peptidases lead to amino acid accumulation (De Vuyst et al., 2017; Papadimitriou et al., 2019).

The main objectives at this stage were, on the one hand, to determine if the addition of sucrose to the cereal slurry had an impact on the evaluated parameters, and on the other hand, to decide on the duration of the fermentation process. Results revealed that the addition of sucrose promoted the growth of the bacterial strains, eboosted the production of organic acids, might have enhanced the antioxidant activity given the increased on the total phenolics content, and, additionally, improved the slurries' texture. In what concerns the fermentation period, the fact that the major metabolic activity takes place during the first 8 h of fermentation was taken into account and a shorter process was envisaged for the next steps in this research endeavour. Based on these rationale, further experiments will be carried out in the presence of sucrose, and over 8 h of fermentation.

Table 4.20 Concentrations of total amino acids (mg of amino acid/ g of lyophilized slurry) in dry-milled pearl millet slurries in water suspension (no added sucrose) or in sucrose suspension, before and after 24 h of fermentation carried out by *W. confusa/cibaria* C2, *W. confusa/cibaria* 32LABPT05 and *W. confusa* 2LABPT05, at 30 ºC and 200 rpm, in an orbital incubator.

		W. confusa/cibaria											
		C2				**2LABPT05**				**32LABPT05**			
		Dry-milled Millet in water suspension		**Dry-milled Millet in sucrose suspension**		**Dry-milled Millet in water suspension**		**Dry-milled Millet in sucrose suspension**		**Dry-milled Millet in water suspension**		**Dry-milled Millet in sucrose suspension**	
		0	24	0	24	0	24	0	24	0	24	0	24
Concentration of amino acids (aas) (mg of aa / g lyophilized slurry)	Aspartic acid	9.3 ±0.3^{a}	4.1 ±0.5^{b}	9.3 ±0.3^{a}	11.0 ±0.5^{a}	9.3 ±0.3^{a}	12 ±2^{a}	9.3 ±0.3^{a}	11.0 ±0.5^{b}	9.3 ±0.3^{a}	13.5 ±0.5^{b}	9.3 ±0.3^{a}	16 ±4^{a}
	Glutamic acid	14.9 ±0.6^{a}	5.6 ±0.4^{b}	14.9 ±0.6^{a}	15.1 ±0.9^{a}	14.9 ±0.6^{a}	17 ±3^{a}	14.9 ±0.6^{a}	19 ±1^{b}	14.9 ±0.6^{a}	18.6 ±0.8^{b}	14.9 ±0.6^{a}	21 ±1^{b}
	Serine	4.39 ±0.04^{a}	1.8 ±0.3^{b}	4.39 ±0.04^{a}	5.0 ±0.2^{a}	4.39 ±0.04^{a}	6 ±1^{a}	4.39 ±0.04^{a}	6.4 ±0.5^{b}	4.39 ±0.04^{a}	4.4 ±0.1^{a}	4.39 ±0.04^{a}	6 ±1^{a}
	Threonine	5.11 ±0.02^{a}	2.2 ±0.2^{b}	5.11 ±0.02^{a}	6.4 ±0.2^{b}	5.11 ±0.02^{a}	6 ±2^{a}	5.11 ±0.02^{a}	8.8 ±0.5^{b}	5.11 ±0.02^{a}	4.3 ±0.1^{b}	5.11 ±0.02^{a}	5 ±1^{a}
	Arginine	5.75 ±0.06^{a}	3.0 ±0.6^{b}	5.75 ±0.06^{a}	8.3 ±0.4^{b}	5.75 ±0.06^{a}	8 ±1^{a}	5.75 ±0.06^{a}	7.3 ±0.3^{b}	5.75 ±0.06^{a}	5.6 ±0.2^{a}	5.75 ±0.06^{a}	7 ±3^{a}
	Alanine	12.3 ±0.2^{a}	5.4 ±0.5^{b}	12.3 ±0.2^{a}	14.0 ±0.9^{a}	12.3 ±0.2^{a}	12.2 ±0.9^{a}	12.3 ±0.2^{a}	10.8 ±0.2^{b}	12.3 ±0.2^{a}	6.0 ±0.2^{b}	12.3 ±0.2^{a}	8 ±2^{b}
	Tyrosine	2.66 ±0.02^{a}	0.9 ±0.2^{b}	2.66 ±0.02^{a}	3.0 ±0.2^{a}	2.66 ±0.02^{a}	3.3 ±0.6^{a}	2.66 ±0.02^{a}	3.52 ±0.08^{b}	2.66 ±0.02^{a}	2.90 ±0.09^{a}	2.66 ±0.02^{a}	3.6 ±0.8^{a}
	Valine	5.10 ±0.01^{a}	1.9 ±0.4^{b}	5.10 ±0.01^{a}	5.8 ±0.3^{a}	5.10 ±0.01^{a}	6 ±1^{a}	5.10 ±0.01^{a}	6.6 ±0.4^{b}	5.10 ±0.01^{a}	5.8 ±0.2^{b}	5.10 ±0.01^{a}	7 ±2^{a}
	Phenylalanine	4.22 ±0.06^{a}	1.5 ±0.3^{b}	4.22 ±0.06^{a}	4.1 ±0.9^{a}	4.22 ±0.06^{a}	4.0 ±0.3^{a}	4.22 ±0.06^{a}	3.6 ±0.2^{b}	4.22 ±0.06^{a}	2.87 ±0.06^{b}	4.22 ±0.06^{a}	3.8 ±0.7^{a}
	Isoleucine	4.26 ±0.03^{a}	1.6 ±0.3^{b}	4.26 ±0.03^{a}	4.5 ±0.3^{a}	4.26 ±0.03^{a}	4.3 ±0.6^{a}	4.26 ±0.03^{a}	4.5 ±0.6^{a}	4.26 ±0.03^{a}	3.9 ±0.2^{a}	4.26 ±0.03^{a}	5.0 ±0.8^{a}
	Leucine	10.37 ±0.05^{a}	3.8 ±0.5^{b}	10.37 ±0.05^{a}	10 ±1^{a}	10.37 ±0.05^{a}	8.8 ±0.5^{b}	10.37 ±0.05^{a}	8.2 ±0.6^{a}	10.37 ±0.05^{a}	6.1 ±0.3^{b}	10.37 ±0.05^{a}	8 ±1^{a}

Values are expressed as the mean ± standard deviation of independent replicate experiments.

Different letters within each line and suspension type are significantly different ($p < 0.05$), using paired-samples T-tests.

4.5. PHASE 3 – CO-FERMENTATION OF AFRICAN WHOLE GRAINS BY *LACTIPLANTIBACILLUS* AND *WEISSELLA* STRAINS

4.5.1 Fermentative capacity - microbial growth and acidification rates

In this third phase, co-cultures of *Lactiplantibacillus* and *Weissella* strains were explored. Co-fermentation of bacterial strains may aid in the release of bound nutrients/bioactives or in the production of added-value compounds due to complementary metabolic activities. The ultimate aim of such co-fermentation, using a co-culture containing an exopolysaccharide-producer strain and a probiotic strain, was the formulation, development and full characterization of a functional fermented African cereal-based beverage. Yet, before moving on to this step it was important to study the fermentation factors affecting the beverage to ascertain required starter culture and cereal matrix which would support its production.

The evolution of pH values of dry-milled sorghum, pearl and finger millets slurries, inoculated with the plain or combined cultures, throughout the 8-h fermentation is presented in Figure 4.9. Reduction in pH values was shown to be more effective for pearl and finger millets (ca. 2 pH units) than for sorghum (ca. 0.5 pH units in case of 2LABPT05 and 32LABPT05 strains, and a reduction of 1 pH unit observed for *W. confusa*/*cibaria* C2). Besides a higher acidification capacity, pearl and finger millets also enabled bacterial strains to grow effectively (at least 2 log cycles) achieving final viable cell numbers around 10^8-10^9 CFU/mL, for all the indigenous strains (Figure 4.10), revealing their prebiotic potential. In contrast, in sorghum's fermentations only *W. confusa*/*cibaria* C2 was able to trigger acidification, yet no growth was observed. As previously mentioned, sorghum is a cereal matrix rich in tannins that might inhibit the growth and limit the metabolism of the selected strains. It has been reported that *L. plantarum* 299v possesses tannase (esterase) activity (Osawa et al., 2000), demonstrated by its capacity to breakdown these phenolic compounds. This characteristic gives this microorganism the ability to ferment sorghum matrices, as observed by the results presented in sub-chapter 4.3.1. On the contrary, there is no evidence that *Weissella* strains have this capacity, and thus, they may be more sensitive to the presence of tannins. Nevertheless, according to the present results, no differences were observed even when co-cultured with the probiotic strain in the fermentation of sorghum.

In terms of pH evolution, there were no statistically significant differences between the type of culture (plain or combined), considering finger millet for all strains, and considering pearl millet for the strain *W. confusa*/*cibaria* C2 ($p > 0.05$). The fermentation of pearl millet by the strains *W. confusa* 2LABPT05 or *W. confusa*/*cibaria* 32LABPT05 in plain culture has resulted in higher acidification, over time ($p < 0.05$). Generally, the final pH at 8 h of fermentation was lower in plain cultures than in co-cultures, more evidently in pearl millet fermentation for all strains and in finger millet for *W. confusa* 2LABPT05 and *W. confusa*/*cibaria* 32LABPT05, showing that *Weissella* strains produced a more acidic environment when fermenting the cereal slurries alone, without any eventual competition of other bacteria. The establishment of a final pH around 4-5 was the

target defined to ensure the inhibition of pathogenic and spoilage organisms, contributing to the safety and stability of the final fermented product (Nout & Ngoddy, 1997).

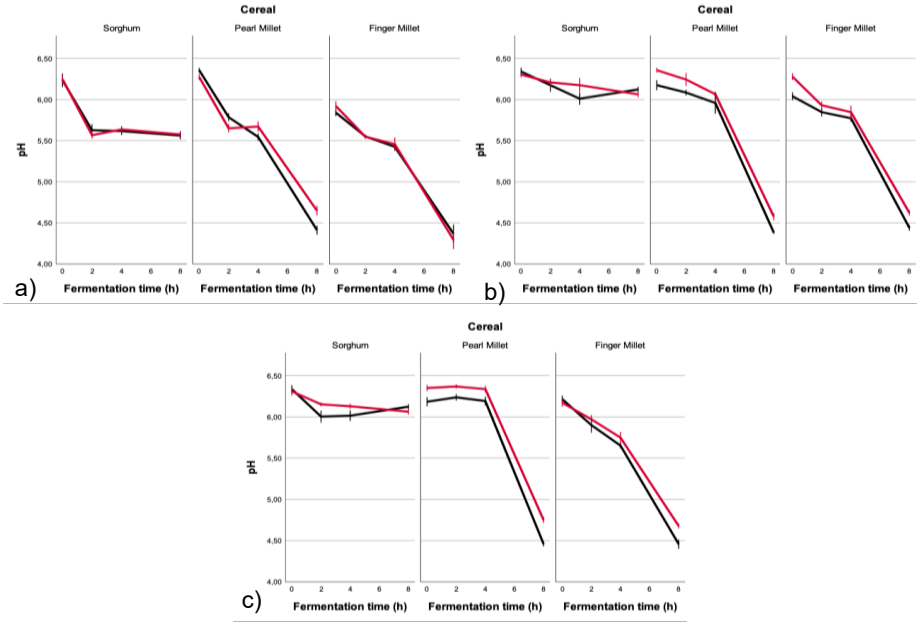


Figure 4.9 Evolution of pH values of dry-milled sorghum, pearl and finger millets slurries, inoculated with *W*. *confusa*/*cibaria* C2 (a), *W*. *confusa* 2LABPT05 (b) and *W*. *confusa*/*cibaria* 32LABPT05 (c) in plain cultures (black lines) or in combined cultures (red lines) with *L*. *plantarum* 299v, over 8 h of fermentation at 30 °C and 200 rpm, in an orbital incubator. Error bars represent the 95 % confidence intervals of independent replicate experiments.

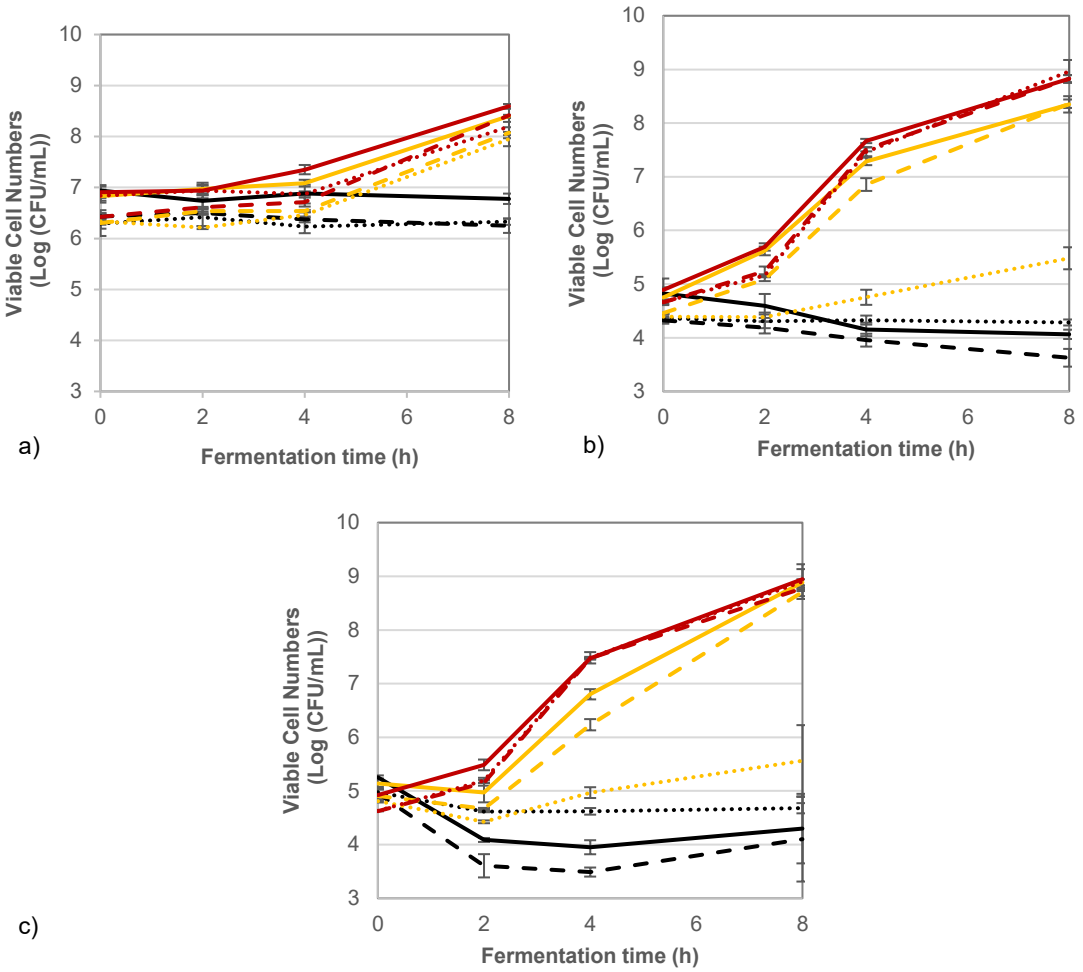


Figure 4.10 Evolution of the viable cell numbers of *W. confusa*/*cibaria* C2 (a), *W. confusa* 2LABPT05 (b) and *W. confusa*/*cibaria* 32LABPT05 (c) inoculated in plain cultures (full lines) or in combined cultures (the indigenous *Weissella* strains are represented in dashed lines and the *Lactiplantibacillus* strain in dotted lines) in dry-milled sorghum (black lines), pearl millet (yellow lines) and finger millet (red lines), over 8 h of fermentation at 30 °C and 200 rpm, in an orbital incubator. Error bars represent the standard deviation of independent replicate experiments.

The observed delta growth ($\triangle t_8 - t_{0,\ Log\ CFU/mL}$) for the indigenous *Weissella* strains was considered similar between plain and combined cultures, for almost all the cases (except for fermentation of pearl millet by *W. confusa*/*cibaria* 32LABPT05 and finger millet by *W. confusa*/*cibaria* C2 and 32LABPT05, $p < 0.05$) showing that the presence of the probiotic strain did not influence much its performance. On average, *L. plantarum* 299v reported a growth increase between one and two log cycles, during the fermentation process. The fact that both strains were able to grow in co-culture indicates the potential for their use as a mixed starter culture in co-fermentation. Table 4.21 presents the absolute difference of growth between the beginning and the end of fermentation for each cereal and strains' combination.

In terms of co-cultures, there were no differences, between both millets tested, in the growth of the strain *W*. *confusa* 2LABPT05, while the strains *W*. *confusa*/*cibaria* C2 and 32LABPT05 seemed to grow better in finger millet ($p \leq 0.05$). *Lactiplantibacillus plantarum* 299v registered a similar growth in both millets, when in combination with *W*. *confusa*/*cibaria* C2 and *W*. *confusa* 2LABPT05, however when combined with *W*. *confusa*/*cibaria* 32LABPT05, the observed growth was more impactful. Nevertheless, when looking for differences between strains' combination, in both millets, no differences between the three combinations were observed.

Table 4.21 Absolute differences of the viable cell numbers (Log (CFU/mL)) between the beginning and the end of fermentation (over 8 h, at 30 °C and 200 rpm, in an orbital incubator) of dry-milled sorghum, pearl millet, and finger millet, inoculated with *W*. *confusa*/*cibaria* C2, *W*. *confusa* 2LABPT05, or *W*. *confusa*/*cibaria* 32LABPT05 in plain cultures or in combination with the probiotic bacterium *L*. *plantarum* 299v.

Culture	Cereal	IB[1]	$\triangle t_8 - t_0$ CFUs IB			Culture	Cereal	IB combination	$\triangle t_8 - t_0$ CFUs IB			$\triangle t_8 - t_0$ CFUs PB[2]		
Plain	Sorghum	C2	0.2 ± 0.1	a	x	Combined	Sorghum	C2	0.18 ± 0.01	a	x	0.022 ± 0.006	a	x
		2LABPT05	1.0 ± 0.6	b	x			2LABPT05	0.9 ± 0.8	a	x	0.33 ± 0.08	b	x
		32LABPT05	0.76 ± 0.09	a,b	x			32LABPT05	0.7 ± 0.2	a	x	0.08 ± 0.06	a	x
	Pearl millet	C2	1.57 ± 0.02	a	y		Pearl millet	C2	1.6 ± 0.1	a	y	1.6 ± 0.1	a	y
		2LABPT05	3.74 ± 0.06	b	y			2LABPT05	3.80 ± 0.08	b	y	1.3 ± 0.1	a,b	y
		32LABPT05	3.6 ± 0.1	b	y			32LABPT05	3.90 ± 0.08	b	y	1.1 ± 0.2	b	y
	Finger millet	C2	1.69 ± 0.04	a	y		Finger millet	C2	2.0 ± 0.1	a	z	1.4 ± 0.2	a	y
		2LABPT05	4.1 ± 0.1	b	y			2LABPT05	4.2 ± 0.2	b	y	1.6 ± 0.3	a	y
		32LABPT05	3.95 ± 0.05	b	z			32LABPT05	4.2 ± 0.1	b	z	1.7 ± 0.1	a	z

[1]IB - Indigenous bacterium strain;

[2]PB – Probiotic bacterium.

For a given type of culture and cereal, different letters (a and b) indicate significant differences between IB strain/IB strain combination ($p \leq 0.05$), and for a type of culture and IB strain/IB combination, different letters (x, y, and z) indicate significant differences between cereals ($p \leq 0.05$), using one-way ANOVA.

4.5.2 Fermentative capacity – Sugars consumption and organic acids production

Metabolic activity was followed by measuring sugars consumption and subsequent organic acids production profiles. Results are presented in Table 4.22 only for pearl and finger millets, which were the cereals where metabolic activity was significant; given its lack of impact on growth and acidification capacity, sorghum was not pursued any further. Lactic acid was the main organic acid produced upon 8 h fermentation, however the production of acetic acid was observed in the case of *W. confusa*/*cibaria* 32LABPT05 in plain cultures. It would be expectable that the production of acetic acid was also performed by the other strains and, also, in combined cultures, given its natural production by both bacteria, however, it was not detected (limit of detection = 0.05 g/L) in those cases. Nevertheless, even within the same bacterial species, different strains can produce metabolites to different extents (Galle et al., 2010), which may be the case in the present experiment. Wang *et al*. (2019), who used a pearl millet fermentable matrix with *Weissella confusa* as the starter culture, reported lower quantities of lactic acid over 24-h fermentation, but the production of acetic acid was observed, even in low amounts. Despite the absence of statistically significant differences either between strain combinations for the same cereal or between cereals for the same strain combination, clear variances in metabolic performance were observed. When comparing plain with combined cultures, it is possible to observe that the final lactic acid concentrations were higher in the former, which is in agreement with the higher acidification observed in these plain cultures. Differences in pearl and finger millets were observed; fermentation of finger millet generated a more acidic environment, when compared with that of pearl millet, for both culture types.

As expected, and also observed in the previous sub-chapter, the levels of sucrose and glucose decreased during fermentation, due to its utilization by the microorganisms, either for growth or for EPS production, specially by the *Weissella* strains. The discussion on this topic can be found above, in sub-chapter 4.4.2.

Table 4.22 Sugars' and organic acids' concentrations (g/kg of slurry) in dry-milled pearl and finger millets' slurries, fermented by *W. confusa*/*cibaria* C2, *W. confusa* 2LABPT05 and *W. confusa*/*cibaria* 32LABPT05, inoculated in plain cultures, or combined with *L. plantarum* 299v, before (0 h) and after (8 h) fermentation at 30 °C and 200 rpm, in an orbital incubator.

			C2		2LABPT05		32LABPT05	
Culture	**Cereal**	**Metabolite (g/kg)**	**0 h**	**8 h**	**0 h**	**8 h**	**0 h**	**8 h**
Plain	**Pearl millet**	Lactic Acid	0.089±0.002	0.267±0.006	0.088±0.002	0.72±0.03	0.08±0.01	0.64±0.04
		Acetic Acid	< LOD[1]	< LOD	< LOD	< LOD	< LOD	0.24±0.06
		Sucrose	83±7	35±28	83±7	5.70±0.03	83±7	8±2
		Glucose	14±4	13±9	14±4	13±1	14±4	6±1
		Fructose	3.9±0.8	7±4	3.9±0.8	31±2	3.9±0.8	30±3
	Finger millet	Lactic Acid	0.088±0.003	0.42±0.01	0.094±0.002	1.0±0.1	0.12±0.03	1.0±0.1
		Acetic Acid	< LOD	< LOD	< LOD	< LOD	< LOD	0.7±0.2
		Sucrose	90±5	23.4±0.8	90±5	2.3±0.4	90±5	2.4±0.5
		Glucose	18±2	3.3±0.1	18±2	2.4±0.5	18±2	2.2±0.3
		Fructose	5±1	1.32±0.05	5±1	26±2	5±1	28±3
Combined	**Pearl millet**	Lactic Acid	0.089±0.004	0.2±0.05	0.089±0.002	0.268±0.006	0.087±0.001	0.27±0.02
		Acetic Acid	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD
		Sucrose	83±7	19±11	83±7	2.2±0.3	83±7	7.1±0.3
		Glucose	14±4	7±4	14±4	4.8±0.6	14±4	3.3±0.9
		Fructose	3.9±0.8	4±2	3.9±0.8	11±1	3.9±0.8	12±2
	Finger millet	Lactic Acid	0.089±0.003	0.455±0.004	0.10±0.02	0.7±0.4	0.10±0.01	0.474±0.009
		Acetic Acid	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD
		Sucrose	90±5	26±1	90±5	5±3	90±5	3±1
		Glucose	18±2	3.8±0.2	18±2	1.4±0.8	18±2	1.0±0.1
		Fructose	5±1	1.3±0.2	5±1	19±10	5±1	11.71±0.09

[1] LOD: Limit of detection (Acetic acid: 0.05 g/L).

4.5.3 Viscosity – qualitative analysis

After 6 h of fermentation and more clearly at the end of fermentation (8 h), it was possible to observe differences in what concerns the texture of the slurries, due to the production of EPS. Moreover, it was possible to observe clear differences in terms of texture, as a consequence of different strain's behaviour. Analogously to what was observed in the previous phase, the native strains from Burkina Faso (*W. confusa*/*cibaria* 32LABPT05 and *W. confusa* 2LABPT05), but now either in plain or combined cultures, were the ones that revealed a more intense viscous texture of the cereal slurry. When comparing plain and combined cultures, it was possible to detect a less viscous texture of the product when the probiotic strain was present, yet it remained very slimy. Table 4.23 shows the intensity of the observed viscosity generated by the different strains, in plain or combined cultures, inoculated in both millet slurries. The previous sub-chapter 4.4.3. presents the discussion on the EPS production and research in terms of cereal-based products, thereby no deep discussion will be addressed here in order to avoid repetition.

Table 4.23 Intensity of the viscosity of dry-milled pearl and finger millet slurries, consequence of the EPS produced by *W. confusa/cibaria* C2, *W. confusa* 2LABPT05 or *W. confusa/cibaria* 32LABPT05 inoculated in plain cultures, or combined with *L. plantarum* 299v, after 8 h of fermentation, at 30 °C and 200 rpm, in an orbital incubator.

	Viscosity intensity			
Strain/Code	**Pearl millet**		**Finger millet**	
	Plain culture	**Combined culture**	**Plain culture**	**Combined culture**
C2	++	+	++	+
2LABPT05	+++	++	+++	++
32LABPT05	+++	++	+++	++

4.5.4 Total phenolics content

Concentrations of total phenolics at the beginning, and after 8 h of fermentation, in the different milled millet slurries, for plain and combined cultures, are presented in Table 4.24. In agreement with previous results, sorghum slurries were shown to be richer in total phenolics than pearl and finger millets' counterparts ($p < 0.05$) (data not shown). Moreover, in spite of not being statistically different, finger millet presented higher content in total phenolics than pearl millet. Generally, the concentration of total phenolics increased after an 8-h fermentation process ($p < 0.05$).

Table 4.24 Total phenolics content (TPC) (mg GAE[1]/kg of slurry) in dry-milled pearl and finger millets slurries, fermented by *W. confusa*/*cibaria* C2, *W. confusa* 2LABPT05 or *W. confusa*/*cibaria* 32LABPT05, inoculated in plain cultures, or combined with *L. plantarum* 299v, before (0 h) and after (8 h) fermentation, at 30 ºC and 200 rpm, in an orbital incubator.

	Concentration of Gallic Acid Equivalents (mg GAE/kg)							
Culture	**Plain**				**Combined**			
Cereal	**Pearl millet**		**Finger millet**		**Pearl millet**		**Finger millet**	
	0 h	**8 h**	**0 h**	**8 h**	**0 h**	**8 h**	**0 h**	**8 h**
C2	83 ± 7 [a]	90 ± 8 [a]	99 ± 6 [a]	118 ± 9 [b]	100 ± 11 [a]	112 ± 15 [a]	94 ± 6 [a]	130 ± 11 [b]
2LABPT05	108 ± 8 [a]	157 ± 39 [b]	112 ± 7 [a]	149 ± 14 [b]	94 ± 11 [a]	155 ± 54 [a]	98 ± 10 [a]	133 ± 16 [b]
32LABPT05	105 ± 12 [a]	223 ± 60 [b]	122 ± 20 [a]	149 ± 14 [b]	98 ± 6 [a]	161 ± 25 [b]	115 ± 7 [a]	137 ± 10 [b]

[1] GAE: Gallic Acid Equivalents.

Values are expressed as the mean ± standard deviation of independent replicate experiments.

For a given culture type, cereal and bacterial strain, different leters indicate significant differences ($p \leq 0.05$) between times, using paired-samples T-tests.

Generally, the obtained increase ($\triangle t_8 - t_0$) of total phenolics, over the 8-h fermentation, was not considered different among the three different strain combinations, for the same cereal, either in plain or combined culture or between cereals for the same strain, either in plain or combined culture (except for the following cases: the strain *W. confusa*/*cibaria* 32LABPT05, for pearl millet, in plain culture, which demonstrated a higher delta compared to *W. confusa* 2LABPT05 and *W. confusa*/*cibaria* C2; and the strain *W. confusa*/*cibaria* C2, in combined culture, for finger millet, in which cereal matrix the phenolics delta was higher). As alrealy deeply discussed in previous sub-chapters, the effect of fermentation on total phenolics is not very clear, as in the literature controversial results are found. In order to not be exhaustive in what concerns these results, and also because no differences were observed, in this sub-chapter no further discussion will be addressed to this topic. Nevertheless, it would be expected that the presence of the *Lactiplantibacillus* strain, in the combined culture would incite a reduction in the phenolics content as previously observed (see Table 4.16), given its eventual possession of tannase activity, however such did not happen. Nonetheless, such reductions, after 8 h of fermentation were not very marked. The presence of a *Weissella* strain could also have somehow interfered with this potential characteristic of the probiotic strain. Recall that such complementarity or antagonism were previously discussed in sub-chapter 4.3.3.

Based on the main purpose of this phase being the selection of the best bacterial consortium and the cereal matrix that showed higher prebiotic potential, both combinations of *L. plantarum* 299v with either *W. confusa* 2LAPT05 or *W. confusa/cibaria* 32LABPT05 showed good potential for further product development. In what concerns the fermenting cereal, results showed that millets revealed a higher prebiotic potential since they promoted a more impactful growth for both types of strains, when compared to sorghum. In terms of nutritional properties, finger millet has a richer-fibre and lower-fat profile than pearl millet. Besides, this cereal has a higher content in phenolic compounds and also in some important micronutrients such as calcium, potassium, and thiamine (Vila-Real et al., 2017). The

abovementioned bacterial combinations showed similar bacterial growth in either millet, nevertheless, the production of lactic acid seemed to be higher when the *L*. *plantarum* was co-cultured with the *W*. *confusa* 2LABPT05 strain, in finger millet. Based on these rationale, the starter culture selected for the next and final phase was composed by *W*. *confusa* 2LABPT05 and *L*. *plantarum* 299v, and the selected fermenting cereal matrix was finger millet.

PART III – NOVEL SYNBIOTIC FERMENTED CEREAL-BASED PRODUCTS

4.6. VISCOUS FERMENTED FINGER MILLET-BASED PRODUCT

4.6.1. Fermentative capacity - microbial growth, acidification rates and sugars consumption, and organic acids production

After the selection of the best combination match between bacterial consortium and cereal matrix, a new fermentation run, under the same operational conditions, was carried out for the development of the fermented finger-millet based product.

Before stepping into the fermentation experiment, the growth of both microorganisms in MRS culture medium was followed during 24 h, at 37 °C. Both strains reached their maximum optical density (OD_{max}) upon 12 h of incubation. The strains' behaviour was similar, although apparently *L. plantarum* 299v enters exponential growth phase at a faster rate (shorter lag phase) than *W. confusa* 2LABPT05 (Figure 4.11).

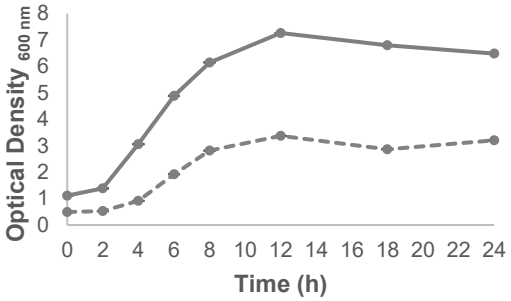


Figure 4.11 Growth of *L. plantarum* 299v (full line) and *W. confusa* 2LABPT05 (dashed line) in MRS medium incubated at 37 °C, over 24 h.

For this final fermentation process, strains were inoculated at the same percentage (1 %, v/v) but not balanced in terms of similar initial viable cell numbers. Previous results (sub-chapter 4.5), showed that when the *L. plantarum* 299v strain was diluted in order to be co-cultured at the same level of growth as the *Weissella* strain, final viable cell numbers were generally found below 10^7 CFU/mL. Thereby, for this final experiment, in order to guarantee greater concentration levels of the probiotic strain, no dilution of the overnight culture inoculum was performed.

Fermentation of finger millet with the selected co-culture shortened the fermentation period similarly to other reported studies in literature for finger millet (6-10 h) (Fasreen et al., 2017; Mugocha et al., 2000). Furthermore, it enabled *L. plantarum* 299v to increase one log cycle and reach final viable cell numbers around 10^8 CFU/mL (above the minimum required threshold of 10^7 CFU/g (Gomes et al., 2017)). On the other hand, *W. confusa* 2LABPT05 achieved a much more impactful increment of about four log cycles, proving that it is not negatively affected by the probiotic strain's presence; on the contrary, its growth and metabolic activities are enhanced and it can still perform its crucial technological role

(Figure 4.12). It is possible to observe that the period between the fourth and the sixth hour of fermentation was important in terms of the *Weissella* strain's metabolism.

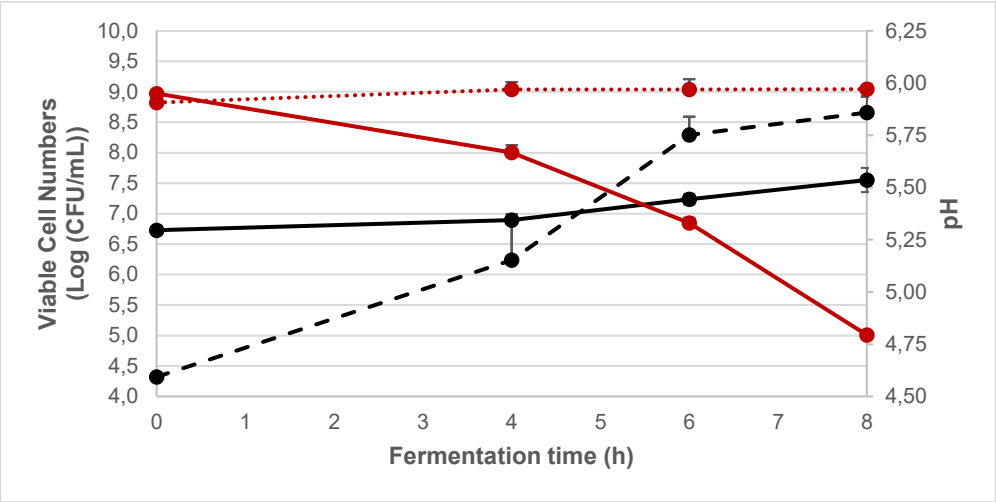


Figure 4.12 Evolution of acidification (red lines) and bacterial growth (black lines) of dry-milled finger millet slurry, inoculated with *W. confusa* 2LABPT05 (dashed black line) co-cultured with *L. plantarum* 299v (full black line), over an 8-h fermentation period, at 30 °C and 200 rpm, in an orbital incubator. A pH control is represented by the dotted red line. Error bars represent the 95 % confidence intervals of independent replicate experiments.

Bacterial metabolic activity followed the growth behaviour trends and the production of lactic and acetic acids was detected in the fermented product (Table 4.25). It is well-known that these fermentation end-products have antimicrobial effects, although, such activity is not equal to all organic acids; specifically, acetic acid has a higher potential to inhibit yeast, moulds, and bacteria than lactic acid providing good product preservative properties (Liptáková et al., 2017). The production of acetic acid reported in this final fermented product, compared to the previous phase for the selection of the best strain combination, may be justified by the presence of a higher initial number (~10^7 CFU/mL *vs*. ~10^4 CFU/mL, respectively) of *L. plantarum* 299v cells. In the work of Lorusso *et al*. (2018), in which quinoa flour was fermented by *L. plantarum* T6B10 (ca.10^7 CFU/mL) for 20 h, acetic acid concentration was slightly higher (1.5 fold) than the present results; such differences may be related with the different fermentation times (8 h *vs*. 20 h) or with the cereal matrix itself. In fact, in the study of Coda *et al*. (2011), in which emmer flour was fermented for 4 h by *L. plantarum* A6, very low concentrations of acetic acid (around 0.7 mM) were found. The higher values of acetic acid obtained in the present study may be related to the presence of *W. confusa as* well, which is also known for the production of this metabolite. In fact, in the abovementioned study of Lorusso *et al*. (2018), when the fermentation was performed singly by *W. confusa* higher values of acetic acid were obtained, when compared with the plain cultures of *L. plantarum* and *L. rhamnosus*. As observed previously, sucrose was utilized by the microorganisms resulting in the release of fructose, as it is possible to observe by the analysis of Table 4.25. In the work of Zannini *et al*. (2013) higher levels of fructose were also observed at the end of fermentation, and the

authors mentioned that fructose formation by *W. cibaria* MG1 was positively correlated with the initial concentration of sucrose. Also, Katina *et al*. (2009) observed significant amounts of fructose in the final sourdough fermented by *W. confusa*, which microorganism has proven to not use fructose for growth, EPS or mannitol production during the fermentations.

Table 4.25 Sugars and organic acids concentrations (g/kg of slurry) in dry-milled finger millet slurry, fermented by *W. confusa* 2LABPT05 co-cultured with *L. plantarum* 299v, over 8 h, at 30 °C and 200 rpm, in an orbital incubator.

	Fermentation time (h)		
Metabolite (g/kg)	**0**	**6**	**8**
Lactic Acid	< LOD	2.32 ± 0.07 [a]	2.69 ± 0.09 [a]
Acetic Acid	< LOD	0.42 ± 0.04 [a]	0.70 ± 0.08 [b]
Sucrose	64 ± 8 [a]	65 ± 5 [a]	38 ± 6 [a]
Glucose	10 ± 2 [a]	7.2 ± 0.2 [a]	7.0 ± 0.8 [a]
Fructose	4.9 ± 0.8 [a]	15.8 ± 0.7 [b]	21 ± 3 [c]

For a given metabolite, different letters indicate significant differences ($p \leq 0.05$), between times, using the Paired T-test or Wilcoxon test.

4.6.2. Biological activity

4.6.2.1. Total phenolics content and antioxidant activity

Fermentation was shown to cause a 35 % increase in the TPC (Table 4.26). In contrast, the DPPH scavenging activity decreased within the 8-h fermentation, from 26.7 ± 0.6 % of inhibition to 23 ± 1 % (corresponding to the initial, 180 ± 11 mg TE/kg of YLB, and the final, 153 ± 11 mg TE/kg, of YLB concentrations, respectively). The ABTS radical scavenging activity of the fermented yoghurt-like beverage (F-YLB) was slightly lower as well, but not statistically different, varying from 27.2 ± 0.9 % to 25.6 ± 0.6 %. As previously discussed, during fermentation enzymatic (from the cereal matrix and the existent microbiota) and non-enzymatic processes might be behind the reduction of phenolic compounds (Adebo & Medina-Meza, 2020; Gabaza et al., 2016). Specifically in these analyses, at this phase, EPS were precipitated prior to the assessment of the phenolics content and the antioxidant activities. Thereby, the samples in question here were the supernatants, EPS-free, in order to observe the compounds released to the medium. On the one hand, this might justify the increased total phenolics content, and, on the other hand, the decrease in the antioxidant activity, which in this case seems to be associated with the presence of EPS.

4.6.2.2. Antidiabetic activity –α-glucosidase inhibitory activity

The α-glucosidase inhibitory activity was improved, by 7 %, after fermentation (Table 4.26). This augmented inhibitory activity might be related with the increase in TPC, and the release of α - glucosidase inhibitory phytochemicals; in their original form these may be found bound to proteins, and consequently with hampered activity (Kim et al., 2010). Furthermore, the three-fold increase of the GABA amino acid, after an 8-h fermentation period (see sub-chapter 4.6.3.1), may also explain these

results. Han and Lee (2017) found a relation between the yeast's content of GABA and their demonstrated α-glucosidase inhibitory activity. The cell-free extract from the two highest GABA-producing yeasts had the highest anti-hyperglycaemic α-glucosidase inhibitory activity, of 72.3 %, and 69.9 %. These authors further explored the anti-hyperglycaemic action of the same two cell-free extract-containing GABA in normal and diabetic rats, and observed a decrease in the blood glucose levels, similarly to the positive control (acarbose); nevertheless high doses were required (Han & Lee, 2017). Chen *et al*. (2014) observed that some LAB strains (namely the cell-free extracts and supernatants of *L*. *plantarum*) have demonstrated potential on the α-glucosidase inhibitory activity, but of a lower order of magnitude. Some other authors (Ramchandran & Shah, 2009) suggest that this activity might come from the production of EPS by LAB, which mechanism may also support results of the present study, given the production of EPS mainly by the *Weissella* strain.

Table 4.26 Total phenolics content (TPC) (mg GAE[1]/kg of yoghurt-like beverage (YLB)), antioxidant activities (DPPH, mg TE[2]/kg of YLB; ABTS mg AAE[3]/kg of YLB) and α-glucosidase inhibitory activity of dry-milled finger millet unfermented slurry and the fermented yoghurt-like beverage, carried out by *L*. *plantarum* 299v co-cultured with *W*. *confusa* 2LABPT05, at 30 ºC and 200 rpm, over 8 h, in an orbital incubator.

	Unfermented slurry	**Fermented YLB (F-YLB)**
TPC (mg GAE/kg YLB)	181 ± 11 [a]	244 ± 11 [b]
DPPH (mg TE/kg YLB)	180 ± 11 [a]	153 ± 11 [b]
ABTS (mg AAE/kg YLB)	239 ± 15 [a]	238 ± 8 [a]
α-glucosidase inhibitory activity (%)	14 ± 2 [a]	21 ± 2 [b]

[1] GAE: Gallic Acid Equivalent;

[2] TE: Trolox Equivalent;

[3] AAE: Ascorbic Acid Equivalent.

For a given parameter, different letters indicate significant differences ($p \leq 0.05$), between samples, using the Paired T-test or Wilcoxon test.

4.6.3. Nutritional Properties

4.6.3.1. Free-amino acids content

The amino acids present at the highest concentrations in finger millet were aspartic acid, glutamic acid and asparagine, and their contents decreased over the 8-h fermentation process ($p < 0.05$); tyrosine content also decreased during fermentation (Figure 4.13). The essential amino acids, methionine, phenylalanine, isoleucine and leucine, revealed a slightly different trend: their contents increased between two- to eleven-fold upon 6-h fermentation, but were then depleted over the next 2 h from the fermented slurries. As previously mentioned, in the sub-chapter 4.4.5, in the research work of Garvie (1967) dextran-forming strain groups require few amino acids, among which the more relevant are glutamic acid, isoleucine, and leucine, a trend that may justify the observed decrease of those amino acids after the 6 h (EPS were produced during this period as discussed latter on in sub-chapter 4.6.3.6). Nonetheless, other amino acids contents were also reduced and, apparently, are not specially required by these EPS-producing strains. On the other hand, threonine, arginine, GABA, and glutamine increased

one- to three-fold upon 8-h fermentation. These amino acids have shown a positive impact on the maintenance of intestinal mucosal integrity and barrier function (threonine), modulation of GI motility (GABA), athletic improvement (arginine and glutamine), and other important body functions namely, in the reproductive, cardiovascular, pulmonary, renal, gastrointestinal, and immune systems, besides the enhancement of insulin sensitivity, and the maintenance of tissue integrity (arginine) (Auteri et al., 2015; Mahan & Escott-Stump, 2008; Mao et al., 2011; Popolo et al., 2014). Nevertheless, pathogenic involvements have been described for arginine metabolites, and thus the protective *vs*. pathogenetic role needs further investigation (Mondanelli et al., 2019). The increased availability of free amino acids plays a beneficial role on the host nutritional status, particularly if a deficiency in endogenous protease production is present (Houngbédji et al., 2021), upholding the important contribution of this fermented yoghurt-like beverage to human health promotion.

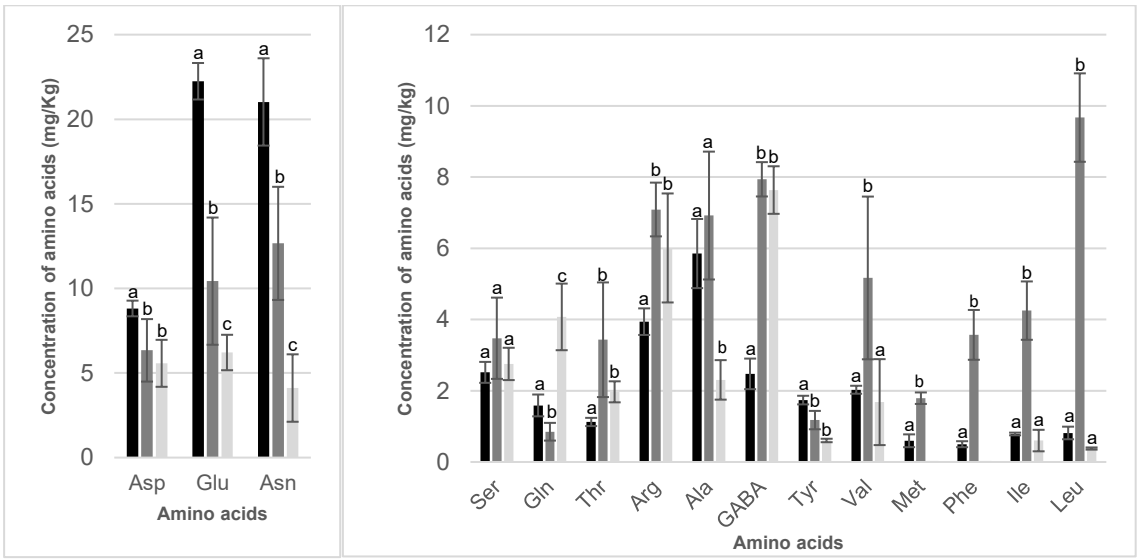


Figure 4.13 Free amino acids content (mg/kg of slurry) of dry-milled finger millet slurry, fermented by *W*. *confusa* 2LABPT05 co-cultured with *L*. *plantarum* 299v, before fermentation (black columns) and 6 h (dark grey columns) and 8 h (light grey columns) after fermentation, at 30 °C and 200 rpm, in an orbital incubator. Error bars represent the 95 % confidence intervals of independent replicate experiments. (Asp: Aspartic acid; Glu: Glutamic acid; Asn: Asparagine; Ser: Serine; Gln: Glutamine; Thr: Threonine; Arg: Arginine; Ala: Alanine; Tyr: Tyrosine; Val: Valine; Met: Methionine; Phe: Phenylalanine; Ile: Isoleucine; Leu: Leucine). For a given amino acid, different letters indicate significant differences ($p \leq 0.05$) between times, using the Paired sample T-test or Wilcoxon test.

4.6.3.2. Protein digestibility

The *in vitro* protein digestibility (IVPD) of the fermented product (64 %) was enhanced when compared to the unfermented flour (25 %) (Table 4.27). Fermentation of sorghum, pearl and finger millets and legume flours impacts positively protein digestibility, as reviewed by Nkhata *et al*. (2018). Pranoto and colleagues observed an increase of protein digestibility, from 41.81 % to 80.31 %, when sorghum was fermented by *L*. *plantarum* over 36 h and mentioned that the *L*. *plantarum* tannase activity

might have been a justification for this increase, due to the release of previously complexed proteins to tannins (Pranoto et al., 2013). The work of Arte and co-authors (2015) showed that controlled fermentation has also led to more intense proteolysis and strongly impacted the IVPD. Furthermore, Ogodo and collaborators (2018) reported that LAB fermentation of soybean flour had a positive impact on protein digestibility. Explanations for such potentially improving effect include the fact that pH reduction triggers protein breakdown into smaller biopeptides, with potential added biological value, a consequence of augmented proteolytic enzymes activity of fermenting bacteria (Hesseltine, 1983; Sripriya et al., 1997). Furthermore, the reduction in levels of antinutrients, such as tannins, which are negatively involved in protein metabolism, allow digestive enzymes to gain easier access to proteins, resulting in increased protein bioavailability and digestibility (Bhattarai et al., 2017; Gilani et al., 2012; Joye, 2019; Kostekli & Karakaya, 2017). This higher IVPD complemented with the increase of some limiting amino acids, two parameters used to assess a product's nutritive value (Lorusso et al., 2018), makes the fermented yoghurt-like beverage more interesting in terms of nutritional profile and more beneficial to consumers relative to the starting flour.

Table 4.27 Physicochemical characterization of unfermented and fermented dry-milled finger millet yoghurt-like beverage (YLB), by *W. confusa* 2LABPT05 co-cultured with *L. plantarum* 299v, over 8 h, at 30 °C and 200 rpm, in an orbital incubator.

	Unfermented slurry	**Fermented YLB (F-YLB)**
Lactic Acid (g/kg YLB)	< LOD	2.69 ± 0.09
Acetic Acid (g/kg YLB)	< LOD	0.70 ± 0.08
Sucrose (g/kg YLB)	64 ± 8 [a]	38 ± 6 [a]
Glucose (g/kg YLB)	10 ± 2 [a]	7.0 ± 0.8 [a]
Fructose (g/kg YLB)	4.9 ± 0.8 [a]	21 ± 3 [b]
Protein digestibility (%)	25 ± 2 [a]	66 ± 2 [b]
Fe (mg/kg YLB)	4.74 ± 0.09 [a]	4.6 ± 0.3 [a]
Mg (mg/kg YLB)	134 ± 1 [a]	126 ± 1 [b]
Mn (mg/kg YLB)	18.3 ± 0.7 [a]	17.1 ± 0.1 [a]
K (mg/kg YLB)	303 ± 2 [a]	312 ± 8 [a]
Na (mg/kg YLB)	3.4 ± 0.1 [a]	32 ± 2 [b]
P (mg/kg YLB)	244 ± 3 [a]	238 ± 2 [b]
Ca (mg/kg YLB)	365 ± 9 [a]	319 ± 2 [b]
Zn (mg/kg YLB)	1.13 ± 0.04 [a]	0.95 ± 0.01 [b]
Apparent viscosity (mPa.s), 20 ºC	11.9 ± 0.4 [a]	35 ± 2 [b]
Apparent viscosity (mPa.s), 8 ºC	13.2 ± 0.4 [a]	102 ± 35 [b]
Average Dextran (%), dry weight	0.3 ± 0.09 [a]	16.1 ± 0.9 [b]

For a given parameter, different letters (a and b) indicate significant different ($p \leq 0.05$) between samples, using the Paired sample T-test or Wilcoxon test.

4.6.3.3. Mineral content

In general, the 8-h fermentation period slightly decreased the mineral content, except for potassium and sodium (Table 4.27). Given the alleged reduction of antinutrients mediated by fermentation, the bioavailability of divalent or trivalent minerals would be expectably enhanced (Gupta et al., 2013; Nkhata et al., 2018) however, such was not observed. Phytate reduction is driven by phytase activity, which might be cereal endogenous or produced by microorganisms. In the present work, the non-increase in the mineral content might be justified by the non-degradation of phytate. This could have happened mainly for two reasons, either because the intrinsic cereal phytases were inactivated (by autoclaving), or because the bacterial strains employed do not produce intra- or extracellular phytases (Reale et al., 2007). Reale *et al*. (2007), who screened 50 LAB, namely *L*. *plantarum*, *L*. *amylovorus*, *L*. *acidophilus*, *L*. *sanfranciscensis*, *Leuconostoc mesenteroides* strains, for intra- and extracellular phytate- degrading activity, concluded that all strains lacked extracellular phytase activity and intracellular phytase activity was not significant. In other studies, in which an effective reduction of antinutrients was noticed, cereal matrices were not subject to heat treatment. Nevertheless, the work of Sripriya *et al*. (1997) showed that even in single cultures or sequential inoculation, probiotic fermentation with *Lactiplantibacillus* strains resulted in a reduction of phytic acid levels. Another issue that may interfere in the action of the phytase enzyme is the process temperature. It is well-studied that phytase activity is optimum at temperatures around 35-45 ºC, a range which does not include the fermentation temperature used in the present work. Other research works (Rasane et al., 2015; Sindhu & Khetarpaul, 2001), revealed that when the food matrices were heat-treated, low levels of phytic acid were obtained with LAB fermentation, at 37 ºC. It must be highlighted, however, that these are all possibilities that cannot be confirmed since phytase activity was not quantified in the present work.

4.6.3.4. Proximate composition

The resultant fermented fresh YLB offers an improved nutritional profile benefiting many consumers (Table 4.28). The high-fibre (7g/100 kcal), fat-free, and low-salt (< 0.1 g/100g) nutritional claims, characteristic of the product, enable an improved food market availability in African and European countries, characterized in many cases by products rich in fat, sugars, and salt, the major contributors to chronic diseases. Moreover, the lactose- and gluten-free matrix makes this product an excellent option for lactose or gluten intolerants.

Table 4.28 Nutritional characterization and list of ingredients of the fermented yoghurt-like beverage (YLB), after 8 h of fermentation at 30 °C and 200 rpm, in an orbital incubator.

	Per 100 g of cereal YLB
Energy (kcal/kJ)	57
Fat (g)	0.1
saturated	< 0.01
Carbohydrates	15.2
sugars (g)	6.2
Fibre (g)	4.0
Protein (g)	0.7
Salt (g)	< 0.1

List of ingredients: Water, whole grain finger millet (*Eleusine coracana* (L.) *Gaertn*) flour, sucrose, live microorganisms (*Lactiplantibacillus plantarum* 299v and *Weissella confusa* 2LABPT05*)*.

4.6.3.5. Apparent viscosity – quantitative analysis

The 8-h fermentation had a considerable impact on the apparent viscosity of the product, which increased from 12 to 35 mPa.s (292 %), measured at 20 ºC (Table 4.27). This texture modification is a consequence of EPS production, mainly generated by the *W. confusa* 2LABPT05 strain, as supported by the results described in the following sub-chapter. The increase of viscosity after fermentation carried out by EPS-producing strains was also observed by other authors, both in dough and sourdough preparation (Galli et al., 2020; Katina et al., 2009; Xu, Coda, et al., 2017; Xu, Wang, et al., 2017), and in the preparation of cereal beverages (Coda et al., 2011, 2012; Lorusso et al., 2018; Zannini et al., 2013). The study of Coda and colleagues, in which a co-fermentation of a *Weissella* strain (*W. cibaria* WC4) and a *Lactiplantibacillus* (*L. plantarum* 6E*)* strain was performed, did not reveal an increase in viscosity, when compared to the plain *Weissella* fermentation (Coda et al., 2011). After refrigeration at 4-6 ºC for approximately 12 h, viscosity underwent a ten-fold increase, showing the impact of temperature on viscosity (Table 4.27). During cooling, starch retrogradation (realignment of amylose and amylopectin hydrogen bonds and recovery of crystalline structure (Kubo et al., 2010)) occurs leading to increased viscosity. This YLB revealed to be a dilatant or shear-thickening fluid, as apparent viscosity increases with increasing shear rate (Figure 4.14) (Rapp, 2017). Once a shear rate is applied to a fluid in which solid particles are suspended in a liquid, these particles need to reorder. If a small shear rate is applied they have enough time to reorder, however, if a higher shear rate is applied those particles do not have enough time to reorganize and consequently, there is a formation of aggregates of particles, increasing the viscosity of the fluid (Rapp, 2017).

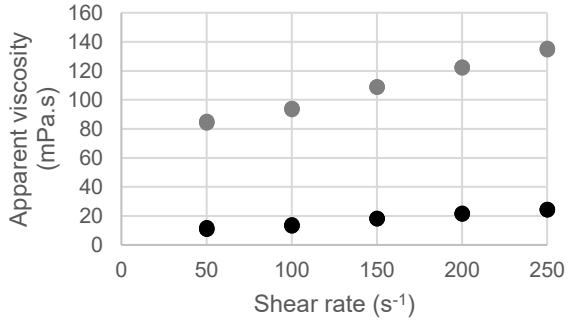


Figure 4.14 Apparent viscosity as a function of shear rate of the non-fermented slurry (black colour) and the fermented yoghurt-like beverage (F-YLB) (grey colour), at 8 °C, for 10 seconds.

4.6.3.6. EPS analysis

Dextran structural characterization

The analysis of the 1D ^{1}H spectrum of the EPS produced by *W. confusa* 2LABPT05 reveals two different distribution peaks' zones (Figure 4.15). Downfield of 4.9–5.3 ppm, with a typical dextran with main chain α–(1 → 6)-linked-D-glucopyranosyl residues, with the anomeric proton signal at 4.98 ppm, and a low intensity anomeric signal is observed at 5.32 ppm, which corresponds to α–(1 → 3)-linked-D-glucopyranosyl residues (Cheetham et al., 1990; Maina et al., 2008; Seymour et al., 1980). The intensity (%) of both peaks, obtained from the integration of relative intensity of the signals, is 97 %, of α–(1 → 6),

and 3 % of α–(1 → 3). Thereby, this EPS is mainly composed by α–(1 → 6) glycosidic linkages in the main chain and few α–(1 → 3) branched linkages. These results are aligned with those obtained by other authors (Ahmed et al., 2012; Galli et al., 2020; Maina et al., 2008; Netsopa et al., 2018; Wang et al., 2019), in which EPS produced by *W. cibaria* CMGDEX3, *W. confusa* Ck15, *W. confusa* E392, *W. confusa* R003, and *W. confusa* A16 respectively, were analysed. The other peaks' zone, upfield of 3.5 – 4.0 ppm, is characterized by bulk region protons, corresponding to ^{6b}H-, ^{5}H, ^{6a}H, ^{3}H, ^{2}H, and ^{4}H of the α-(1→6)-linked glucosyl main chain, respectively (Wang et al., 2019).

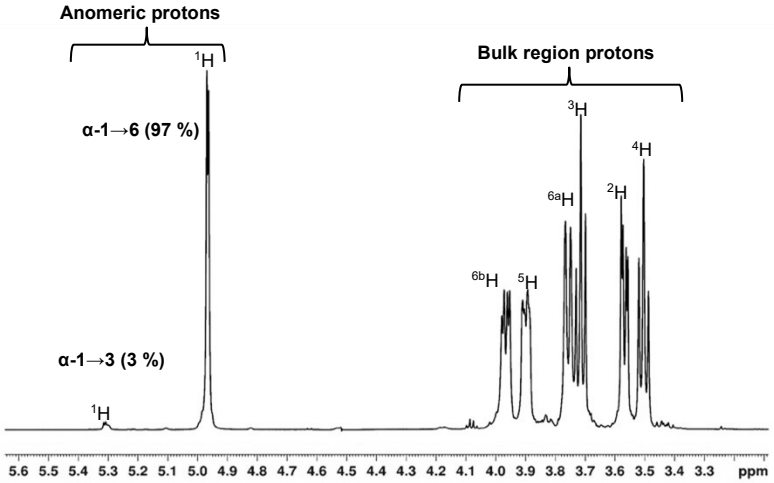


Figure 4.15 The 1D ^{1}H nuclear magnetic resonance spectrum of EPS produced by *W. confusa* 2LABPT05) recorded at 600 MHz in D_20 at 50 ºC. The peaks are referenced to internal acetone (^{1}H = 2.225 ppm).

Dextran quantification

The fermented beverage showed high levels of dextran (16.1 ± 0.9 %, dry-weight, or 3 g/100 g fresh fermented YLB), in comparison to the non-fermented slurry (0.30 ± 0.09 %, dry-weight), with a considerable *in situ* production of this compound by the *Weissella* strain (Table 4.27). Regarding the evolution of sucrose over fermentation and the growth kinetics of *W. confusa* 2LABPT05 (Figure 4.11), it seems that sucrose utilization for EPS production might have happened mostly at the late exponential phase. Galli and colleagues (2020) also observed that the production of EPS started during the exponential phase, and thereby, they concluded that LAB might use sucrose simultaneously for both the growth and for EPS production. Nevertheless, according to other authors (Han et al., 2014), the viscosity increase was higher during the stationary phase, regardless of having previously increased during the late exponential phase. Nonetheless, this is not possible to confirm by the present work, since fermentation ends after 8 h, *id est* before entering the stationary phase.

Regardless of the fact that in this study only the EPS produced by *Weissella* were evaluated, it has been shown that several strains of *L. plantarum* also have the ability to produce EPS (Bachtarzi et al., 2020; Haroun et al., 2013; Liu et al., 2019; Min et al., 2019; Xu et al., 2019); however the structure of its EPS is quite different, sometimes composed by different sugars, such as glucose, fructose,

rhamnose and galactose, and also by either α- or β-type configurations (Oleksy & Klewicka, 2018). All of these studies, except that of Bachtarzi *et al*. (2020), extracted the EPS from the growth medium, and no publications were found in which the EPS produced from *L*. *plantarum* were extracted and applied in cereal matrices (Bachtarzi et al., 2020). Nevertheless, Di Cagno *et al*. (2006) observed the production of HoPS, glucans and fructans, by *L*. *plantarum* LP9, however the production of HoPS, compared to HePS, by *Lactiplantibacillus plantarum* seems to be more scarce (Angelin & Kavitha, 2020).

EPS yield is influenced by several factors including, pH, the food matrix and the presence of oxygen (Kaditzky & Vogel, 2008; Katina et al., 2009). In addition, Pintado *et al*. (2006) concluded that different media influence both the quantity and the composition of the produced EPS. It has been shown that pH around 4.7-4.8 (coinciding with the pH value of the final hour of the present fermentation) leads to a higher production of EPS, even when the involved enzymes have shown to have optimal activity at different pHs (Kaditzky & Vogel, 2008). Furthermore, the influence of the cereal matrix on EPS yield may be related to the buffering capacity of the substrate (Kaditzky & Vogel, 2008). Mennah-Govela *et al*. (2020) concluded that the protein content and the initial pH value of a food product are the major influencers on the buffering capacity. In what concerns the product developed in this work, the protein content seems to have no major influence, given its low value; however the levels of aspartic and glutamic acids, in particular, may have affected buffering capacity in an acidic environment (Mennah-Govela et al., 2020). Although their levels were reduced upon fermentation, they remained the majority among the amino acids detected. Upon fermentation, a pH below 5, and the increased organic acids levels may have contributed to the finger millet YLB buffering capacity (Mennah-Govela et al., 2020). Lastly, the aeration, improved by the slurry agitation over fermentation, leads to higher growth rates of the involved microorganisms (Katina et al., 2009), and thereby, may also have influenced positively the EPS yield.

4.6.4. Sensory Analysis

Fermentation is a process with important implications in quality attributes such as appearance, taste, odour and texture which need to be described for each new product developed. Furthermore, the involvement of different strains will necessarily influence sensory attributes given their complementary metabolic activity, hence sensory evaluation is a very important step in any product development research pipeline.

In this study, the mean age of the panel of consumers was 34 ± 7 years-old, with 73 % being women (n=22). Globally, the unflavoured and flavoured YLBs were well appreciated (‘like slightly’), with texture being the less well rated parameter (Table 4.29 and Figure 4.16). This attribute was explored in terms of its relationship with global appreciation and certainty of purchase, and for that, based on the median texture’s classification it was divided into two classes, namely the respondents who classified texture with less than 4 points (53.5%), including 4 (‘dislike slightly’) and those who classified texture with more than 4 points. The latter group tended to classify product global appreciation better and classify more equally the product consumed with or without yoghurt, but no statistical differences were observed. Consequently, those who liked the texture more would buy the product with more certainty. Regarding appearance, colour, and consistency 50 % of the sample ‘liked slightly’ these parameters as

they were presented (median = 6, on a scale from 1 to 9, 1 corresponds to dislike extremely and 9 to like extremely).

Table 4.29 Medians and extreme classifications for the four parameters evaluated regarding the non-flavoured yoghurt-like beverage (YLB) and for the global appreciation of each flavoured YLB.

	Parameter	Minimum	Median	Maximum
Unflavoured YLB	Appearance	3	6	9
	Colour	3	6	8
	Consistency	2	6	8
	Texture	2	4	8

Flavoured YLB		**No added yoghurt**			**With added yoghurt**		
		Minimum	**Median**	**Maximum**	**Minimum**	**Median**	**Maximum**
Coffee	Global appreciation	3	6	8	3	6	8
Hazelnut		3	6.5	9	3	6	9
Chocolate		2	6	8	2	5.5	9
Blueberry		2	6	8	2	5.5	8

For all flavoured cereal YLBs, sweetness and acidity were classified as 'As I like' by 50 % of the sample, however, extreme classifications (insufficient and excessive) were also scored. Flavour was also evaluated for the flavoured samples. The best classified flavour was Hazelnut (min=3; median=7; max=9), however, no statistical differences were observed ($p > 0.05$). Half of the panel would buy the product if it was offered with yoghurt, and when no yoghurt was added, the certainty of the purchase decreased.

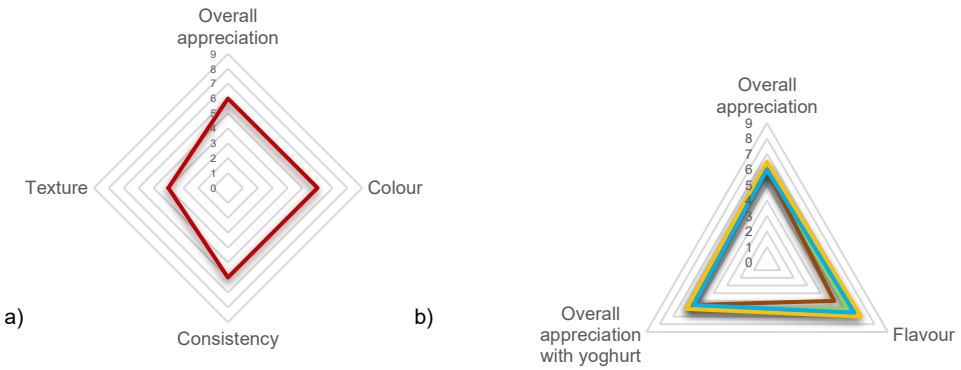


Figure 4.16 Spider diagram of the sensory analysis for the non-flavoured finger millet fermented yoghurt-like beverage (YLB) (a) and the aroma-flavoured (brown line: chocolate; green line: coffee; blue line: blueberry; yellow line: hazelnut) finger millet fermented YLB, incorporated or not in natural yoghurt (b). Hedonic scale from 1 (to dislike extremely) to 9 (to like extremely). The results are expressed in medians.

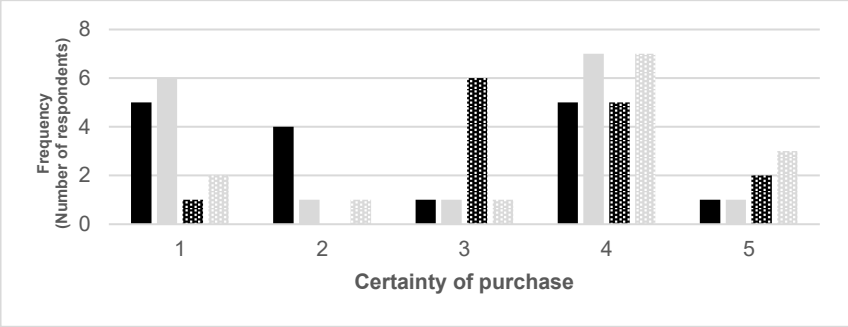


Figure 4.17 Distribution of consumers according to their certainty about the purchase of the product (1 - Certainly would not buy and 5 - Certainly would buy) with (grey bars) or without yoghurt (black bars), and considering their texture classification (solid fill bars - less than 4 points: n=32; and pattern fill bars - more than 4 points: n=28).

Concerning the quality of the aromas, there was a difference in the classification by the panel ($p < 0.05$). In terms of mean rank, the hazelnut aroma was the highest-rated aroma registering the highest value (70.2), blueberry was the second most favourite (62.1), coffee followed (56.1) and the lowest value was registered for chocolate (53.6). The initial establishment of the aroma's concentration was based on technical references, given by the supplier. However, considering the specificity of the cereal matrix (with a characteristic flavour), it would be useful to evaluate the adequacy of this concentration; for example, there was a need to increase the amount of chocolate aroma added against the supplier's recommendation and even so, the chocolate aroma was the least preferred probably due to a low-intensity threshold. The influence of gender and age in the classifications of different variables was also studied, however, no statically significant differences were observed ($p > 0.05$).

For some respondents adding the yoghurt improved the product in terms of texture, however, the aroma was not so well perceived. The mixing of the cereal YLB with another food product, namely yoghurt, was planned in order to broaden market positioning and enable reaching a wider number of population groups including children and the elderly. The combination of both products could be more easily accepted and successful in terms of consumer appreciation. Despite the important results obtained with this sensory evaluation, it would be advisable to increase the panel of tasters, and also to have a trained panel for the appreciation of the product in terms of attributes of interest, capable of having an impactful input.

4.6.5. Stability throughout storage – fresh fermented yoghurt-like beverage

Fermentation also contributes positively to shelf-life, an important asset if product development is to be successful regardless of market placement. The resultant fermented YLB replicates were placed under refrigeration (3-4 °C) and the stability of several parameters was studied. In general, both strains maintained their viability throughout storage with slight differences between them, viz. *Lactiplantibacillus plantarum* 299v underwent a slight increase in viable cell numbers (+ 0.8 log cycles) between 0 and 7 d

of storage, which were then maintained up to 21 d of storage, whereas viable cell numbers of *Weissella confusa* 2LABPT05, remained stable up to 14 d of storage and underwent a one log cycle decrease between 14 d and 21 d of storage, yet were still above 10^8 CFU/mL, showing that the storage period and conditions were not critical for their survival (Table 4.30). During storage, metabolic activity apparently continued during the first 7 d, since lactic and acetic acids contents increased, while sugars' levels reduced, possibly due to a non-immediate cooling; contents stabilised thereafter.

Table 4.30 Bacteriological and chemical characteristics of the fermented finger millet yoghurt-like beverage (YLB), inoculated with the bacterial consortium composed of the EPS-producing strain *W. confusa* 2LABPT05 and the probiotic strain *L. plantarum* 299v, after 8-h fermentation at 30 °C, 200 rpm, in an orbital incubator (0 d), and after 7, 14 and 21 d of storage at 4°C.

	0 d	**7 d**	**14 d**	**21 d**
Indigenous *Weissella* (Log (CFU/mL))	8.7 ± 0.1 [a]	9.1 ± 0.1 [b]	9.07± 0.02 [b]	8.1 ± 0.2 [c]
Probiotic *Lactobacillus* (Log (CFU/mL))	7.6 ± 0.2 [a]	8.4 ± 0.1 [b]	8.47 ± 0.09 [b,c]	8.5 ± 0.1 [c]
pH	4.79 ± 0.07 [a]	3.92 ± 0.03 [b]	3.745 ± 0.007 [c]	3.639 ± 0.005 [d]
Lactic acid (g/kg YLB)	2.69 ±0.09 [a]	5.2±0.4 [b]	6.7±0.3 [b]	7.3±0.4 [b]
Acetic acid (g/kg YLB)	0.70±0.08 [a]	1.40±0.04 [b]	1.6±0.2 [b]	1.8±0.2 [b]
Sucrose (g/kg YLB)	38±6 [a]	5.5±0.6 [b,c]	6.2±0.5 [b]	4.4±0.1 [c]
Glucose (g/kg YLB)	7.0±0.8 [a]	2.9±0.5 [b]	3.5±0.2 [b]	3.0±0.1 [b]
Fructose (g/kg YLB)	21±3 [a]	26±3 [a,c]	30.8±0.9 [b]	28.0±0.4 [c]

Values are expressed as the mean ± standard deviation of independent replicate experiments. Different letters within each row indicate significant differences ($p \leq 0.05$) over time, using Paired-samples T-tests.

4.7. POWDERED FERMENTED CEREAL

4.7.1. Preliminary test – Spray-drying, Tray-drying and Freeze-drying

The production of long shelf-life, concentrated dried fermented cereal product is of paramount importance for the food industry. In a first test to produce the fermented cereal powder, part of the fermented product obtained in a previous fermentation experiment was dried using three different processes. The aim was to understand which would be the best drying process to be used considering the feasibility, yield and cost. Freeze-drying was the only process that made it possible to obtain a fine powder, with a reasonable yield (ratio between real moisture, from drying at atmospheric pressure at 102 °C and process obtained moisture, calculated by weight difference); the powder was not darkened, and the process reached 99% of drying yield. Spray-drying was not effective due to sample texture and heterogeneity and, consequently, it was not possible to have a final dried product. Tray-drying showed a drying yield around 98 %, however, the dried product darkened during processing and the final texture was not desirable. Consequently, based on these findings, freeze-drying was the selected method to produce the fermented cereal powder. Freezing may be highly detrimental to cell viability especially due to osmotic shock and membrane injury resulting from intracellular ice formation and recrystallization. In order to protect the cells from such damage, cryoprotectant agents including glycerol compounds, mono-, oligo- and, polysaccharides, polyalcohols, sugar alcohols, proteins and polyethylene glycol are used. Hence, as a first step, different cryoprotectants were tested in order to study the pertinence of their addition beforehand. Figure 4.18 presents the viable cell numbers in each situation before and after the processing, aiming to analyse the effect of freeze-drying on strain viability.

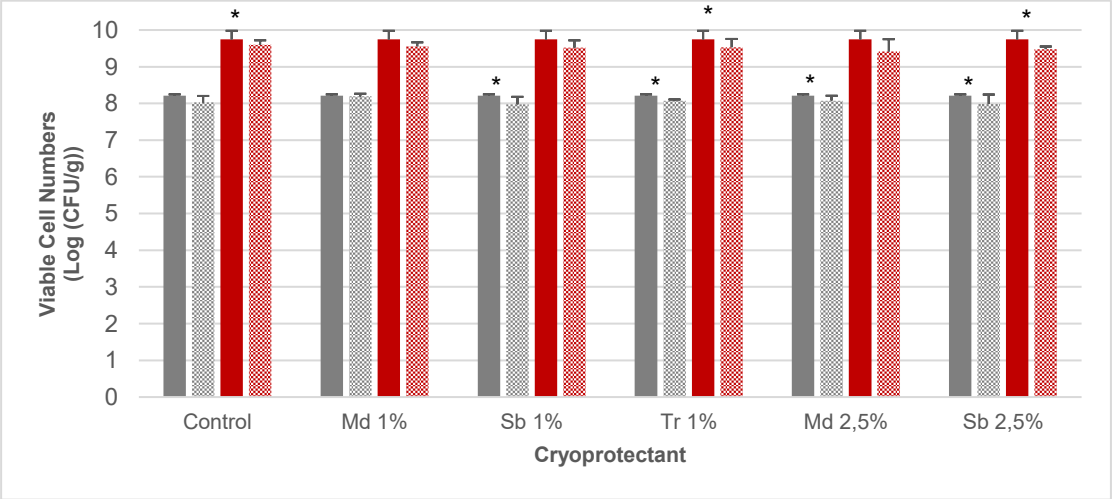


Figure 4.18 Bacterial growth before (full columns) and after (dotted columns) freeze-drying (48 h) for both strains *L. plantarum* 299v (grey columns) and *W. confusa* 2LABPT05 (red columns) in the presence of three different cryoprotectants (Md: maltodextrin, Sb: Sorbitol and Tr: Trehalose) in different concentrations (1 % and 2.5 %) and absence of cryoprotectant (control). Error bars represent 95 % confidence intervals of means. The symbol * indicates significant differences ($p \leq 0.05$) after the drying process for the indicated cryoprotectant, using Paired-samples T-test.

The effect of the freeze-drying process on both strains was cryoprotectant-dependent (Figure 4.18). *Lactiplantibacillus* strain has retained 68 % of viability and *Weissella* 70 %, without any protectant. The observed viability of the probiotic strain with different protectants was 95 %, 59 % and 73 % for Md, Sb and Tr at 1 % and 74 % and 62 % for Md and Sb at 2.5 %, respectively. Regarding the indigenous strain, viability after processing was of 64 %, 59 % and 61 % for Md, Sb and Tr at 1 % and 48 % and 53 % for Md and Sb at 2.5 %, respectively. According to these results, it seems that maltodextrin at 1 % seemed to be the most effective cryoprotectant, however, no statistically significant differences were observed between all the cryoprotectants ($p > 0.05$), in case of *W. confusa* 2LABPT05. Nonetheless, for *L. plantarum* 299v Md at 1% retained a higher cell viability in comparison to Sb at 1% and at 2.5 % ($p \leq 0.05$). The augment of protectant concentration did not imply an increase in strain viability. Carvalho *et al*. (2002) reported that sorbitol was the most effective among the tested cryoprotectants.

Besides the evaluation of the drying process impact, strain stability was monitored throughout storage, mainly because this is a critical phase for strain survival, as described in the review of Carvalho *et al*. (2002). Comparing the viable cell numbers before treatment and after the 10 wks of storage, significantly lower ($p < 0.05$) values were obtained for all the cryoprotectants, for both strains. However, strains behaviour during storage was different, since *W. confusa* seemed to be more resistant, showing higher levels of viability. For this strain, the viable cell numbers were not impacted during the 10-wk storage, when in the presence of Sb 1 % and Md 2.5 % (Figure 4.19). *L. plantarum* showed to be more sensitive to storage time, however, in the presence of Sb 1 % no statistically significant differences in the viable cell numbers were observed from the 3-wk storage point onwards (Figure 4.19). When viability was compared for each cryoprotectant, at each time, some statistical differences were observed (Table 4.31). From the results obtained in this experiment, it was possible to conclude that the use of sorbitol at 1 % seemed to be the best option in what concerns the protection of both strains. Previous studies have concluded that the most favourable cryoprotectants to achieve higher viabilities of *L. plantarum* TISTR 2075 was a combination of cryoprotectants, such as 15 % protein plus 5 % of trehalose/maltodextrin (survival rates of 98.13 % and 97.58%, respectively) (Savedboworn et al., 2017), or 10 % rice protein and 5 % fructooligosaccharides (survival rate of 71.34 %) (Savedboworn et al., 2019). On the other hand, Carvalho *et al*. (2002) reported that sorbitol (12 g/L) was the most effective cryoprotectant for *L. plantarum* and *L. rhamnosus* during storage, among myo-inositol, fructose, monosodium glutamate (all at 12.5 g/L), trehalose (3.5 g/L), and propyl gallate (7 g/L) protectants.

Table 4.31 Percentage viability (relative to the numbers after freeze-drying) of *L. plantarum* 299v and *W. confusa* 2LABPT05 after 3, 7 and 10 wk of storage, at room temperature, in the dark, in a desiccator.

	***L. plantarum* 299v**			***W. confusa* 2LABPT05**		
Cryoprotectant	**3 w**	**7 w**	**10 w**	**3 w**	**7 w**	**10 w**
Control	22 ± 4 ^{a,x}	26 ± 17 ^{a,x,y}	14 ± 7 ^{a,x,y}	26 ± 4 ^{a,x}	42 ± 4 ^{b,x}	34 ± 13 ^{a,b,x}
Maltodextrin 1 %	13 ± 4 ^{a,x}	10 ± 1 ^{a,x}	4 ± 2 ^{b,x}	35 ± 8 ^{a,x}	51 ± 11 ^{a,x,y}	46 ± 14 ^{a,x}
Maltodextrin 2.5 %	15 ± 3 ^{a,x}	9 ± 1 ^{b,x}	15 ± 10 ^{a,b,x,y}	71 ± 6 ^{a,y,z}	61 ± 13 ^{a,x,y}	56 ± 28 ^{a,x}
Sorbitol 1 %	38 ± 13 ^{a,y}	30 ± 2 ^{a,y}	26 ± 3 ^{a,y}	58 ± 9 ^{a,y,z}	59 ± 17 ^{a,x,y}	66 ± 9 ^{a,x}
Sorbitol 2.5 %	45 ± 4 ^{a,y}	22 ± 5 ^{b,x,y}	9 ± 5 ^{b,x,}	77 ± 8 ^{a,z}	67 ± 6 ^{a,y}	62 ± 11 ^{a,x}
Trehalose 1 %	14 ± 2 ^{a,x}	10 ± 3 ^{b,x}	6 ± 2 ^{c,x}	57 ± 11 ^{a,y}	57 ± 4 ^{a,x,y}	65 ± 10 ^{a,x}

For a given strain, and cryoprotectant, different letters (a, b, c) indicate significant differences over time, using Paired samples T-tests.

For a given strain, and time, different letters (x,y,z) indicate significant differences between cryoprotectants, using one-way ANOVA.

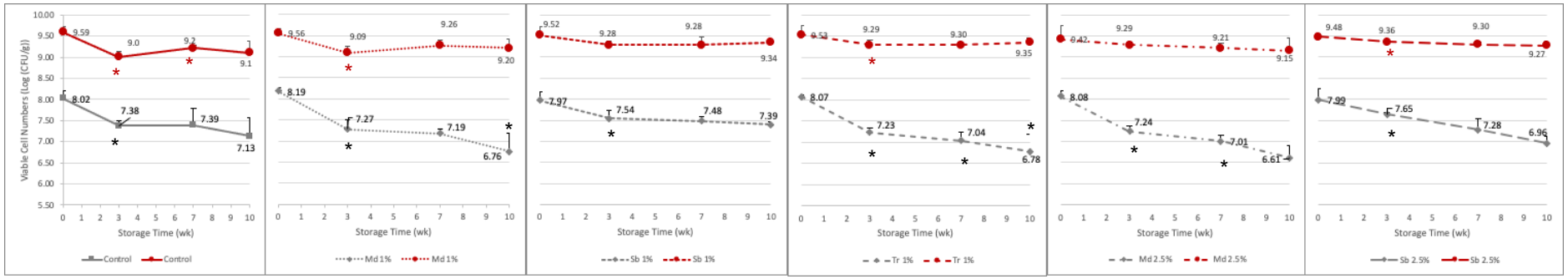


Figure 4.19 Survival of freeze-dried *L. plantarum* 299v (grey lines) *W. confusa* 2LABPT05 (red lines) during storage in the presence of three cryoprotectants (Md: maltodextrin, Sb: Sorbitol at 1 % and 2.5 % and Tr: Trehalose at 1 %) and absence of cryoprotectant (control). Error bars represent 95 % confidence intervals of means. The symbol * indicates significant differences ($p < 0.05$) in reference to the previous time point, using Paired samples T-tests.

4.7.2. Stability throughout storage – powdered fermented yoghurt-like beverage

Since powdered formulations have advantages in terms of extended shelf-life, quality preservation and are more economical in terms of transport and storage logistics, stability throughout storage of the freeze-dried fermented beverage was also tested. Freeze-drying led to a slight reduction (Figure 4.20) in viable cell numbers, specially of *W. confusa* 2 LABPT05 but of no statistical relevance, *id est* it dropped by ca. 0.1 and 0.15 log cycles relative to the fresh counterpart, independently of the presence or absence of sorbitol ($p > 0.05$). It is important to note that the presence of remaining sucrose in the medium might have also acted as a protector, contributing to the strains' survival, since sucrose is known to preserve intact cells during this dehydration process (Chang et al., 2005; Leslie et al., 1995).

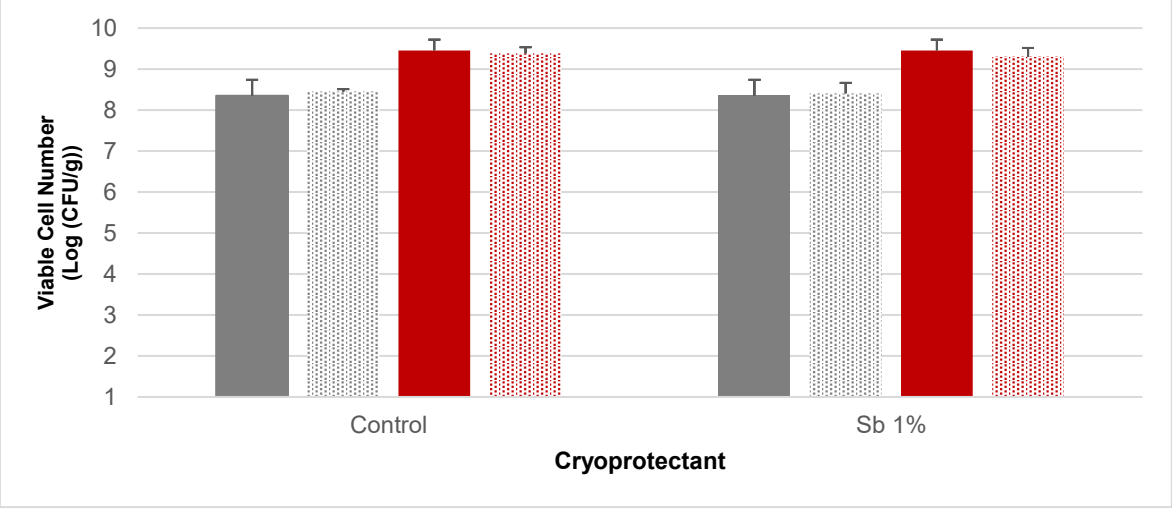


Figure 4.20 Bacterial growth before (full columns) and after (dotted columns) freeze-drying (48 h) for *L. plantarum* 299v (grey columns) and *W. confusa* 2LABPT05 (red columns) in the presence and absence of the cryoprotectant sorbitol at 1 %. Error bars represent 95 % confidence intervals of means.

Similarly to the procedure during the preliminary test, besides the evaluation of the drying process impact, strain stability was monitored throughout a 12-wk storage period, mainly because this is a critical phase for strain survival, as described in the work of Carvalho *et al*. (2002), as mentioned before. Sorbitol at 1 % protected both strains, although the effect was more prominent for *L. plantarum* 299v (reduction of ca. 1.29 versus 0.40 log cycles in the absence and presence of sorbitol, respectively) than for *W. confusa* 2LABPT05 (reduction of ca. 0.33 versus 0.16 log cycles, respectively), which seems to be more resistant to the established storage conditions ($p \leq 0.05$) (Figure 4.21). The presence of sorbitol enabled the viable cell numbers of both strains to remain above 10^7 CFU/mL throughout storage. Similarly, the study of Carvalho *et al*. (2002) revealed that sorbitol had a great protective effect on the survival of *Lactiplantibacillus plantarum* during storage, but not necessarily during the freeze-drying process.

Some proposed mechanisms attempt to explain the protection effect during storage given by sorbitol. On the one hand, there is the 'water substitute hypothesis', in which sorbitol substitutes the space between lipids in the cellular membrane, once occupied by water, replacing its thermodynamic stabilization function by the formation of hydrogen bonds at specific sites on the surface of the proteins (Chang et al., 2005). This leads to the native structure preservation of the membrane. On the other hand, there is the 'glass dynamics hypothesis', in which the presence of a stabilizer, such as sorbitol, forms a rigid and inert matrix, in which mobilization is limited and, in consequence, bimolecular interactions, such as unfolding and chemical degradation are slowed (Chang et al., 2005).

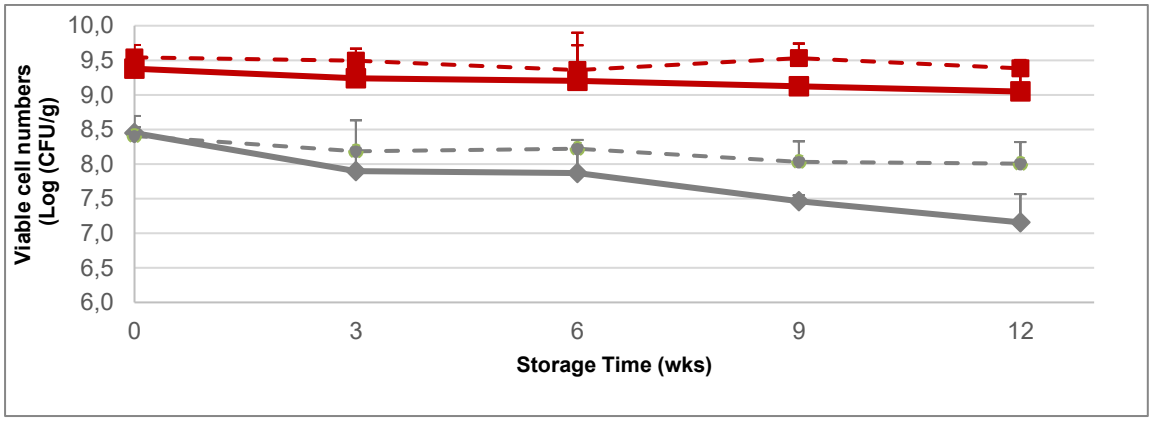


Figure 4.21 *L. plantarum* 299v (grey lines) and *W. confusa* 2LABPT05 (red lines) survival (in log (CFU/mL)) in the presence (dashed lines) and absence (full lines) of the cryoprotectant sorbitol at 1 %, during 12 wks of storage at room temperature in a desiccator, away from light. Error bars represent 95 % confidence intervals of means.

During the 12-wk storage, no differences in acidification or the effect of the cryoprotectant on pH values were observed ($p > 0.05$). Concentrations of organic acids and sugars remained stable throughout storage (Table 4.32). Considering the free amino acids content during storage, only from the ninth week onwards it is possible to observe statistically significant differences between the presence and absence of the cryoprotectant (Table 4.33). At 12 wk of storage, higher contents were observed in the samples with sorbitol, namely for threonine, alanine, tyrosine, GABA, valine, and isoleucine. It is interesting to notice that arginine levels were greatly increased after freeze-drying. Given its known hygroscopicity property, in the liquid fresh matrix, it might have been bonded to water and after freeze-drying, once the water was eliminated, this amino acid became free and more easily detectable in the medium.

Table 4.32 Chemical composition of the dry-milled finger millet slurry, fermented by *W. confusa* 2LABPT05 co-cultured with *L. plantarum* 299v, after fermentation and of the yoghurt-like beverage powdered slurry after 3, 6, 9 and 12 wks of storage without (control) and with 1% sorbitol as cryoprotectant, at room temperature, in a desiccator.

				End of fermentation	Storage Time (wk)			
					3	6	9	12
Cryoprotectant	Control	pH		4.79 ± 0.07 ^{a}	4.58 ± 0.05 $^{a,b\ x}$	4.62 ± 0.03 $^{a,b\ x}$	4.62 ± 0.02 $^{a,b\ x}$	4.57 ± 0.05 $^{b,\ x}$
		Lactic acid		2.69 ±0.09 ^{a}	2.9 ± 0.5 $^{a,\ x}$	2.7 ± 0.2 $^{a,\ x}$	2.4 ± 0.2 $^{a,\ x}$	2.5 ± 0.4 $^{a,\ x}$
		Acetic acid		0.70±0.08 ^{a}	0.67 ± 0.03 $^{a,\ x}$	0.52 ± 0.02 $^{a,b,\ x}$	0.44 ± 0.06 $^{a,b,\ x}$	0.49 ± 0.04 $^{b,\ x}$
		Sucrose		38±6 ^{a}	18 ± 2 $^{b,\ x}$	19.2 ± 0.9 $^{b,\ x}$	18 ± 2 $^{b,\ x}$	21.0 ± 0.2 $^{b,\ x}$
		Glucose		7.0±0.8 ^{a}	4.1 ± 0.2 $^{b,c\ x}$	4.0 ± 0.1 $^{b,\ x}$	3.8 ± 0.3 $^{b,c,\ x}$	4.32 ± 0.01 $^{c,\ x}$
		Fructose		21±3 ^{a}	13.6 ± 0.7 $^{a,\ x}$	14 ± 1 $^{a,\ x}$	13 ± 1 $^{a,\ x}$	13.9 ± 0.6 $^{a,\ x}$
	Sorbitol 1 %	pH		4.79 ± 0.07 ^{a}	4.55 ± 0.06 $^{a,b\ x}$	4.60 ± 0.05 $^{a,b\ x}$	4.55 ± 0.03 $^{b,\ x}$	4.58 ± 0.06 $^{a,b\ x}$
		Lactic acid		2.69 ±0.09 ^{a}	2.8 ± 0.3 $^{a,\ x}$	3 0 ± 0.4 $^{a,\ x}$	3 0 ± 0.3 $^{a,\ y}$	2.9 ± 0.2 $^{a,\ x}$
		Acetic acid		0.70±0.08 ^{a}	0.66±0.03 $^{a,\ x}$	0.55±0.06 $^{a,\ x}$	0.54±0.06 $^{a,\ x}$	0.63±0.06 $^{a,\ y}$
		Sucrose		38±6 ^{a}	17±1 $^{b,\ x}$	18±1 $^{c,\ x}$	22±2 $^{b,c,d\ x}$	21.7±0.2 $^{d,\ y}$
		Glucose		7.0±0.8 ^{a}	3.8±0.3 $^{b,\ x}$	4.1±0.2 $^{b,\ x}$	4.2±0.1 $^{b,\ x}$	4.48±0.09 $^{b,\ y}$
		Fructose		21±3 ^{a}	12.1±0.6 $^{b,\ y}$	13.1±0.8 $^{a,b\ x}$	15.1±0.8 $^{b,\ x}$	14.5±0.7 $^{b,\ x}$

Values are expressed as the mean ± standard deviation of independent replicate experiments.

For a given cryoprotectant and parameter, different letters (a, b, c and d) indicate significant differences ($p \leq 0.05$) over time, using Paired-samples T-tests.

For a given parameter and time, different letters (x and y) indicate significant differences ($p \leq 0.05$) between cryoprotectants, using independent T-student tests.

Concentrations of sugars (sucrose, glucose and fructose) and organic acids (lactic and acetic acids) are expressed in g/kg of YLB.

Table 4.33 Free amino acids content (mg/kg yoghurt-like beverage (YLB)) of the dry-milled finger millet slurry, fermented by *W. confusa* 2LABPT05 co-cultured with *L. plantarum* 299v, after fermentation and of the yoghurt-like beverage powdered slurry after 3, 6, 9 and 12 wks of storage without (control) and with 1% sorbitol as cryoprotectant, at room temperature, in a desiccator.

Concentration of amino acids (mg/kg)	Amino acids	End of fermentation	Storage Time (wk)			
			3	6	9	12
	Asp (C[1])	5.6 ± 0.7	10.1±0.7 $^{a;x}$	11± 1 $^{a;x}$	13.7±0.6 $^{b;x}$	7.9±0.6 $^{c;x}$
	Asp (S[2])		9±1 $^{a;x}$	9.8±0.7 $^{a,b;x}$	10.3±0.6 $^{a,b;y}$	14±2 $^{b;x}$
	Glu (C)	6.2 ± 0.5	20.4±0.3 $^{a;x}$	22±1 $^{a;x}$	16±2 b$^{;x}$	22.3±1.2 $^{a;x}$
	Glu (S)		19.2±0.3 $^{a;y}$	19.8±0.2 $^{a;x}$	22.3±0.6 $^{b;y}$	23±2 $^{a,b;x}$
	Asn (C)	4 ± 1	5.0±0.6 $^{a;x}$	4.8±0.4 $^{a;x}$	7.1±0.2 $^{b;x}$	4.5±0.4 $^{a;x}$
	Asn (S)		4.4±0.4 $^{a;x}$	4.43±0.08 $^{a,b;x}$	4.5±0.4 $^{a;y}$	4.9±0.2 $^{b;x}$
	Se (C)	2.8 ± 0.2	9.2±0.2 $^{a;x}$	9.0±0.3 $^{a;x}$	12.1±0.6 $^{b;x}$	8.8±0.2 $^{a;x}$
	Se (S)		9.0±0.4 $^{a;x}$	8.7±0.1 $^{a;x}$	8.8±0.2 $^{a;y}$	10±1 $^{a;x}$
	Gln (C)	4.1 ± 0.4	2.0±0.4 $^{a;x}$	2.63±0.03 $^{a,c;x}$	3.8±0.4 $^{b;x}$	2.9±0.3 $^{c;x}$
	Gln (S)		2.28±0.08 $^{a,b;x}$	2.35±0.05 $^{a;y}$	2.9±0.3 $^{b;y}$	3.1±0.8 $^{a,b;x}$
	Thr (C)	2.0 ± 0.2	5.8±0.2 $^{a;x}$	5.7±0.1 $^{a,c;x}$	7.9±0.8 $^{b;x}$	5.5±0.2 $^{a;x}$
	The (S)		5.7±0.2 $^{a;x}$	5.5±0.2 $^{b;x}$	5.5±0.2 $^{a,b;y}$	6.5±0.1 $^{c;y}$
	Arg (C)	6.0 ± 0.8	51±2 $^{a;x}$	51.4±0.7 $^{a;x}$	70±3 $^{b;x}$	50.4±0.8 $^{a;x}$
	Arg (S)		50±2 $^{a;x}$	49±2 $^{a;x}$	50.4±0.9 $^{a;y}$	56±4 $^{a;x}$
	Ala (C)	2.3 ± 0.3	12.4±0.7 $^{a;x}$	14±1 $^{a;x}$	14±1 $^{a;x}$	13±1 $^{a;x}$
	Ala (S)		13±1 $^{a;x}$	13±2 $^{a;x}$	13±1 $^{a;x}$	18±1 $^{b;y}$
	GABA (C)	7.6 ± 0.3	7.2±0.3 $^{a;x}$	6.6±0.4 $^{a,c;x}$	11±1 $^{b;x}$	5.8±0.6 $^{c;x}$
	GABA (S)		6.5±0.4 $^{a;x}$	5.6±0.8 $^{a;x}$	5.8±0.6 $^{a;y}$	35±6 $^{b;y}$
	Tyr (C)	0.60 ± 0.02	3.5±0.1 $^{a;x}$	3.27±0.06 $^{a,b;x}$	4.5±0.9 $^{a,b;x}$	3.2±0.2 $^{b;x}$
	Tyr (S)		3.36±0.05 $^{a;x}$	3.2±0.2 $^{a;x}$	3.2±0.2 $^{a;x}$	3.6±0.1 $^{a;y}$
	Val (C)	1.7 ± 0.6	11.8±0.3 $^{a;x}$	10.9±0.3 $^{a,c;x}$	14.5±0.6 $^{b;x}$	10.8±0.3 $^{c;x}$
	Val (S)		11.6±0.4 $^{a;x}$	10.8±0.1 $^{b;x}$	10.8±0.3 $^{a,b;y}$	13±1 $^{a;y}$
	Met (C)	< LOD	3.45±0.09 $^{a;x}$	3.44±0.08 $^{a;x}$	4.8±0.2 $^{b;x}$	3.4±0.1 $^{a;x}$
	Met (S)		3.52±0.05 $^{a;x}$	3.4±0.1 $^{a;x}$	3.4±0.1 $^{a;y}$	3.9±0.4 $^{a;x}$
	Phe (C)	< LOD	7.8±0.2 $^{a;x}$	8.7±0.1 $^{b;x}$	8.7±0.9 $^{a,b;x}$	8.6±0.2 $^{b;x}$
	Phe (S)		8.2±0.2 $^{a,b;x}$	8.4±0.5 $^{a,b;x}$	8.6±0.2 $^{a;x}$	8.0±0.2 $^{b;y}$
	Ile (C)	0.7 ± 0.1	8.6±0.4 $^{a;x}$	7.8±0.2 $^{b;x}$	9.4±0.3 $^{a;x}$	7.8±0.3 $^{b;x}$
	Ile (S)		8.1±0.2 $^{a;x}$	7.8±0.3 $^{b;x}$	7.8±0.3 $^{a,b,c;y}$	9.1±0.3 $^{c;y}$
	Leu (C)	0.4 ± 0.02	12.9±0.3 $^{a;x}$	12.9±0.2 $^{a;x}$	11±1 $^{a;x}$	13.0±0.5 $^{a;x}$
	Leu (S)		12.4±0.4 $^{a,b;x}$	12.4±0.4 $^{a,b;x}$	13.0±0.5 $^{a;x}$	12.0±0.6 $^{b;x}$

[1] C: Control, no cryoprotectant;

[2] S: Sorbitol, 1 %.

Values are expressed as the mean ± standard deviation of independent replicate experiments.

For a given amino acid and cryoprotectant, different letters (a, b, and c) indicate significant differences ($p \leq 0.05$) over time, using Paired-samples T-tests.

For a given amino acid and time, different letters (x and y) indicate significant differences ($p \leq 0.05$) between cryoprotectants, using independent T-student tests.

4.8. *IN VITRO* GASTROINTESTINAL DIGESTION SIMULATION AND GUT MICROBIOTA IMPACT

4.8.1. *In vitro* simulation of gastrointestinal digestion

4.8.1.1. Simulation of the digestion in the gastrointestinal tract

The microbiological results concerning the simulation of the GI digestion are presented in Figure 4.22. It is possible to observe that while in the free form, namely, not embedded in any matrix, both *L. plantarum* 299v and *W. confusa* 2LABPT05 decreased ca. 0.5 log cycles during passage through the GI simulated conditions of both gastric and intestinal phases. It is well recognised that *L. plantarum* 299v is able to survive to the passage through the gastrointestinal tract (GRAS Notification for Lactobacillus Plantarum Strain 299v, 2016; Johansson et al., 1998). Also, some *Weissella* strains (*W. confusa* or *W. cibaria*) have shown higher survival rates in the presence of bile salts (0.3 %) and in an acidic environment (pH = 3), showing promising capability to successfully pass through the GI tract (Patel et al., 2014). Nonetheless, when inoculated in the fermented YLB, results were slightly different. It seems that the cereal matrix offered a protective environment for both strains, enhancing viable cell numbers, particularly in the first hour of gastric phase (t=1 h) ($p < 0.05$). This shows the important role of the cereal for the bacterial delivery (Kalui et al., 2010). As shown by Patel and colleagues (2004), the physicochemical characteristics and pH of the food carrier has a significant impact on the strains viability.

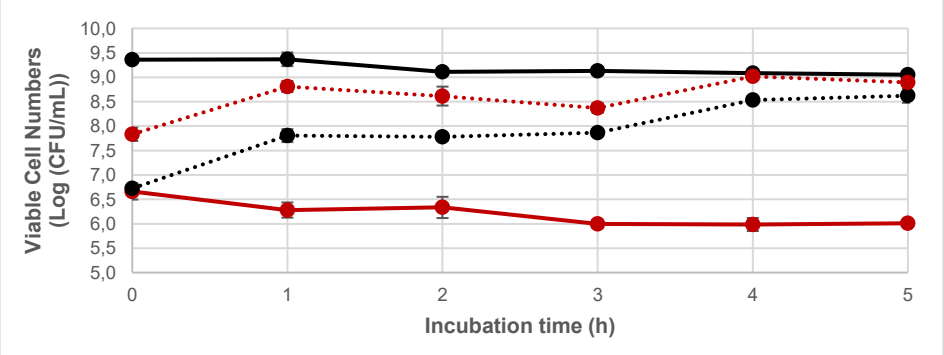


Figure 4.22 Evolution of viability of *W. confusa* 2LABPT05 (red lines) and *L. plantarum* 299v (black lines). Full lines are relative to the individual behaviour of the strains in deionised water, whereas dashed lines are relative to the behaviour of the strains inoculated in combination in the finger millet slurry, during the simulation of the digestion in the gastrointestinal tract process, at 37 °C, 200 rpm, over 5 h, in an orbital incubator. Error bars represent the standard deviation of independent replicate experiments.

4.8.2. Faecal fermentations for studying gut microbiota impact

4.8.2.1. Subjects

A total of five healthy subjects were recruited as faeces samples donors, of whom two were female and three were males, with ages between 27 and 33 years-old.

4.8.2.2. Faecal fermentations conditions and procedure

After the simulation of the small intestinal absorption, through dialysis, the digested fermented YLB (DF-YLB) was freeze-dried and used for the faecal culture fermentations, established for 24 h, in which the DF-YLB was placed in contact with the human faecal samples, to study the effect upon the human microbiota modulation. Aliquots from different locations on the stools were targeted, since the taxa concentration might varies from the peripheries to the centre of the stool specimen (Thomas et al., 2015).

4.8.3. Bacterial enumeration and organic acids production

4.8.3.1. Bacterial enumeration using real-time qPCR

After DNA extraction and equilibration to the final concentration of 20 ng/μL, real-time qPCR was used to determine the number of copies of the 16S rRNA gene in the DF-YLB and controls. The evolution of the number of copies of the 16S rRNA gene (in logarithmic scale), in the DF-YLB and controls, during faecal fermentations, relative to the target bacterial groups are presented in Figure 4.23 and Table 4.34.

There is a lack of data about the impact of the consumption of fermented cereals on the gut microbiota (Dimidi et al., 2019; Tsafrakidou et al., 2020), being more common the study of native cereals, in general, or of some compounds generated or augmented during fermentation, such as EPS or polyphenols (Tsafrakidou et al., 2020). The potential gut microbiota modulation, with increased levels of *Bifidobacterium*, *Lactobacillus* and *Bacteroides* and reduction of *Clostridium*, by the consumption of whole grain oat has been demonstrated by *in vitro* fermentation models and animal studies (Kristek et al., 2019). More recently, the prebiotic effect of oat bran was also observed, but not of its bioactive compounds separately, β-glucans or polyphenols, suggesting that this effect might come from the harmonious combination of all oat compounds and cereal matrix; such synergy between compounds has also been explored for other food matrices (Kristek et al., 2019).

On the other hand, the review of Dimidi *et al*. (2019) summarizes the main recent publications on this topic focused on fermented foods, such as Kefir, Kombucha, Tempeh, Natto, Kimchi and sourdough bread, concluding that Kefir is the most investigated fermented food in what concerns gut microbiota impact and it has demonstrated good results in promoting the growth of healthy bacterial communities, in *H. pylori* erradication as well as benefits for lactose malabsorption patients. Natto and Kimchi have also revealed increased levels of *Bifidobacterium* or *Lactobacillus* in *in vivo* studies, but no evidence exists for Kombucha and some controversial results were found for sourdough bread.

A recent study explored the impact of the consumption of fermented foods, with focus on plants with modifications in the human gut microbiome, using as sample a subset of the American Gut Project cohort, finding an overall difference in the gut microbial communities between consumers and non-consumers, and that consumers had higher concentrations of bacterial groups more associated with fermented foods (Taylor et al., 2020).

Bifidobacterium spp.

Bifidobacterium spp. belong to the Actinobacteria phylum, one of the five most abundant phyla in the human gut microbiota. Concerning the present results, it was possible to observe a positive impact of the DF-YLB on its number of copies of the 16S rRNA gene, after 6 h of fermentation ($p < 0.05$). However, this effect is not maintained until the end of the process, due to the high variability resultant from one of the five donors, whose faeces showed a different behaviour during the 24 h. Once this donor would be excluded, a consistent effect would have been observed over time. Nevertheless, in terms of statistics that donor was not considered an outlier, reason why it was included in the final sample. Therefore, it seems that DF-YLB has a potential bifidogenic effect. This trend was partially followed by FOS, which had also shown a significant increase during the first 6 h, and the positive impact was maintained over the fermentation time. Nevertheless, the increment during the first 6 h was more impactful for the DF-YLB, but no statistical differences were observed. For the negative control no differences were observed over time. When comparing the increment in the number of copies of the 16S rRNA gene between the DF-YLB and the controls, DF-YLB and FOS had similar increments over time (with the exception at 24 h), and higher than the negative control, however not statistically different. FOS is a recognisable prebiotic (Gibson & Roberfroid, 1995) that stimulates the growth of *Bifidobacterium* and this fructooligosaccharide has been used as a positive control in the same type of *in vitro* studies, also showing a positive impact on the target bacterial group (Campos et al., 2020; Mota de Carvalho et al., 2019; Ribeiro et al., 2020). The presence of EPS in the DF-YLB might be behind this bifidogenic effect, as microbial EPS have been hypothesised as having important prebiotic effect, depending on their molecular mass and type of bonds (Amaretti et al., 2020; Lynch, Zannini, et al., 2018; Olano-Martin et al., 2000). It is expected that EPS predominantly composed by α-1,6-glycosidic bonds would escape the digestion by the intestinal lumen enzymes, thereby reaching the colon partly intact to be metabolized by the microbiota present (Olano-Martin et al., 2000).

Lactobacillus spp.

The baseline presence of *Lactobacillus* spp. in the sampled faeces was lower compared to other bacterial groups. *Lactobacillus* belong to the phyla Firmicutes, one of the major resident phyla in the gut microbiota, however the genus represents only 0.3 % of total bacteria in the colon and 6 % in the duodenum (Heeney et al., 2018). Nevertheless, *Lactobacillus* even in smaller concentrations are correlated with the absence of several diseases (Heeney et al., 2018). When in the presence of the DF-YLB, despite the fact that no increase of the genus was observed over time, significantly

higher concentrations compared to the positive and negative controls were noticed from the beginning and over 12 h of fermentation ($p < 0.05$). This shows the potentiality of the DF-YLB as carrier of *Lactobacillus* into the gut colonization. In consequence, the increased number of lactobacilli in the gut microbiota may result in potential health benefits, since they are health promoting bacteria. Regarding the positive control, an increase in those numbers was observed however, there was no statistically significant differences over fermentation, given the high inter-individual variability. On the contrary, in the negative control no changes were observed over time. In the study of Amaretti *et al*. (2020), in which the prebiotic effect of low-molecular mass EPS was evaluated and compared with inulin, the genus *Lactobacillus* did not reach the limit of detection, either for the EPS or inulin, suggesting that both substrates do not support their growth. Also, Connolly *et al*. (2012), using an *in vitro* model, tested raw/toasted/partially toasted wheat flakes, FOS and cellulose and only raw wheat promoted the growth of this genus.

Clostridium leptum subgroup and *Faecalibacterium prausnitzii*

Clostridium leptum subgroup, also known as *Clostridium* cluster IV, represents between 16 - 25 % of the faecal microbiota in adult humans, and it includes *Faecalibacterium prausnitzii* and certain species of *Eubacterium* and *Ruminococcus* (Kabeerdoss et al., 2013). In this study, the DF-YLB and the positive control have induced a decrease in the numbers of *C. leptum* subgroup and *F. prausnitzii*, however those differences were not considered statistically significant. When no carbon source was added (negative control) the results followed the same trend. In the study of Mao *et al*. (2015), that evaluated the effects of FOS on gut bacteria in mice, FOS in low doses (5 %), similar to the present study (2 %), only resulted in higher concentrations of *Clostridium* after 4 wk of feeding, nevertheless those increases were not significant, and their abundance remained low. Similarly to the present results, in the work of Amaretti *et al*. (2020), *F. prausnitzii* was not positively affected by inulin, over 24-h fermentation. On the contrary, the work of Ramirez-Farias *et al*. (2009), revealed the increase of *F.* p*rausnitzii* on adult faecal samples, after ingestion of inulin (10 g/d) for a 15 d period. These bacteria have been associated with inflammatory conditions, namely the inflammatory bowel diseases, with Crohn's disease's and ulcerative colitis' patients demonstrating *C. leptum* group species' numbers reduced, compared to healthy controls (Kabeerdoss et al., 2013). In this perspective, it would have been desired that the DF-YLB had had a positive impact on *Clostridium leptum* subgroup numbers.

Roseburia spp.

For this bacterial group, belonging to the *Clostridium* cluster XIVa, a decrease over time was observed in the presence of the DF-YLB ($p < 0.05$), similarly to the positive control (FOS), although for the latter no statistically significant differences were observed until 12 h of fermentation. For the negative control, despite the observed reduced levels, no differences were observed in statistical terms ($p > 0.05$). On the contrary, the study of Vanegas and colleagues observed a positive impact of the consumption of whole grain wheat compared to the control, *id est* refined wheat, on the *Roseburia* abundance after a follow-up of 8 wk (Vanegas et al., 2017). Also, in the study of Mota de

Carvalho *et al*. (2018), in which the *in vitro* assessment of the impact of an edible insect on the gut microbiota was carried out, *Roseburia's* number of copies of the 16S rRNA gene showed an increase while fermenting FOS over 24 h, and experienced a decrease after that period, and a decrease during the fermentation of the digested *Tenebrio molitor* insect flour (Mota de Carvalho et al., 2019). *Roseburia* is recognized for using as energy source resistant starch (Umu et al., 2017), mostly of type 3, and also it was found to utilizes mucin (Lordan et al., 2020). This might suggest that *Roseburia* bacteria have preferences for the metabolism of higher molecular mass compounds.

Bacteroides spp.

Bacteroides are saccharolytic fermenters, known for utilizing complex carbohydrates (Amaretti et al., 2020), in which genus healthy and harmful bacteria are found (Gibson & Roberfroid, 1995). In the present study, a decrease of the number of copies of the 16S rRNA gene over time was observed in the presence of the DF-YLB, however it was not considered statistically different ($p > 0.05$). Nevertheless, in the presence of the DF-YLB absolute values were significantly lower when compared to both controls, suggesting a bacterial competition may be present between the endogenous LAB strains and the *Bacteroides* genus bacteria. Connolly and collaborators (2012) observed an increase in *Bacteroides* spp. promoted by the toasted wheat and, despite the higher values obtained when fermenting FOS, no significant differences were considered. On the contrary, in other studies, fermenting FOS resulted in lower *Bacteroides*' number of 16S gene copies (Campos et al., 2020; Mota de Carvalho et al., 2019; Ribeiro et al., 2020). Some studies revealed that when in the presence of inulin no differences over time are observed (Amaretti et al., 2020; Ramirez-Farias et al., 2009) while in the presence of the dextran, synthetized by *W*. *confusa*, *Bacteroides* spp. increased its numbers, a trend which was not observed in the present study.

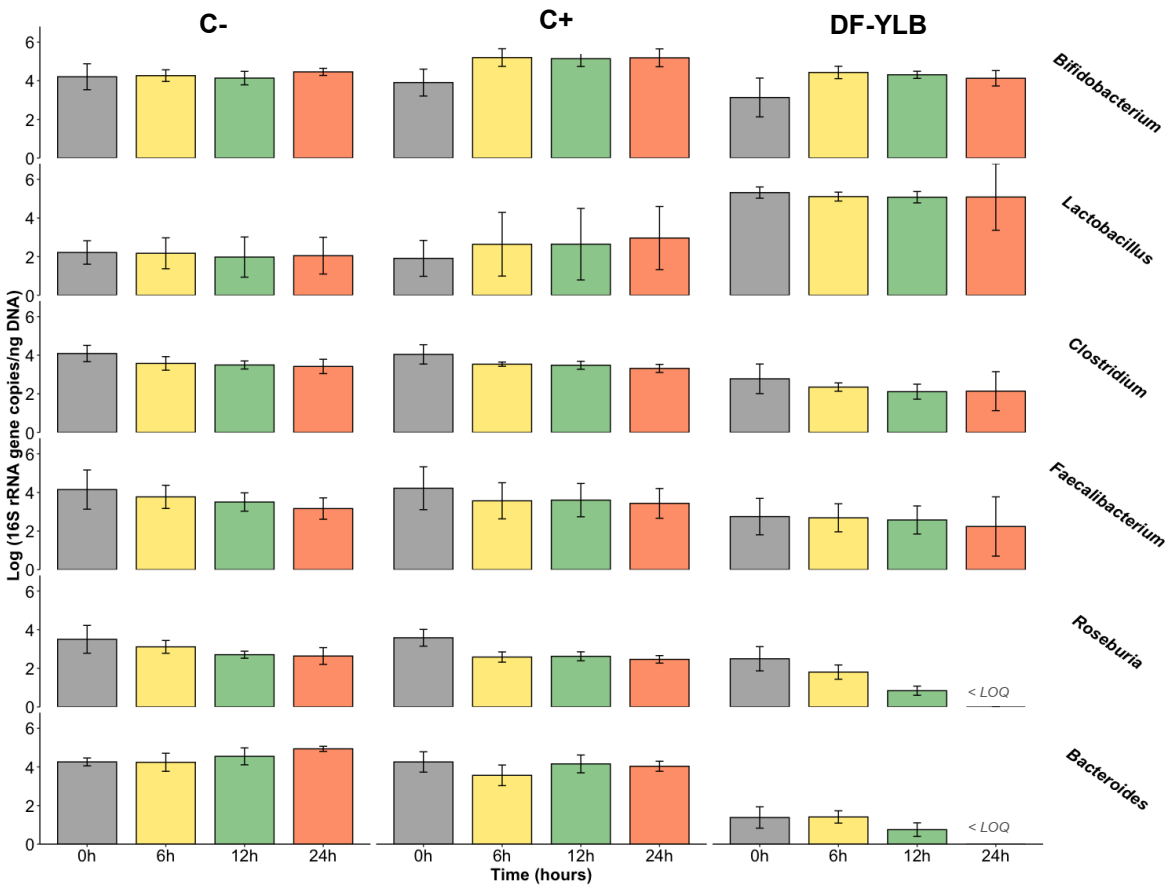


Figure 4.23 Quantification of the bacterial groups (expressed as log (16S rRNA gene copies/ng of DNA); mean ± standard deviation) detected by qPCR in faecal samples (negative control (C-), positive control (C+; FOS), digested fermented YLB (DF-YLB)) of five donors. LOQ: Limit of quantification.

Table 4.34 Quantification of the bacterial groups (expressed as log (16S rRNA gene copies/ng of DNA); mean ± standard deviation) detected by qPCR in faecal samples (negative control (C-), positive control (C+; FOS), digested F-YLB (DF-YLB)) of five donors.

		Log (16S rRNA gene copies/ng of DNA)							
		C-			C+ (FOS)			DF-YLB	
		Absolute concentration		Increment (+) /Reduction (-)	Absolute concentration		Increment (+) /Reduction (-)	Absolute concentration		Increment (+) /Reduction (-)
***Bifidobacterium* spp.**	0h	4.3 ± 0.7	a,x		3.9 ± 0.7	a,x,y		3.1 ± 0.9	a,y	
	6h	4.3 ± 0.3	a,x	+1.4	5.2 ± 0.5	b,x	+33.1	4.2 ± 0.3	b,x	+41.3
	12h	4.1 ± 0.3	a,x	-1.6	5.1 ± 0.4	b,x	+31.7	4.3 ± 0.2	b,x	+37.5
	24h	4.5 ± 0.2	a,x,y	+6.0	5.2 ± 0.5	b,x	+32.8	4.1 ± 0.2	a,b,y	+31.7
***Lactobacillus* spp.**	0h	2.2 ± 0.6	a,x		1.9 ± 0.9	a,x		5.3 ± 0.2	a,y	
	6h	2.2 ± 0.8	a,x	-1.9	2.6 ± 1.6	a,x	+38.2	5.1 ± 0.2	a,y	-3.9
	12h	2.0 ± 1.0	a,x	-10.7	2.6 ± 1.6	a,x	+38.4	5.1 ± 0.3	a,y	-4.5
	24h	2. 1 ± 0.9	a,x	-7.4	3.0 ± 1.6	a,x,y	+55.1	5.1 ± 0.2	a,y	-4.3
***Clostridium leptum* subgroup**	0h	4.1 ± 0.4	a,x		4.0 ± 0.5	a,x		2.8 ± 0.7	b,y	
	6h	3.6 ± 0.3	a,x	-12.5	3.5 ± 0.1	a,x	-12.5	2.4 ± 0.2	b,y	-15.4
	12h	3.5 ± 0.2	a,x	-14.5	3.5 ± 0.2	a,x	-13.9	2.1 ± 0.4	b,y	-24.0
	24h	3.4 ± 0.4	a,x	-16.4	3.3 ± 0.2	a,x	-18.0	2.1 ± 0.5	b,y	-23.0
***Roseburia* spp.**	0h	3.4 ± 0.7	a,x		3.5 ± 0.4	a,x		2.3 ± 0.6	a,y	
	6h	2.9 ± 0.4	a,x	-11.1	2.6 ± 0.2	a,b,x,y	-27.9	1.7 ± 0.4	a,b,y	-27.7
	12h	2.5 ± 0.4	a,x	-22,8	2.6 ± 0.2	a,b,x	-26.8	0.7 ± 0.5	b,y	-66.3
	24h	2.4 ± 0.6	a,x	-24.7	2.4 ± 0.2	b,x	-31.2	< LOQ		n.a.[1]
Faecalibacterium prausnitzii	0h	4.1 ± 1.0	a,x		4.2 ± 1.1	a,x		2.8 ± 1.0	a,x	n.a.
	6h	3.8 ± 0.6	a,x	-9.1	3.6 ± 0.9	a,x	-15.5.	2.7 ± 0.7	a,x	-2.3
	12h	3.5 ± 0.5	a,x	-15.5	3.6 ± 0.9	a,x	-14.6	2.6 ± 0.7	a,x	-6.4
	24h	3.2 ± 0.5	a,x	-23.7	3.4 ± 0.7	a,x	-18.6	2.2 ± 0.9	a,x	-18.7
***Bacteroides* spp.**	0h	3.9 ± 0.7	a,x		4.1 ± 0.6	a,x		1.3 ± 0.6	b,y	
	6h	3.9 ± 0.8	a,x	-0.5	3.4 ± 0.6	a,x	-16.3	1.3 ± 0.3	b,y	2.0
	12h	4.3 ± 0.6	a,x	+6.8	3.9 ± 0.8	a,x	-2.4	0.5 ± 0.3	b,y	-45.7
	24h	4.7 ± 0.6	a,x	+15.9	3.7 ± 0.8	a,x	-5.2	< LOQ		n.a.

[1] n.a.: not applicable.

For each bacterial group, for a given sample, different letters (a, b, and c) indicate significant differences between times ($p \leq 0.05$), and for a given time, different letters (x, y, and z) indicate significant differences between samples ($p \leq 0.05$), using one-way ANOVA. The increment/reduction values are expressed in %.

4.8.3.3. Acidification and SCFA production

The previous bacterial enumeration results were supported by the acidification observed for the DF-YLB's and FOS's fermentation, along with the sugars consumption and the subsequent production of organic acids (Table 4.35). The major organic acid produced was lactic acid, followed by the SCFA acetic, propionic and butyric acids. Succinic acid was also considerably produced.

Production profiles were similar for both positive control (FOS) and DF-YLB by 12-h and 24 - h fermentation ($p > 0.05$).

Lactic acid, mainly produced by LAB, despite not being considered a SCFA, it has several benefits for the host. Its major production over time ($p < 0.05$) is justified by the higher levels observed for the *Lactobacillus* genus. For the DF-YLB a progressive increase ($p < 0.05$) until 12 h of fermentation was observed, while for FOS, the more impactful period was the first 6 h ($p < 0.05$), remaining constant until the end of fermentation ($p > 0.05$). Lactate has been associated to several bioactive properties, besides the well-recognized organoleptic and antimicrobial properties, among which can be highlighted the capacity to reduce the innate response activation and the proinflammatory cytokine levels and also the ability to reinforce the gut barrier function (Garrote et al., 2015).

In both substrates, FOS and the DF-YLB, the production of acetate was observed over time. However, during the first 6 h, the fermentation of FOS resulted in almost twice as high as that observed for the DF-YLB, nevertheless acetate concentrations after 24 h was considered similar between both. The bacterial groups mostly responsible for the production of acetic acid are Bacteroidetes, *Bifidobacteria* and also Firmicutes (Lordan et al., 2020). Acetate can be produced either via acetyl Co-A or H_2 and CO_2 or formate, using hexoses or pentoses as source of energy (Lordan et al., 2020). This SCFA has been associated to weight management, however controversial results, depending on the species or the GI location where the acetate is released, have been achieved (Hernández et al., 2019). Other effects that derive from acetate production acts on appetite regulation, adipose tissue lipolysis regulation and also in blood pressure regulation (Chambers et al., 2018). It has also been demonstrated that acetate promotes plasma membrane re-localization of MCT-1 (a SCFA transporter), triggers changes in the glucose metabolism and induces apoptotic cell death in colorectal cancer cells, involving lysosomal membrane permeabilization and the release of Cathepsin D, associated with mitochondria dysfunction (Gomes et al., 2020).

Once acetate is produced, this SCFA is also available for cross-feeding interactions, resulting in the production of butyrate, via butyryl-CoA:acetate CoA-transferase, by butyrate-producers colonic bacteria, such as bacteria belonging to the *Clostridium leptum* subgroup, with *Faecalibacterium* being one of the most relevant (Kabeerdoss et al., 2013; Lordan et al., 2020; Louis & Flint, 2009) . Nevertheless, butyrate is generated as well through the formation of butyryl-P from carbohydrates (Lordan et al., 2020). Butyrate was the SCFA found in lowest concentrations in the present study. Such trend was expected since the growth of the butyrate-producing bacterial groups was not promoted neither by FOS nor the DF-YLB. Another reason that might justify the lower production of butyrate is the low pH reached after 6 h of fermentation, since butyrogenic bacteria are sensitive to this pH (Baxter et al., 2019; Lordan et al., 2020). In the negative control, in which not so low pH values were obtained, butyrate was produced in higher concentrations. Butyrate has revealed an important role in the immune system, beneficial effects in the GI tract, namely the epithelial barrier, colorectal cancer protective role, and also it has been associated to an increase of satiety (Baxter et al., 2019; Chambers et al., 2018).

Propionate is another SCFA with interesting health promotion properties. In the present study the concentrations of propionate increased in the presence of both, the DF-YLB and the positive control. The main bacterial groups responsible for its production are Bacteroidetes, but also Firmicutes and *Clostridium* cluster IV, and different routes are used for propionate formation. Propionate is mainly generated via the succinate pathway, however, the acrylate pathway, in which lactate is involved in, and the propanediol pathway, in which deoxyhexose sugars are utilized, are alternatives (Lordan et al., 2020). The positive health effects associated to this SCFA are linked to the reduction of food intake and the prevention of long-term weight gain, due to, on the one hand, the modulation of gut hormones release, and, on the other hand, the direct neural gut-brain signalling via free-fatty acids receptors (Chambers et al., 2018). Moreover it has been associated to the improvement of the pancreatic function and the reduction of hepatic lipid storage (Chambers et al., 2018; De Vadder et al., 2016).

Succinate is not a SCFA, but it can be produced during microbial fermentation in the gut. Succinic acid is usually generated by the reversal of partial tricarboxylic acid cycle reactions, and this cycle is the main pathway to produce the metabolic intermediate succinate (Chambers et al., 2018). Succinate is traditionally associated to a less positive effect on the human host, specifically acting as pro-inflammatory onto the regulation of local stress, tissue damage and immune response (Fernández-Veledo & Vendrell, 2019). However, this SCFA has demonstrated an interesting performance in the regulation of intestinal gluconeogenesis and thermogenesis and also a key role in obesity-associated inflammation (De Vadder et al., 2016; Fernández-Veledo & Vendrell, 2019). Considering the present results, its concentrations increased after 6 h of fermentation in the presence of FOS, but not with DF-YLB, given the initial quantities present. Moreover, in the study of Amaretti *et al*. (2020), succinic acid was produced over the first 6 h and decreased over the remaining period, until 24 h of fermentation (Amaretti et al., 2020).

In the negative control, an increase in acetate (only significantly different at 24 h), butyrate and propionate (only significantly different after 12 h) production was observed. Propionate increase might be due to the high presence of *Bacteroides*, which contributes to most of the propionic acid produced in the colon (Louis & Flint, 2009). The observed increase in butyrate and acetate levels was not expected given the lack of growth of the main producing strains of the bacterial groups, *Clostridium leptum* subgroup and Bifidobacterium, respectively.

Gut microbiota modulation is transient, given its primarily influence by dietary intake and non-digestible carbohydrates availability to the microbiota, which will affect the balance between saccharolytic fermentation and proteolytic fermentation (Chambers et al., 2018). Consequently, people with different dietary patterns, closely linked to living conditions, place of residence, financial availability and purchasing capacity, are expected to have different gut microbiota composition profiles. The study of De Fillipo and colleagues (2010) compared faecal microbiota of European children, with a westernized diet, and that of rural children from Burkina Faso, with a diet rich in fibre, and low in fat and protein, observing that African children had an enrichment in Bacteroidetes, *Prevotela*, Xylanibacter and general SCFA, while the European showed higher representativeness of Firmicutes and Enterobacteriaceae (Shigella and Escherichia) (De Filippo et al., 2010). Besides

individual diet, gut microbiota is influenced by other intra-individual factors, such as the infant transitions (birth- and diet-related), age, presence of diseases, and consumption of antibiotics (Rinninella et al., 2019). On the other hand, inter-individual factors such as ethnicity, gender, body mass index, dietary habits, and cultural habits are also motive for gut microbiota differences (Rinninella et al., 2019). In the present study, factors such as age, ethnicity, presence of diseases or the consumption of antibiotics did not influence the composition profiles, given the transversality between donors. On the other hand, the factors that apparently may have affected most the different results between donors were gender, body mass index (not shown), and dietary habits; however no specific influence should be inferred, given the small sample size, one of the limitations of the present research work. Lack of stirring during fermentation and also of pH control did not allow a more realistic recreation of the intestinal conditions. Nonetheless, *in vitro* studies like the one reported herein are important and useful for the early development of potentially prebiotic food products, towards an ultimate validation of prebiotic activity which can only be concluded by *in vivo* feeding studies.

Table 4.35 Concentration (mean in g/kg ± standard deviation) of consumed sugars and produced organic acids during the fermentation over 24 hours in faecal samples (negative control (C-), positive control (C+; FOS), digested F-YLB (DF-YLB)) of five donors. The presented values are the mean of the five donors, considering one injection for each sample.

	Time (h)	C-		C+ (FOS)		DF-YLB	
pH	0	6.947 ± 0.008	a,x	6.94 ± 0.02	ax	6.61 ± 0.02	ax
	6	6.6 ± 0.1	a,x	4.5 ± 0.3	b,y	4.8 ± 0.4	b,y
	12	6.58 ± 0.07	a,x	4.2 ± 0.3	b,y	4.6 ± 0.3	b,y
	24	6.6 ± 0.1	a,x	4.0 ± 0.3	b,y	4.5 ± 0.3	b,z
		Sugars and organic acids (g/kg)					
Sucrose	0	< LOD		< LOD		3.1 ± 0.1	a
	6	< LOD		< LOD		2.1 ± 0.3	b
	12	< LOD		< LOD		2.1 ± 0.5	b
	24	< LOD		< LOD		1.9 ± 0.3	b
Glucose	0	< LOD		< LOD		2.9 ± 0.1	a
	6	< LOD		< LOD		2.3 ± 0.3	b
	12	< LOD		< LOD		2.1 ± 0.3	b
	24	< LOD		< LOD		2.0 ± 1.4	b
Fructose	0	< LOD		2.1 ± 0.3	a,x	1.8 ± 0.2	a,x
	6	< LOD		2.4 ± 1.0	a,x	1.3 ± 0.5	a,x
	12	< LOD		2.4 ± 0.9	a,x	1.0 ± 0.4	a,y
	24	< LOD		2.6 ± 1.2	a,x	1.0 ± 0.3	a,y
Succinic acid	0	0.20 ± 0.04	a,x	< LOD		0.7 ± 0.1	a,y
	6	0.17 ± 0.03	a,x	0.6 ± 0.3	a,y	0.8 ± 0.1	a,y
	12	0.13 ± 0.04	a,x	0.6 ± 0.4	a,y	0.8 ± 0.1	a,y
	24	< LOD		0.6 ± 0.2	a,x	0.8 ± 0.2	a,x
Lactic acid	0	< LOD		< LOD		0.9 ± 0.1	a
	6	< LOD		1.8 ± 0.6	a,x	1.6 ± 0.3	a,x
	12	< LOD		2.0 ± 0.9	a,x	2.3 ± 0.8	b,x
	24	< LOD		2.2 ± 0.9	a,x	2.3 ± 0.7	b,x
Acetic acid	0	0.0485 ± 0.007	a,x	< LOD	a,x	< LOD	a,x
	6	0.17 ± 0.06	a,b,x	1.0 ± 0.5	b,y	0.59 ± 0.07	b,x,y
	12	0.40 ± 0.07	a,b,x	0.9 ± 0.3	b,x	0.8 ± 0.2	b,x
	24	0.59 ± 0.06	b,x	1.0 ± 0.3	b,x	0..8 ± 0.2	b,x
Propionic acid	0	0.04 ± 0.03	a	< LOD		< LOD	
	6	0.16 ± 0.03	a,x	0.3 ± 0.1	a,y	0.4 ± 0.2	a,z
	12	0.25 ± 0.05	b,x	0.27 ± 0.09	a,x	0.4 ± 0.1	a,x
	24	0.37 ± 0.05	b,x	0.26 ± 0.07	a,x	0.4 ± 0.1	a,x
Butyric acid	0	< LOD		< LOD		< LOD	
	6	0.16 ± 0.07	a,x	0.3 ± 0.1	a,y	0.19 ± 0.07	a,y
	12	0.29 ± 0.09	a,b,x	0.2 ± 0.1	a,x	0.20± 0.05	a,x
	24	0.42 ± 0.09	b,x	0.2 ± 0.1	a,y	0.20 ± 0.06	a,y

For each bacterial group, for a given sample, different letters (a, b, and c) indicate significant differences between times ($p \leq 0.05$), and for a given time, different letters (x, y, and z) indicate significant differences between samples ($p \leq 0.05$), using one-way ANOVA. LOD: Limit of detection (Sucrose: 0.05 g/L; Glucose: 0.1 g/L; Fructose: 0.1 g/L; Succinic acid: 0.05 g/L; Lactic acid: 0.05 g/L; Acetic acid: 0.05 g/L; Propionic acid: 0.01 g/L; Butyric acid: 0.01 g/L.)

CHAPTER 5.

GENERAL CONCLUSIONS

"*It's the time you spent on your rose that makes your rose so important.*"

Antoine Saint-Exupéry, 1943

The two-fold research Europe-Africa bridge strategy proposed by this Ph.D. thesis has been successfully fulfilled and has brought pioneering novelty and increased knowledge to the following areas of research:

- epidemiological studies carried out in African populations, development and validity of research tools to be used in the future;
- fermentation of traditional whole grains (sorghum, pearl and finger millets) with high nutritional value;
- development of new added-value, nutritionally balanced products containing bacterial strains with probiotic and technological potential, EPS-producers, a suitable natural alternative to chemical food additives;
- impact of fermented finger millet-based synbiotic products on gut microbiota modulation.

In the first part of the work, a comprehensive procedure was followed with the purpose of developing a culturally adapted semi-quantitative FFQ. As a result, a 123-food item-FFQ was developed and validated. Although it tended to overestimate intakes, and for that reason, it might not be a precise instrument to measure absolute intakes, this FFQ showed to be, clearly, a valuable tool for ranking individuals according to their nutrient intake. Moreover, according to literature this dietary tool is the only validated questionnaire adapted to the Kenyan adult urban population currently available.

Thereby, the developed FFQ was used to study the dietary intake of urban Kenyan adults, which revealed to be in agreement with WHO/FAO dietary guidelines in what concerns macronutrients' intake ranges. Also, this FFQ allowed to explore the food sources of some nutrients. Nevertheless, this dietary intake survey revealed that the Kenyan diet is very high in sugar and that sugary and salty snacks have an extreme contribution to the total energy intake, carbohydrates, sugars and fats, mainly in women, students and unemployed people. The food group of cereal products was the main contributor to energy and all macronutrients, especially in men, youngsters, and people with lower EI. The contribution of meat and eggs to protein and total fat was much higher than fish, and people with higher levels of education had higher contributions from this food group and dairy to both nutrients. Nevertheless, a considerable part of the protein came from plant sources. The dietary fibre came from different food groups, with a major influence of cereal products for men, people with lower EI and older people, of vegetables, for women, people with a higher education level, and with lower EI and fruits for women and people with higher EI. The information generated in the present study can work as an incentive to the formulation and implementation of nutritional interventional strategies by national authorities, aiming the improvement of the dietary habits and, consequently, the nutritional profile of Kenyan adults, towards the reduction of NCD prevalence. Also, this FFQ is available for future epidemiological studies to accurately rank urban Kenyan adults, according to their dietary intake allowing, for instance, the investigation of associations of diet with risk of disease in urban Kenya.

In the second and third parts of this research work, in relation to the first phase targeting the fermentation of African whole grains, *Lactiplantibacillus plantarum* 299v was revealed as the best

performer in what concerns fermentation of sorghum and finger millet flours. Interesting results were found during fermentation of dry-milled cereals, especially for millet slurry, and during the first 8 h of incubation. These results were important in order to understand which probiotic strain, among the three studied species, had the most impactful fermentative capacity, but also to pin-point the most preferable milling process, aiming further successful development of new cereal-based beverages.

Since the major goal was to identify a starter culture which included a probiotic strain and an EPS-producer strain, the second step was the selection of the latter strain, which would have an important technological role in the fermentation process. Thereby, three different *Weissella* strains isolated from native African spontaneously fermented products, and previously screened for EPS production, were studied for the fermentative capacity of sorghum and pearl millet. The studied *Weissella* strains did not reveal the capacity to ferment sorghum slurries. On the contrary, pearl millet slurries fermentation was successfully carried out, either in the presence or absence of sucrose, with clear induced biochemical changes in the cereal matrices. In the presence of sucrose, the increase in viscosity, consequence of EPS production, was notoriously more evident than in the water-based formulation, perceptible by the slimy texture obtained after fermentation, especially for the native strains from Burkina Faso (2LABPT05 and 32LABPT05). Results from this part of the research work allowed to conclude that the indigenous strains revealed promising properties with potential benefits for health and for improving the nutritional and sensory profiles of the inherent cereal matrix, enhancing the possibility to be used as starter cultures for the development of novel fermented functional and nutritious cereal-based products.

The next step was dedicated to the study of combined cultures using the *L. plantarum* 299v and the three *Weissella* strains towards the selection of the best performer bacterial consortium and the most interesting cereal matrix. From these results, interesting combinations between the probiotic strain *L. plantarum* 299v and Burkinabé *Weissella* strains, namely 2LABPT05 and 32LABPT05, were found. No statistically significant differences were observed for the analysed parameters between both bacterial consortia. In what concerns the fermenting cereal, results showed that millets revealed a higher prebiotic potential since they promoted a more impactful growth of both types of strains. In terms of nutritional properties, finger millet has a richer-fibre and lower-fat profile than pearl millet. Besides, this cereal has a higher content in total phenolic compounds and also in some important micronutrients such as calcium, potassium, and thiamine. Based on these rationale, the selected strain combination to be used as starter culture for product development, was composed by the *W. confusa* 2LABPT05 strain and the probiotic *L. plantarum* 299v, and the selected whole grain was finger millet.

This synbiotic consortium allowed the successful development of a novel functional finger millet-based product, characterized not only by high viable numbers of probiotic microorganisms totally within the required standards (> 10^7 CFU/g), which were maintained throughout the storage period (both for the liquid and the powdered versions of the product), but also achieving an interesting slimy texture, naturally improved by the *in situ* production of EPS. The resulting product had a very interesting nutritional composition, including high-fibre, low-fat, high phenolic compounds content, increased protein digestibility, higher content of some amino acids, and also significantly higher

content in dextran, than the native unfermented flour. Moreover, the YLB was organoleptically acceptable, either *per si* or when incorporated in a dairy matrix. Furthermore, the final product not only revealed antidiabetic properties, given its demonstrated α-glucosidase inhibitory activity, but also had the capacity to protect probiotic co-cultures during passage through a gastrointestinal environment and promote *Bifidobacterium* growth and the increase of the lactobacilli numbers in the *in vitro* gut microbiota modulation assay, which upholds its potential positive effect on the probiotic microbiota and the continuous production of beneficial organic acids. A dried version of the YLB was also explored, envisaging longer shelf-life and facilitated transport (particularly, but not only, if African populations are targeted). Freeze-drying of YLB using sorbitol at the concentration of 1 % as cryoprotectant was the most effective drying treatment, based on the viability of both strains and the impact on the product physical properties. The freeze-dried version of the YLB behaved as planned having shown important stability at room temperature over a 3-month period. Additional characteristics to the envisaged longer shelf-life, and facilitated transport are the possibility to target different international markets, and enable more versatility in terms of consumption (added to water, yoghurt, dessert, fruit puree, or ice-cream) and new product development. Concluding, there is an apparent beneficial impact resulting from the consumption of this innovative functional fermented product in what concerns the nutrient intake status, the blood glucose levels and the positive modulation of the human gut microbiota.

CHAPTER 6.

FUTURE PROSPECTS

"*Nothing in life is to be feared, it is only to be understood.*"

Marie Skłodowska-Curie, 1951

The research studies carried out along this Ph.D. project and compiled in the present thesis covered a wide range of topics within different scientific areas, and contributed to advance science, pave new perspectives and innovations and open up exciting avenues of research. Hence future work should be performed in order to address several challenges that stemmed from the Ph.D. project activities and help clarify and support the achieved results.

Concerning Part I, Burkina Faso, the second African country initially involved in the ERAfrica project, should also be considered for the dietary intake study in order to assess its nutrient intake status. Burkina Faso is a poor country, with an HDI of 0.434, also facing a double burden of malnutrition, and this study would have a great impact on its population, bridging the scarcity of epidemiology studies, which could be used for the establishment of pertinent associations between diet and risk of disease in this country.

Relative to the application of the FFQ, an improvement of the standard portions' perception should be addressed; a possible good alternative will be to assess its application together with a photographic album with portions sizes for the most critical items. Regarding the exploration of the dietary data, it would be useful to do a deeper analysis in what concerns the contributions of different foods, and not only the food groups. This would allow the identification of more important or more critical food items for a specific nutrient, and thereby taking conclusions about the diversity within each food group. Moreover, the exploration of whole grain food items' consumption would increase the knowledge about the intake of this type of items towards the establishment of strategies in the food sector.

Regarding Part II, the study of more probiotic strains (other strains of *Lactobacillus acidophilus* or *Lacticaseibacillus rhamnosus*, *Limosilactobacillus fermentum* or *Lacticaseibacillus casei* and other genera of *Bifidobacterium* (*B. bifidum, B. longum, B. animalis*)), at either 30 °C and/or 37 °C, would enable a wider behavioural spectrum from which to select the best performer, contributing to the validation of achieved results. In what concerns the indigenous *Weissella* strains, the assessment of toxicity and antimicrobial potential would be beneficial to validate the expected safety and biological activity of developed products in terms of ensuring consumers' health.

Evaluation of the impact of fermentation on the levels of phytate levels is also desirable, in order to understand how fermentation affects this antinutrient, and also, to explore its possible association with reported antioxidant activities and mineral contents. Some microorganisms produce phytase enzyme, and therefore, the involved microorganisms should also be tested for phytase activity.

Moreover, the profile of phenolic compounds and the determination of tannins were not included, but both analyses should be planned in order to achieve a better characterization of the bioactive compounds in the fermented product and also to understand the impact of fermentation on such molecules.

Finally, in what concerns Part III, evaluation on the antioxidant activity of the fermented product using additional assays, such as ORAC (Oxygen radical absorbance capacity), would be useful in order to better understand if the antioxidant activity might be enhanced or not by the fermentation. This assay involves hydrogen atom transfer reactions, contrary to ABTS and DPPH

which are both based on electron transfer reactions, and by that it would complement the reported analysis.

A full validation of the biological potential (antioxidant and antimicrobial activities) of the EPS produced during the fermentation process is also of importance. The antimicrobial potential against GI infection agents should be targeted, thus exploring the possibility that we are developing a food product that carries functional metabolites, capable of preventing diseases of the GI tract. This would increase the knowledge about the strains and their generated metabolites, providing an overall validation of the YLB and enabling additional added value applications.

Furthermore, the potential prebiotic effect of YLB should be confirmed using a broader assay, involving a larger number of donors sample. Once proven, towards the exploration of this association, it would be an additional gain to design *in vivo* clinical trials (short- and long-term), as required for health claim applications. It would also be important to establish dose-effect relationships in these human intervention studies. Regarding the sensory evaluation of this product, an improvement in texture should be explored aiming a finer and smooth mouthfeel of the fermented product. After this achievement, a consumer study should be performed.

Other food product presentations could be explored, for instance an ice-creamed matrix or a baby food, such as fruit pulp, aiming to target specific populational groups, such as children. This would require a new study on product and strain stability under freezing conditions.

In a future perspective, a scale up, from the lab to a pilot scale would help the understanding about the product's ultimate potential. Nevertheless, for this a step-by-step validation is required, since there are countless issues that might affect the outcome, for instance the milling process, the fermentation process conditions, such as the mixing time, and the strains' behaviour in a different environment. In short, a myriad of aspects would have to be optimized from a process engineering point of view.

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APPENDICES

Appendix A – Food photographic album

Content

- **Introduction**
- **Food Portions**
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  - Fruits
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- **Household Measures**
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Introduction

This photographic album intends to combine illustrative photos of food portion sizes from different food groups, in order to facilitate the estimation of food portions by Kenyan population. These images were collected during visits to households, at the Nairobi county, under the FIBRE-PRO Project.

All the foods included in this album are part of the traditional Kenyan diet. The selection of these foods was based on the availability of foods at the studied households.

These portions represent the individual consumption, and therefore they should by applied at individual level.

Food Portions

Cereals and Grain Products

Breakfast Cereals

1 bowl (113 g)

1 bowl (72 g)

Bread and Chapatti

2 slices (34 g each)

2 units (150 g)

Food Portions

Cereals and Grain Products

Ugali

285 g

375 g

Potatoes and Chips

Big plate
290 g

Small plate
300 g

Food Portions

Cereals and Grain Products

Pasta and Rice

4 serving spoons
300g

½ Small Plate or 1/3 Big Plate
160 g

1 Small Plate or ½ Big plate
190 g

1 Big Plate
325 g

Food Portions

Vegetables

Cabbage and Kales

Small Plate
122g

Small Plate
86 g

Spinach

Small Plate
80 g of each

Big Plate
76 g

Food Portions

Fruits

Pawpaw

1 Bowl
180g

Apple

1 medium unit
130 g

Orange

1 medium unit
120 g

Banana

1 medium unit
75 g

Food Portions

Fruits

Mangoes

1 Small Unit

1 Small Unit and 1 Medium unit

Food Portions

Meat and Fish

Beef, Chicken and Pork

1 Small plate
150g

¼ Chicken

Fish (Tilapia)

1 piece
75g

1 piece
240 g

Food Portions

Meat and Fish

Fish (Omena)

1 big plate
50g

1 cup
50g

Sausages and Samosas

3 units
35 g each

1 unit
46 g

Food Portions

Legumes

Green Grams (*Ndengu*)

1/3 Bowl
145g

1 Small deep plate
270g

1 Big deep Plate
313g

Beans

1 Big deep Plate
254g

Food Portions

Composite Dishes

Beans Potatoes and Carrot Stew

2 serving spoons
510 g

Githeri

2 serving spoons
421 g

Mukimo

2 serving spoons
570 g

Pilau

3 serving spoons or a 1 Small Plate
290 g

Food Portions

Sweets

Mandazi

1 unit
47 g

Household Measures

Glass and Cups

180 ml

200 ml

250 ml

350 ml

Household Measures

Cutlery

Serving Spoon type 1

Serving Spoon type 2

Serving Spoon type 3

Household Measures

Dishes

Small Plate

Big Plate

Small Bowl (Breakfast Cereals)

Medium Bowl

Food Portion Photographic Album

Kenya

Appendix B – Statement of Consent, used in the validity study.

STATEMENT OF CONSENT

According to the Declaration of Helsinki of the World Medical Association (WMA) (Helsínki 1964; Tokyo 1975; Venice 1983, Hong Kong 1989, Somerset West 1996, Edinburgh 2000, Washington 2002, Tokyo 2004, Seoul 2008, Fortaleza 2013)

Designation of the Study: FIBRE-PRO Project – Dietary Survey

I, _____, the undersigned, under the FIBRE-PRO Project – Dietary Survey of which I will be part, agree in participating, during which I will provide the required information over five different interviews, the purpose of which will be the study of the eating habits of Kenyan population.

I understood the entire explanation that was given to me in order to participate in this study, namely the voluntary nature of my collaboration. Furthermore, I was given the opportunity to ask the questions that I judged necessary and was always provided with decent answers.

I understand that, according to the recommendations of the Declaration of Helsinki, the explanation that was given to me detailed the objectives, the methodology, the foreseen benefits, and that the researchers guarantee complete confidentiality of data.

It became also clear that I can refuse to continue to be part of this research at any time, without arising any disadvantages. Therefore, I accept providing all the necessary information regarding my dietary habits, which will be used for the mentioned purposes of the study.

Nairobi, _____ 2016

Signature:

_____

By the responsible researchers,

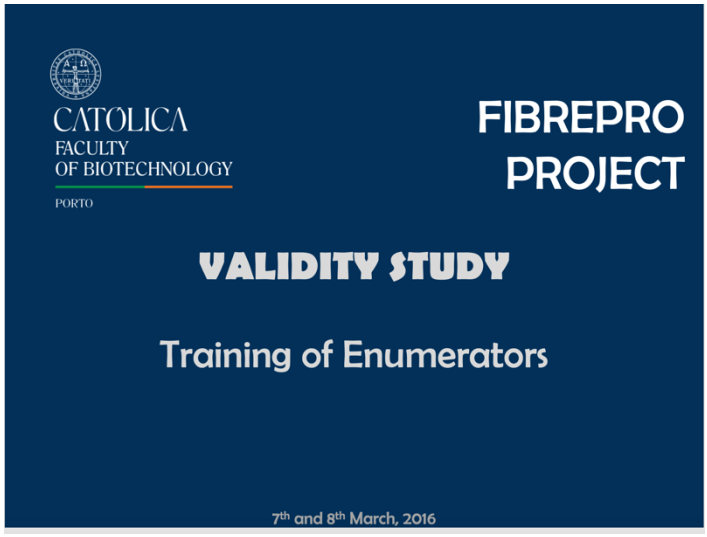
Signature:

_____

FIBREPRO Project
Contacts: cvreal@porto.ucp.pt | 0712 392 036

Site: blogs.helsinki.fi/fibrepro-project

Appendix C – Presentation prepared for the training of interviewers, used in the validity study.



Agenda

- ✓ Who am I?
- ✓ Objectives of the project and study
- ✓ Study site and Sample
- ✓ Dietary Tools
  - Semi-Quantitative Food Frequency Questionnaire
  - 24-hour Recall
- ✓ Schedule
- ✓ Important Information to give/to ask to the respondents
- ✓ How to assure quality data?
- ✓ Step by Step

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WHO AM I?

CATARINA VILA REAL, from Portugal

B.Sc. Nutritional Sciences

Master Food Engineering

PhD student (FIBRE-PRO)

Research Assistant in FIBRE-PRO

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OBJECTIVES

Objective

Evaluating dietary consumption of Kenyan adult population

Specific Objectives

S.O. 1

Validation Study and Reproducibility Study, among 75 people

S.O. 2

Dietary Survey, among 500 people

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OBJECTIVES | Validation Study

Validity → "The degree to which the questionnaires actually measures the aspect of diet that it was designed to measure." (Willet, 2013)

HOW?

Comparing with a superior, although always imperfect, standard.

FFQ VS 3 non-consecutive 24 hour-Recall (24hR)

The plan:

- 1st FFQ Interview
- 3 non-consecutive 24hR Interviews (1 weekend day + 2 week days)
- 2nd FFQ Interview

Total of 5 face-to-face interviews per respondent

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Study site and Sample

Kabete Campus

Main Campus

Apartment Complex

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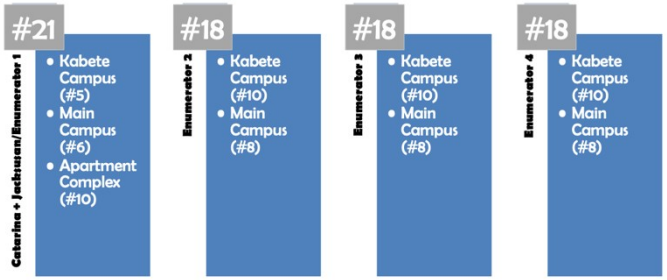
Study site and Sample

TOTAL SAMPLE → 75 People



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Study site and Sample

Characteristics of the sample

	Men	Women	Students (≥18y)*	Teachers, Staff and others professions (≥18y-60y)*	Low/ Medium social Strata	High social Strata
Kabete Campus	15	20	8 men and 10 women	7 men and 10 women	20	15
Main Campus	15	15	8 men and 10 women	7 men and 10 women	15	15
Apartments	5	5	5	5	-	10

* Excluding people with Nutrition background

Not necessarily this, but the closest possible

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Dietary Tools

Food Frequency Questionnaire

- to measure a participant's usual food intake during a specific period of time.
- limited check-list of food and beverages with a frequency response section for subjects to report how often each item is consumed over a specific periods of time.
- In semi quantitative FFQ the quantification is made by comparison, where a medium portion is given to the respondent and is asked to him to refer if his portion is bigger or smaller than that standard one.

24-hour Recall

- to identify and report all foods and beverages consumed in the preceding 24 hours, since he/she woke up the day before until she/he wakes up in the current day.
- Besides foods/beverages other information are need to be asked:
  - hour of consumption
  - preparation method
  - portion sizes
- - We can used the "Food Portion Photographic Album" in order to help respondents to estimate food portions

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FIBREPRO PROJECT

SEMI-QUANTITATIVE FOOD FREQUENCY QUESTIONNAIRE

Interviewer's Name _____

Code |__|__| |__|

The questionnaire aims to identify your food consumption during the previous month. So, for each food you should indicate how many times a day/week /month on average you ate each of the foods mentioned in this list, over the last month, and if the consumed quantity was smaller, equal or bigger to the correspondent standard portion.

Food Group / Food items (# 124 food items)	Frequency: Never	1-3 per month	1 per week	2-4 per week	5-6 per week	1 per day	2-3 per day	4-6 per day	+6 per day	Amount: Standard Portion	S	E	B
CEREALS AND GRAIN PRODUCTS (22 FOOD ITEMS)													
Maize Ugali	□	□	□	□	□	□	□	□	□	1 small plate (330g)	□	□	□
Millet Ugali	□	□	□	□	□	□	□	□	□	1 small plate (330g)	□	□	□
Sorghum Ugali	□	□	□	□	□	□	□	□	□	1 small plate (330g)	□	□	□
Mixed Ugali	□	□	□	□	□	□	□	□	□	1 small plate (330g)	□	□	□
Maize Porridge	□	□	□	□	□	□	□	□	□	1 cup (250 ml)	□	□	□

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Schedule

Month	MARCH								
Days	5	6	7	8	9	10	11	12	13
Local	APARTMENTS		KABETE					APARTMENTS	
Enumerators	Catarina + Jacksuan		Training of enumerators		All in the field			Catarina+ Jacksuan	
8a.m.-12a.m	10 FFQ (household)		2 FFQ as example (1/day)		3 FFQ (Enumerator 2 + Catarina)	3 FFQ (Enumerator 4 + Catarina)	5 recalls(1st) + 4 FFQ+ 2 recalls (1st) +6 FFQ+ 2 recalls (1st) +6 FFQ+ 2 recalls (1st)	10 recalls (1st) (household)	
1p.m.-5p.m.					3 FFQ (Enumerator 3 + Catarina)	3 FFQ+3 FFQ+3 FFQ			

MARCH													
14	15	16	17	18	19	20	21	22	23	24	25		
KABETE	CENTER	KABETE	CENTER/KABETE		APARTMENTS		CENTER	KABETE	CENTER	KABETE	CENTER		
All in the field					Catarina + Jacksuan		All in the field						
5 recalls(2nd) + 8 recalls(1st) +8 recalls (1st) +8 recalls (1st)	6 FFQ + 6 FFQ +6 FFQ +6 FFQ	5 recalls(3rd) + 10 recalls(2nd) +10 recalls (2nd) +10 recalls (2nd)	6 Recalls (1st) + 2 FFQ+ 5 recalls(1st) +2 FFQ+5 recalls(1st) +2 FFQ+5 recalls(1st)	CENTER: 3 recalls(1st) +3recalls(1st) + 3 recalls(1st) KABETE: 5 FFQ + 5 recalls(3rd) +5 recalls (3rd) +5 recalls (3rd)	10 recalls (3rd) (household)		6 Recalls (2nd) +8 recalls(2nd) +8 recalls(2nd) + 8 recalls(2nd)	5 recalls(3rd)+4FFQ +5 recalls (3rd)+4FFQ +5 recalls (3rd)+4FFQ	6 Recalls (3rd) +8 recalls(3rd) +8 recalls(3rd) + 8 recalls(3rd)	6FFQ +6FFQ +6FFQ	6FFQ +8FFQ +8FFQ +8FFQ		

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Schedule

Month									
Days	5	6	7	8	9	10	11	12	13
Local	APARTMENTS		KABETE					APARTMENTS	
Catarina + Jacksuan/ Enumerator 1	5 FFQ Hous.	5 FFQ Hous.	2 FFQ	Training	Supervision	Supervision + 3FFQ	5 recalls(1st)	5 recalls(1st)	5 recalls(1st)
Enumerator 2	X	X	Training	Training	3FFQ	3FFQ	4FFQ + 2 recalls (1st)	X	X
Enumerator 3	X	X	Training	Training	3FFQ	3FFQ	4FFQ + 2 recalls (1st)	X	X
Enumerator 4	X	X	Training	Training	X	6FFQ	4FFQ + 2 recalls (1st)	X	X

MARCH												
14	15	16	17	18	19	20	21	22	23	24	25	
KABETE	CENTER	KABETE	CENTER/KABETE		APARTMENTS		CENTER	KABETE	CENTER	KABETE	CENTER	
5 recalls(2nd) + 2 recalls Hous.(2nd)	6FFQ + 2 recalls Hous. (2nd)	5 recalls(3rd) + 2 recalls Hous.(2nd)	6 recalls(1st) + 2 recalls Hous.(2nd)	5 FFQ + 2 recalls Hous. (2nd)	5 recalls(3rd)	5 recalls(3rd)	6 recalls(2nd) + 2 FFQ Hous.	2 FFQ Hous.	6 recalls(3rd) + 2 FFQ Hous.	2 FFQ Hous.	6FFQ + 2FFQ Hous.	
8 recalls(1st)	6FFQ	8 recalls(1st)	2 FFQ + 5 recalls(1st)	3 recalls (1st	X	X	8 recalls(2nd	5 recalls(3rd) + 4 F	8 recalls(3rd)	6FFQ	8FFQ	
8 recalls(1st)	6FFQ	8 recalls(1st)	2 FFQ + 5 recalls(1st)	3 recalls (1st	X	X	8 recalls(2nd	5 recalls(3rd) + 4 F	8 recalls(3rd)	6FFQ	8FFQ	
8 recalls(1st)	6FFQ	8 recalls(1st)	2 FFQ + 5 recalls(1st)	3 recalls (1st	X	X	8 recalls(2nd	5 recalls(3rd) + 4 F	8 recalls(3rd)	6FFQ	8FFQ	

+ Send daily reports of the work done (to Portuguese Team)

Each one of us has daily objectives, however if we can speed up the process, perfet!

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Important Information to give/to ask to the respondents

To give

- Statement of Consent (reinforce: anonymity; 5 interviews)
- They coud be contacted by phone after the interview

To ask

- Week availability for being interviewed

They cannot know in advance in which day they will be interviewed

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How to ensure quality data?

- ✓ Ask for individual consumption
- ✓ Ask for edible portions
- ✓ Do not influence respondent's answers
- ✓ Do not rush during the interviews (neither take too long)
- ✓ Ask for any doubt you might have, at any time
- ✓ Be the most complete you can during the collection of data
- ✓ Send daily reports of the work done (to Portuguese Team)
- ✓ **Give your best** ☺

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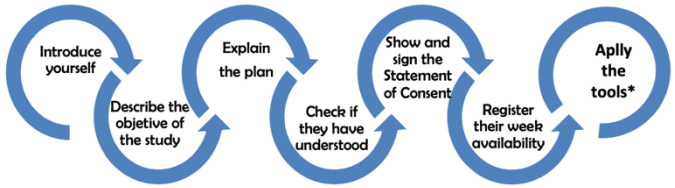
Step by Step



*
1st interview: General Questionnaire + Semi-Quantitative FFQ
2nd interview: 1st 24hR
3rd interview: 2nd 24hR
4th interview: 3rd 24hR
5th interview: Semi-Quantitative FFQ

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PORTO

Thank you for your attention

Catarina: cvreal@porto.ucp.pt | 0712 392 036
Prof. Elisabete Pinto: epinto@porto.ucp.pt

Appendix D – General Questionnaire, used in the validity study.

QUESTIONNAIRE

Interviewer's Name _____

Code |__|__| |__|

Respondent's address _____

Respondent's contact _____

Sex

Female |__| Male |__|

Age |__|__|years-old

Education level

Primary |__| Tertiary |__|

Secondary |__| None |__|

Occupation/Profession

Unemployed |__| Formally employed |__|

Self-employed |__| Casual labourer |__|

Marital Status

Single |__| Married|__| Widow |__|

Household Number

|__|__|people, which |__|__|are children.

Anthropometric Data

Weight |__||__|kg Height |__|,|__||__|m

Lifestyle Habits

Do you smoke?

Yes |__| No|__|

Do you usually consume alcoholic beverages?

Yes |__| No|__|

How many meals do you eat per day?

|__||__|

Appendix E – Semi-quantitative Food Frequency Questionnaire.

SEMI-QUANTITATIVE FOOD FREQUENCY QUESTIONNAIRE

Interviewer's Name _____

Code |_|_| |1|

The questionnaire aims to identify your food consumption during the previous month. So, for each food you should indicate how many times a day/week /month on average you ate each of the foods mentioned in this list, over the last month, and if the consumed quantity was smaller, equal or bigger than the correspondent standard portion.

	Frequency									Amount			
Food Group Food items (# 124 food items)	Never	1-3 per month	1 per week	2-4 per week	5-6 per week	1 per day	2-3 per day	4-6 per day	+6 per day	Standard Portion	S	E	B
CEREALS AND GRAIN PRODUCTS (22 FOOD ITEMS)													
Maize Ugali	□	□	□	□	□	□	□	□	□	1 small plate (330g)	□	□	□
Millet Ugali	□	□	□	□	□	□	□	□	□	1 small plate (330g)	□	□	□
Sorghum Ugali	□	□	□	□	□	□	□	□	□	1 small plate (330g)	□	□	□
Mixed Ugali	□	□	□	□	□	□	□	□	□	1 small plate (330g)	□	□	□
Maize Porridge	□	□	□	□	□	□	□	□	□	1 cup (250 ml)	□	□	□
Millet Porridge	□	□	□	□	□	□	□	□	□	1 cup (250 ml)	□	□	□
Sorghum Porridge	□	□	□	□	□	□	□	□	□	1 cup (250 ml)	□	□	□
Mixed Porridge	□	□	□	□	□	□	□	□	□	1 cup (250 ml)	□	□	□
White Rice	□	□	□	□	□	□	□	□	□	2 serving spoons (180g)	□	□	□
Brown Rice	□	□	□	□	□	□	□	□	□	2 serving spoons (180g)	□	□	□
Maize (Boiled, Roasted)	□	□	□	□	□	□	□	□	□	1 medium ear	□	□	□
Potatoes (Boiled, stewed)	□	□	□	□	□	□	□	□	□	1 medium unit (100g)	□	□	□
French Fries	□	□	□	□	□	□	□	□	□	1/2 big plate (200g)	□	□	□
Potato (sweet)	□	□	□	□	□	□	□	□	□	1 medium unit (200g)	□	□	□
Cassava	□	□	□	□	□	□	□	□	□	1/2 small root	□	□	□
Spaghetti and macaroni	□	□	□	□	□	□	□	□	□	1/2 big plate (200g)	□	□	□

Food Group Food items	Frequency									Amount			
	Never	1-3 per month	1 per week	2-4 per week	5-6 per week	1 per day	2-3 per day	4-6 per day	+6 per day	Standard Portion	S	E	B
Weetabix wholegrain cereals	□	□	□	□	□	□	□	□	□	2 biscuits (37,5g)	□	□	□
Other type of Breakfast Cereals	□	□	□	□	□	□	□	□	□	1 bowl (90g)	□	□	□
White Bread	□	□	□	□	□	□	□	□	□	2 slices (70g)	□	□	□
Brown Bread	□	□	□	□	□	□	□	□	□	2 slices (70g)	□	□	□
White Chapatti	□	□	□	□	□	□	□	□	□	2 units (180g)	□	□	□
Brown Chapatti	□	□	□	□	□	□	□	□	□	2 units (180g)	□	□	□
VEGETABLES (21 food items)													
Carrots	□	□	□	□	□	□	□	□	□	1 medium unit (90g)	□	□	□
Pumpkin/butternut	□	□	□	□	□	□	□	□	□	1 medium portion (100 g)	□	□	□
Sweet Pepper, Green Pepper	□	□	□	□	□	□	□	□	□	1/4 medium unit(40g)	□	□	□
Kales (Sukuma Wiki), cooked	□	□	□	□	□	□	□	□	□	1/2 big plate or 2 serving spoons (150g)	□	□	□
Cabbages, cooked	□	□	□	□	□	□	□	□	□	1/2 big plate or 2 serving spoons (150g)	□	□	□
Spinach, cooked	□	□	□	□	□	□	□	□	□	1/2 big plate or 2 serving spoons (150g)	□	□	□
Black Night Shades (Managu), cooked	□	□	□	□	□	□	□	□	□	1/4 big plate or 1 serving spoon (75g)	□	□	□
Amaranthus (Terere), cooked	□	□	□	□	□	□	□	□	□	1/4 big plate or 1 serving spoon (75g)	□	□	□
Pumpkin leaves (Seveve, malenge leaves) , cooked	□	□	□	□	□	□	□	□	□	1/4 big plate or 1 serving spoon (75g)	□	□	□
Comfrey (Mabaki), cooked	□	□	□	□	□	□	□	□	□	1/4 big plate or 1 serving spoon (75g)	□	□	□

	Frequency									Amount			
Food Group Food items	Never	1-3 per month	1 per week	2-4 per week	5-6 per week	1 per day	2-3 per day	4-6 per day	+6 per day	Standard Portion	S	E	B
Bacella alba (Nderema), cooked	□	□	□	□	□	□	□	□	□	1/4 big plate or 1 serving spoon (75g)	□	□	□
Spider Weed (Sargiet), cooked	□	□	□	□	□	□	□	□	□	1/4 big plate or 1 serving spoon (75g)	□	□	□
Cowpeas Leaves (Kunde), cooked	□	□	□	□	□	□	□	□	□	1/4 big plate or 1 serving spoon(75g)	□	□	□
Jute Leaves (Mrenda), cooked	□	□	□	□	□	□	□	□	□	1/4 big plate or 1 serving spoons (75g)	□	□	□
French Beans, cooked	□	□	□	□	□	□	□	□	□	1/4 big plate (50g)	□	□	□
Courgettes	□	□	□	□	□	□	□	□	□	1/2 cup or 1 medium unit (200g)	□	□	□
Onions	□	□	□	□	□	□	□	□	□	1 medium unit (90g)	□	□	□
Tomatoes	□	□	□	□	□	□	□	□	□	2 medium units (100 g each)	□	□	□
Garlic	□	□	□	□	□	□	□	□	□	1 clove (2g)	□	□	□
Mushrooms	□	□	□	□	□	□	□	□	□	1 serving spoon (40g)	□	□	□
Salad Vegetables (Lettuce, watercress)	□	□	□	□	□	□	□	□	□	1/2 big plate	□	□	□
LEGUMES, PULSES, SEEDS, AND NUTS (5 food items)													
Beans	□	□	□	□	□	□	□	□	□	2 serving spoons (300g) or 1/2 big plate	□	□	□
Lentils (Kamande)	□	□	□	□	□	□	□	□	□	2 serving spoons (280g) or 1/2 big plate	□	□	□
Green Peas	□	□	□	□	□	□	□	□	□	1/2 cup (80g)	□	□	□
Green Grams (Ndengu)	□	□	□	□	□	□	□	□	□	2 serving spoons (280g) or 1 small plate	□	□	□
Nuts, groundnuts and seeds	□	□	□	□	□	□	□	□	□	1 table spoon (20g)	□	□	□

Food Group Food items	Frequency									Amount			
	Never	1-3 per month	1 per week	2-4 per week	5-6 per week	1 per day	2-3 per day	4-6 per day	+6 per day	Standard Portion	S	E	B
FRUITS (15 food items)													
Orange/Tangerine	□	□	□	□	□	□	□	□	□	1 medium unit (130g)	□	□	□
Melon (Watermelon, Thon melon)	□	□	□	□	□	□	□	□	□	1 small bowl (140g)	□	□	□
Grapes	□	□	□	□	□	□	□	□	□	1 medium bunch (125g)	□	□	□
Passion Fruit	□	□	□	□	□	□	□	□	□	2 medium units (65g each)	□	□	□
Pawpaw	□	□	□	□	□	□	□	□	□	1 small bowl (200g)	□	□	□
Apple	□	□	□	□	□	□	□	□	□	1 medium unit (130g)	□	□	□
Pears	□	□	□	□	□	□	□	□	□	1 medium unit (130g)	□	□	□
Avocado	□	□	□	□	□	□	□	□	□	1 medium unit (300g)	□	□	□
Mangoes	□	□	□	□	□	□	□	□	□	1 medium portion (180g)	□	□	□
Loquats	□	□	□	□	□	□	□	□	□	1 medium unit (60g)	□	□	□
Pineapple	□	□	□	□	□	□	□	□	□	1 medium portion (200g)	□	□	□
Plums	□	□	□	□	□	□	□	□	□	1 medium unit (60g)	□	□	□
Berries	□	□	□	□	□	□	□	□	□	1/2 cup	□	□	□
Banana, ripe	□	□	□	□	□	□	□	□	□	1 medium unit (70g)	□	□	□
Banana (Matoke)	□	□	□	□	□	□	□	□	□	4 medium units (70g)	□	□	□
MEAT, FISH AND EGGS (18 food items)													
Beef	□	□	□	□	□	□	□	□	□	1 medium portion (100g)	□	□	□
Pork	□	□	□	□	□	□	□	□	□	1 medium portion (100g)	□	□	□
Chicken	□	□	□	□	□	□	□	□	□	1/4 chicken or 2 pieces	□	□	□
Goat meat	□	□	□	□	□	□	□	□	□	1 medium portion (100g)	□	□	□
Lamb meat	□	□	□	□	□	□	□	□	□	1 medium portion (100g)	□	□	□

Food Group / Food items	Frequency									Amount			
	Never	1-3 per month	1 per week	2-4 per week	5-6 per week	1 per day	2-3 per day	4-6 per day	+6 per day	Standard Portion	S	E	B
Bush meat (wild animals)	□	□	□	□	□	□	□	□	□	1 medium portion (100g)	□	□	□
Sausages	□	□	□	□	□	□	□	□	□	2 medium units (35g each)	□	□	□
Smokies	□	□	□	□	□	□	□	□	□	2 medium units (35g each)	□	□	□
Intestines (Matumbo)	□	□	□	□	□	□	□	□	□	1 medium portion (100g)	□	□	□
Other type of offals (liver, kidney, heart)	□	□	□	□	□	□	□	□	□	1 medium portion (100g)	□	□	□
Samosas (meat)	□	□	□	□	□	□	□	□	□	1 medium unit (46g)	□	□	□
Batter dipped fried chicken (e.g. KFC)	□	□	□	□	□	□	□	□	□	2 pieces or 3 hot wings	□	□	□
Omena	□	□	□	□	□	□	□	□	□	1 cup (50g)	□	□	□
Tilapia	□	□	□	□	□	□	□	□	□	1 fillet (100g) or 1 medium portion (250g)	□	□	□
Nile Perch	□	□	□	□	□	□	□	□	□	1 fillet (100g)	□	□	□
Other type of fish: tuna sardines/pilchard, swordfish/marlin, mackerel, salmon, herring, etc.	□	□	□	□	□	□	□	□	□	1 fillet (100g)	□	□	□
Canned fish	□	□	□	□	□	□	□	□	□	1/2 can (130g each)	□	□	□
Eggs (boiled, fried, scrambled)	□	□	□	□	□	□	□	□	□	1 egg (60g)	□	□	□
DAIRY PRODUCTS (7 food items)													
Whole Milk (cow)	□	□	□	□	□	□	□	□	□	1 cup (250ml)	□	□	□
Skimmed milk (cow)	□	□	□	□	□	□	□	□	□	1 cup (250ml)	□	□	□
Other types of milk (Soy Milk, Lactose-free milk, fortified milk)	□	□	□	□	□	□	□	□	□	1 cup (250ml)	□	□	□
Cheese	□	□	□	□	□	□	□	□	□	1 medium slice (30g)	□	□	□
Yogurt	□	□	□	□	□	□	□	□	□	1 pack (250 ml)	□	□	□
Lala/Mala	□	□	□	□	□	□	□	□	□	1 cup (250ml)	□	□	□

Food Group / Food items	Frequency									Amount			
	Never	1-3 per month	1 per week	2-4 per week	5-6 per week	1 per day	2-3 per day	4-6 per day	+6 per day	Standard Portion	S	E	B
Powder Milk/Milk substitute (Mido, Cremora,Safari Land)	□	□	□	□	□	□	□	□	□	1 cup (250ml)	□	□	□
FATS AND OILS (6 food items)													
Butter	□	□	□	□	□	□	□	□	□	1 tea spoon	□	□	□
Margarine (brick, tube)	□	□	□	□	□	□	□	□	□	1 tea spoon	□	□	□
Olive Oil	□	□	□	□	□	□	□	□	□	1 tea spoon	□	□	□
Soybean Oil	□	□	□	□	□	□	□	□	□	1 tea spoon	□	□	□
Corn Oil	□	□	□	□	□	□	□	□	□	1 tea spoon	□	□	□
Other types of oil	□	□	□	□	□	□	□	□	□	1 tea spoon	□	□	□
SAUCES, SEASONINGS AND FLAVOURINGS (7 food items)													
Meat Extracts (Bovril, Marmite)	□	□	□	□	□	□	□	□	□	1 tea spoon	□	□	□
Savoury Spreads (Pâté, Cream cheese)	□	□	□	□	□	□	□	□	□	1 tea spoon	□	□	□
Peanut Butter	□	□	□	□	□	□	□	□	□	1 tea spoon	□	□	□
Sweet spreads (honey, jam, atchar)	□	□	□	□	□	□	□	□	□	1 tea spoon	□	□	□
Tomato sauce/Chili Sauce, etc.	□	□	□	□	□	□	□	□	□	1 tea spoon	□	□	□
Mayonnaise, Salad Dressing	□	□	□	□	□	□	□	□	□	1 tea spoon	□	□	□
Spices and Herbs (such as Royco, Coriander)	□	□	□	□	□	□	□	□	□	1 tea spoon (5g) or 1 small bunch (18 g)	□	□	□
SUGAR, SYRUPS, SWEETS AND SNACKS (9 food items)													
Low sugar cookies, Rusks (Marie, Family Cookies)	□	□	□	□	□	□	□	□	□	3 units (5g each)	□	□	□
High-sugar cookies and biscuits (Ginger Cookies)	□	□	□	□	□	□	□	□	□	3 units	□	□	□
Cakes, Pies, Pudding	□	□	□	□	□	□	□	□	□	1 slice (65g) or 1 pudding (80g)	□	□	□
Muffins/Scones, Pancakes/Waffles	□	□	□	□	□	□	□	□	□	1 medium unit (75g)	□	□	□
Mandazi, or Doughnuts	□	□	□	□	□	□	□	□	□	1 medium unit (50g)	□	□	□
Popcorn, Crips	□	□	□	□	□	□	□	□	□	1 bowl popped (12,5g) or 1 pack (32,5g)	□	□	□

Food Group / Food items	Frequency									Amount			
	Never	1-3 per month	1 per week	2-4 per week	5-6 per week	1 per day	2-3 per day	4-6 per day	+6 per day	Standard Portion	S	E	B
Ice cream	□	□	□	□	□	□	□	□	□	2 balls (120g)	□	□	□
Chocolates(snacks such as Mars, Snickers, Kit Kat, etc)	□	□	□	□	□	□	□	□	□	1 unit (45g)	□	□	□
Sugar (white, Brown)	□	□	□	□	□	□	□	□	□	2 tea spoons (15g)	□	□	□
BEVERAGES (10 food items)													
Carbonated Cold Drinks (Coca-Cola, Fanta, Sprite etc.)	□	□	□	□	□	□	□	□	□	1 bottle (500ml)	□	□	□
Diet cold drinks (Coca-Cola zero)	□	□	□	□	□	□	□	□	□	1 bottle (500ml)	□	□	□
Energy drinks (RedBull, Monster, etc.)	□	□	□	□	□	□	□	□	□	1 can (250ml)	□	□	□
Coffee	□	□	□	□	□	□	□	□	□	1 cup (250ml)	□	□	□
Tea (with milk) Black Tea	□	□	□	□	□	□	□	□	□	1 cup (250ml)	□	□	□
Water	□	□	□	□	□	□	□	□	□	1,5 L	□	□	□
Beer	□	□	□	□	□	□	□	□	□	1 can (500ml) or 1 bottle (330ml)	□	□	□
Wine	□	□	□	□	□	□	□	□	□	1 glass (125 ml)	□	□	□
Fruit Juices (Fresh fruit)	□	□	□	□	□	□	□	□	□	1 glass (250ml)	□	□	□
Sweetened Fruit-Flavoured Drinks (Del Monte, Pick& Pell, Quencher etc.)	□	□	□	□	□	□	□	□	□	1 glass (250ml)	□	□	□
COMPOSITE DISHES (3 food items)													
Pilau	□	□	□	□	□	□	□	□	□	3 serving spoons or 1/2 big plate (300g)	□	□	□
Githeri	□	□	□	□	□	□	□	□	□	2 serving spoons (420g)	□	□	□
Mukimo	□	□	□	□	□	□	□	□	□	2 serving spoons or 1/2 big plate (320g)	□	□	□

In addition to the foods mentioned, there is something else that is part of your eating habits and I did not mention (pizza, kachumbari, arrowroot, sugarcane, drinking chocolate, whisky, etc.)**?**

Appendix F – Twenty-four-hour Recall template.

TWENTY-FOUR HOUR RECALL

Interviewer's Name _____

Code |__|__| |__|

Interview Date, Week day: _____

Please, give detailed information about everything you ate and drank in the last 24 hours, since you woke up yesterday until you wake up today. Please mention all the foods and beverages items you consumed during this period.

Hour	Food/Beverages Items	Preparation Method	Portion Size

ADDITIONAL QUESTIONS

1. Is there any food/beverage that you have consumed, between meals for instance, but you didn't mention on the recall?
   _____
2. Did you add sugar in consumed foods/beverages?
   If so, you should add this item in the previous table
3. Did you consume any sauce? If so, with which ingredients this sauce was cooked?
   If so, you should add this item in the previous table.
4. Did you consume sweets/snacks?
   If so, you should add this item in the previous table.
5. Did you consume alcohol?
   If so, you should add this item in the previous table.
6. How much water did you drink?
   If so, you should add this item in the previous table.
7. The day you have recalled is typical of your food consumption?
   _____
8. If it is an unusual day could you give some reasons to justify this and refer which are the major differences?
   _____

Appendix G – Manual of Codification.

MANUAL OF CODIFICATION

(Food Processor® Version 11.0.3)

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CEREALS AND GRAIN PRODUCTS

- *UGALI*

FOOD ITEM	SOFTWARE DESCRIPTION	CREATED CODE	ESHA CODE	STANDARD PORTION
Maize Ugali	Cereal, simply maize	K001	52612	1 small plate = 330 g
	Water		21105	
Millet Ugali	Millet flour	K002	28421	1 small plate = 330 g
	Water		21105	
Sorghum Ugali	Sorghum, flour, whole grain	K003	28455	1 small plate = 330 g
	Water		21105	
Mixed Ugali	Cereal, simple maize	K004	52612	1 small plate = 330 g
	Millet flour		28421	
	Sorghum, flour, whole grain		28455	
	Cassava flour		6064	
	Water		21105	
Mixed Ugali (maize + millet)	Cereal, simply maize	K096	52612	1 small plate = 330 g
	Millet flour		28421	
	Water		21105	
Mixed Ugali (maize + cassava)	Cereal, simply maize	K099	52612	1 small plate = 330 g
	Cassava flour		6064	
	Water		21105	

VEGETABLES

- *GREEN VEGETABLES*

FOOD ITEM	SOFTWARE DESCRIPTION	CREATED CODE	ESHA CODE	STANDARD PORTION
Cabbage, cooked	Cabbage, savoy, cooked, with salt, drained, shredded	-	5881	1/2 big plate or 2 serving spoons = 150 g
Spinach, cooked	Mustard spinach, tendergreen, cooked, with salt, drained	-	5929	1/2 big plate or 2 serving spoons = 150 g
Black Night shades (Managu) cooked	-	K017	-	1/4 big plate or 1 serving spoon = 75 g
Amaranthus (Terere)	Amaranth Greens, cooked, with salt, drained	-	5837	1/4 big plate or 1 serving spoon = 75 g
Pumpkin Leaves	-		-	1/4 big plate or 1 serving spoon = 75 g
Comfrey (Mabaki) cooked	Comfrey, leaf	-	26305	1/4 big plate or 1 serving spoon = 75 g
Bacella Alba (Nderema), cooked	Spinach, malabar, cooked	-	7946	1/4 big plate or 1 serving spoon = 75 g
Spider weed (Sarget), cooked	Spider weed (sarget) ("Dek Spider Herb"), with salt	K018	-	1/4 big plate or 1 serving spoon = 75 g
Cowpeas Leaves (kunde), cooked	Cowpeas leaves (kunde), raw, with salt	K019	-	1/4 big plate or 1 serving spoon = 75 g
Jute Leaves (Mrenda), cooked	Jute leaves, cooked, with salt, drained	-	5914	1/4 big plate or 1 serving spoon = 75 g
Salad Vegetables (Lettuce, watercress)	Lettuce, bibb, fresh, leaf	-	4867	1/2 big plate = 15 g
Brocolli	brocolli	-	84435	1 side = 25 g

Appendix H – Statement of Consent, used in the main survey.

STATEMENT OF CONSENT

According to the Declaration of Helsinki of the World Medical Association (WMA) (Helsínki 1964; Tokyo 1975; Venice 1983, Hong Kong 1989, Somerset West 1996, Edinburgh 2000, Washington 2002, Tokyo 2004, Seoul 2008, Fortaleza 2013)

Designation of the Study: FIBRE-PRO Project – Dietary Survey

I, _____, the undersigned, under the FIBRE-PRO Project – Dietary Survey of which I will be part, agree in participating, during which I will provide the required information in one interview, the purpose of which will be the study of the eating habits of Kenyan population.

I understood the entire explanation that was given to me in order to participate in this study, namely the voluntary nature of my collaboration. Furthermore, I was given the opportunity to ask the questions that I judged necessary and was always provided with decent answers.

I understand that, according to the recommendations of the Declaration of Helsinki, the explanation that was given to me detailed the objectives, the methodology, the foreseen benefits, and that the researchers guarantee complete confidentiality of data.

It became also clear that I can refuse to continue to be part of this research at any time, without arising any disadvantages. Therefore, I accept providing all the necessary information regarding my dietary habits, which will be used for the mentioned purposes of the study.

Nairobi, _____ 2016

Signature:

_____

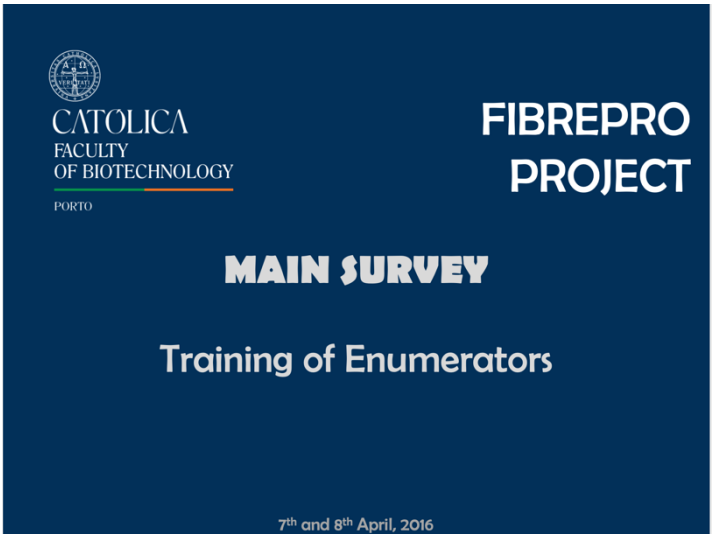
By the responsible researchers,

Signature:

_____

FIBREPRO Project
Contacts: cvreal@porto.ucp.pt | 0712 392 036
Site: blogs.helsinki.fi/fibrepro-project

Appendix I – Presentation prepared for the training of interviewers, used in the main survey.



Agenda

- ✓ Who am I?
- ✓ Objectives of the project and study
- ✓ Study coverage and Sample
- ✓ General Questionnaire
- ✓ Dietary Tool
  - Semi-Quantitative Food Frequency Questionnaire
- ✓ Schedule
- ✓ How to assure quality data?
- ✓ Step by Step
- ✓ Interview Proccess

CATOLICA FACULTY OF BIOTECHNOLOGY PORTO

WHO AM I?

CATARINA VILA REAL, from Portugal

B.Sc. Nutritional Sciences

Master Food Engineering

PhD student (FIBRE-PRO)

Research Assistant in FIBRE-PRO

CATOLICA FACULTY OF BIOTECHNOLOGY PORTO

OBJECTIVES

Objective

To assess dietary intake of Kenyan adult population

Specific Objectives

S.O. 1

Validation Study and Reproducibility Study, among 75 people ✓

S.O. 2

Dietary Survey, among 500 people

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OBJECTIVES | Main Survey

To assess dietary intake, of adult Kenyan people, using a validated Semi-Quantitative Food Frequency Questionnaire (FFQ)

The plan:

1) To apply a general questionnaire, in 500 Kenyan adults
2) To apply the FFQ, in 500 Kenyan adults

1 interview per person

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Study coverage and Sample

General Motors

Sarit Center
Greenspan Mall

Karioko Market
Open Air Market (in Kangemi)
Indoor Market (in Parklands)

ACK St. Joseph, in Kabete
PCEA St. Andreas, Nyerere Rd.

Kibera
Mathare

Representative Sample
Different ages and gender
Different incomes
Different environments
Different lifestyles

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Study coverage and Sample

TOTAL SAMPLE → 500 People

Each enumerator can do, on average, at least **5 interviews per day**

In a day we can do, on average, **50 interviews**

↓

~ 10-15 days
(10 week days + 2 sundays)

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Characteristics of the sample

	Men	Women	Low/ Medium social Strata	High social Strata
General Motors	25	25	10	40
Sarit Center	25	25	20	30
Greenspan Mall	25	25	20	30
Karioko Market	25	25	25	25
Open Air Market (in Kangemi)	25	25	50	-
Indoor Market (in Parklands)	25	25	25	25
ACK St. Joseph Church	25	25	25	25
PCEA St. Andreas Church	25	25	-	50
Kibera Slum	25	25	50	-
Mathare Slum	25	25	50	-
TOTAL	**250**	**250**	**275**	**225**

* Excluding people with Nutrition background, people in particular situations (pregnant).

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General Questionnaire

FIBREPRO PROJECT

GENERAL QUESTIONNAIRE

Interviewer's Name _____

Code |__|__| |Q|

SSex Female |__| Male |__|

Age |__|__|years-old

Education level Primary |__| Tertiary |__| Secondary |__| None |__|

Occupation/Profession Student/Unemployed |__| Formally employed |__|
Self-employed |__| Casual labourer |__|

Marital Status Single |__| Married |__| Widow |__|

Household Number |__|__|people, which |__|__|are children

Anthropometric Data Weight |__|__|.|__|kg Height |__|.|__|__|m/ft

Lifestyle Habits

- Do you smoke? Yes |__| No |__|
- Do you usually consume alcoholic beverages? Yes |__| No |__|
- How many meals do you eat per day? |__|__|

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- Do you practice physical activity? If so, which type of exercise and how often do you do it?

Exercise_____

Frequency (times per week)_____

Duration (minutes)_____

Health Status

Do you have any chronic disease, such as:

	Yes	No	
Diabetes	__	__	
Dyslipidaemia	__	__	
Hypertension	__	__	
Coronary disease	__	__	
Heart failure	__	__	
Other heart disease	__	__	If yes, what? _____
Stroke	__	__	
Respiratory diseases	__	__	If yes, what? _____
Allergies	__	__	If yes, what? _____
Cancer	__	__	If yes, what? _____
Others.	__	__	If yes, what? _____

In the last year how often did you go to the doctor in non-urgent medical appointment?

_____

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Dietary Tool

Food Frequency Questionnaire

- To measure a participant's usual food intake during a specific period of time.
- Limited check-list of food and beverages with a frequency response section for subjects to report how often each item is consumed over a specific period of time.
- In semi quantitative FFQ the quantification is made by comparison, where a medium portion is given to the respondent and it is asked to him to refer if usually he/she consumes this portion, a smaller or a bigger portion.

Semi Quantitative FFQ

Organized by food groups

Standard Portion Sizes for each food

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"I will enumerate several foods and for each one you should refer the frequency of consumption in the last month (4 previous weeks). In the cases that you have not eaten, you answer "never". For the ones you consumed I will refer a standard portion and you will tell me if your portion is smaller/equal/bigger than the standard one."

FIBREPRO PROJECT

SEMI-QUANTITATIVE FOOD FREQUENCY QUESTIONNAIRE

Interviewer's Name _____

Code ___ ___

The questionnaire aims to identify your food consumption during the previous month. So, for each food you should indicate how many times a day/week /month on average you ate each of the foods mentioned in this list, over the last month, and if the consumed quantity was smaller, equal or bigger to the correspondent standard portion.

	Frequency									Amount			
Food Group Food items **(# 124 food items)**	Never	1-3 per month	1 per week	2-4 per week	5-6 per week	1 per day	2-3 per day	4-6 per day	+6 per day	Standard Portion	S	E	B
CEREALS AND GRAIN PRODUCTS (22 FOOD ITEMS)													
Maize Ugali	□	□	□	□	□	□	□	□	□	1 small plate (330g)	□	□	□
Millet Ugali	□	□	□	□	□	□	□	□	□	1 small plate (330g)	□	□	□

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In addition to the foods mentioned, there is something else that is part of your eating habits and I did not mention?

IMPORTANT

Ask for the consumption of the previous 4 weeks (the previous month)

Ask for individual consumption

Ask for edible portions

Do not ask for fats/oils used for cooking (just what they add in salads, vegetables,etc.)

If the respondent refers the consumed quantity you can add it in the FFQ

In the last question you can give examples: foods such as *pizza, snaks, arrowroot, kachumbary, sugarcane, whisky* etc.

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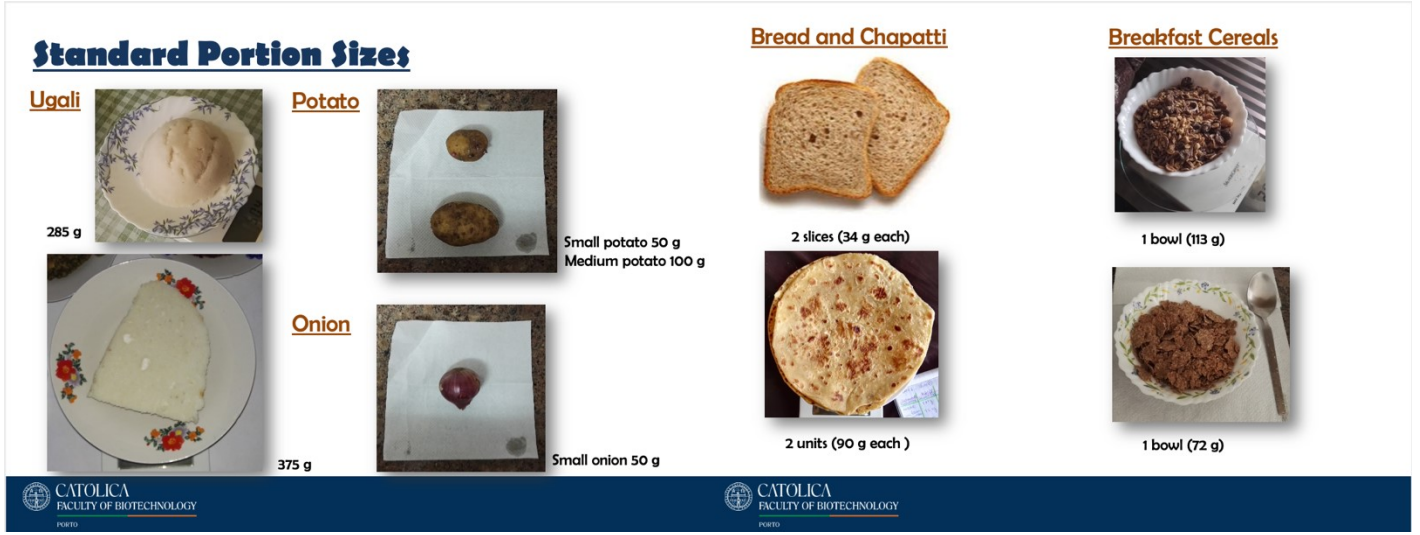
Standard Portion Sizes
Ugali
285 g
375 g
Potato
Small potato 50 g
Medium potato 100 g
Onion
Small onion 50 g
Bread and Chapatti
2 slices (34 g each)
2 units (90 g each)
Breakfast Cereals
1 bowl (113 g)
1 bowl (72 g)
CATOLICA
FACULTY OF BIOTECHNOLOGY
PORTO
Pawpaw
1 Bowl
180g
1 piece of KES 20 ≈ 200g
Mangoes
1 Small Unit and 1 Medium unit
1 Medium unit (60 g)
Fish (Tilapia and Omena)
1 piece
250 g
1 cup (not cooked)
50g
Carrot
1 medium unit
90 g
Orange
1 medium unit
120 g
Banana
1 medium unit
75 g
Apple
1 medium unit
130 g
Watermelon
200g ≈ 1 piece of KES 20
Pineapple
150g ≈ 1 piece of KES 20
Sausages and Samosas
1 medium unit
35 g
1 medium unit
46 g
Tomato
1 medium unit
100 g

Mandazi

1 medium unit
47 g

Chocolates

45-50 g ≈ 1 pack ≈ 5-6 pieces

Popcorns

45 g ≈ 1 pack

Queen Cake / Heart Cake

50 g ≈ 1 unit / 60 g ≈ 1 unit

Groundnuts

25 g ≈ 1 small pack
10 g ≈ 1 table spoon

Coriander

18 g ≈ 1 bunch of KES 5
50 g ≈ 1 bunch of KES 15

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Household Measures

250 ml

200 ml

375 ml

Serving Spoon

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Household Measures

Small Plate

Big Plate

Small Bowl

Medium Bowl

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Schedule

Month	APRIL												
Days	7	8	10	11	12	12	14	15	17	18	19	20	
Local	KABETE	KABETE	ST. ANDREAS CHURCH	KANGEMI MARKET	PARKLANDS MARKET	KARIOKO MARKET	KIBERA SLUM	SARIT CENTER	ST. JOSEPH'S CHURCH	GENERAL MOTORS	MATHERE SLUM	GREENSPAN MALL	
8a.m.-12a.m	Training Enumerators		2 FFQ / per enumerator	2 FFQ / per enumerator	2 FFQ / per enumerator	2 FFQ / per enumerator	2 FFQ / per enumerator	3 FFQ / per enumerator	2 FFQ / per enumerator	3 FFQ / per enumerator	2 FFQ / per enumerator	2 FFQ / per enumerator	
1p.m.-4p.m.			2 FFQ / per enumerator	2 FFQ / per enumerator	2 FFQ / per enumerator	2 FFQ / per enumerator	2 FFQ / per enumerator	2 FFQ / per enumerator	2 FFQ / per enumerator	2 FFQ / per enumerator	2 FFQ / per enumerator	2 FFQ / per enumerator	
4p.m.-5p.m.	Discussion of the results between the whole team												
Total per enumerator	X	X	4	4	4	4	4	5	4	5	4	4	42
Total	X	X	48	48	48	48	48	60	48	60	48	48	504

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How to ensure quality data?

- ✓ Ask for individual consumption
- ✓ Ask for edible portions
- ✓ Do not influence respondent's answers
- ✓ Do not rush during the interviews (neither take too long)
- ✓ Be the most complete you can during the collection of data
- ✓ Give daily reports of the work done (to Portuguese Team)
- ✓ Ask for any doubt you might have, at any time
- ✓ **Give your best** ☺

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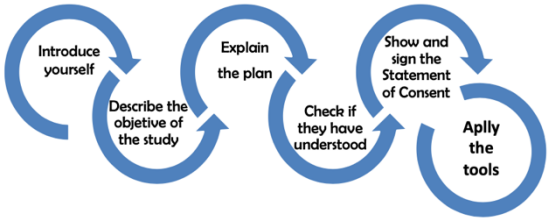
Step by Step



Tools: General Questionnaire + FFQ

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Interview Process

1st phase)
Hello,
My name is _____, and I am part of a team from the UON, which is doing a study whose main objective is to assess dietary intake of adult Kenyan population. For that we are doing a dietary survey, which includes one interview and we would like to know if you are interested in participating, being one of the respondents for this study. If so, we would like to explain to you the plan.

2nd phase) *(If people don't agree, we thank for their time and we try another one)*
In this interview we want to do some general questions and to apply a Food Frequency Questionnaire, in which we will read a list of foods and you will have to indicate, on average, how many times (per day/week/month) you ate each food during the previous month and if the quantity you ate is smaller, equal or bigger than the correspondent standard portion.

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Interview Process

3rd phase) *(If people don't agree, we thank for their time and we try another one)*
In order to ensure that you really understood our explanation and you agree to participate voluntarily, we need that you sign this informed consent. We will sign two papers, one for you and other for the research team.
Can we do it now?

4th phase) *(If there is no chance to start in that time the interview, you can schedule for another time in that day)*
Let's start with the General questionnaire and then we apply the FFQ.

Here we explain that we are asking for these information in order to characterize our sample, but no data will be connect with the person, due to anonymity.

We need to explain again the objective of this questionnaire, reinforcing that is for individual consumption. Remember that the standard portion is always referent to edible portions. It is important to explain that the mentioned amount should be the consumed quantity (not what they have bought or cooked).

5th phase) Thank you very much for your participation and for your time.

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PORTO
Thank you
for your attention
Catarina: cvreal@porto.ucp.pt | 0712 392 036
Prof. Elisabete Pinto: epinto@porto.ucp.pt

Appendix J – General Questionnaire, used in the main survey.

GENERAL QUESTIONNAIRE

Interviewer's Name

Code |__|__| |0|
5Sex Female |__| Male |__|
Age |__|__| years-old
Education level Primary |__| Tertiary |__| Secondary |__| None |__|
Occupation/Profession Student/Unemployed |__| Formally employed |__|
Self-employed |__| Casual labourer |__|

Marital Status Single |__| Married |__| Widow |__|
Household Number |__|__| people, which |__|__| are children
Anthropometric Data Weight |__|__|,|__| kg Height |__|,|__|__| m/ft
Lifestyle Habits

- Do you smoke? Yes |__| No |__|
- Do you usually consume alcoholic beverages? Yes |__| No |__|
- How many meals do you eat per day? |__|__|
- Do you practice physical activity? If so, which type of exercise and how often do you do it?

Exercise

Frequency (times per week)

Duration (minutes)

Health Status
Do you have any chronic disease, such as:

	Yes	No	
Diabetes	|__|	|__|	
Dyslipidaemia	|__|	|__|	
Hypertension	|__|	|__|	
Coronary disease	|__|	|__|	
Heart failure	|__|	|__|	
Other heart disease	|__|	|__|	If yes, what?
Stroke	|__|	|__|	
Respiratory diseases	|__|	|__|	If yes, what?
Allergies	|__|	|__|	If yes, what?
Cancer	|__|	|__|	If yes, what?
Others	|__|	|__|	If yes, what?

In the last year how often did you go to the doctor in non-urgent medical appointment?

Appendix K – Questionnaires used for the sensory analysis of the fermented yoghurt-like beverage.

QUESTIONÁRIO PARA AVALIAÇÃO SENSORIAL DE *SMOOTHIE* FERMENTADO

Muito obrigada por participar nesta prova de "SMOOTHIES" fermentados com propriedades funcionais que se enquadra no âmbito do doutoramento em Biotecnologia e do Programa de Mentorado Comendador Arménio Miranda *by Frulact Academy*.

O *smoothie* tem como base cereal integral (isento em glúten), o qual foi fermentado com bactérias lácticas e aromatizado com quatro diferentes aromas separadamente: café, chocolate, avelã e mirtilo.

Esta prova é dividida em duas fases:

1) Avaliação do produto não aromatizado;
2) Avaliação de 4 amostras com diferentes aromas sem e com iogurte.

Prove as amostras na ordem que lhe é apresentada e entre cada uma beba água.

Caso tenha alguma alergia/intolerância a algum dos ingredientes, por favor não faça a prova. É importante que responda com a maior sinceridade e seriedade às questões que se seguem. As respostas serão apenas utilizadas para efeitos de pesquisa. Não hesite em solicitar ajuda se tiver alguma dúvida ou se precisar de qualquer tipo de assistência.

Muito obrigada.

Género: Feminino ☐ Masculino ☐

Idade: _____

Profissão:

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SMOOTHIE NÃO AROMATIZADO

Por favor prove o *smoothie* e classifique-o em relação aos seguintes parâmetros:

	Aparência Global	Cor	Consistência	Textura
Gosto Extremamente	☐	☐	☐	☐
Gosto Muito	☐	☐	☐	☐
Gosto	☐	☐	☐	☐
Gosto Ligeiramente	☐	☐	☐	☐
Não Gosto Nem Desgosto	☐	☐	☐	☐
Desgosto Ligeiramente	☐	☐	☐	☐
Não Gosto	☐	☐	☐	☐
Desgosto Muito	☐	☐	☐	☐
Desgosto Extremamente	☐	☐	☐	☐

Caso não tenha gostado da consistência ou da textura, diga porquê:

_____

_____

_____

_____

SMOOTHIE COM AROMA DE CAFÉ/MIRTILO/CHOCOLATE/AVELÃ

Por favor prove o *smoothie* e indique ☒ o quanto gostou ou não do produto, globalmente:

☐ - Gosto extremamente
☐ - Gosto muito
☐ - Gosto
☐ - Gosto ligeiramente
☐ - Não gosto nem desgosto
☐ - Desgosto ligeiramente
☐ - Não gosto
☐ - Desgosto muito
☐ - Desgosto extremamente

Dê-nos, agora, a sua opinião ☒ relativamente à doçura e acidez do *smoothie:*

	Insuficiente		Como gosto		Excessivo
Doçura	☐	☐	☐	☐	☐
Acidez	☐	☐	☐	☐	☐

Por favor avalie a qualidade do aroma a café:

☐ – Muito má (não sabe/cheira a café)
☐
☐
☐
☐ – Muito boa (sabe/cheira a café)

Classifique ☒ a sua opinião relativamente ao sabor do *smoothie*:

☐ - Gosto extremamente
☐ - Gosto muito
☐ - Gosto
☐ - Gosto ligeiramente
☐ - Não gosto nem desgosto
☐ - Desgosto ligeiramente
☐ - Não gosto
☐ - Desgosto muito
☐ - Desgosto extremamente

Finalmente, misture com a colher o *smoothie* com iogurte, prove e indique ☒ o quanto gostou ou não do produto:

☐ - Gosto extremamente
☐ - Gosto muito
☐ - Gosto
☐ - Gosto ligeiramente
☐ - Não gosto nem desgosto
☐ - Desgosto ligeiramente
☐ - Não gosto
☐ - Desgosto muito
☐ - Desgosto extremamente

Por favor, responda às últimas duas questões, independentemente do aroma do produto.

Se encontrasse este *smoothie* no mercado a um preço conveniente, comprá-lo-ia?

☐ – Certamente não compraria

☐

☐

☐

☐ – Certamente compraria

Caso o *smoothie* fosse conjugado com iogurte e mantivesse um preço conveniente, comprá-lo-ia?

☐ – Certamente não compraria

☐

☐

☐

☐ – Certamente compraria

Associa este produto a algum produto já existente no mercado?

☐ - Não

☐ - Sim

Se sim, qual? _____

Comentários ou observações:

_____
_____
_____
_____
_____
_____

Muito Obrigado pela participação ☺

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Appendix L – Statement of consent, used for the fermentability study (stool donation).

FORMULÁRIO DE CONSENTIMENTO INFORMADO

Título do projecto de investigação: Dietary fibre intake and tailored fermentation toward the development of functional cereal fibre-rich food products: bridge between Africa and Europe	
Nome do investigador: Catarina Pereira de Melo Vila Real	
A completar pelo participante 1. Leu a ficha informativa deste estudo?	SIM / NÃO
2. Se colocou questões, recebeu respostas adequadas?	SIM / NÃO / Não aplicável
3. Compreende que é livre de abandonar este estudo, sem necessidade de dar uma justificação?	SIM / NÃO
4. Aceita participar neste estudo?	SIM / NÃO
Assinatura (participante):	Data:
Nome do participante (letras maiúsculas):	
Assinatura do investigador:	Data:

Este projecto é supervisionado por: Prof. Dra. Ana Gomes/ Prof. Dra Elisabete Pinto
Contactos: Escola Superior de Biotecnologia da Universidade Católica Portuguesa do Porto (ESB-UCP); +351 916752200 (Catarina Vila Real)

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Appendix M – Fermentability study information and Instructions for Stool collection.

KIT Doação de fezes

O presente kit é composto por:

- Formulário de consentimento informado;
- Informação do estudo, critérios de elegibilidade e instruções;
- 1 Caixa de vácuo;
- 1 Saco de plástico;
- 1 saqueta de anaerobiose;
- 1 par de luvas;
- 1 rolo de película aderente;
- 2 elásticos de borracha.

Informações do estudo

O presente estudo tem como objectivo avaliar o efeito prebiótico de matrizes alimentares (cereais integrais fermentados) na microbiota intestinal humana. Para isso, após simulção das condições gastrointestinais, proceder-se-à a fermentação *in vitro* de fezes humanas com as matrizes alimentares selecionadas. Por fim, será avaliado o crescimento de determinados grupos bacterianos, bem como alterações na composição da microbiota.

Critérios de elegibilidade

Os participantes no presente estudo devem preencher os seguintes requisitos:

- Ser saudável;
- Ter entre 18 e 40 anos de idade;
- Não ter intolerâncias/alergias alimentares;
- Não ter ingerido suplementos prebioticos, probioticos (incluindo “iogurtes com bifidus”) ou antibióticos nos últimos 6 meses;

Instruções para colheita

Na colheita de fezes, é importante ter em consideração que a urina, a água ou o papel higiénico podem contaminar a amostra. Assim, este procedimento deve seguir as seguintes etapas:

1. Calçar as luvas;
2. Levantar ao tampa e o aro da sanita, e cobri-la com película aderente, deixando uma folga, para a amostra assentar;
3. Baixar o aro da sanita, e defecar em cima da película aderente;

4. Segurando nas bordas da película, levantar o aro da sanita e colocar a película com a amostra dentro do saco de plástico;
5. Colocar o saco de plástico, as luvas dentro da caixa de vácuo;
6. Abrir a embalagem da saqueta de anaerobiose e colocá-la dentro da caixa de vácuo;
7. Fechar a caixa de vácuo, colocando o elástico à sua volta;
8. Colocar a caixa de vácuo e o rolo de película aderente dentro do saco de plástico.

ANNEXES

Annex I – NZYTech gDNA isolation protocol

nzytech
genes & enzymes

NZY Tissue gDNA Isolation kit

Catalogue numbers:

MB13502, 50 columns
MB13503, 200 columns

Description

NZY Tissue gDNA Isolation kits are designed for the simple and rapid small-scale preparation of highly pure genomic DNA from a variety of sample sources including animal cells and tissues, Gram-positive and Gram-negative bacteria, mouse tails, yeast, forensic samples and clinical samples. The method is spin column silica-based and requires no phenol or chloroform extraction. This kit uses optimized lysis buffers containing Proteinase K and SDS to release DNA from cells. After preparing the lysate, DNA is selectively absorbed into the NZYSpin Tissue Column and others impurities such as proteins and salts are removed during the washing steps. The eluted genomic DNA has a $A_{260/280}$ ratio between 1.7 and 1.9 what makes it ready to use in applications like sequencing, PCR, multiplex-PCR, genotyping and a wide range of other enzymatic manipulations.

The NZY Tissue gDNA Isolation kit is optimized to isolate up to 35 µg of DNA from up to 25 mg of tissue samples or 10^7 cells. We suggest not using more than the recommended starting material to prevent reduction in yield and purity of DNA isolated. For samples with very high RNA and protein contents (e.g. liver or spleen tissues), use only up to 15 mg of the sample. This kit is suitable for isolation of DNA from human or animal blood.

Storage conditions and reagents preparation

All kit components can be stored at room temperature (20-25 °C) and are stable till the expiry date. Before use, add 1.35 mL (MB13502/3) of Proteinase buffer to each vial of Proteinase K and vortex. Proteinase K solution is stable at -20 °C for up to 6 months. Add 28 mL (MB13502) or 100 mL (MB13503) of ethanol (96-100%) to each bottle of buffer NW2. Buffers NL and NW1 contain guanidine hydrochloride. Wear gloves and goggles when using this kit.

System Components

Component	50 columns	200 columns
Buffer NT1	20 mL	80 mL
Buffer NL	15 mL	60 mL
Buffer NW1	30 mL	120 mL
Buffer NW2 (concentrate)	2 x 7 mL	2 x 25 mL
Buffer NE	15 mL	60 mL
Proteinase K (lyophilized)	30 mg	4 x 30 mg
Proteinase buffer	1.8 mL	7 mL
NZYSpin Tissue columns (light green ring)	50	200
Collection tubes (2 mL)	100	400

Standard protocol for isolating genomic DNA from animal tissues, cultured cells and bacteria cells

1. Sample preparation

Animal Tissues: Cut up to 25 mg tissue sample into small pieces, and place it in a microcentrifuge tube. Proceed with step 2.

Notes: *Tissue samples can be ground under liquid nitrogen for more efficient lysis.*
For rodent tails, place one (for rat) or two (for mouse) 0.6 cm-long pieces in a 1.5 mL tube.

Cultured Cells: Re-suspend up to 10^7 cells in 200 µL Buffer NT1. Add 25 µL Proteinase K solution and 200 µL Buffer NL*. Mix thoroughly by vortex, and incubate at 56 °C for 10-15 min. Vortex occasionally during incubation. Proceed with step 5.

**Mix Buffer NL thoroughly by shaking before use.*

Bacteria Cells: Pellet up to 1 mL bacteria culture for 5 min at 8,000 x*g*. Discard supernatant. Re-suspend cell pellet in 180 µL Buffer NT1 by pipetting up and down. Add 25 µL Proteinase K solution and vortex vigorously. Incubate at 56 °C for 1-3 hours. Mix occasionally during incubation. Proceed with step 3.

For other sample sources see the support procedures available in https://www.nzytech.com/products-services/kits-genomic-dna-purification/mb135/.

2. Pre-lysis of sample

Add 180 µL of Buffer NT1 and 25 µL Proteinase K solution to the sample. Mix thoroughly by vortex. Incubate at 56 °C for 1-3 hours and vortex occasionally during incubation.

Note: *Samples that are difficult to lyse can be incubated overnight as well*.

3. Removal of RNA (optional)

If RNA-free DNA is required, add 10 µL of RNase A (40 mg/mL) solution (not included, available as MB18701 – NZY RNAse A, 100 mg) to each sample. Mix and incubate for 5 min at room temperature.

4. Lysis of sample

Vortex the sample. Add 200 µL Buffer NL* to the sample, and mix by vortex for 10 seconds.

Notes: *If insoluble particles are visible, centrifuge for 5 min at full speed and transfer the supernatant to a new microcentrifuge tube. *Mix Buffer NL thoroughly by shaking before use.*

5. Addition of ethanol

Add 210 µL of 100% ethanol to the sample and mix immediately by vortex.

6. DNA binding

Transfer the mixture from step 5 into a NZYSpin Tissue Column placed in a 2 mL collection tube. Centrifuge for 1 min at > 11,000 x*g*. Discard flow-through and place the column in a new collection tube.

7. Wash silica membrane

Add 500 µL of Buffer NW1 to the column. Centrifuge for 1 min at > 11,000 x*g*. Discard flow-through and place the column back into the collection tube.

Add 600 µL of Buffer NW2 (make sure ethanol was previously added) to the column, and centrifuge for 1 min at > 11,000 x*g*. Discard flow-through.

Note: *For isolations of viral DNA from stool, we recommend to repeat the wash silica membrane step with Buffer NW2*. Add more 600 µL of Buffer NW2 to the column, and centrifuge for 1 min at > 11,000 x*g*. Discard flow-through.

8. Dry silica membrane

Place the NZYSpin Tissue Column back into the collection tube and centrifuge for 2 min at > 11,000 x*g*.

9. Elute DNA

Place the column into a clean microcentrifuge tube and add 100 µL of Buffer NE, TE buffer or sterile water (preheating of elution buffer to 70 °C may improve yield) directly in the membrane column. Incubate 1 min at room temperature and centrifuge at >11,000 x*g* for 2 min to elute DNA. The genomic DNA can be stored at 4 °C or -20 °C.

Data:

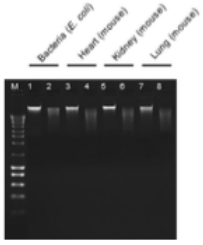

Fig. 1. Genomic DNA from various sources isolated with NZY Tissue gDNA Isolation kit. 0.25 µg of each isolated DNA was analysed on a 0.8% agarose gel. Lanes 2, 4, 6 and 8: gDNA digested with *Eco*R I. Lanes 1, 3, 5 and 7: undigested gDNA. M: NZYDNA Ladder III (MB0440); 1, 2: gDNA from bacteria (*E. coli*); 3, 4: gDNA from mouse heart; 5, 6: gDNA from mouse kidney; 7, 8: gDNA from mouse lung.

Quality control assay

Functional assay

All components of NZY Tissue gDNA Isolation kit are tested following the isolation protocol described above. The purification system must isolate 25-35 µg of gDNA/column.

V1902

Certificate of Analysis

Test	Result
Functional assay	Pass

Approved by:

Patrícia Ponte
Senior Manager, Quality Systems

For research use only

nzytech
genes & enzymes

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