CATÓLICA
ESCOLA SUPERIOR DE BIOTECNOLOGIA
PORTO

EVALUATION OF THE PREBIOTIC POTENTIAL OF AKPAN - A CEREAL-BASED FERMENTED PRODUCT AND A BY-PRODUCT OF CASSAVA BEER

by

Miguel Fernando Ribeiro Pereira

[November 2017]

CATÓLICA
ESCOLA SUPERIOR DE BIOTECNOLOGIA
PORTO

EVALUATION OF THE PREBIOTIC POTENTIAL OF AKPAN - A CEREAL-BASED FERMENTED PRODUCT AND A BY-PRODUCT OF CASSAVA

AVALIAÇÃO DO POTENCIAL PREBIÓTICO DE AKPAN - PRODUTO FERMENTADO À BASE DE CEREAIS E UM SUB-PRODUTO DE CERVEJA DE MANDIOCA

Thesis presented to *Escola Superior de Biotecnologia* of the *Universidade Católica Portuguesa* to fulfill the requirements of Master of Science degree in Applied Microbiology

by

Miguel Fernando Ribeiro Pereira

Place: CBQF/ Escola Superior de Biotecnologia da Universidade Católica Portuguesa

Supervision: Professor Doutora Maria Manuela Pintado
Co-supervision: Doutora Beatriz Gullón

[November 2017]

"Science knows no country, because knowledge belongs to humanity,

and is the torch which illuminates the world."

Louis Pasteur

"The most beautiful thing we can experience is the mysterious.

It is the source of all true art and science."

Albert Einstein

Resumo

Os alimentos fermentados à base de cereais são amplamente consumidos em todo o Mundo, especialmente em muitos países africanos. Para além dos nutrientes tradicionais estes alimentos contêm compostos que promovem a saúde. O Akpan é um produto tradicional Africano fermentado à base de amido de cereais, milho e/ou sorgo, e consumido como bebida refrescante no Benin.

Hoje em dia, as principais cervejeiras de África substituem parcialmente a cevada importada por outras culturas locais, porque são mais baratas e são produzidas localmente. Devido ao alto teor em hidratos de carbono e amido na mandioca, esta cultura é uma alternativa adequada à indústria cervejeira. Durante o processo de produção de cerveja de mandioca, são gerados subprodutos, e um deles é o resíduo de mandioca (BSC), um material lignocelulósico rico em fibras e proteínas.

Atualmente, a importância da microbiota intestinal humana na manutenção da saúde do hospedeiro é bem conhecida. Uma das estratégias para estimular a proliferação de bactérias intestinais benéficas é o consumo de prebióticos. Atualmente, existem vários hidratos de carbono prebióticos no mercado, mas há um interesse crescente no desenvolvimento de novos produtos. Assim, este trabalho teve como objetivo avaliar, *in vitro*, o potencial prebiótico do Akpan e do BSG.

Para atingir este objetivo, foi realizada uma fermentação *in vitro* de Akpan e BSG utilizando como inóculo, estirpes probióticas (*Lactobacillus casei* L431, *Lactobacillus casei* L01, *Bifidobacterium lactis* B94 e *Bifidobacterium animalis* Bb12) e amostras fecais de três dadores saudáveis. Neste último caso, a atividade prebiótica foi avaliada através da quantificação da produção de ácidos gordos de cadeia curta (SCFA), a evolução do valor de pH e a dinâmica da população bacteriana por hibridação fluorescente *in situ* (FISH). Os resultados demonstraram que o Akpan e o BSC foram metabolizados após 44 h de fermentação, exercendo um efeito prebiótico, semelhante ao observado para os frutooligosacarídeos (FOS). Todos os grupos bacterianos considerados aumentaram significativamente após a fermentação de Akpan, BSC e FOS, contudo, observou-se que o Akpan e o BSC promoveram um efeito mais pronunciado no crescimento de bifidobactérias e lactobacilos do que FOS. Sob as condições testadas, verificou-se que o BSC origina uma maior produção de SCFA, mas não foram observadas diferenças significativas entre a concentração de SCFA produzida no Akpan e FOS, porém o perfil de SCFA foi diferente.

Os resultados obtidos contribuem para suportar a utilização de Akpan como alimento funcional e BSC como um potencial ingrediente funcional com potenciais efeitos benéficos na saúde gastrointestinal.

Palavras chave: Akpan, Cereais, Alimentos Funcionais, Prebióticos, Resíduo de Mandioca

Abstract

Cereal-based fermented foods are widely consumed worldwide, especially in many African countries. Generally, these contain health-promoting compounds beyond traditional nutrients. Akpan is a traditional fermented product made from cereal starch, maize and/or sorghum, and consumed as a thirst-quenching beverage in Benin.

Nowadays, all major brewers in Africa partially replace imported barley by other local crops, because they are cheaper and close produced. Due to the high content in carbohydrates and starch in cassava, this crop is a suitable alternative in brewing industry. During the brewing process by-products are generated, and one of these is brewers' spent cassava (BSC), a lignocellulosic material rich in fibre and in protein.

Currently, the importance of human intestinal microbiota in maintaining the host health is well-known. One of the strategies to stimulate the proliferation of beneficial intestinal bacteria is the consumption of prebiotics. Currently, there is a range of prebiotic carbohydrates on the market, but there is an increasing interest in the development of new prebiotics. Thus, this work aimed to evaluate, *in vitro*, prebiotic potential of Akpan and BSG.

In order to accomplish this objective an *in vitro* fermentation of Akpan and BSG using as inoculum single probiotic strains (*Lactobacillus casei* L431, *Lactobacillus casei* L01, *Bifidobacterium lactis* B94 and *Bifidobacterium animalis* Bb12) and faecal samples from three healthy donors was performed. In this latter case, the prebiotic activity was assessed through the quantification of short chain fatty acid (SCFA) production, the evolution of the pH value and the dynamic bacterial population by fluorescent *in situ* hybridization (FISH). Results demonstrated that Akpan and BSC was metabolized after 44 h of fermentation, exerting a prebiotic effect, similar to that observed for fructooligosaccharides (FOS). All the considered bacterial groups significantly increased after Akpan, BSC and FOS fermentation, however it was observed that Akpan and BSC promoted a stronger effect on the growth of bifidobacteria and lactobacilli than FOS. Under the tested conditions, it was observed that BSC have a higher production of SCFA, while no significant differences between the SCFA concentration produced by Akpan and FOS were observed, however the SCFA profile was different.

The results obtained contribute to support the utilization of Akpan as a functional food and BSC as a potential functional ingredient with potential beneficial effects on gastrointestinal health.

Keywords: Akpan, Cereal, Functional Food, Prebiotic, Brewers' Spent Cassava

Acknowledgements

I would like to recognise the important role played by Escola Superior de Biotecnologia not only in my education but also in my interest in scientific research meanwhile opening my horizons to new points of view. It is also important to underline the fact that Escola Superior de Biotecnologia gave me opportunities, allowing me to pursue different projects that enriched my path no only as a scientist but as a person.

First of all, I would like to thank to my supervisor, Professor Manuela Pintado for the encouragement during this troubled journey and for believing in my capacities and in myself even when I didn't. I also appreciate the patience that Professor Manuela had with me.

I want to thank my co-supervisor Beatriz Gullón for her unconditional support, for always being available to help me even at late hours, for keeping me motivated and not letting me sink when this journey wasn't going well and for being a shelter. I have to thank you for everything.

I also want to thank the Department of Chemical Engineering, Faculty of Science, University of Vigo (Campus Ourense) where the *in vitro* fermentation assays with human faecal microbiota were performed.

I want to use this opportunity to thank my dear laboratory friends. I will start with the one that I've known the longest, Débora Campos, thank you for making me laugh, being so patience with me, protecting me and never allowing me to forget my goal. You know that for me our friendship is very important, and I will always be here when you need. Joana Odila you were the person that heard me most because you were so patience, so available to spend time with me in long conversations about everything, diners, coffees, thanks for becoming the older sister I never had, and allowing me to be an uncle. José Soares I also thank to you for allowing me to be an uncle and for being so patient and a good friend. Ezequiel Coscueta, you came from Argentina and presented me with a little of Argentinian culture and way of life, but at after all you ended up becoming Portuguese, thank you for everything. Sérgio Sousa thanks for all the support and help that you gave me and for the companionship during the long days that we shared in the laboratory. Manuela Amorim and Ana Oliveira thanks for always reminding me of the thesis, and for caring about me. Pedro Castro, you were the last to enter the brotherhood,

but you arrived in time to cheer, even more, our days and became a good friend, thank you for everything.

I also want to thank my other friends, Eugénia, Luísa, Victor, Luís for always pushing me forward. Vasco, Luisa, Ana, you know that as “Legs” you have the function to support, and you did, so thanks for that. João, with you I passed good moments and bad moments, but there were good moments that I will always remember and be proud. Carla during this journey you had an important project and you wanted me to be by your side, thanks for letting me be part of it even with my time restrictions.

I would like to thank my parents, the most important persons in my life, thanks for the sacrifice, effort, commitment that you made, that allowed me to achieve my goals. I want to apologise for the times that I wasn’t well. These Thesis is for you. Thanks for everything that you made and we know that all of it was worth it.

I also want to thank to my aunts for being my mother’s all these years, thanks for everything that you made for me. Luis, you know that we are brothers of different parents but during all these years you have been my brother, and were always here to protect and support me, thanks for everything. I could not pass this opportunity to thank destiny for giving me two lovely dogs, that completely change my life, thanks Nuty and Goa for being my companions in “loneliness”.

And finally, to my dear girlfriend, Paloma Matias, you were my safe house, the person that was always there supporting me, always pushing me forward, you have always believed in me even when I doubted. You are my anchor, my soulmate, my passion, my life. Thanks for making my life better and happier, thanks for everything.

Table of contents

Figure Index

1. Introduction ……...…….

3. Results and discussion ……

Table Index

1. Introduction ……

3. Results and discussion ……

List of Abbreviations

AXOS …… Arabinoxylooligosaccharides

BSC …… Brewers' spent cassava

BSCF …… Brewers' spent cassava flour

BSG …… Brewers' spent grain

CFU …… Colony forming units

CIRAD …… Centre de Coopération Internationale en Recherche Agronomique pour le Développement

DAPI …… 4',6-diamidino-2-phenylindole

FI …… Faecal inoculum

FISH …… Fluorescent *in situ* hybridization

FOS …… Fructooligosaccharides

GI …… Gastrointestinal

GOS …… Galactooligosaccharides

GRAS …… Generally recognized as safe

HPLC …… High performance liquid chromatography

MRS …… de Man, Rogosa and Sharpe

MWCO …… Molecular weight cut-off

OD …… Optical density

PCR …… Polymerase chain reaction

RI …… Refractive index

RPS …… Reduced physiological salt solution

SCFA …… Short chain fatty acids

SPSS …… Statistical package for the social science

TSB …… Trypticase soya broth

YNB ……Yeast nitrogen base

1. Introduction

In the world dietary culture there are three distinct traditional food habits based on cereal diets: (1) cooked-rice eaters of Eastern food culture, (2) wheat/barley-based breads/loves of Western and Australian food culture, and (3) sorghum/maize porridges of African and South American food culture. In the diet of numerous societies worldwide, traditional fermented foods still play a major role. The African dietary culture includes both fermented and non-fermented sorghum, maize, millet and cassava products, wild legume seeds and tubers, but also meat, milk products, and alcoholic beverages. Low-cost food processing procedures and, where possible, "low-tech" have an importance to all rural societies making them also affordable for the poor (Franz *et al.*, 2014).

1.1. Akpan

In developing countries street foods, Akpan plays an important role in the catering business, particularly in urban areas. Among the variety of products usually referred to as street food, traditional yoghurt-like beverages made from cereals play an important role in the diet of local populations. Production of cereals contributes to over 60% of world food production, providing dietary fibre, proteins, energy, minerals, and vitamins required for human health. In many African countries is widespread the use of cereal grains as a source of fermented beverages for human consumption (Akissoé *et al.*, 2014; Sacca *et al.*, 2012).

In the diet of Beninese traditional cereal beverages, such as Akpan, have high importance. Akpan is a starchy fermented cereal beverage, prepared from Ogi, named vegetable milk or non-dairy yoghurt-like product that is consumed mixed with milk, sugar and ice becoming a thirst-quenching beverage consumed throughout the year. This is the reason why in the local markets there is a high demand, especially during the dry season, for the product, that is ready-to-serve beverage, being so popular and widely consumed. (Akissoé *et al.*, 2014; Sacca *et al.*, 2012).

1.1.1. Processing of Akpan

1.1.1.1. Raw materials

Akpan traditionally is prepared from Ogi, a gruel obtained by fermentation of wet-

milled cereal, commonly maize, although sorghum or millet are also employed as substrate for fermentation (Nago *et al.*, 1998; Sacca *et al.*, 2012). Ogi is consumed as a cooked porridge, which in West Africa is considered the most important weaning food for infants although for adults it is an important cereal breakfast (Blandino *et al.*, 2003; Sacca *et al.*, 2012).

The most common raw materials in Akpan production include maize (*Zea mays*) and sorghum (*Sorghum vulgare*), singly or combined, although the maize is the preferred grain used, follow by sorghum and a mixture of maize and sorghum. According Sacca *et al.*, (2012) the most important quality criterion of the raw material for Akpan production is the colour. In the case of maize-based Akpan producers/sellers, 98% prefer to use white maize varieties whereas for the sorghum-based Akpan producer/sellers all prefer the red sorghum varieties (Sacca *et al.*, 2012). Other requirements for Akpan raw materials include the need of grains be mainly free from weevils and stones and well dried. In order to produce higher starch yields the grains must be floury (Sacca *et al.*, 2012).

1.1.1.2. Production of Akpan

The processing technology of Akpan based on the Ogi process is long-established however, in recent years' new forms of Akpan have been developed. In these new forms there are slight variations in processing technology and raw materials used, namely fermented kneaded sorghum and/or maize flour (Sacca *et al.*, 2012).

According to Sacca *et al.*, (2012), independently of the raw materials, in Benin there are two principal processing technologies to produce Akpan: Akpan made from Ogi, using submerged fermentation or Akpan made from kneaded flour, using solid-state fermentation (**Figure 1.1**), however Akpan from Ogi are the predominant technology.

In Akpan prepared from Ogi mash, the grains are cleaned of dust, sand, and plant debris. The following step, steeping the grains can be done by three different procedures: the cold procedure, in which the grains are steeped for 3 days in cold water (steeping liquor is changed every day); the second procedure is the "Fon" method, in which maize grains are steeped in water at 85 ºC for 24 h; and the mostly used in Benin, the "Goun" procedure, in which grains are cooked in boiling water for 10 minutes and then steeped at ambient temperature for 12-48 h (Nago *et al.*, 1998). Despite the use of hot or warm

water, the purpose is to enhance the softness of the kernels and to reduce the steeping time (Sacca *et al.*, 2012). Then the grains are wet milled and wet sieved, to separate flour from bran, using a muslin cloth, and let decanting for 15 minutes, followed by submerged fermentation for 24-72 h.

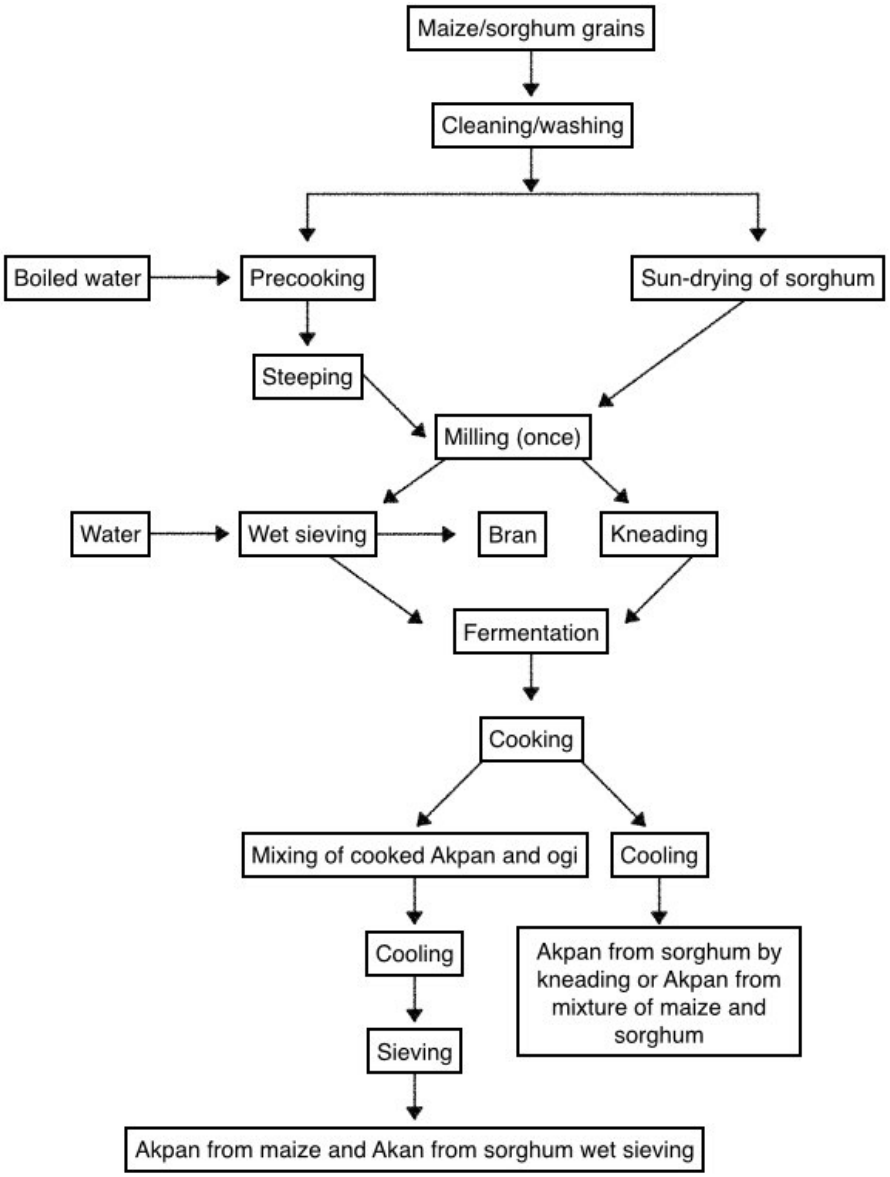

Figure 1.1: Diagram of Akpan processing (Sacca *et al.*, 2012).

The native microorganisms responsible for the fermentation are lactic acid bacteria, yeast and moulds, however the *Lactobacillus plantarum* is the predominant microorganism and the predominant responsible for lactic acid production. Besides lactic acid bacteria, the others bacteria's involved in the fermentation are *Aerobacter* and *Corynebacterium* species the main responsible for maize starch hydrolysis. The main

yeasts present in the fermentation are *Rhodotorula spp.*, *Candida mycoderma* and *Saccharomyces cerevisiae*, and the last two are involved in the flavour development. The moulds present in the fermentation are *Fusarium*, *Aspergillus*, *Penicillium* and *Cephalosporium* (Blandino *et al.*, 2003; Osungbaro, 2009).

Sacca *et al.*, (2012) reported two main procedures for Akpan production: in the first procedure, the Ogi mash is made into slurry and it is slightly cooked, with low levels of gelatinized starch. In the second way, Ogi mash is split into two parts: one part is made into slurry, cooked, and then mixed with the raw Ogi (uncooked part). At the end a free flowing gruel is obtained as final product, which can be consumed plain or adding sugar, milk and ice.

The procedure to produce Akpan from kneaded flour involves the grains being cleaned as described above and washed and dried before milling. Thereafter, the flour is kneaded with tap water and undergoes fermentation for 18-24 h. The obtained dough is made into slurry and precooked into gruel, which is treated as described above (Sacca *et al.*, 2012).

1.2. Brewer's spent cassava

1.2.1. Cassava

Cassava (*Manihot esculenta,* Crantz) also known as *mandioca* is historically native from Latin America and the Caribean and was taken to Africa and Asia by Portuguese traders in the sixteenth century (Ford 2015; Rosemary *et al.*, 2013).

Cassava is one of the dominant food and feed plants in the world and is a key staple root crop in many tropical and subtropical developing countries, mainly in West Africa (Montagnac *et al.*, 2009b). Between 1994 and 2004 the production of cassava increased about 65%, as show the **Figure 1.2**, which represents more 100 million tons, achieving 270 million tons in 2014.

Most of the cassava production is located in three regions, West Africa, and Congo Basin, tropical South America, and South East Asia (Ukwuru and Egbonu, 2013), wherein Africa, particularly in Nigeria, Democratic Republic of the Congo and Ghana, undertake a major role in the global production of Cassava (Tewe and Lutaladio, 2004)

as we can see in **Figure 1.3**.

Figure 1.2: Quantities of Cassava production in the World between 1994 and 2014.

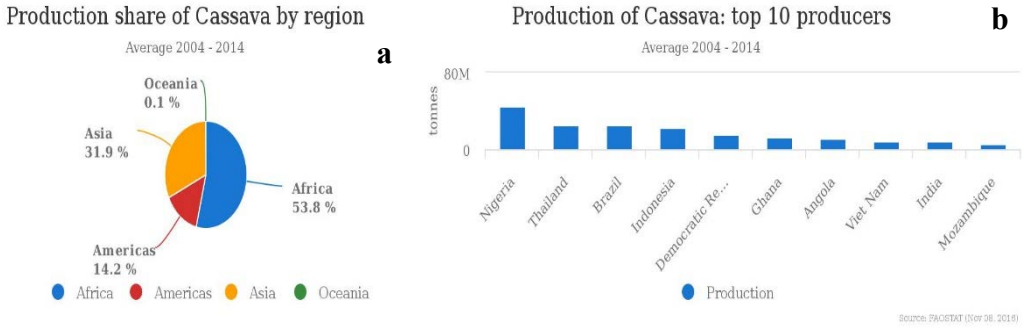

Figure 1.3: a) Production of Cassava by Continents between 2004 - 2014; b) Top 10 countries producers of Cassava between 2004 and 2014.

Cassava as a food crop own important characteristics which that make it an attractive crop because it requires low input of labour to produce more carbohydrates per hectare than the main cereal crops with lower cost of production and its available all year round (Montagnac *et al.*, 2009a; Rosemary *et al.*, 2013; Ukwuru and Egbonu, 2013). Cassava is also a very hardy crop that can grow in a wide range of conditions, it is tolerant to soil infertility, and is resistant to drought, pests and diseases. Beyond these characteristics, cassava tubers are a great source of energy that can be stored underground, prior to harvest, up to two years without water and still retain nutritional value, that make

cassava a strategic famine crop (Montagnac *et al.*, 2009b; Rosemary *et al.*, 2013; Ukwuru and Egbonu, 2013).

1.2.1.1. Nutritional value of cassava

Cassava plant has two nutritionally valuable parts, roots and leaves, which constitute, respectively, 50 and 6% of the mature cassava plant (Tewe and Lutaladio, 2004). Cassava root is a physiological energy reserve with high carbohydrate content which varies from 80 to 90% on a dry weight (Manz *et al.*, 1996) basis. Starch represents 80% of the carbohydrates present (Gil and Buitrago, 2002); 83% is in form of amylopectin and 17% is amylose (Rawel and Kroll, 2003). The protein content in cassava roots is low at 1 to 3% on a dry matter basis (Buitrago, 1990). About 50% of the crude protein in the roots corresponds to whole protein and the other 50% represents free amino acids (mainly aspartic and glutamic acids) and non-protein components such as nitrite, nitrate and cyanogenic compounds. Cassava roots exhibit a deficit of some essential amino acids, such as methionine, cysteine and tryptophan whereas the roots contain an excess of arginine, glutamic and aspartic acid (Buitrago, 1990). The lipid content in cassava roots ranges from 0,2 to 0,6% on a fresh weight basis. The extractable lipids are mainly polar in nature, with the galactosyl/ diglycerides being the principal group (Tewe and Lutaladio, 2004). The fiber content in cassava roots does not exceed 1,5% in fresh root and 4% in root flour (Gil and Buitrago, 2002).

1.2.2. Beer

Beer is a non-distilled alcoholic beverage made from partially germinated cereal grains, generally commonly known as malt. Its origin is related to the Mesopotamians 6000 or 7000 years ago, however northern Europe is linked with the historical development of brewing industry, because the cold conditions inhibited the development of viticulture (Waites *et al.*, 2001; Anderson, 2006).

With an annual world beer production, above 180 million tonnes in 2014 (FAO, 2016). Among alcohol per capita consumption, beer is the second most consumed group, 34,8%, behind spirits 50,1% and in front of wine, 8,0% and other alcoholic beverages, 7,1%. However, “other” beverages are the most popular beverages type in Africa where represent 51,6% of consumption while beer represents 33,7% (WHO, 2014).

Most beer is made from four essential ingredients: (1) Water, (2) fermentable carbohydrates such as barley malt, starch, and sugar adjuncts, (3) hops, and (4) yeast. Among these major groups of ingredients different ingredients can be used and in multiple different combinations (Anderson, 2006).

1.2.2.1. Cassava application in brewing industry

Since 1977, Rajagopal had evaluated the possibility of use cassava in beer production and concluded that is possible to use cassava as source of carbohydrate for beer production. Later, in 1988 the Nigerian government restricted importation of malted barley, forcing the brewing industry to look for alternative sources of fermentable carbohydrates, such other cereals, like sorghum or maize, or locally produced crops like cassava. Nowadays, all major brewers in Africa partially replace imported barley by sorghum and other crops produced locally because they are cheaper, close produced and help to create employment opportunities in cereals and crops plantations (Goode *et al.*, 2002; Rajagopal, 1977; Rosemary *et al.*, 2013). These adjustment to local resources serves as a stimulus for the development of local supply chains that could trigger agricultural production in Africa (van Wijk and Kwakkenbos, 2011).

The use of cassava as local raw material in brewing industry, despite the high content of carbohydrates and starch, can increase employment in rural areas. This provide the opportunity to small scale farmers migrate into cash-crop farming increasing their income, and allow to local farmers being part of the brewery value chain. The use of cassava represents a more affordable cost alternative to imported cereals, and help the industry to reduce costs (Graham, 2015).

1.2.2.2. Brewer's spent grain

The brewing industry inevitably involves generation of various residues and by-products which are generated from the beer-brewing main raw materials, the barley malt, hops and yeast. The most common by-products of the beer-brewing process are spent grains, spent hops and surplus yeast (Mussatto, 2009).

The most abundant by-product in brewing process is spent grains, representing around 85% of the total solid by-products generated, which represents an annual

worldwide production above 34 million tonnes of wet brewers' spent grains (BSG), which is equivalent to ca. 8.5 million tonnes of dry BSG due to its high water content (75-80%) (Xiros and Christakopoulos, 2012). Around 18-20 kg of wet BSG are produced per 100 L of beer (Briggs *et al.*, 2004). BSG is available, in large volumes throughout the year, at a low cost. Until nowadays BSG main utilization has been animal feed (Lynch *et al.*, 2016).

1.2.2.2.1. BSG generation

Brewing process begins adding water to the milled barley malt in a mash tun, and slowly increasing the temperature from 37 to 78 ºC promoting enzymatic hydrolysis of malt constituents. At this stage, the malt starch is converted to fermentable sugars (maltose and maltotriose), non-fermentable sugars (dextrins) and proteins are partially degraded to polypeptides and amino acids. This stage (mashing) originate a sweet liquid known as wort. The insoluble, undegraded malted barley grain components sediment at the mash tun to form a bed in which the wort is filtered through it. The liquid fraction is the medium which will be fermented to produce beer. The solid fraction obtained at these stage is constituted by the spent grains. The **Figure 1.4** represents the brewing process using malted barley, at the end of mashing stage the insoluble part of the adjuncts is separated with the undegraded malted barley grain components (Mussatto *et al.*, 2006).

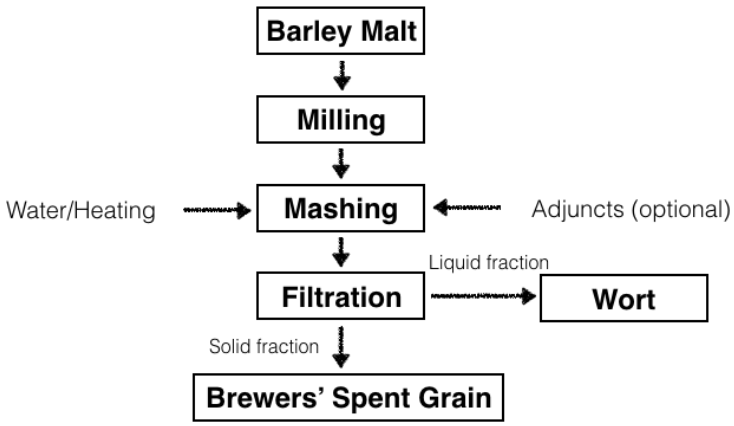

Figure 1.4: Overview of the process to obtain BSG from malted barley (Mussatto *et al.*, 2006).

1.2.2.2.2. BSG potential application as foods ingredient

Nowadays, industry is under great social and political pressure with the goal to reduce pollution from their activities. In that way, companies are modifying their processes in which their residues can be recycled, starting to consider their residues not a waste but a raw material for obtaining other new products (Mussatto *et al.*, 2006).

BSG is a heterogeneous substance, because their compositions varies according to barley variety, time of harvesting, malting and mashing conditions, and the quality and type of adjuncts added during the brewing process (Santos *et al.*, 2003). BSG is basically a lignocellulosic material, which main components are fibre (hemicellulose, cellulose and lignin) and protein. Fractions of hemicellulose and cellulose are composed by sugars, wherein the most representative in BSG are xylose, arabinose and glucose. Around half of the BSG composition on a dry weight basis is represented by sugars (Mussatto, 2014). During the mashing stage, most of the barley starch is removed, leading to a high concentration of protein and fibre in spent grain (Kissell *et al.*, 1979; Mussatto *et al.*, 2006).

As previous referred BSG main utilisation is cattle feed. However, BSG is rich in sugars and protein and are available in large amounts all year at a low price make this a by-product of great interest to evaluate the application different areas (Mussatto, 2014). For a long time has been evaluated the potential application of BSG in the food field, either as animal feed and in human diet, and, more results have been proven that the ingestion of BSG compounds have health benefits (Mussatto, 2014).

1.2.2.2.3. BSG in human diet

Due to the relatively high fibre and protein content in BSG, investigations have been carried out to evaluate the possibility of BSG improve the nutritional value of different foods. Ingestion of BSG provides several health benefits, like accelerated transit time, increased faecal weight and fat excretion, decreased gallstones and reduce plasma cholesterol and postprandial serum glucose levels (Brennan and Cleary, 2005; Fastnaught, 2001). These beneficial effects were attributed to the presence in BSG of glutamine-rich protein, non-cellulosic polysaccharides (arabinoxylan) and soluble dietary fibres including (1 → 3,1 → 4)-β –glucan (McCleary and Nurthen, 1986; Viëtor *et al.*, 1993).

Due to the previous mentioned benefits, various investigation has been performed to evaluate the possibility of use BSG in the manufacture of different food products like bakery products such as breads, biscuits, cookies, muffins, cakes, waffles, pancakes, tortillas, snacks, doughnuts and brownies and frankfurters (Huige, 2006; Mussatto, 2014). **Table 1.1** exhibit examples of studies that have incorporated BSG in new food products. BSG is too granular for direct application, and to be used it must be converted to flour (Mussatto *et al.*, 2006). Incorporation of BSG in foods lead to increases of protein, fibre and amino acids contents while decrease the calorie content of the final product, imparts higher water absorption and lower fat absorption capacities to the final product (Huige, 2006; Kissell *et al.*, 1979; Ktenioudaki *et al.*, 2012; Prentice and D'Appolonia, 1977; Waters *et al.*, 2012). Nevertheless, there are some limitations in the use of BSG flour in human food, due to its flavour and brownish colour, it is only recommended for application in coloured products such as some breads, cookies, cakes, or spaghetti. Furthermore, it is recommended incorporation of small quantities (5-10%) in food formulations to avoid alterations in the flavour and physical properties (e.g. texture) of the final products (Ktenioudaki *et al.*, 2012; Mussatto *et al.*, 2006; Waters *et al.*, 2012).

Table 1.1: Application of BSG in food products

Food Product	Form of incorporated BSG (or component)	Reference
Bread	Oven dried at various temperatures, milled	(Prentice and D'Appolonia, 1977)
Cookies	Oven dried at various temperatures, milled and fractionated into specific flour components	(Kissell *et al.*, 1979)
Extruded snack (chickpea based)	Extruded	(Ainsworth *et al.*, 2007)
Bread (wheat), baker's yeast, kefir, ***Lactobacillus casei*** immobilised on BSG	Untreated	(Plessas *et al.*, 2007)
Ready-to-eat snacks of various bases	Flour	(Stojceska *et al.*, 2008)
Ready-to-eat (extruded) snacks	Dried and milled	(Stojceska *et al.*, 2009)
Frankfurters	Milled and sieved to various fractions	(Özvural *et al.*, 2009)
Breadsticks	Flour	(Ktenioudaki *et al.*, 2012)
Bread (Wheat)	Untreated flour (BSG) Flour	(Waters *et al.*, 2012)

	fermented with *Lactobacillus plantarum*	
Fruit beverages	Hydroxycinnamic acid (phenolic extracts), liquid	(McCarthy *et al.*, 2013)
Dough (Wheat)	Flour	(Ktenioudaki *et al.*, 2013b)
Baked snacks (crispy-slices)	Flour	(Ktenioudaki *et al.*, 2013a)
Bread (wheat)	Untreated flour (BSG)Flour fermented with sourdough starter ± xylanase or dough conditioner	(Ktenioudaki *et al.*, 2015)

1.2.2.3. Brewers' spent cassava

Application of high quality cassava flour in brewing produce by-products called brewers' spent cassava (BSC), which can be an interesting material for application in food and in other industrial purposes (Ha *et al.*, 2014; Omidiran *et al.*, 2016) resembling the BSG. BSC is considered also a lignocellulosic material rich in fibre (ca. 70) and protein (20%) (Ha *et al.*, 2014). When BSC is converted to flour can be a low-cost ingredient as source of fibre and protein (Ha *et al.*, 2014; Omidiran *et al.*, 2016).

Ha *et al.*, (2014) developed a method to use brewers' spent cassava flour (BSCF) to produce a snack and he reported that with 4% of BSCF there is no change in the extruded snack quality. Omidirian (2016) developed a snack including 32% of BSCF to wheat flour, and when compared with a control snack produced only with wheat flour it exhibited higher protein content, lower oil uptake, better crispiness and required less force to break.

1.3. Functional foods

The main function of diet is to supply the required amount of nutrients for the metabolic requirements while offer to the consumer a sense of pleasure and well-being. However, there is nowadays knowledge that support the theory that diet besides fulfil the nutritional requirements, can modulate some physiological functions and in some diseases may play a prejudicial or beneficial role (Koletzko *et al.*, 1998). In the past of nutrition sciences their concepts were focused on survival, hunger satisfaction and prevention of adverse effects, and nowadays the concepts in nutrition are evolving and focusing in the use of foods to promote a state of well-being, improve health and reduce the risk of diseases. The increasing cost of health care, the increase in life expectancy

and the desire for improve quality of life, mainly among the older people, make the nutrition concepts very important (Roberfroid, 2000).

Traditionally, the nutritional profile of populations is characterized by high consumption of sugars, salt, saturated and trans-fatty acids, and low consumption of fibres, vitamins, and essential minerals, which represents one of the main causing problems of non-transmissible chronic-degenerative diseases. Therefore, with the aim of reduce the risk of chronic diseases has been proposed the development of new food products containing biologically active substances (Granato *et al.*, 2010a; Roberfroid, 2002). In the food industry, functional foods are one of the most attractive areas of research and innovation, with an increased popularity and with a higher commercial interest in exploiting the different types of functionality (Annunziata and Vecchio, 2013). The term "functional food", which refers to food products fortified with special constituents that possess advantageous physiological effects, was used for the first time in the 1980s, in Japan (Hardy; Kaur and Das, 2011; Kwak and Jukes, 2001; Stanton *et al.*, 2005). Nowadays, functional food is defined as "natural or processed foods that contains known or unknown biologically-active compounds; which, in defined, effective non-toxic amounts, provide a clinically proven and documented health benefit for the prevention, management, or treatment of chronic disease (Martirosyan and Singh, 2015). In **Table 1.2** are summarised some functional compounds commonly making part of functional foods and the most relevant sources.

Table 1.2: Functional compounds and their sources (IFIC, 2011)

Class/Components	Source
Carotenoids	Carrots; spinach; citrus fruits; tomatoes; broccoli; asparagus; kale; collards
Dietary (functional and total) fiber	Oat, wheat and corn bran; barley; rye; psyllium seed husks, peas, beans, apples
Fatty acids	Tree nuts, olive and canola oil, walnuts, flaxseeds, salmon, tuna, marine and other fish oils,
Flavonoids	Berries, cherries, red grapes, tea, cocoa, apples, chocolate, red wine, strawberries
Isothiocyanates	Cauliflower, broccoli, broccoli sprouts, cabbage, kale, horseradish

Minerals	Sardines, spinach, yogurt, pumpkin seeds, whole grain breads and cereals, halibut, beans, potatoes, red meat, garlic
Phenolic acids	Apples, pears, citrus fruits, some vegetables, whole grains, coffee
Plant stanols/sterols	Corn, soy, wheat, fortified foods and beverages, stanol ester dietary supplements
Polyols	Some chewing gums and other food applications
Prebiotics	Whole grains, onions, some fruits, garlic, honey, leeks, banana, fortified foods and beverages
Probiotics	Certain yogurts and other cultured dairy and non-dairy applications
Phytoestrogens	Soybeans and soy-based foods, flax seeds, rye, some vegetables, seeds and nuts, lentils, triticale, broccoli, cauliflower, carrot
Soy protein	Soybeans and soy-based foods like milk, yogurt, cheese and tofu

1.3.1. Microbiota of the gastrointestinal tract

The human gastrointestinal (GI) tract microbiota represents a complex ecosystem (Wilson and Blitchington, 1996). The colon is the most important site of microbial colonization and, where typically the indigenous microbiota is made up of more than 500 different species of bacteria.

However, the numbers and diversity of bacteria present in the different regions of the gastrointestinal tract may be influenced by factors such as pH, nutrient availability, host health, bacterial adhesion, transit time, among others (Kerckhoffs *et al.*, 2006). A representation of the basic gut anatomy is shown in **Figure 1.5**, where the different regions of the gut being colonized by different types of microbial community (either in species diversity or numbers) can be observed. In healthy adults, the stomach and the duodenum have a pH as low as 2 and 4 respectively due to the secretion of HCl, which leads to a low bacterial population in these regions, 10^2 and 10^4 colony forming units per mL (CFU/mL), respectively. In the more distal parts of the small intestine is observed an increase in the bacterial population density, 10^6 to 10^8 CFU/mL and even more in the colon reaching a maximal concentration of up to 10^{12} CFU/mL. In the various regions of the GI tract differences are found in the bacterial population groups with respect to their

diversity and numerical importance. The bacteria have adapted optimally to the conditions of respective regions. The colon is the preferred site of bacterial fermentation (Roberfroid *et al.*, 2010b).

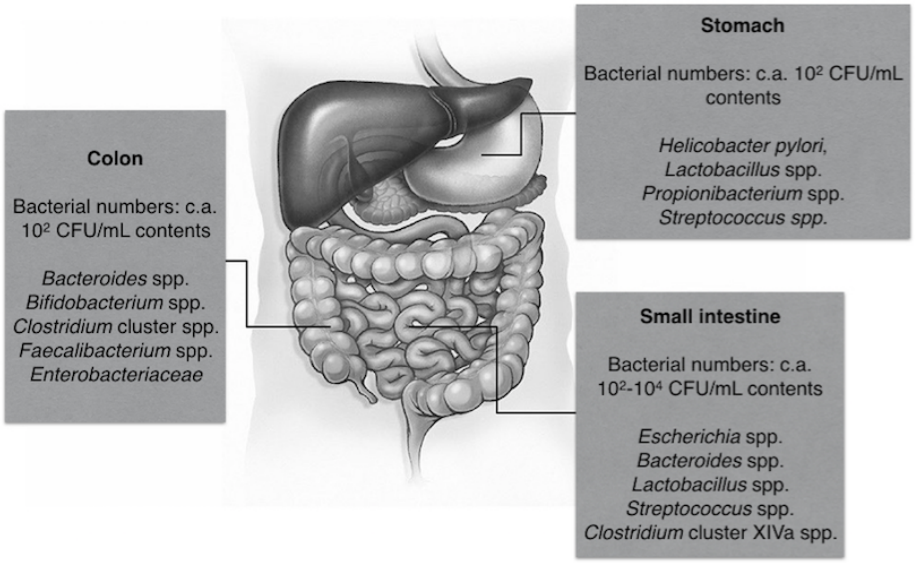

Figure 1.5: Basic gastrointestinal tract anatomy and different types of microbial community that colonize the different regions (Rivière *et al.*, 2016).

It is known besides the knowledge on the complexity of the gut microflora that certain bacteria, belonging to groups of clostridia and bacteroides, are associated with toxin formation, pathogenicity when they become dominant, and others are associated with carcinogen generation and the metabolism of other xenobiotics. Due to the symptoms of overt or latent pathogens the knowledge on these organisms has markedly advanced, however there is less consensus on what characterises potentially harmful bacteria, without direct pathogenicity, and potentially healthy bacteria. Potentially healthy bacterial groups are characterised by absence of toxin production, a beneficial metabolism to the host through the production of short chain fatty acids (SCFA), production of defensins or vitamin synthesis. These potentially healthy bacteria, through a multiplicity of mechanisms can also inhibit pathogens. These mechanisms can be: competition for receptor sites on the gut wall, competition for nutrients, release of antimicrobial substances and stimulation of immunological defence systems (Roberfroid *et al.*, 2010b). Recognized examples of beneficial bacteria inhabiting the gut are bifidobacteria and lactobacilli. The genera streptococci, enterococci, eubacteria and bacteroides have also been classified as beneficial to health, however some species of these genera can be potentially harmful. However, for the most recently identified genera

in the major phyla (*Firmicutes*, *Actinobacteria* and *Bacteroidetes*) the classification as potentially beneficial to health or potentially harmful remains to be made (Roberfroid *et al.*, 2010b). **Figure 1.6** exhibit the main bacteria present in the adult gut.

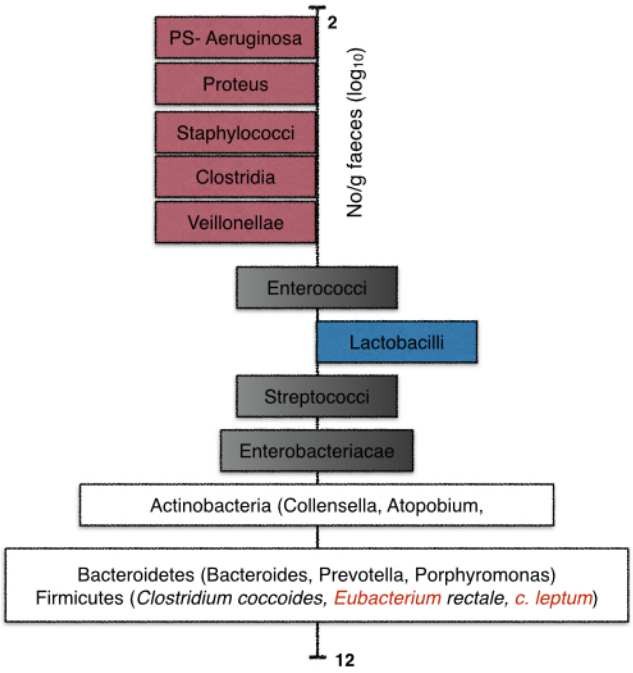

Figure 1.6: Schematic representation of an adult microbiota. The major phyla and genera are located on a logarithmic scale as number of CFU/g of faeces. Genera on pink are likely to be potentially harmful, those on blue are potentially beneficial to health, those on black contain species that are potentially harmful and potentially beneficial to health and those on white are genera/species that still need to be classified (Roberfroid *et al.*, 2010b).

In the last decades, the acceptance by the consumers the use of foods to improve health and wellbeing is increasing (Muthaiyan *et al.*, 2012). Nowadays, there is a great variety of compounds with a specific functional activity susceptible to be used in food industry as functional ingredients or dietary supplements. Among these, those who have attracted more attention are prebiotics and probiotics due their influence on the human intestinal function. As it is believed that alterations in composition of intestinal microbiota and, or metabolic activity are related with some diseases, an important role in the maintenance of health is attributed to colon microbiota (Van der Meulen *et al.*, 2006).

The concept of probiotics emerged long before that of prebiotics. Probiotics are living microorganisms considered to have several beneficial effects on health such as, reduction of diarrhoea, antimicrobial and antitumor activities, or the enhancement of the immunological status (Altieri *et al.*, 2011; Cronin *et al.*, 2011; Malaguarnera *et al.*, 2012; McFarland, 2007). On the other hand, prebiotics are non-digestible substances that are selectively fermented which results in changes in the composition and/or activity of the colonic microbiota (Gibson *et al.*, 2004).

1.4. Prebiotics

In 1995 the term prebiotic was defined for the first time by Gibson and Roberfroid as "A non-digestible food ingredient that beneficially affects the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon, and thus improving host health". In 2008 this definition was revised and the International Scientific Association for Probiotics and Prebiotics (ISAPP), proposed that "A dietary prebiotic is a selectively fermented ingredient that results in specific changes, in the composition and/or activity of the GI microbiota, thus conferring benefit(s) upon host health" (ISAPP, 2008).

After the concept of prebiotics appeared it has aroused much of scientific and industrial interest. Although many food component, especially many food oligosaccharides and polysaccharides (including dietary fiber), have been alleged to have prebiotic activity, these allegations don't take in consideration the required criteria (Roberfroid, 2007).

Not all dietary carbohydrates are prebiotics, thus to classify a food ingredient as a prebiotic was necessary to establish clear criteria. This classification requires scientific demonstration that the ingredient is resistant to gastric acidity, enzymatic hydrolysis and gastrointestinal absorption; be fermented by the intestinal microflora; selectively stimulates the growth and/or activity of intestinal bacteria potentially associated with health and well-being (Gibson *et al.*, 2004).

1.4.1. Types and sources of prebiotics

A dietary ingredient that intact (or partly so) achieves the colon is a potential candidate to be considered prebiotic. The prebiotics most commonly known are fructooligosaccharides (FOS), galactooligosaccharides (GOS) and inulin. GOS are derived from lactose that is naturally present in mammalian milk consist of chains of galactose monomers, and are non-digestible. Inulin and inulin-type fructans are known soluble fibres (Roberfroid, 2005). Furthermore, dietary fibre containing several non-starch polysaccharides, like cellulose, dextrins, pectins, beta-glucans, waxes, and lignin can adjust the transfer time through the gut, therefore providing the same beneficial effects as those of inulin-type fructans (Napolitano *et al.*, 2009). There are prebiotics occurring naturally that can be found in various foods, such as asparagus, chicory, tomatoes and wheat, and it is a natural constituent of breast milk. Several types of prebiotics and their sources are resumed in **Table 1.3**.

Table 1.3: Types and respective sources of prebiotics (Al-Sheraji *et al.*, 2013)

Type of prebiotic	Sources of prebiotic
Fructooligosaccharides	Asparagus, sugar beet, garlic, chicory, onion, Jerusalem artichoke, wheat, honey, banana, barley, tomato and rye
Isomaltulose	Honey, sugarcane juice
Xylooligosaccharides	Bamboo shoots, fruits, vegetables, milk, honey and wheat bran
Galactooligosaccharides	Human's milk and cow's milk
Cyclodextrins	Water-soluble glucans
Raffinose oligosaccharides	Seeds of legumes, lentils, peas, beans, chickpeas, mallow composite, and mustard
Soybean oligosaccharide	Soybean
Lactulose	Lactose (Milk)
Lactosucrose	Lactose
Isomaltulose	Sucrose
Palatinose	Sucrose
Maltooligosaccharides	Starch
Isomaltooligosaccharides	Starch
Arabinoxylooligosaccharides (AXOS)	Wheat bran
Enzyme-resistant dextrin	Potato starch

1.4.2. *In vitro* evaluation of prebiotic properties

In order to obtain reliable and biological meaningful data on different prebiotics, potential molecules must be rigorously tested using standardized methodologies. Each candidate prebiotic, should demonstrate under these methodologies resistance to gastric acidity, hydrolysis by mammalian enzymes and GI absorption, fermentation by intestinal microflora, and selective stimulation of growth and/or activity of intestinal bacteria.

1.4.2.1. Criteria 1: Prebiotic resistance to gastric acidity, hydrolysis by mammalian enzymes, and gastrointestinal absorption

In vitro methods include determining resistance to acidic conditions (for example, those that occur in the stomach) and enzymatic (saliva, pancreatic and small intestinal) hydrolysis (Molis *et al.*, 1996; Nilsson and Björck, 1988; Oku *et al.*, 1984; Ziesenitz and Siebert, 1987).

1.4.2.2. Criteria 2: Fermentation by intestinal microflora

The methodologies for studying functionality of prebiotics benefits has improved throughout the years because the interest on these benefits has increased, particularly improvements in the change of microbiota composition as a response to selective fermentation has been seen (Gibson *et al.*, 2004; Roberfroid, 2007). The main methods used in the study of the prebiotic properties are:

(a) Pure cultures. Early studies performed on pure cultures have been described in literature. Typically, it involves the selection of a range of strains of *Bifidobacterium* spp., *Lactobacillus* spp. and other bacteria such as *Bacteroides* spp., *Clostridium* spp., *Eubacterium* spp. and *Escherichia coli*. This is the simplest method used to study the prebiotic properties of a substrate. In this method, tested bacteria are grown in a basal medium without carbohydrate source, and the tested substrate is added to this medium, and are incubated under anaerobic conditions at 37 ºC (Gibson and Wang, 1994; Gibson *et al.*, 2004). The most usual methods to evaluate the bacterial growth is by measuring the optical density (OD) or via enumeration of viable microorganisms (CFU/mL). In this type of studies, using pure cultures, they are not representative of the colonic microbiota and does not take into account the possible interactions between bacteria, therefore these studies aren't able to evaluate if the carbohydrate is metabolised selectively. However,

these studies give an idea of how the fermentation occurs, thus this type of studies should be used for an initial screening (Gibson *et al.*, 2004; Roberfroid, 2007).

(b) Mixed cultures. Studies using mixed cultures can be carried out by two types, studies on mixed pure cultures and studies using faecal samples.

(b.1.) Mixed pure cultures. These studies are characterized by using a certain number of selected species, both beneficial and harmful, of the gastrointestinal tract. However, this approach does not reflect every interaction that occur in the colonic microbiota in physiological conditions, but it does introduce competition between the microorganisms. Although, these types of studies are very helpful to evaluate how the prebiotic substrate influences the growth of pathogenic species.

(b.2.) Human faecal inoculum. In *in vitro* prebiotic studies, using faecal inoculum are more significant methods, in which can be established if the fermentation is selective or not, because these studies evaluate the change in population of selected species. In these studies, it is ensured that the test carbohydrate acts over a representative range of bacterial species. However, the use of faeces only represents accurately the events occurred in the distal colon. It is known that depending on the region being sampled the composition and activities of the indigenous microbiota of the colon varies. Thus, to overcome this, complex gut models, which replicate different anatomical areas, should be used in concert with human trials (Gibson *et al.*, 2004; Roberfroid, 2007).

(c) Models of the gastrointestinal tract. Human microbiota of GI tract is a highly dynamic ecosystem inhabit by a complex bacterial community (Roberfroid *et al.*, 2010). There are only few species of bacteria adhering to the epithelia and some other bacteria in transit in stomach and small intestine. Upper tract exhibits a scarcity of bacteria which is attributed to the composition of the luminal medium (acid, bile, pancreatic secretion), which kills most ingested microorganisms, and because the phasic propulsive motor activity towards ileum, impedes stable colonisation of bacteria in the lumen. On the other hand, the large intestine contains a complex and dynamic microbial ecosystem with high densities of living bacteria. Furthermore, different physiological conditions are found in different regions of the colon. The proximal region, exhibits an intense fermentation activity with high production of SCFAs, an acidic pH (5-6) and rapid bacterial growth. By contrast, in the distal colon the substrate is less available, the bacterial growth slows,

the pH is close to neutral and putrefactive processes become quantitatively more important (Guarner and Malagelada, 2003). Due to this GI tract characteristics, multistage chemostats have been developed where each vessel represents the different physicochemical regions of the intestine. These multistage chemostats are used efficiently as gut models (Gibson and Fuller, 2000; Macfarlane *et al.*, 1998).

1.4.2.3. Criteria 3: Selective stimulation of growth and/or activity of intestinal bacteria

The field of prebiotics was developed in parallel with the methodology for investigating functionality, particularly, changes in flora composition as a response to the selective fermentation. Much of the early works describes studies performed on pure cultures. Typically, in these studies, take place a selection of a range of strains of *Bifidobacterium* spp., *Lactobacillus* spp. and other bacteria such as *Bacteroides* spp., *Clostridium* spp., *Eubacterium* spp. and *Escherichia coli*. According to different reports the number of strains tested varies. However, the select strains cannot be truly considered representative of the colonic microbiota represents a problem to this approach.
In some studies, authors have used a wide range of bifidobacteria and lactobacilli while they only used one or two strains of the 'undesirable' species. Such studies should be used as initial screening because they cannot establish that the test carbohydrate is selectively metabolised.

As previous referred, the use of faecal inoculum is the most meaningful *in vitro* method for studying prebiotic oligosaccharides, because it ensures that the test material is exposed to a representative range of bacterial species. To establish if fermentation is selective is needed to study the changes in populations of selected genera or species. An accurate representation of events in the distal colon can probably be given using faeces. However, more proximal areas will have a more saccharolytic nature, depending on the region being sampled the composition and activities of the indigenous microbiota to the colon are variable. To overcome this constraint complex gut models, which replicate different anatomical areas, should be used in concert with human trials.

Culture on selective media. Using faecal inoculum entails problems in identifying the genera and species present. Traditionally, this has been carried out by culturing on a

range selective agars followed by morphological and biochemical tests, which aims to confirm culture identities (Finegold *et al.*, 1974; van Houte and Gibbons, 1966). This method is suitable to establish that a prebiotic selectively enriches defined 'desirable' organisms and depletes 'undesirable' organisms. However, this approach does not give an accurate perspective of the population changes occurring because it is estimated that about 60 to 80% of the gut microbial diversity has not been cultivated (Suau *et al.*, 1999).

The use of molecular methods of bacteria identification is a trustworthy approach, once these have advantages over culture-based methods in that they have improved reliability and can embrace the full flora diversity. **Table 1.4** summarizes the principal molecular procedures used in bacterial identification.

Table 1.4: Principal molecular procedures of bacterial identification (Gibson *et al.,* 2004; Roberfroid, 2007)

Method	Description	Advantages	Disadvantages
Fluorescence *in situ* hybridization (FISH)	Group-specific oligonucleotide probes target highly conserved regions of the rRNA molecule allowing specific groups of bacteria can be distinguished from others in mixed culture	Can be used on unculturable as well culturable bacteria. Highly specific.	Probes limited to known bacteria. Time consuming.
Polymerase chain reaction (PCR)	The 16S subunits of the bacterial ribosomes are codifying by genes with conserved and variable regions. Sequencing of the 16S rRNA gene allows bacterial identifications. Using PCR, segments of this gene are amplified to a level whereby their sequence can be determined.	High reliability, allows placement of previously unidentified bacteria. Can be used on unculturable as well culturable bacteria	Time consuming and expensive. Subject to bias in the PCR process
Direct community analysis	Characterizes the 16S rRNA diversity of the sample. Total bacterial DNA is extracted and partial 16S rDNA genes are amplified by PCR. The purified PCR products are cloned. Clones containing	Culture-independent. Clarify the diversity of entire samples.	The bias introduced by PCR can lead to some loss of bacterial diversity.

	the 16S rDNA inserts are sequenced and identified by comparison with databases.		
Denaturation and temperature-gradient gel electrophoresis	A PCR is carried out to the whole community and from the different bacterial species presents partial 16S rDNA sequences are amplified. Separation is based in the decreased electrophoretic mobility of the partially melted, double-stranded DNA molecule in polyacrylamide gels containing a temperature or chemical denaturant gradient. Identification can be made by sequencing excised fragments from the gel or comparing their motility with that	Can be used on unculturable as well culturable bacteria. Rapid.	Qualitative rather than quantitative. The bias introduced by PCR can lead to some loss of bacterial diversity.

1.4.3. Beneficial health effects related to the ingestion of prebiotics

In the past, many studies investigated the health-promoting effects of prebiotics. However, some of the suggested effects have not been fully demonstrated, the data suggest clinically significant effects that justify further study and explanation (Venter, 2007). The main health benefits of prebiotic oligosaccharides are shown in **Table 1.5**

When evaluating the potential benefits related to the ingestion of prebiotics it must be taken in to account that there is a dose-effect relationship, although in generally is difficult to establish a minimum effective dose. In fact, the main factor that quantitatively controls the prebiotic effect is the number of targeted bacteria genus/species per gram of faeces that the volunteers exhibit previously to the supplementation with the target compound presumed to show a prebiotic effect (Roberfroid *et al.*, 2010a).

Table 1.5: Main health benefits of prebiotic oligosaccharides (Mussatto and Mancilha, 2007)

Health benefit	Description
Modification of the colonic microbiota	Stimulation of the growth and proliferation of beneficial bacteria (*Lactobacillus* and *Bifidobacterium*).

Decrease of pH value in the colon	The production of SCFAs by these microorganisms results in a pH decrease and thus inhibition of the growth of pathogenic bacteria.
Nutrient production	Production of vitamins of the B complex (B1, B2, B6 and B12, nicotinic and folic acid).
Increase in faecal dry weight excretion	Related to the increased number of bacteria resulting from the extensive fermentation of prebiotics.
Constipation relief due to faecal bulking and possibly effects on intestinal motility	The ingestion of prebiotic oligosaccharides has demonstrated to prevent constipation. The end products of prebiotics fermentation by colonic bacteria, the SCFAs, are efficiently absorbed and utilized by the human colonic epithelial cells, stimulating their growth as well as salt and water absorption, increasing thus the humidity of the faecal bolus improves the intestinal motility.
Inhibition of diarrhoea	Especially when it is associated with intestinal infections. This may be directly related to the possible inhibitory effect of bifidobacteria both on Gram+ and Gram – bacteria.
Protective effect against infection in the gastrointestinal, respiratory and urogenital tracts	Due to their capacity to inhibit the adhesion of bacteria to the epithelial surfaces (initial stage of the infective process).
Increase in absorption of minerals	The increased absorption of iron, calcium, and magnesium is related to the binding/sequestering capacity of the prebiotic oligosaccharides. The minerals that are bound/sequestered and, consequently, are not absorbed in the small intestine reach the colon, where they are released from the carbohydrate matrix and absorbed. The increase on calcium absorption reduces the risk of osteoporosis since this mineral promotes an increase in the bone density and bone mass. The hypotheses most frequently proposed to explain this enhancing effect of prebiotics on mineral absorption are the osmotic effect, acidification of the colonic content due to fermentation and production of SCFA, formation of calcium and magnesium salts of these acids, hypertrophy of the colon wall.

Beneficial effect on carbohydrates and lipids metabolism	The ingestion of prebiotic oligosaccharides decreases the blood concentration of cholesterol, triglycerides and phospholipids, reducing the risk of diabetes and obesity. Changes in the concentration of serum cholesterol have been related with changes in the intestinal microflora. Some strains of *Lactobacillus acidophilus* assimilate the cholesterol present in the medium, while others appear to inhibit the absorption of cholesterol through the intestinal wall. On the other, changes in lipid metabolism were suggested to be a consequence of a metabolic adaptation of the liver induced by SCFAs.
Reduction of cancer risk	This anticarcinogenic effect appears to be related to an increase in cellular immunity, the components of the cell wall and the extra-cellular components of bifidobacteria. Faecal physiological parameters such as pH, ammonia, p-cresol, and indole are considered to be risk factors not only for colon cancer development but also for systemic disorders. It has been demonstrated in a human study that the intake of transgalactosylated disaccharides reduces the faecal pH as well as ammonia, p-cresol and indole concentrations with an increase in bifidobacteria and lactobacilli and a decrease in *Bacteroidaceae* populations. These alterations may be beneficial in reducing the risk of cancer development. A low colonic pH may also aid in the excretion of carcinogens.

1.5. Probiotics

1.5.1. Definition of probiotics

The term “probiotics” has origins in Greek, which means “for life”. Various definitions of probiotics have been proposed by various investigators and were modified until a more specific and appropriate definition. The first description of probiotics by Lilly and Stillwell (1965) was “substances secreted by one microorganism which stimulates the growth of another”, this description contrasted with the definition of antibiotic. Parker (1974) define the term probiotic as “organisms and substances, which

contribute to intestinal microbial balance". This definition had a wide connotation that included antibiotics due to the use of the word substances. In 1989 Fuller revised the definition of probiotics, as "a live microbial feed supplement, which beneficially affects the host animal by improving its intestinal microbial balance". However, this definition did not mention if the probiotics beneficial effect is also regarding to humans or only to animal.

Definition of probiotics was extended to "A viable mono-or mixed culture of microorganisms which applied to animal or man, beneficially affects the host by improving the properties of the indigenous microflora" (Havenaar and Huis In't Veld, 1992). Salminen (1996) defined probiotic as "a live microbial culture or cultured dairy product, which beneficially influences the health and nutrition of the host". According to Schaafsma (1996) probiotic may be defined as "oral probiotics are living microorganisms which upon ingestion in certain numbers exert health effects beyond inherent basic nutrition". Schrezenmeir and de Vrese (2001) reviewed probiotic definition as, "A preparation of or a product containing viable, defined microorganisms in sufficient numbers, which alter the microflora (by implantation or colonization) in a compartment of the host and by that exert beneficial health effects in this host.

In 2001 probiotic definition is redefined as "live micro-organisms which when administered in adequate amounts confer a health benefit on the host" (FAO/WHO, 2001).

1.5.2. Selection criteria for probiotic cultures

Probiotic strains have some ideal properties that benefit human health and could be useful in the probiotics industry. Included in these properties are, resistance to acid and bile; attachment to the human gut epithelial cells; colonization in the human intestine; production of antimicrobial substances, including bacteriocins; good growth characteristics and beneficial effects on the human health. It is of great importance that a probiotic strain must be nonpathogenic and generally recognized as safe (GRAS). Probiotics should exhibit some desirable characteristics, such as maintenance of viability during processing and storage, easily applicable in products, and resistance to the physicochemical processing of the food (Prado *et al.*, 2008). Probiotic strains can not be pathogenic, toxic, mutagenic, or carcinogenic in the host organism, they must have

antagonistic activity against pathogens and must be genetically stable without a plasmid transfer mechanism, especially related to antibiotic resistance; they should be capable of survive during digestion and they can adhere and colonize the gut mucosa, promoting immuno-stimulation without inflammatory effects (Saarela *et al.*, 2000). The minimum amount of probiotic bacteria present in a dairy food should be 10^6 CFU/g or the daily intake should be about 10^8 CFU/g, because the possible reduction in the number of microorganisms during the passage through gastrointestinal tract must be compensated (Shah, 2007). The most used and extensively studied probiotics are the lactic acid bacteria, especially the *Lactobacillus* and *Bifidobacterium* spp. as mentioned in **Table 1.6**.

Table 1.6: The most commonly used probiotics (Holzapfel and Schillinger, 2002; Jeon *et al.*, 2012; Kumar *et al.*, 2012; Maldonado *et al.*, 2012; Mombelli and Gismondo, 2000; Thomas *et al.*, 2012)

***Lactobacillus* species**	***Bifidobacterium* species**	**Other species**
L. acidophilus	*B. bifidum*	*Bacillus cereus* (toyoi)
L. casei	*B. lactis*	*Saccharomyces cerevisiae*
L. crispatus	*B. longum*	*Saccharomyces boulardii*
L. plantarum	*B. breve*	*Lactococcus lactis*
L. rhamnosis GG	*B. infantis*	*Stryptomyces* (yeast)
L. salivarius	*B. adolescentis*	*Escherichia coli* Nissle
L. gasseri	*B. animalis*	*Streptococcus phocae*
L. buchneri	*B. gallicum*	*Enterococcus faecium*
L. johnsonii	*B. asteroides*	
L. reuteri	*B. merycycum*	
L. fermentum	*B. magnum*	
L. delbrueckii	*B. indicum*	
L. acetotolerans	*B. gallinarum*	
L. acidifarinae	*B. cuniculi*	
L. zymae	*B. boum*	
L. agilis	*B. coryneforme*	
L. alimentarius	*B. choerium*	
	B. ruminale	
	B. minimum	
	B. subtile	
	B. thermaacidophilum.	

B. psuedocatenulapum

1.5.3. Mechanisms of action of probiotics

With the goal to explain how probiotics exert their beneficial effects several mechanisms of action have been proposed. However, the knowledge on probiotics' mechanisms of action is only preliminary, and it must be considered that these mechanisms may be multifactorial and it is clear that significant differences exist between how the probiotic strains affect the host. There are some strains that are capable of transiently colonise and modulate the indigenous microbiota despite, in general, probiotics do not colonise the human intestinal tract permanently (Shinde, 2012) . **Table 1.7** exhibit the main mechanisms of action of probiotics.

Table 1.7: Mechanisms of action of probiotics (Ng *et al.*, 2009)

Antimicrobial Activity	**Enhancement of Barrier Function**	**Immunomodulation**
Decrease luminal pH by the production of short chain fatty acids; Secrete antimicrobials peptides Inhibit bacterial invasion; Block bacterial adhesion to epithelial cells.	Increase mucus production; Enhance barrier integrity.	Effects on epithelial cells Effects on dendritic cells Effects on monocytes/macrophage Effects on lymphocytes – B lymphocytes – NK cells – T cells – T cell redistribution

1.5.4. Health benefits of probiotics

Currently, there is an emerging demand for products that provide basic nutrition and can enhance health, because consumers are conscious of the link between lifestyle, diet and good health. The health benefits attributed to functional food continues to increase and among those researchers have demonstrated therapeutic evidence, probiotics are one of the fastest growing group (Soccol *et al.*, 2010). Some health claims of probiotics proposed by their authors were compiled by Fijan (2014) and are listed in **Table 1.8**.

Table 1.8: Health claims of probiotics proposed by Fijan (2014)

Health claims of probiotics
Stimulation of components of the immune system gut immune response and intestinal homeostasis
Prevention and treatment of diarrhoea
Improvement of faecal properties and microbiota
Treatment of irritable bowel syndrome, inflammatory bowel disease and constipation
Prevention and treatment of *Clostridium difficile*-associated diarrhoea in adults and children
Alleviation of the symptoms of lactose intolerance and other food allergies
Prevention of necrotising enterocolitis in preterm infants
Decrease the cholesterol level in plasma
Improvement of *Helicobacter pylori* eradication regimens
Therapeutic effects by supporting the immune response of HIV-infected children and adults
Anti-proliferative activity on tumour cells
Reduction of viral-associated pulmonary damage through controlling immune-coagulative responses and clearing respiratory viruses
Immune-stimulatory properties of low molecular mass molecules produced by probiotic bacteria

To validate a health claim the trials must have a sufficient sample size, heterogeneity of study designs (targeted population) must be enabled and a precise identification of strains should be made (Fijan, 2014).

According to Fijan (2014) probiotic studies performed with animals exhibited positive effects of probiotic administration which consequential can lead to a reduction of antibiotic consumption in animals used in food production. Therefore, the presence of drugs and multi-resistant organisms in the environment (including drinking water) will decrease.

1.5.5. Food application of probiotics

It is widespread in western dietary culture ferment milk into yogurt, sour milk or cheese. In Europe, due to this practice there is a high popularity of probiotic products presented as fermented milk, specifically sour milk and yogurt. However, in developed countries industries are inventing other delivery forms for probiotics, including cheese, whey dairy products, desserts, ice cream, breads, confectionary and soy products as well as meats and fermented vegetables (De Bellis *et al.*, 2010; Gawkowski and Chikindas,

2013; Gomes da Cruz *et al.*, 2009; Mäkeläinen *et al.*, 2009; Sharp *et al.*, 2008; Zoellner *et al.*, 2009).

1.5.5.1. Non-dairy probiotic products

In general, dairy products are the main vehicles to deliver probiotic bacteria to human, due to their inherent properties. However, due to the increase of vegetarian consumers and a high prevalence of lactose intolerance around the world, the vegetarian probiotic products became more important. Vegetarians and lactose intolerant customers exhibit a high interest in non-dairy probiotic products. In the way that non-dairy probiotic food products have to meet the consumer's concerns about health benefits, the development of these products appears to be a great challenge (Charalampopoulos *et al.*, 2002; Stanton *et al.*, 2003). **Table 1.9** exhibit some non-dairy probiotic foods recently developed.

Table 1.9: Recently developed non-dairy probiotic products. Adapted from (Granato *et al.*, 2010b)

Category	Product
Fruit and vegetable based	Vegetable-based drinks
	Fermented banana pulp
	Fermented banana
	Beets-based drink
	Tomato-based drink
	Many dried fruits
	Green coconut water
	Peanut milk
	Cranberry, pineapple and orange juices
	Ginger juice
	Grape and passion fruit juices
	Cabbage juice
	Carrot juice
	Noni juice
	Onion
	Probiotic banana puree
	Nonfermented fruit juice beverages
	Blackcurrant juice
	Nonfermented soy-based frozen deserts

Soy based	Fermented soymilk drink
	Soy-based stirred yogurt-like drinks
Cereal based	Cereal-based puddings
	Rice-based yogurt
	Oat-based drink
	Oat-based products
	Yosa (oat-bran pudding)
	Mahewu (fermented maize beverage)
	Maize-based beverage
	Wheat, rye, millet, maize and other cereals fermented probiotic beverages
	Malt-based drink
	Boza (fermented cereals)
	Millet or sorghum flour fermented probiotic beverage
Other non-dairy foods	Starch-sacchariffied probiotic drink
	Probiotic cassava-flour product
	Meat products
	Dosa (rice and Bengal gram)

1.5.6. Recent trends in probiotics

The process that leads to the development of probiotic food is expensive and is a multistage process that considers many factors, for instance sensory acceptance, physical and microbial stability, chemical and other intrinsic functional properties and price to be successful in the marketplace. Furthermore, must be understood and taken into account the consumer expectation regarding the product. For functional foods, especially for probiotic products, to have viability and success in the marketplace, they are depending on several elements; however, consumer acceptance (food safety, sensory appeal, brand, marketing, and others) is a key factor (Granato *et al.*, 2010a). Consumers need to be convinced about any health claim through clear, truthful and unambiguous message in order to agree to pay the associated cost of functional foods. In the functional food market, probiotic products are one of the largest player in the market (Granato *et al.*, 2010b). Regarding to the dairy probiotic products, they have been widely explored by industry and scientific researchers because of their health claims and continuously increasing interest by consumers. Despite that the non-dairy probiotic sector continuously growth with the soy probiotic products sales and marketing increasing in the last decades

exposing a trend to the development of new products (Granato *et al.*, 2010b). Representing an enormous growth potential for the food industry, probiotic foods may be widely explored via the development of innovative ingredients, processes, and products. Nevertheless, is challenging combining both the consumer's eating desires with the potential providing health benefits, when developing probiotic and other functional foods. It is of the utter most importance to identify specific sensory attributes that will drive the product to acceptance, although this alone will not guarantee the successful placement of the product in the marketplace (Granato *et al.*, 2010a).

1.6. Objectives

Research studies on Akpan are very limited and focused on its basic description, sensory quality and nutritional attributes.

Regarding to BSC there are also scarce research studies, however there are few studies of BSG, a similar by-product. In both cases no study has been performed until now to explore its prebiotic potential.

So, the aim of the present thesis was to evaluate the prebiotic potential of Akpan as a functional food and of BSG as potential functional ingredient. To achieve this general objective, we have established several specific objectives:

1. Assessment of Akpan and BSC as a fermentable substrate for probiotic bacteria and its influence on their growth and metabolic activity.
2. Evaluate the impact of Akpan and BSC on gut microbiota by evaluating the dynamics of selected microbial populations using the fluorescent *in situ* hybridization (FISH) technique and evaluate the production of short-chain fatty acids (SCFAs).

The final objective of this study was to contribute to the promotion of consumption of Akpan and the application of BSC in the food industry.

2. Material and methods

2.1. Akpan and BSC

Akpan was prepared in the Centre de Coopération Internationale en Recherche Agronomique pour le Développement (CIRAD), Food Process Engineering Research Unit (France) using white maize grains provided by Abomey-Calavi University (Cotonou, Benin) as raw material. The traditional process for making Akpan from white maize ogi was described by Sacca *et al.*, (2012). It involves the followings steps: i) the grains were steeped in boiling water for 5 min and left in water for 24 h until it reached room temperature (grain/water ratio: 1/2), ii) the steeped grains were milled using a grinder (Thermomix Vorwerk; for 1.5 min, depending on the amount) and iii) the wet maize meal was sifted through a 315 µm sieve using a large amount of water (wet flour/ water ratio: 1/3) for separating flour and bran. Then, the slurry collected under the sieve was transferred to a crystallizer and a lactic acid starter (*Lactobacillus casei* CNCM I-4592, Lesaffre Company, Marcq-en-Baroeul, France) was added. Finally, the mixture was fermented for 20-24 h in an oven at 35 ºC. The starter was previously activated at 35 ºC for 5-7 h in a small amount of maize flour slurry obtained as described above. The supernatant of fermented product was separated. Part of it (85%) was boiled; the other part (15%) was remixed with fermented flour (*ogi*). The boiled supernatant was added to part of the *ogi* (70%), which was cooked for 15 min at a temperature of 80-90 ºC. The 30% of raw *ogi* put aside was added to the cooked *ogi* after cooling and thus, Akpan, a partially cooked product, was obtained. The Akpan was then freeze dried and stored at refrigeration temperature.

BSC was provided by SABMiller PLC (United Kingdom) and it was obtained from a batch of brewing, that has been frozen and shipped to our facilities and kept frozen until use.

2.2. Stimulated *in vitro* digestion of Akpan and BSC

The digestibility of BSC and Akpan through the gastrointestinal tract were studied using an *in vitro* model that chemically simulates the physiological conditions following the method described by Mills *et al.*, (2008). Briefly, water (85 mL) was added to 15 g of sample (BSC or Akpan), and the mixture was stomached for 2 min. The sample solution

was transferred to a glass screw topped bottle, mixed with α-amylase (7.3 mg) in $CaCl_2$ (1 mmol/L), pH 7.0; 1.6 mL) and incubated at 37 ºC for 30 min. The pH value was decreased to ca. 2 with 6 mol/L HCl and pepsin (1.0 g) dissolved in HCl (0.1 mol/L; 6.25 mL) was added. The sample was incubated at 37 ºC for 2 h. The pH value was increased to 7.0 with 6 mol/L NaOH, and pancreatin (0.21 g) and bile salts (1.3 g) in sodium bicarbonate (0.5 mol/L; 31 mL) were added. The sample solution was transferred to 1 kDa MWCO (molecular weight cut-off) dialysis tubing and dialysed for 2 days at 20 ºC to remove low molecular mass digestion products. The dialysis fluid was changed and dialysis continued for an additional 2 h. Finally, the sample was freeze dried until further utilisation in fermentation experiments (see below).

2.3. Evaluation of Akpan and BSC as carbon source for probiotic bacteria growth

2.3.1. Microorganisms and growth conditions

The bacterial strains used to evaluate prebiotic activity of BSC and Akpan were four commercial probiotic strains: *Lactobacillus casei* L431 and *Bifidobacterium lactis* B94 that were kindly provided by DELVO PRO LAFTI (DSM, Netherlands); *Lactobacillus casei* L01 and *Bifidobacterium lactis* Bb12 that were supplied by CHR HANSEN (Denmark).

Strains were stored at -80 ºC in Man-Rogosa-Sharpe (MRS) broth (Biokar Diagnostics, France) with 30% (v/v) glycerol. Before the assays, the strains were grown in a static culture with MRS broth (supplemented with cysteine hydrochloride in the case of *Bifidobacterium*) at 37 ºC for 16 h under aerobic conditions for the *Lactobacillus* and under anaerobic conditions for *Bifidobacterium*.

2.3.2. Media

Based on MRS five different basal media were prepared, i.e. MRS without carbon source, MRS with 1% (w/v) glucose (Fluka) and MRS with 1% (w/v) of FOS (Orafti, Oreye, Belgium; positive prebiotic control) and MRS with 1% (v/v) BSC or Akpan.

The basal medium used was MRS broth prepared by addition of the different compounds, in order to enable carbon source substitution: 10 g/L of protease peptone, 10 g/L of beef extract, 5 g/L of yeast extract, 1 g/L of Tween 80, 2 g/L of ammonium citrate, 5 g/L of sodium acetate, 0.1 g/L of magnesium sulfate, 0.05 g/L of manganese sulfate, 2 g/L of dipotassium sulfate, 0.05 g/L of cysteine hydrochloride (before inoculation in the case of anaerobic strain) and 1% (w/v) of carbon source (BSC, Akpan or FOS). The media were sterilized at 110 °C for 20 min.

2.3.3. *In vitro* fermentation assays

The basal medium containing each of the tested compounds was inoculated with a 2% (v/v) inoculum of each probiotic strain. Inoculum were obtained as previously described in Section 2.3. The assay was performed in duplicate.

The different glass bottles were placed in the orbital incubator at 37 °C with stirring of 100 rpm. For the confirmation of fermentable activity, samples were drawn from the media at 0, 5, 10, 24 and 48 h. Growth curves were monitored by enumeration of viable cells and bacteria metabolism was assessed by pH measurement and determination of organic acids by high performance liquid chromatography (HPLC) analysis. Viable cells were quantified by the drop count method described by (Miles *et al.*, 1938) wherein 100 µl of sample where collected and diluted using peptone water through serial decimal dilutions and 20 µl of each dilution were plated, in duplicate, on MRS agar (supplemented with cysteine hydrochloride and under anaerobic conditions in the case of *Bifidobacterium*) and incubation at 37 °C for 48 h. The pH was determined with a pH electrode model Crison 52-02 (Crison Barcelona, Spain).

2.3.3.1. Determination of organic acids by high performance liquid chromatography (HPLC) analysis

After collecting the sample for enumeration of viable cell and measuring the pH the tube sample were centrifuged (8973 × g for 10 min), and supernatants were collected and filtered through 0.20 µm cellulose acetate membranes. Aliquots of the filtered samples were assayed for organic acids (lactic, formic, acetic, propionic and butyric acids) using an Agilent 1200 series HPLC system with a RI detector (Agilent, Germany)

operated at 50 ºC. Other analysis conditions were as follows: Aminex HPX-87H column (BioRad, Hercules, CA); mobile phase, 0.003 mol/L H_2SO_4; flow, 0.6 mL min^{-1}.

2.4. Evaluation of probiotic effect of Akpan and BSC with mixed cultures (human faecal inoculum)

2.4.1. Faecal inoculum

Faecal samples were obtained from three healthy human volunteers, who usually ingested a normal diet, presented no digestive diseases and did not receive antibiotics for, at least, 3 months. Faeces were collected into sterile vials, kept in an anaerobic cabinet and used within a maximum of 2h after collection. Faecal inoculum (FI) was prepared by dilution in a reduced physiological salt solution (RPS; cysteine-HCl 0.5 g/L and NaCl 8.5 g/L) at a ratio of 10% (w/v). Before use, and during preparation of FI, anaerobiosis and pH (6.8) were maintained by continuous bubbling of CO_2 and N_2. The slurry was mixed and homogenized for 2 min under a CO_2 and N_2 stream. Blended, diluted faeces were filtered through four layers of surgical gauze to remove non-digested materials and transferred to serum bottles (Gullón *et al.*, 2014; Hartemink and Rombouts, 1999).

2.4.1.1. Fermentation media

The nutrient base medium used in fermentation experiments was prepared as described previously (Gullón *et al.*, 2014). Thus, a solution containing 5.0 g/L trypticase soya broth (TSB) without dextrose (BBL, Lockeysville, MD), 5.0 g/L bactopeptone (Amersham, Buckinghamshire, U.K.), 5.0 g/L yeast nitrogen base (YNB; Difco, Detroit, MI), 0.5 g/L cysteine hydrochloride (Merck, Darmstadt, Germany), 1.0% (v/v) salt solution A (100.0 g/L NH_4Cl, 10.0 g/L $MgCl_2 \cdot 6H_2O$, 10.0 g/L $CaCl_2 \cdot 2H_2O$), trace mineral solution, 0.2% (v/v) salt solution B (200.0 g/L $K_2HPO_4 \cdot 3H_2O$), and 0.2% (v/v) 0.5 g/L resazurin solution was prepared in distilled water. After deoxygenation with CO_2 and N_2 and pH adjustment to 6.8, aliquots were distributed into airtight anaerobic serum bottles, which were capped with butyl rubber stoppers and sealed with aluminium caps before autoclave sterilization.

Stock solutions of yeast nitrogen base (YNB) and FOS from chicory (Sigma-Aldrich Co., St. Louis, USA) were sterilised under syringe filters of 0.20 µm (Chromafils,

Macherey-Nagel, Düren, Germany) into sterile airtight serum bottles, whereas the solutions of BSC and Akpan were pasteurised at 100 °C for 15 min.

YNB, BSC, Akpan and FOS solutions were aseptically added to the anaerobic serum bottles with nutrient base medium (using syringes and needles under anaerobic conditions in a cabinet) before inoculation, to achieve a final concentration of 5 g YNB/L and 10.0 g carbohydrate sources/L. The serum bottles with the fermentation media were inoculated with 0.2 mL of faecal slurry dilution (2% v/v) and incubated at 37 °C for 48 h without shaking. At each sampling time (0, 5, 10, 24 and 48 h), tubes were withdrawn and cultures were centrifuged (8973 × g for 10 min), and pellets and supernatants were collected for further analysis. All additions and inoculations were carried out under anaerobic conditions, in a cabinet flushed with a gas stream containing 5% H_2, 10% CO_2 and 85% N_2.

2.4.1.2. Determination of fermentation products in cell-free supernatants

The determination of fermentation products in cell-free supernatants was performed by HPLC as described in section 2.3.3.1. Supernatants from the batch cultures were filtered through 0.20 µm cellulose acetate membranes. Aliquots of the filtered samples were assayed for organic acids (lactic, formic, acetic, propionic and butyric acids) using an Agilent 1200 series HPLC system with a RI detector (Agilent, Germany) operated at 50 ºC. Other analysis conditions were as follows: Aminex HPX-87H column (BioRad, Hercules, CA); mobile phase, 0.003 mol/L H_2SO_4; flow, 0.6 mL min^{-1}.

2.4.1.3. Fluorescent *in situ* hybridization (FISH) assays

FISH was performed as described previously by (Gullón *et al.*, 2011). Synthetic oligonucleotide probes (TIB MOLBIOL, S.L., Spain) targeting specific regions of the 16S rRNA molecule and labelled with the fluorescent dye Cy3 (Sigma) were utilized for the enumeration of bacterial groups: Bif164, specific for the *Bifidobacterium* genus, (Langendijk *et al.*, 1995); Lab158, for the *Lactobacillus–Enterococcus* group (Hermie J. M. Harmsen, 1999); Bac303, specific for the *Bacteroides-Prevotella* group (Manz *et al.*, 1996); His150, for the *Clostridium histolyticum* subgroup (Franks *et al.*, 1998); Erec482

for the *Ruminococcus–Eubacterium–Clostridium* cluster (Franks *et al.*, 1998); and Fpra655 for the *Faecalibacterium prausnitzii* and relatives (Hold *et al.*, 2003).

At fixed fermentation times, samples were centrifuged at 15,000 × g for 2 min to separate particulate matter, and fixed overnight in paraformaldehyde (4 % w/v) at 4 ºC (volume ratio of sample/paraformaldehyde solution, 1:3). Cells were washed with phosphate-buffered saline (0.1 M, pH 7.0), resuspended in 150 µL phosphate-buffered saline plus 150 µL ethanol, and stored at -20 ºC for at least 1 h before further processing. In 1.5 mL microcentrifuge tubes, 200 µL of filtered hybridization buffer (40 mmol/L Tris-HCl pH 7.2, 1.8 mol/L NaCl and 20 mL/L of a solution of 100 g sodium dodecyl sulphate/L), 64 µL of deionized distilled water and 16 µL of the fixed cells were added. In a 0.5 mL microcentrifuge tubes, 5 µL of the probe (50 ng/µL) was mixed with 45 µL of the above hybridization solution, and the mixture was shaken and incubated overnight at the temperature recommended for each bacterial group.

In a 10 mL centrifuge tube, 5.0 mL of prewarmed hybridization buffer (20 mmol/L Tris-HCl pH 7.2, 0.9 mol/L NaCl) and 20 µL of nucleic acid stain 4',6-diamidino-2-phenylindole (DAPI 500 ng/mL) were added. The mixture was returned to the oven at the adequate temperature for 30 min. The washing mixture was filtered through a 0.20 µm pore size filters (Whatman, Nuclepore Policarbonate) under vacuum. The filters were then placed on slides and covered with a coverslip, after addition of 5 µL of antifade reagent (polyvinyl alcohol mounting medium with DABCO antifading, Sigma). Slides were stored in the dark at 4 ºC for a maximum of 3 days, and during this period were examined using an epifluorescence microscope (Olympus BX41) equipped with Fluor 100 lenses. A minimum of 15 fields were counted per sample analysed.

2.5. Statistical analysis

Statistical analysis was performed using SPSS for Windows version 21.0 (IBM SPSS, Chicago, IL). Univariate analysis of variance (Penna *et al.*) was used to determine the significance of the effect of carbon source on bacterial group populations and SCFA production. Pair wise comparisons were done using the posthoc Tukey test. Differences were considered significant at the 5% level of significance.

3. Results and discussion

Diet is considered a major driver for changes in the compositional and functional relationship between microbiota and the host. In fact, dietary components are susceptible to the metabolism of the intestinal microbial ecosystem, particularly influencing the growth and the metabolic activity of the dynamic bacterial populations thriving in the human colon (Laparra and Sanz, 2010; Maccaferri *et al.*, 2012).

Several dietary fibres including some non-starch polysaccharides, such as cellulose, dextrins, chitins, pectins, beta-glucans, waxes, and lignin can modulate the transit time through the gut providing similar beneficial effects as those of inulin-type fructans. These compounds are found in many foods such as cereal, nuts, etc. They are also partially susceptible to bacterial fermentation and may induce changes in bacterial populations, particularly in number of bifidobacteria and lactobacilli. These dietary soluble fibres have been shown to exert additional beneficial effects, for instance improving gut barrier function *in vitro* (Napolitano *et al.*, 2009) and *in vivo* (Gómez-Conde *et al.*, 2007; Laparra and Sanz, 2010), which could be partially a consequence of their effect on the microbiota composition.

In the present study, we aimed at investigating the impact of two carbohydrates sources (Akpan and BSC) characterized by a naturally high content in dietary fibres. *In vitro* fermentation of prebiotic oligosaccharides provides an easy, fast and cheap alternative to assess the fermentation of different substrates at the laboratory scale on a comparative basis (Madhukumar and Muralikrishna, 2010; Moura *et al.*, 2008). In this work, the prebiotic potential of both freeze-dried Akpan and BSC were evaluated directly by studying the effect on growth of single probiotic stain and by *in vitro* fermentation with human faecal microbiota following methods reported in literature by assessment of their impact on the human intestinal microbial ecosystem in terms of metabolism and bacterial population (Barry *et al.*, 2007). Fermentations were followed by measuring the assimilation of the carbon sources, the accumulation of SCFA (acetate, propionate and butyrate) and lactate in the media, the pH shifts and evolution of the microbial populations. For comparative purposes, the same type of information was obtained in experiments with FOS (which is accepted as the gold standard of a prebiotic ingredient) (Meyer and Stasse-Wolthuis, 2009), and for media without carbon source (negative control).

3.1. Effects of Akpan and BSC on the probiotic growth

In order to evaluate the suitability of Akpan and BSG as a fermentable carbohydrate source for probiotic bacteria, *in vitro* fermentation assays were carried out using two *Lactobacillus* strains (*L. casei* L431 and *L. casei* L01) and two *Bifidobacterium* strains (*B. animalis* Bb12 and *B. lactis* B94). **Figure 3.1** exhibit the evolution of the viable cells numbers and pH value in the fermentation media along 48 h for the different strains tested. As expected, a very limited growth was observed in the negative control assay confirming that the growth enhancement was a consequence of the carbohydrates added, Akpan, BSG and FOS.

Regarding the *Lactobacillus* strains, no growth enhancement was observed for Akpan (**Figure 3.1**), showing similar profiles to the observed in the negative control. Regarding the BSG it was observed a growth enhancement in both strains. However, in the assays of the strain *L. casei* L431 the number of viable cells at the end of the fermentation was slightly lower in media containing BSG than the observed in media containing FOS (8.61 log CFU/mL and 9.27 log CFU/mL for BSG and FOS, respectively), while in the case of *L. casei* L01 at the end of fermentation it was observed that the number of viable cells was slightly higher in media containing BSG (9.03 log CFU/mL and 8.94 log CFU/mL for BSG and FOS, respectively). However, the decrease in pH value was similar for BSG, Akpan and FOS in the assays using *L. casei* L01, after 48 h of fermentation, pH reached values of 6 for BSG and Akpan and 6.3 for FOS. In the case of *L. casei* L431, after 48 h the pH value decreased to 4 for the media containing FOS, when cultured in media containing BSG the pH reached values of 6.2. When *L. casei* L431 was cultured in media containing Akpan the pH remained almost constant decreasing from 6.8 at the beginning of fermentation, until to 6.5 at the end of fermentation.

Regarding to the *Bifidobacteria* strains, an enhancement effect on the growth was observed after 48 h using BSG, Akpan and FOS. In the assays performed with *B. animalis* Bb12 the results show a similar profile for BSG, Akpan and FOS, however at the end of fermentation the number of viable cells in media containing FOS was slightly higher (7.79 log CFU/mL, 7.31 log CFU/mL and 7.35 log CFU/mL for FOS, BSG and Akpan, respectively). However, some differences were observed in the case of *B. lactis* B94 when

compared the results in media containing BSG or Akpan, the number of viable cells at the end of fermentation was slightly higher in media containing BSG (8.53 log CFU/mL and 8.24 log CFU/mL for BSG and Akpan, respectively), but in these both media the number of viable cells was slightly lo than in media containing FOS (8.75 log CFU/mL), but did not show significant differences ($p>0.05$). Regarding the pH values in media, between the three carbon sources, BSG, Akpan and FOS, at the end of fermentation, it was observed the highest pH value in media containing BSG for both strains (final pH = 6.4 and 5.9 for *B. animalis* Bb12 and *B. lactis* b94, respectively) (**Figure 3.1**). In the case of *B. animalis* Bb12, the pH exhibits a similar trend for Akpan and FOS (final pH=5.5 and 5.9, respectively) (**Figure 3.1**). By contrast, when *B. lactis* B94 was cultured in media containing FOS, a higher reduction in pH was observed in comparison with BSG and Akpan (final pH = 4.2, 5.9 and 5.2 for FOS, BSG and Akpan, respectively). These results are in agreement with those obtained for the viable cell numbers (*B. lactis* B94 reached higher values in media containing FOS).

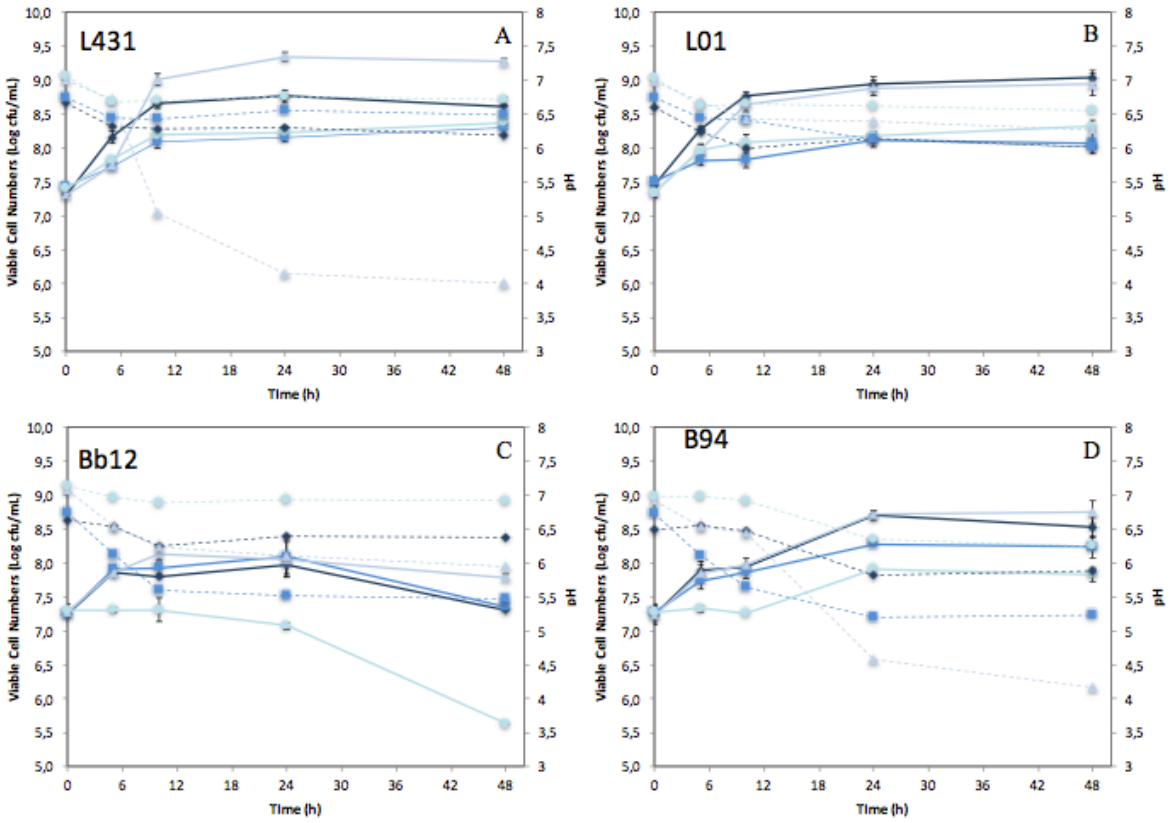

Figure 3.1: Viable cell numbers (log CFU/mL) (—) of *Lactobacillus casei* L431 (A), *Lactobacillus casei* L01 (B), *Bifidobacterium animalis* Bb12 (C) and *Bifidobacterium lactis* B94 (D) and pH (--) values throughout incubation time (48 h) at 37 ºC growing in MRS-medium supplemented with 1% (w/v) of BSG (◆), 1% (w/v) of Akpan (■),

1%(w/v) (▲) and MRS medium without fermentable carbohydrates (●). Values are expressed as the mean ± standard deviation (Kurdi and Hansawasdi) of independent duplicate assays.

HPLC analysis confirmed the presence of lactate, formate and acetate as fermentation products in the cell-free culture supernatants. However, variations in the production of these acids for various strains and substrates were observed (**Table 3.1**). In general, strains *L. casei* L431 and *B. lactis* B94 grown in media containing FOS produced higher lactic acid concentration than those achieved when Akpan and BSG were use as substrate. Between Akpan and BSG, when BSG is used as substrate the lower concentration of lactic acid were achieved. Acetic acid levels were higher in media containing Akpan in comparison with BSG and FOS, for the four strains, however *L. casei* L01 achieve higher concentration, while *B. animalis* Bb12 and *B. lactis* B94 produce similar amounts. When the media is supplemented with BSG higher concentrations of formic acid for the fours strains were achieved, although the *L. casei* L01 and *B. lactis* B94 produced higher levels of these acid. These results are in agreement with the obtained for the pH measurements where the control exhibit the higher pH values and lower concentration of lactate, formate and acetate.

Previous results are in agreement with a number of studies in which is demonstrated that the ability and the final products of lactobacilli and bifidobacteria fermentation of prebiotic carbohydrates depends on the substrate in addition to tested bacterial strain (Huebner *et al.*, 2007; Sousa *et al.*, 2015).

Tadayoni *et al.*, (2015) observed that polysaccharides from acorn can be metabolized by *Lactobacillus plantarum* A7. Akpan is obtained from cereal, and contains significant amounts of polysaccharides that act as fermentable carbohydrates for supporting the growth of *Lactobacillus* and *Bifidobacterium* strains, resulting in the production of organic acids. However, this is the first time that Akpan has been reported as prebiotic compound.

Recently, Kurdi and Hansawasdi,(2015) reported that an oligosaccharide mixture obtained from cassava pulp, a by-product of cassava starch production, had the ability to promote the growth of some tested *Lactobacillus* and *Bifidobacterium* strains. However,

no study of beer cassava byproduct has been obtained in terms of its potential prebiotic activity.

Table 3.1: Organic acids concentrations (mM) produced during the fermentations using Akpan, BSG or FOS as substrate in media inoculated with *Lactobacillus casei* L431, *Lactobacillus casei* L01, *Bifidobacterium animalis* Bb12 or *Bifidobacterium lactis* B94

Fermentable carbohydrate	Acid	Time (h)	L431	L01	Bb12	B94
Akpan	Lactic	5	4.67 ± 0.15	4.86 ± 0.42	2.32 ± 0.21	1.15 ± 0.27
		10	11.26 ± 0.24	5.88 ± 0.89	2.17 ± 0.74	1.24 ± 0.34
		24	7.96 ± 1.78	6.58 ± 1.25	0.97 ± 0.12	9.09 ± 1.21
		48	9.07 ± 2.31	6.89 ± 1.33	0.45 ± 0.24	9.45 ± 1.56
	Formic	5	2.26 ± 0.45	1.72 ± 0.48	1.16 ± 0.21	0.00 ± 0.00
		10	5.45 ± 1.23	0.00 ± 0.00	6.71 ± 1.55	3.28 ± 1.21
		24	5.19 ± 0.75	0.00 ± 0.00	7.42 ± 0.33	7.02 ± 0.75
		48	0.00 ± 0.00	0.00 ± 0.00	3.95 ± 1.80	0.00 ± 0.00
	Acetic	5	6.30 ± 1.25	5.90 ± 1.34	7.45 ± 2.34	9.97 ± 1.27
		10	16.23 ± 3.24	12.81 ±3.12	13.47 ± 3.47	13.34 ± 2.87
		24	18.72 ± 4.21	23.78 ± 5.25	11.86 ± 2.41	17.79 ± 3.24
		48	19.81 ± 1.34	24.43 ± 4.85	13.23 ± 1.29	19.24 ± 3.78
BSC	Lactic	5	1.84 ± 0.20	2.07 ± 0.10	0.70 ± 0.98	0.40 ± 0.02
		10	4.61 ± 0.51	3.31 ± 0.27	0.00 ± 0.00	0.59 ± 0.06
		24	3.49 ± 2.29	1.93 ± 0.15	0.00 ± 0.00	3.01 ± 0.28
		48	2.69 ± 0.29	1.66 ± 0.17	0.00 ± 0.00	2.17 ± 0.32
	Formic	5	24.67 ± 1.04	27.67 ± 1.32	23.50 ± 1.54	30.64 ± 1.22
		10	21.04 ± 1.78	36.55 ± 0.94	21.84 ± 2.22	32.23 ± 1.43
		24	22.12 ± 0.87	30.85 ± 1.82	24.14 ± 1.38	32.17 ± 0.56
		48	10.50 ± 1.05	16.31 ± 1.31	21.96 ± 6.82	32.08 ± 0.15
	Acetic	5	3.26 ± 0.22	4.34 ± 0.44	9.35 ± 0.78	6.20 ± 0.99
		10	14.10 ± 1.86	14.36 ± 1.49	9.85 ± 0.51	8.93 ± 1.46
		24	15.73 ± 1.42	12.54 ± 1.23	12.09 ± 0.45	11.17 ± 0.26
		48	2.86 ± 0.49	8.95 ± 0.30	11.91 ± 0.19	10.87 ± 0.46
FOS	Lactic	5	5.90 ± 1.27	6.39 ± 0.78	2.22 ± 0.37	16.62 ± 2.24
		10	59.09 ± 12.45	7.32 ± 0.36	0.00 ± 0.00	31.52 ± 4.34
		24	110.42 ± 14.45	7.05 ± 1.21	0.00 ± 0.00	47.97 ± 8.12
		48	109. 13 ± 7.25	9.54 ± 1.78	0.00 ± 0.00	92.50 ± 7.45
	Formic	5	1.59 ± 0.23	1.05 ± 0.10	4.66 ± 10.35	3.39 ± 0.21
		10	0.00 ± 0.00	5.93 ± 1.00	7.30 ± 0.78	7.56 ± 1.27
		24	0.00 ± 0.00	2.13 ± 0.17	7.09 ± 1.98	0.00 ± 0.00

	Acetic	48	0.00 ± 0.00	0.00 ± 0.00	5.88 ± 0.74	0.00 ± 0.00
		5	3.69 ± 1.25	4.44 ± 0.38	5.34 ± 1.00	9.56 ± 2.48
		10	5.00 ± 1.64	9.29 ± 1.25	5.44 ± 0.87	10.91 ± 1.39
		24	3.79 ± 0.87	15.05 ± 2.38	5.53 ± 1.21	13.22 ± 0.15
		48	9.31 ± 1.21	19.03 ± 3.45	6.99 ± 1.11	17.10 ± 1.72
Control	Lactic	5	5.26 ± 0.64	3.80 ± 0.11	0.00 ± 0.00	0.30 ± 0.02
		10	5.21 ± 0.13	3.53 ± 0.10	0.00 ± 0.00	0.97 ± 0.01
		24	4.11 ± 0.42	2.55 ± 0.16	0.00 ± 0.00	1.41 ± 0.14
		48	4.66 ± 0.23	2.88 ± 0.17	0.00 ± 0.00	1.14 ± 0.29
	Formic	5	5.15 ± 0.31	2.26 ± 0.29	0.69 ± 0.11	2.64 ± 0.37
		10	3.91 ± 0.10	7.57 ± 0.42	7.56 ± 0.79	6.83 ± 1.15
		24	0.00 ± 0.00	3.56 ± 0.32	4.53 ± 0.25	5.47 ± 0.55
		48	0.00 ± 0.00	0.00 ± 0.00	6.11 ± 1.19	5.67 ± 0.39
	Acetic	5	5.25 ± 0.91	7.28 ± 0.55	0.00 ± 0.00	0.00 ± 0.00
		10	6.90 ± 0.74	9.16 ± 0.98	2.50 ± 0.12	4.25 ± 0.21
		24	8.99 ± 0.39	9.16 ± 1.12	2.33 ± 0.09	5.33 ± 0.33
		48	9.66 ± 1.12	6.83 ± 0.33	5.38 ± 0.28	0.00 ± 0.00

Values are expressed as mean ± standard deviation (Kurdi and Hansawasdi) of two replicates.

3.2. Effects of Akpan and BSC on the intestinal microbiota

3.2.1. SCFA and lactate accumulation in faecal cultures

It is known that SCFA are produced by almost all intestinal bacteria as consequence of non-digestible carbohydrates fermentation. **Table 3.2** summarizes the concentrations of SCFAs and lactate along fermentations and the pH value shifts resulting from the generation of acids. Results confirmed that substrates were metabolized by the several species present in the faecal microbiota (Rycroft *et al.*, 2001). The samples were analysed at 0, 6, 24 and 44 h for SCFA content.

Expectedly, SCFA accumulation was considerably higher in cultures containing carbohydrates (**Table 3.2**) when compared with the negative control samples, in which SCFA generation was mainly ascribed to protein degradation by putrefactive bacteria present in the intestinal microbiota (Hartemink and Rombouts, 1999; Rivas *et al.*, 2012). Consequently, low SCFA generation was expected in control cultures, thus indicating that SCFA generation was a result of the addition of external carbon sources. The SCFA resulting from fermentation of prebiotic carbohydrates by colonic bacteria act as electron

sinks of respiration in the anaerobic gut environment, and decrease the intestinal pH, promoting the bio-availability of calcium and magnesium, causing inhibition of harmful bacteria (Broekaert *et al.*, 2011) and allowing numerous trophic effects on the colonic environment.

The SCFA concentration profiles determined in samples containing carbon sources were in agreement with the observed pH value drops, which occurred mainly along the first 24 h of fermentation (see **Table 3.2**). After 44 h of incubation, a significant increase in total SCFA concentration was observed for media containing Akpan, BSC or FOS. BSC resulted in higher SCFA production at 44 h compared to Akpan or FOS ($p \leq 0.05$). The total SCFAs concentrations after 44 h varied in the range 81.83-105.85 mM, confirming that the degree of metabolic modulation varied depending on the carbohydrate type as it was previously established for other carbohydrates by several authors (Cardelle-Cobas *et al.*, 2012; Gullón *et al.*, 2014; Rivas *et al.*, 2012; Salazar *et al.*, 2009). These results are in agreement with those reported by Gullón *et al.*, 2014, where the total SCFA concentration reached similar values (99.7 mM) using arabinooligosaccharides from wheat bran.

It can be noted that the fermentation of all substrates induced the production of acetate. Acetate is the main SCFA in the gut (Oliveira *et al.*, 2012) and it was the most prevalent SCFA in all experiments; BSC produced significantly higher amounts of this acid for all fermentation times in comparison to Akpan or FOS (see **Table 3.2**). The acetic acid concentrations after 44 h varied in the range 44.46-70.83 mM. Acetic acid has been described as a major product of the bifidobacteria pathway following fermentation by human faecal suspensions, although acetic acid is also formed by many anaerobic bacteria from the human gut (Salazar *et al.*, 2009). Thus, it is difficult to relate the production of this acid with one bacterial genus when mixed cultures are studied (Bezkorovainy, 1989). These findings were in agreement with those reported by Connolly *et al.* (2010), who observed that acetic acid was the dominant SCFA produced in faecal fermentations using konjac glucomannan hydrolysate as a carbon source. The acetogenic potential of galactoglucomannans has been confirmed by Rivas *et al.* 2012 using *in vitro* fermentations with human faeces. Acetate largely bypasses colonic and liver metabolism and is metabolized by peripheral tissues (Bourquin *et al.*, 1992).

Lactate was formed in media containing FOS along the first 24 h of fermentation reaching a concentration of 11.07 mM. In the experiments with Akpan and BSC, lactic acid was detected only at 6 h of fermentation and was considerably minor compared to FOS. Lactic and acetic acid are considered products of the fermentation pathway of the *Bifidobacterium* genus. However, lactic acid was a transient metabolite, not observed after 24 h of fermentation, this was expected since lactate is utilized by other bacteria which then produce acetate, butyrate, and propionate as secondary metabolites, suggesting a cross-feeding mechanism (Hughes *et al.*, 2008; Kabel *et al.*, 2002).

A number of studies have confirmed the beneficial effects of butyrate and propionate in gut health (Topping and Clifton, 2001). As a consequence, it is important to identify and characterize prebiotics promoting the growth of bacterial groups able to produce butyrate and propionate selectively.

Propionate is mainly produced by Bacteroides and other bacteria within the Clostridium IX cluster (Maccaferri *et al.*, 2012). Several studies have reported that a reduction in the acetic to propionic acid ratio was linked to inhibition of cholesterol synthesis both in the liver as well as intestinal cholesterol biosynthesis (Delzenne and Kok, 2001). The data in **Table 3.2** show that the highest concentrations of this acid were found in media containing Akpan as the carbon source, followed by the media with BSC and FOS (32.13, 24.09 and 11.16 mmol/L respectively). Interestingly, values for the acetic acid/propionic acid ratio obtained for Akpan and BSC were lower than those obtained for the FOS. In the present study, the average ratio of acetic/propionic at 44 h for Akpan was 1.4, for BSC was 2.9 and for FOS was 4.3. This ratio obtained for Akpan and BSC would be considered to be a propionic acid rich SCFA profile. Our results compared favourably with those reported by Rivas *et al.* (2012), that carried out a study using human faecal inoculum to ferment galactoglucomannans derived from wood mannan, which resulted in an acetic/propionic ratio of 4.9. Connolly *et al.* (2010) carried out a study using batch cultures inoculated with human faeces to ferment konjac glucomannan hydrolysate and resulted in an acetic acid/propionic acid ratio of 1.9 and they reported that this was a high proportion of propionic acid when compared to other SCFAs profiles generated by other carbohydrates (Cardelle-Cobas *et al.*, 2012; Gullón *et al.*, 2010; Salazar *et al.*, 2009; Sarbini *et al.*, 2013). Hence, the fermentation with faecal inoculum in the presence of Akpan and BSC promoted a shift in the SCFAs profile,

causing a decrease of levels of acetic acid and a favourable increase in propionic acid proportions.

Butyrate, mainly produced by eubacteria and some clostridia (Charalampopoulos and Rastall, 2009), it has been studied for its role in nourishing the colonic mucosa and generally regarded as a healthy metabolite, since it positively influences cell growth and differentiation, exerts anti-inflammatory effects and has been linked to reducing colon cancer (Laparra and Sanz, 2010). Moderate increases in butyrate levels were observed in media containing Akpan or BSC (5.23 and 10.92 Mm after 44 h of fermentation). The butyrate accumulation was more pronounced when FOS was employed as the sole carbon source (23.78 mM at 44 h) with a significant difference ($p \leq 0.05$) between samples. Related results were reported in a study dealing with FOS and arabinooligosaccharides in which the media containing FOS resulted in butyrate concentrations higher than the ones obtained with arabinooligosaccharides (Gullón *et al.*, 2014). These results also have been confirmed by Gomez *et al.* (2014) using *in vitro* fermentations with human faecal.

Table 3.2: Mean value of lactic acid and short chain fatty acids concentration (mM) and pH values of fermentation media made with Akpan, BSC or FOS as carbon sources at 6, 24, and 44 h

Carbon source	Time (h)	pH	Lactate	Acetate	Propionate	Butyrate	Total SCFA
Akpan	0	7.2	0.14 (0.07)	1.28 (0.45)	0.05 (0.00)	0.24 (0.13)	2.05 (0.19)
	6	6.5	1.03^{a,b} (0.06)	5.57^{a,b} (1.03)	2.93^{a,b} (0.63)	0.00^{a} (0.00)	8.51^{a} (0.58)
	24	5.8	2.57^{a,b} (0.62)	32.86^{b} (3.47)	26.37^{b} (4.80)	1.75^{a} (1.27)	60.99^{b} (9.51)
	44	5	0.00^{a} (0.00)	44.46^{a,b} (3.43)	32.13^{b} (6.46)	5.23^{a} (0.31)	81.83^{b} (10.05)
BSC	0	7.1	0.12 (0.02)	1.45 (0.23)	0.05 (0.00)	0.33 (0.14)	2.27 (0.12)
	6	6.4	2.73^{b} (1.19)	14.9^{c} (5.45)	5.54^{b} (2.59)	1.45^{b} (0.67)	21.9^{b} (8.35)
	24	5.2	0.00^{a} (0.00)	57.75^{d} (0.91)	19.70^{b} (1.55)	7.38^{b} (1.24)	84.84^{c} (3.54)
	44	4.8	0.00^{a} (0.00)	70.83^{c} (8.24)	24.09^{b} (0.51)	10.92^{b} (2.60)	105.85^{c} (10.31)
FOS	0	7.2	0.18 (0.10)	2.75 (0.89)	0.38 (0.16)	0.45 (0.11)	4.21 (0.25
	6	6.0	2.60^{b} (0.68)	13.34^{b,c} (4.35)	3.15^{a,b} (1.05)	1.04^{a,b} (0.46)	17.5^{a,b} (5.85)
	24	5.0	11.07^{b} (7.43)	46.86^{c} (2.29)	11.35^{a} (2.42)	13.09^{c}(2.77)	71.32^{b,c} (2.73)
	44	4.5	0.68^{a} (0.74)	47.49^{b} (9.12)	11.16^{a} (1.69)	23.78^{c} (0.92)	82.44^{b} (8.13)
Control	0	7.2	0.11 (0.05)	1.45 (0.29)	0.14 (0.09)	0.00 (0.00)	1.83 (0.11)
	6	6.9	0.00^{a} (0.00)	3.17^{a} (0.31)	0.95^{a} (0.2)	0.096^{a} (0.16)	4.22^{a} (0.50)
	24	6.8	0.00^{a} (0.00)	20.38^{a} (2.67)	4.68^{a} (1.83)	3.99^{a,b}(0.59)	29.06^{a} (3.50)
	44	7.2	0.00^{a} (0.00)	29.20^{a} (0.82)	6.21^{a} (1.23)	5.14^{a} (0.19)	40.55^{a} (0.52)

Starting concentration of the test substrates were 1% (w/v). Standard deviation is shown in parentheses (n = 3). Different letters indicate significant differences ($P < 0.05$) for the same acid. Substrates were compared for three points of fermentation (6, 24 and 44 h).

3.2.2. Effect of Akpan and BSC fermentation in bacterial composition of intestinal human microbiota

There are several factors namely genetics, age, health status, nutrition, and diet of the faeces donors that can affect the bacterial composition (Hernandez-Hernandez *et al.*, 2011). The data consistency presented in this work is proved by the low standard deviation showed by the quantitative results obtained for the three donors.

Although it is very difficult to fully characterize all changes occurring in the colonic microbiota, monitoring the populations of selected species provides information suitable for assessing colon health (Woodmansey, 2007). In this work, the dynamics of the bacterial groups along fermentation was assessed using FISH. The changes in the human faecal bacterial populations are summarized in **Table 3.3**.

There is strong evidence that the colonic fermentation of prebiotic carbohydrates is influential in the gut function. Fermentation results in increased bacterial mass, increased stool weight (by osmotic water binding), increased stool frequency, and softer stools (Gómez *et al.*, 2014). The data in **Table 3.3** show that the total cell counts increased markedly with time in media containing suitable carbon sources. In the control experiment, bacterial growth was observed just in the first 6 h of fermentation. In media containing FOS, Akpan, and BSC, the total cell counts increased by 12%, with no significant differences among them ($p > 0.05$). This finding is in agreement with data reported for experiments using other carbon sources, as well as pectic oligosaccharides from apple pomace (Gullón *et al.*, 2011) or alternansucrase raffinose-derived oligosaccharides (Hernandez-Hernandez *et al.*, 2011).

Increased proportions of bifidobacteria and lactobacilli (which are able to convert carbohydrates into acids and generally lack toxicity) are thought to be associated with healthier microbial compositions. The data in **Table 3.3** confirmed a preferential growth of bifidobacteria on the various cultures with respect to the negative control. After 6 h of incubation, the shifts in bifidobacteria counts were similar for media containing FOS, Akpan or BSC ($p > 0.05$). In contrast, the bifidobacteria population increased slightly in media made with Akpan or BSC after 24 h. In comparative terms, Akpan or BSC caused stronger bifidogenic effects than FOS. In the experiments containing carbon sources, the

rapid increase in acetic acid during the early stages of fermentation (0-6 h) correlated well with both the increase of the populations of *Bifidobacterium*.

The results obtained in this work are in agreement with the data reported in related studies. So, Gullón (2014) reported increased levels of *Bifidobacterium* species using arabinoxylooligosaccharides from wheat bran. A similar increment was also found during the fermentation of other carbohydrates sources such as konjac glucomannan (Connolly *et al.*, 2010), feruloylated oligo- and polysaccharides from maize and wheat brans (Yang *et al.*, 2014), linear and α-1,2-branched dextrans (Sarbini *et al.*, 2013).

Regarding *Lactobacillus* group, progressive increases in numbers were observed during fermentations in cultures with all substrates during the first 6 h of fermentation, whereas no significant increases were observed after 6 h in cultures containing FOS. After 24 h of fermentation, the *Lactobacillus* numbers increased by 27% and 25% in cultures containing Akpan and BSC, respectively, whereas less marked effects were observed in media containing FOS (in which the counts increased by 22%). Our results compared favourably with those reported in literature. So, recently (Rodriguez-Colinas *et al.*, 2013) evaluated the *in vitro* fermentation of various purified galactooligosaccharides and observed minor changes in the content of *Lactobacillus* after 24 h of fermentation (increase by 2%).

It is also evident that no substantial changes were observed in Bacteroides and *Eubacterium rectale* group population during fermentations with all carbon sources tested ($p<0.05$). Regarding Bacteroides population, this group was increased similarly with FOS, Akpan and BSC as shown in **Table 3.3**. Bacteroides is one of the predominant bacterial group in gut microbiota, and it is a metabolically versatile group that is able to use many types of carbohydrates and produce acetic acid and propionic acid (Macy *et al.*, 1978). This latter is considered as a healthy compound that can inhibit the synthesis of cholesterol (Delzenne and Kok, 2001). Therefore, this group is important because Bacteroides are major polysaccharides-degrading organisms in the gut. According to the results in **Table 3.3**, the population of Bacteroides in media containing the carbon sources tested increased progressively with time. (Sarbini *et al.*, 2011) reported on the ability of this group to grow on several dextrans with different structure and molecular weight. Hernandez-Hernandez *et al.* (2011) also demonstrated a similar effect during the fermentation by gut bacteria of alternansucrase raffinose-derived oligosaccharides.

Eubacteria rectale plays a physiological role related to their ability to produce relatively large amounts of butyrate from acetate, lactate, and polysaccharides (Cardelle-Cobas *et al.*, 2012). Major increases in counts were observed in the period 0-6 h, with variations of minor importance at longer fermentation times. No significant differences among substrates were observed ($p<0.05$). On average, the numbers of this group of bacteria increased by 16% after 44 h fermentation in media containing FOS, Akpan or BSC. These results are in agreement with the data reported in related studies; so, Gullón *et al.* (2011) reported an increase in the population of *E. rectale* upon fermentation with human faeces using pectic-oligosaccharides mixture from apple pomace as substrates. Conversely, Sarbini *et al.* (2011) did not observe an enhancement of this type of bacteria in fermentation of different dextrans.

Regarding *Faecalibacterium prausnitzii*, this type of bacteria plays a physiological role related to their ability to produce relatively large amounts of butyrate, and have been shown that a deficit of which has been related to the recurrence of inflammatory bowel disease and to ulcerative colitis (Scott *et al.*, 2014). The data in **Table 3.3** show that proliferation of this group was significant in cultures with Akpan and BSC after 24 h of fermentation with respect to FOS. However, the data obtained at 44 h revealed no significant count differences between FOS and BSC cultures, whereas Akpan led to the highest result. In media containing Akpan, the number of *F. prausnitzii* increased by 20% after 24 h of fermentation. The results obtained in this work are not in agreement with the data reported in related studies. Sarbini *et al.* (2011) observed a significant decrease of *F. prausnitzii* when studying the fermentation properties of a series of linear and α(1→2)-branched dextrans. Recently, Rodríguez-Colinas *et al.* (2013) also reported decrease counts of these bacterial group in media containing galactooligosaccharides.

Concerning *Clostridium histolyticum* the data in **Table 3.3** show a significant increase of this group in cultures with FOS during the first 6 h of fermentation with respect to Akpan or BSC. The data obtained after 24 h revealed significant count differences between Akpan or BSC cultures and FOS. Our results are in line with those previously reported by Hernandez-Hernandez *et al.* (2011) and Salazar *et al.* (2009). These authors reported increased counts of these clusters after 10 h in media containing alternansucrase raffinose-derived oligosaccharides and inulin respectively.

Table 3.3: Bacterial populations in faecal batch cultures measured at 0, 6, 24 and 44 h after fermentation onset (expressed as Log cells/mL±SD)

Group		FOS			Akpan			BSC			Control		
	0 h	6 h	24 h	44 h	6 h	24 h	44 h	6 h	24 h	44 h	6 h	24 h	44 h
DAPI	8.13±0.04	$9.04^{a,b}$±0.36	9.87^{b}±0.23	9.66^{b}±0.31	9.13^{b}±0.05	9.61^{b}±0.05	9.89^{b}±0.21	9.09^{b}±0.24	9.52^{b}±0.10	9.93^{b}±0.06	8.50^{a}±0.03	8.52^{a}±0.12	8.37^{a}±0.02
Bif164	7.46±0.11	8.43^{b}±0.34	8.67^{b}±0.04	8.65^{b}±0.14	8.45^{b}±0.05	8.89^{c}±0.06	8.86^{b}±0.06	$8.38^{a,b}$±0.13	8.93^{c}±0.06	8.64^{b}±0.18	7.90^{a}±0.05	7.71^{a}±0.06	7.64^{a}±0.14
Chis150	7.49±0.13	8.89^{c}±0.14	8.83^{b}±0.10	8.81^{b}±0.23	8.64^{b}±0.03	$9.00^{b,c}$±0.08	8.91^{b}±0.15	8.52^{b}±0.05	9.14^{c}±0.05	8.47^{b}±0.29	8.10^{a}±0.05	7.92^{a}±0.05	7.83^{a}±0.12
Lab158	7.01±0.10	8.66^{b}±0.17	8.58^{b}±0.16	8.47^{b}±0.12	8.51^{b}±0.04	8.93^{c}±0.05	8.96^{c}±0.15	8.48^{b}±0.15	$8.75^{b,c}$±0.01	8.66^{b}±0.07	7.87^{a}±0.11	7.69^{a}±0.06	7.40^{a}±0.13
Erec482	7.51±0.03	8.78^{b}±0.21	9.00^{b}±0.07	8.84^{b}±0.20	8.46^{b}±0.09	8.82^{b}±0.08	8.76^{b}±0.08	8.52^{b}±0.11	9.00^{b}±0.15	8.54^{b}±0.05	8.04^{a}±0.05	7.74^{a}±0.05	7.68^{a}±0.06
Bac303	7.88±0.01	8.83^{b}±0.11	8.96^{b}±0.08	8.93^{b}±0.18	8.71^{b}±0.07	8.93^{b}±0.02	8.83^{b}±0.09	8.72^{b}±0.04	9.03^{b}±0.04	8.48^{b}±0.24	8.08^{a}±0.11	7.90^{a}±0.13	7.84^{a}±0.20
Frap655	7.50±0.14	8.60^{b}±0.23	8.78^{b}±0.04	8.62^{b}±0.07	8.81^{b}±0.08	9.00^{c}±0.04	8.87^{c}±0.07	8.66^{b}±0.10	8.95^{c}±0.04	8.50^{b}±0.09	7.92^{a}±0.06	7.99^{a}±0.07	7.86^{a}±0.05

Total bacterial counts were obtained using DAPI and bacterial groups with FISH probes, (Bif164: *Bifidobacterium* genus; Lab158: *Lactobacillus–Enterococcus* group; Bac303: *Bacteroides-Prevotella* group; His150: *Clostridium histolyticum* subgroup; Erec482: *Ruminococcus–Eubacterium–Clostridium* cluster; and Fpra655: *Faecalibacterium prausnitzii* and relatives). Mean bacterial count ± standard deviation (n = 3). Different letters indicate significant differences ($P < 0.05$) for each bacterial group. Substrates were compared in three fermentation sampling times (6, 24 and 44 h).

4. Conclusions

Based on all the results achieved and their analysis, it was possible to conclude that, Akpan and BSC can exert potential prebiotic effects, once they promote the growth of beneficial colonic bacterial populations and the production of SCFA.

Akpan and BSC can enhance the growth of *Bifidobacteria* strains and have a similar effect as FOS. However, Akpan cannot enhance the growth of the *Lactobacillus* strains while BSC can enhance the growth of *L. casei* L01 in the same way as FOS.

Akpan and BSC can stimulate the growth of certain bacterial groups in the human intestinal microbiota in an extent comparable to FOS. It was observed that Akpan and BSC promote a stronger effect on the growth of bifidobacteria and lactobacilli than FOS. These bacterial groups are thought to be associated with healthier microbial compositions.

The fermentation of these carbohydrates resulted in the production of SCFA. BSC was the carbohydrate that during the fermentation promoted a higher production of SCFA, while Akpan and FOS have a similar production of SCFA, however the SCFA profile was different. BSC fermentation result in a remarkable generation of acetate, the main SCFA in the gut, while FOS fermentation led to a higher concentration of butyrate. Akpan and BSG fermentation resulted in propionate rich SCFA profile.

Therefore, the results of this work could support that the consumption of this cereal-based fermented beverage (Akpan), could improve the gut health. Despite BSC being a by-product, exhibit functional properties that made BSC a potential functional ingredient in the food industry.

Nevertheless, further studies are recommended, mainly *in vivo* studies, to confirm these *in vitro* studies.

5. Future works

The present work evaluated the *in vitro* prebiotic potential of Akpan and BSC. However, *in vivo* studies using animal models and human trials with validated methodologies need to be made in order to demonstrate the *in vivo* prebiotic effect of Akpan and BSC.

Furthermore, it will be important a detailed study of the chemical composition of Akpan and BSC, particularly the polysaccharide fractions which provide a better understand of its fermentability. Regarding to BSC, further studies on their chemical composition will allow the study of small fractions and compounds of interest that can be isolated and study their potential biological activities, and applications.

Additionally, it's important evaluate if Akpan or BSC have antimicrobial activity against pathogenic microorganisms, to evaluate if once in the gut these compounds wont promote the growth of pathogenic microorganism. Also, other potential beneficial effects can be assessed for Akpan and BSC, namely antioxidant activity and antihypertensive activity.

As BSG have potential to be an ingredient its important study the development of food products incorporating BSG in their composition, like snacks, biscuits, breads, cakes, flour, etc, after this it is important to evaluate if during the food processing the prebiotic activity is still maintained.

6. References

A ……

Ainsworth, P., İbanoğlu, Ş., Plunkett, A., İbanoğlu, E., Stojceska, V. 2007. Effect of brewers spent grain addition and screw speed on the selected physical and nutritional properties of an extruded snack. Journal of Food Engineering 81(4), 702-709.

Akissoé, N.H., Sacca, C., Declemy, A.-L., Bechoff, A., Anihouvi, V.B., Dalodé, G., Pallet, D., Fliedel, G., Mestres, C., Hounhouigan, J.D., Tomlins, K.I. 2014. Cross-cultural acceptance of a traditional yoghurt-like product made from fermented cereal. Journal of the Science of Food and Agriculture.

Al-Sheraji, S.H., Ismail, A., Manap, M.Y., Mustafa, S., Yusof, R.M., Hassan, F.A. 2013. Prebiotics as functional foods: A review. Journal of Functional Foods 5(4), 1542-1553.

Altieri, C., Bevilacqua, A., Sinigaglia, M. 2011. Prolonging the Viability of Lactobacillus plantarum through the Addition of Prebiotics into the Medium. Journal of Food Science 76(6), M336-M345.

Anderson, G. R. 2006. History of Industrial Brewing. In: Handbook of Brewing (Fergus G. Priest, Graham G. Stewart) Taylor & Francis, Boca Raton, USA. pp. 2-3

Annunziata, A., Vecchio, R. 2013. Consumer perception of functional foods: A conjoint analysis with probiotics. Food Quality and Preference 28(1), 348-355.

B ……

Barry, J.L., Hoebler, C., MacFarlane, G.T., MacFarlane, S., Mathers, J.C., Reed, K.A., Mortensen, P.B., Nordgaard, I., Rowland, I.R., Rumney, C.J. 2007. Estimation of the fermentability of dietary fibre in vitro: a European interlaboratory study. British Journal of Nutrition 74(3), 303-322.

Bezkorovainy, A. 1989. Nutrition and metabolism in Bifidobacteria. In Biochemistry and

Physiology of Bifidobacteria; Bezkorovainy, A., Miller- Catchpole, R., Eds.; CRC Press: Boca Raton, FL, pp 93−129.

Blandino, A., Al-Aseeri, M.E., Pandiella, S.S., Cantero, D., Webb, C. 2003. Cereal-based fermented foods and beverages. Food Research International 36(6), 527-543.

Bourquin, L.D., Titgemeyer, E.C., Garleb, K.A., Fahey, G.C. 1992. Short-Chain Fatty Acid Production and Fiber Degradation by Human Colonic Bacteria: Effects of Substrate and Cell Wall Fractionation Procedures. The Journal of nutrition 122(7), 1508-1520.

Brennan, C.S., Cleary, L.J. 2005. The potential use of cereal (1→ 3, 1→ 4)-β-D-glucans as functional food ingredients. Journal of Cereal Science 42(1), 1-13.

Briggs, D.E., Boulton, C.A., Brookes, P.A. 2004. *Brewing: Science and Practice*. Taylor & Francis.

Broekaert, W., Courtin, C., Delcour, J. 2011. (arabino)xylan oligosaccharide preparation. US 2011/0020498 A1.

Buitrago, J.A. 1990. *La yuca en la alimentación animal*. CIAT.

C .....

Cardelle-Cobas, A., Olano, A., Corzo, N., Villamiel, M., Collins, M., Kolida, S., Rastall, R.A. 2012. In Vitro Fermentation of Lactulose-Derived Oligosaccharides by Mixed Fecal Microbiota. Journal of Agricultural and Food Chemistry 60(8), 2024-2032.

Charalampopoulos, D., Rastall, R.A. 2009. *Prebiotics and probiotics science and technology*. Springer.

Charalampopoulos, D., Wang, R., Pandiella, S.S., Webb, C. 2002. Application of cereals and cereal components in functional foods: a review. International Journal of Food Microbiology 79(1), 131-141.

Connolly, M.L., Lovegrove, J.A., Tuohy, K.M., 2010. Konjac glucomannan hydrolysate beneficially modulates bacterial composition and activity within the faecal microbiota. Journal of Functional Foods 2(3), 219-224.

Cronin, M., Ventura, M., Fitzgerald, G.F., van Sinderen, D. 2011. Progress in genomics, metabolism and biotechnology of bifidobacteria. International Journal of Food Microbiology 149(1), 4-18.

D ……

De Bellis, P., Valerio, F., Sisto, A., Lonigro, S.L., Lavermicocca, P. 2010. Probiotic table olives: Microbial populations adhering on olive surface in fermentation sets inoculated with the probiotic strain Lactobacillus paracasei IMPC2.1 in an industrial plant. International Journal of Food Microbiology 140(1), 6-13.

Delzenne, N.M., Kok, N., 2001. Effects of fructans-type prebiotics on lipid metabolism. The American journal of clinical nutrition 73(2), 456s-458s.

F ……

FAO. 2016. “FAOSTAT”. Available: http://www.fao.org/faostat/en/#data/QD (date visited: 5/01/2017)

FAO/WHO. 2001 Health and Nutritional Properties of Probiotics in Food including Powder Milk with Live Lactic Acid Bacteria. Cordoba, Argentina: Food and Agriculture Organization of the United Nations and World Health Organization Expert Consultation Report

Fastnaught, C. 2001. Barley Fiber, *Handbook of Dietary Fiber*. CRC Press, pp. 503-526.

Fijan, S. 2014. Microorganisms with claimed probiotic properties: an overview of recent literature. International journal of environmental research and public health 11(5), 4745-4767.

Finegold, S.M., Attebery, H.R., Sutter, V.L. 1974. Effect of diet on human fecal flora: comparison of Japanese and American diets. The American journal of clinical nutrition 27(12), 1456-1469.

Ford, D. 2015. Why the Cassava industry Now?. In: Regional Conference on Cassava in the Caribbean and Latin America. Food and Agriculture Organization of the United Nations, Rome. pp.5-7

Franks, A.H., Harmsen, H.J.M., Raangs, G.C., Jansen, G.J., Schut, F., Welling, G.W. 1998. Variations of Bacterial Populations in Human Feces Measured by Fluorescent In Situ Hybridization with Group-Specific 16S rRNA-Targeted Oligonucleotide Probes. Applied and environmental microbiology 64(9), 3336-3345.

Franz, C.M.A.P., Huch, M., Mathara, J.M., Abriouel, H., Benomar, N., Reid, G., Galvez, A., Holzapfel, W.H. 2014. African fermented foods and probiotics. International Journal of Food Microbiology 190(0), 84-96.

Fuller, R. 1989. Probiotics in man and animals. J Appl Bacteriol 66(5), 365-378.

G .....

Gawkowski, D., Chikindas, M.L. 2013. Non-dairy probiotic beverages: the next step into human health. Beneficial Microbes 4(2), 127-142.

Gibson, G., Wang, X. 1994. Bifidogenic properties of different types of fructo-oligosaccharides. Food Microbiology 11(6), 491-498.

Gibson, G.R., Fuller, R. 2000. Aspects of In Vitro and In Vivo Research Approaches Directed Toward Identifying Probiotics and Prebiotics for Human Use. The Journal of nutrition 130(2), 391.

Gibson, G.R., Probert, H.M., Van Loo, J., Rastall, R.A., Roberfroid, M.B. 2004. Dietary modulation of the human colonic microbiota: updating the concept of prebiotics. Nutrition research reviews 17(02), 259-275.

Gil J.L., Buitrago J. A. 2002. La yuca en la alimentacion animal. In: Ospina B, Ceballos H, editors. La yuca en el tercer milenio: sistemas modernos de producción, procesamiento, utilización y comercialización . Cali, Colombia: Centro Internacional de Agricultura Tropical. p527–69.

Gomes da Cruz, A., Alonso Buriti, F.C., Batista de Souza, C.H., Fonseca Faria, J.A., Isay Saad, S.M. 2009. Probiotic cheese: Health benefits, technological and stability aspects. Trends in Food Science & Technology 20(8), 344-354.

Gómez, B., Gullón, B., Remoroza, C., Schols, H.A., Parajó, J.C., Alonso, J.L. 2014. Purification, Characterization, and Prebiotic Properties of Pectic Oligosaccharides from Orange Peel Wastes. Journal of Agricultural and Food Chemistry 62(40), 9769-9782.

Gómez-Conde, M.S., García, J., Chamorro, S., Eiras, P., Rebollar, P.G., Pérez de Rozas, A., Badiola, I., de Blas, C., Carabaño, R. 2007. Neutral detergent-soluble fiber improves gut barrier function in twenty-five-day-old weaned rabbits1. Journal of Animal Science 85(12), 3313-3321.

Goode, D.L., Halbert, C., Arendt, E.K. 2002. Mashing Studies with Unmalted Sorghum and Malted Barley. Journal of the Institute of Brewing 108(4), 465-473.

Grahman, D. 2015 Cassava Use in Beer as an Adjunct Replacement. In: Regional Conference on Cassava in the Caribbean and Latin America. Food and Agriculture Organization of the United Nations, Rome. pp.48

Granato, D., Branco, G.F., Cruz, A.G., Faria, J.d.A.F., Shah, N.P. 2010a. Probiotic Dairy Products as Functional Foods. Comprehensive Reviews in Food Science and Food Safety 9(5), 455-470.

Granato, D., Branco, G.F., Nazzaro, F., Cruz, A.G., Faria, J.A.F. 2010b. Functional Foods and Nondairy Probiotic Food Development: Trends, Concepts, and Products. Comprehensive Reviews in Food Science and Food Safety 9(3), 292-302.

Guarner, F., Malagelada, J.-R. 2003. Gut flora in health and disease. The Lancet 361(9356), 512-519.

Gullón, B., Gullón, P., Sanz, Y., Alonso, J.L., Parajó, J.C. 2011. Prebiotic potential of a refined product containing pectic oligosaccharides. LWT - Food Science and Technology 44(8), 1687-1696.

Gullón, B., Gullón, P., Tavaria, F., Pintado, M., Gomes, A.M., Alonso, J.L., Parajó, J.C. 2014. Structural features and assessment of prebiotic activity of refined arabinoxylooligosaccharides from wheat bran. Journal of Functional Foods 6, 438-449.

Gullón, P., González-Muñoz, M.J., van Gool, M.P., Schols, H.A., Hirsch, J., Ebringerová, A., Parajó, J.C. 2010. Production, Refining, Structural Characterization and Fermentability of Rice Husk Xylooligosaccharides. Journal of Agricultural and Food Chemistry 58(6), 3632-3641.

H

Ha, H.P., Nga, L.H., Phu, T.V., Anh, T.K., Tosch, W. 2014. Development of Method to Produce Snacks Supplemented with Brewer's Spent Cassava. International Proceedings of Chemical, Biological and Environmental Engineering (IPCBEE) 77 (10).

Hardy, G. 2000. Nutraceuticals and functional foods: introduction and meaning. Nutrition 16(7), 688-689.

Hartemink, R., Rombouts, F.M. 1999. Comparison of media for the detection of bifidobacteria, lactobacilli and total anaerobes from faecal samples. Journal of Microbiological Methods 36(3), 181-192.

Havenaar, R., Huis In't Veld, J.H.J. 1992. Probiotics: A General View, in: Wood, B.J.B. (Ed.), *The Lactic Acid Bacteria Volume 1: The Lactic Acid Bacteria in Health and Disease*. Springer US, Boston, MA, pp. 151-170.

Hermie J. M. Harmsen, P.E.F.S.G.W.W. 1999. A 16S rRNA-targeted Probe for Detection of Lactobacilli and Enterococci in Faecal Samples by Fluorescent In Situ Hybridization. Microbial Ecology in Health and Disease 11(1), 3-12.

Hernandez-Hernandez, O., Sanz, M.L., Kolida, S., Rastall, R.A., Moreno, F.J. 2011. In Vitro Fermentation by Human Gut Bacteria of Proteolytically Digested Caseinomacropeptide Nonenzymatically Glycosylated with Prebiotic Carbohydrates. Journal of Agricultural and Food Chemistry 59(22), 11949-11955.

Hold, G.L., Schwiertz, A., Aminov, R.I., Blaut, M., Flint, H.J. 2003. Oligonucleotide Probes That Detect Quantitatively Significant Groups of Butyrate-Producing Bacteria in Human Feces. Applied and environmental microbiology 69(7), 4320-4324.

Holzapfel, W.H., Schillinger, U. 2002. Introduction to pre- and probiotics. Food Research International 35(2–3), 109-116.

Huebner, J., Wehling, R.L., Hutkins, R.W. 2007. Functional activity of commercial prebiotics. International Dairy Journal 17(7), 770-775.

Hughes, S.A., Shewry, P.R., Gibson, G.R., McCleary, B.V., Rastall, R.A. 2008. In vitro fermentation of oat and barley derived β-glucans by human faecal microbiota. FEMS Microbiology Ecology 64(3), 482-493.

Huige, N.J., 2006. Brewery by-products and effluents, *Handbook of Brewing, Second Edition*. CRC Press, pp. 655-713.

I .....

IFIC. 2011. Background on Functional Foods. Retrieved June 29, 2016, from http://www.foodinsight.org/Background_on_Functional_Foods#

ISAPP. 2008. 6th Meeting of the International Scientific Association of Probiotics and Prebiotics, London, Ontario.

J ……

Jeon, S.G., Kayama, H., Ueda, Y., Takahashi, T., Asahara, T., Tsuji, H., Tsuji, N.M., Kiyono, H., Ma, J.S., Kusu, T., Okumura, R., Hara, H., Yoshida, H., Yamamoto, M., Nomoto, K., Takeda, K. 2012. Probiotic Bifidobacterium breve Induces IL-10-Producing Tr1 Cells in the Colon. PLOS Pathogens 8(5), e1002714.

K ……

Kabel, M.A., Kortenoeven, L., Schols, H.A., Voragen, A.G.J. 2002. In Vitro Fermentability of Differently Substituted Xylo-oligosaccharides. Journal of Agricultural and Food Chemistry 50(21), 6205-6210.

Kaur, S., Das, M. 2011. Functional foods: An overview. Food Science and Biotechnology 20(4), 861.

Kerckhoffs, A.M., Samsom, M., van Berge Henegouwen, G., Akkermans, L.A., Nieuwenhuijs, V., Visser, M. 2006. Sampling Microbiota in the Human Gastrointestinal Tract, *Gastrointestinal Microbiology*. CRC Press, pp. 25-50.

Kissell, L., Prentice, N., Lindsay, R. 1979. Protein and fiber enrichment of cookie flour with brewer's spent grain. Cereal Chem 56(4), 261-266.

Koletzko, B., Aggett, P.J., Bindels, J., Bung, P., Ferre, P., Gil, A., Lentze, M.J., Roberfroid, M., Strobel, S. 1998. Growth, development and differentiation: a functional food science approach. British Journal of Nutrition 80(S1), S5-S45.

Ktenioudaki, A., Alvarez-Jubete, L., Smyth, T.J., Kilcawley, K., Rai, D.K., Gallagher, E. 2015. Application of bioprocessing techniques (sourdough fermentation and technological aids) for brewer's spent grain breads. Food Research International 73, 107-116.

Ktenioudaki, A., Chaurin, V., Reis, S.F., Gallagher, E. 2012. Brewer's spent grain as a functional ingredient for breadsticks. International Journal of Food Science & Technology 47(8), 1765-1771.

Ktenioudaki, A., Crofton, E., Scannell, A.G., Hannon, J.A., Kilcawley, K.N., Gallagher, E. 2013a. Sensory properties and aromatic composition of baked snacks containing brewer's spent grain. Journal of Cereal Science 57(3), 384-390.

Ktenioudaki, A., O'Shea, N., Gallagher, E. 2013b. Rheological properties of wheat dough supplemented with functional by-products of food processing: Brewer's spent grain and apple pomace. Journal of Food Engineering 116(2), 362-368.

Kumar, M., Nagpal, R., Kumar, R., Hemalatha, R., Verma, V., Kumar, A., Chakraborty, C., Singh, B., Marotta, F., Jain, S., Yadav, H. 2012. Cholesterol-Lowering Probiotics as Potential Biotherapeutics for Metabolic Diseases. Experimental Diabetes Research 2012, 902917.

Kurdi, P., Hansawasdi, C. 2015. Assessment of the prebiotic potential of oligosaccharide mixtures from rice bran and cassava pulp. LWT - Food Science and Technology 63(2), 1288-1293.

Kwak, N.-S., Jukes, D.J. 2001. Functional foods. Part 1: the development of a regulatory concept. Food Control 12(2), 99-107.

L .....

Langendijk, P.S., Schut, F., Jansen, G.J., Raangs, G.C., Kamphuis, G.R., Wilkinson, M.H., Welling, G.W. 1995. Quantitative fluorescence in situ hybridization of Bifidobacterium spp. with genus-specific 16S rRNA-targeted probes and its application in fecal samples. Applied and environmental microbiology 61(8), 3069-3075.

Laparra, J.M., Sanz, Y. 2010. Interactions of gut microbiota with functional food components and nutraceuticals. Pharmacological Research 61(3), 219-225.

Lilly, D.M., Stillwell, R.H. 1965. Probiotics: Growth-Promoting Factors Produced by Microorganisms. Science 147(3659), 747-748.

Lynch, K.M., Steffen, E.J., Arendt, E.K. 2016. Brewers' spent grain: a review with an emphasis on food and health. Journal of the Institute of Brewing 122(4), 553-568.

M

Maccaferri, S., Klinder, A., Cacciatore, S., Chitarrari, R., Honda, H., Luchinat, C., Bertini, I., Carnevali, P., Gibson, G.R., Brigidi, P., Costabile, A. 2012. In vitro fermentation of potential prebiotic flours from natural sources: Impact on the human colonic microbiota and metabolome. Molecular Nutrition & Food Research 56(8), 1342-1352.

Macfarlane, G.T., Macfarlane, S., Gibson, G.R. 1998. Validation of a Three-Stage Compound Continuous Culture System for Investigating the Effect of Retention Time on the Ecology and Metabolism of Bacteria in the Human Colon. Microbial Ecology 35(2), 180-187.

Macy, J.M., Ljungdahl, L.G., Gottschalk, G. 1978. Pathway of Succinate and Propionate Formation in Bacteroides fragilis. Journal of Bacteriology 134(1), 84-91.

Madhukumar, M.S., Muralikrishna, G. 2010. Structural characterisation and determination of prebiotic activity of purified xylo-oligosaccharides obtained from Bengal gram husk (Cicer arietinum L.) and wheat bran (Triticum aestivum). Food Chemistry 118(2), 215-223.

Mäkeläinen, H., Forssten, S., Olli, K., Granlund, L., Rautonen, N., Ouwehand, A.C. 2009. Probiotic lactobacilli in a semi-soft cheese survive in the simulated human gastrointestinal tract. International Dairy Journal 19(11), 675-683.

Malaguarnera, G., Leggio, F., Vacante, M., Motta, M., Giordano, M., Biondi, A., Basile, F., Mastrojeni, S., Mistretta, A., Malaguarnera, M., Toscano, M.A., Salmeri, M. 2012. Probiotics in the gastrointestinal diseases of the elderly. The journal of nutrition, health & aging 16(4), 402-410.

Maldonado, J., Cañabate, F., Sempere, L., Vela, F., Sánchez, A.R., Narbona, E., López-Huertas, E., Geerlings, A., Valero, A.D., Olivares, M., Lara-Villoslada, F. 2012. Human Milk Probiotic Lactobacillus fermentum CECT5716 Reduces the Incidence of Gastrointestinal and Upper

Respiratory Tract Infections in Infants. Journal of Pediatric Gastroenterology and Nutrition 54(1), 55-61.

Manz, W., Amann, R., Ludwig, W., Vancanneyt, M., Schleifer, K.-H. 1996. Application of a suite of 16S rRNA-specific oligonucleotide probes designed to investigate bacteria of the phylum cytophaga-flavobacter-bacteroides in the natural environment. Microbiology 142(5), 1097-1106.

Martirosyan, D.M., Singh, J. 2015. A new definition of functional food by FFC: what makes a new definition unique? Functional Foods in health and disease 5(6), 209-223.

McCarthy, A.L., O'Callaghan, Y.C., Neugart, S., Piggott, C.O., Connolly, A., Jansen, M.A., Krumbein, A., Schreiner, M., FitzGerald, R.J., O'Brien, N.M. 2013. The hydroxycinnamic acid content of barley and brewers' spent grain (BSG) and the potential to incorporate phenolic extracts of BSG as antioxidants into fruit beverages. Food Chemistry 141(3), 2567-2574.

McCleary, B.V., Nurthen, E. 1986. Measurement of (1→ 3)(1→ 4)-β-d-glucan in malt, wort and beer. Journal of the Institute of Brewing 92(2), 168-173.

McFarland, L.V. 2007. Meta-analysis of probiotics for the prevention of traveler's diarrhea. Travel Medicine and Infectious Disease 5(2), 97-105.

Meyer, D., Stasse-Wolthuis, M. 2009. The bifidogenic effect of inulin and oligofructose and its consequences for gut health. Eur J Clin Nutr 63(11), 1277-1289.

Miles, A.A., Misra, S.S., Irwin, J.O. 1938. The estimation of the bactericidal power of the blood. The Journal of Hygiene 38(6), 732-749.

Mills, D.J.S., Tuohy, K.M., Booth, J., Buck, M., Crabbe, M.J.C., Gibson, G.R., Ames, J.M. 2008. Dietary glycated protein modulates the colonic microbiota towards a more detrimental composition in ulcerative colitis patients and non-ulcerative colitis subjects. Journal of Applied Microbiology 105(3), 706-714.

Molis, C., Flourie, B., Ouarne, F., Gailing, M.-F., Lartigue, S., Guibert, A., Bornet, F., Galmiche, J. 1996. Digestion, excretion, and energy value of fructooligosaccharides in healthy humans. The American journal of clinical nutrition 64(3), 324-328.

Mombelli, B., Gismondo, M.R. 2000. The use of probiotics in medical practice. International Journal of Antimicrobial Agents 16(4), 531-536.

Montagnac, J.A., Davis, C.R., Tanumihardjo, S.A. 2009a. Nutritional value of cassava for use as a staple food and recent advances for improvement. Comprehensive Reviews in Food Science and Food Safety 8(3), 181-194.

Montagnac, J.A., Davis, C.R., Tanumihardjo, S.A. 2009b. Processing techniques to reduce toxicity and antinutrients of cassava for use as a staple food. Comprehensive Reviews in Food Science and Food Safety 8(1), 17-27.

Moura, P., Cabanas, S., Lourenço, P., Gírio, F., Loureiro-Dias, M.C., Esteves, M.P. 2008. In vitro fermentation of selected xylo-oligosaccharides by piglet intestinal microbiota. LWT - Food Science and Technology 41(10), 1952-1961.

Mussatto, S.I. 2009. Biotechnological Potential of Brewing Industry By-Products, in: Singh nee' Nigam, P., Pandey, A. (Eds.), *Biotechnology for Agro-Industrial Residues Utilisation: Utilisation of Agro-Residues*. Springer Netherlands, Dordrecht, pp. 313-326.

Mussatto, S.I. 2014. Brewer's spent grain: a valuable feedstock for industrial applications. Journal of the Science of Food and Agriculture 94(7), 1264-1275.

Mussatto, S.I., Dragone, G., Roberto, I.C. 2006. Brewers' spent grain: generation, characteristics and potential applications. Journal of Cereal Science 43(1), 1-14.

Mussatto, S.I., Mancilha, I.M. 2007. Non-digestible oligosaccharides: a review. Carbohydrate polymers 68(3), 587-597.

Muthaiyan, A., Hernandez-Hernandez, O., Moreno, F.J., Sanz, M.L., Ricke, S.C. 2012. Hydrolyzed Caseinomacropeptide Conjugated Galactooligosaccharides Support the Growth

and Enhance the Bile Tolerance in Lactobacillus Strains. Journal of Agricultural and Food Chemistry 60(27), 6839-6845.

N .....

Nago, M.C., Hounhouigan, J.D., Akissoe, N., Zanou, E., Mestres, C. 1998. Characterization of the Beninese traditional ogi, a fermented maize slurry: physicochemical and microbiological aspects. International Journal of Food Science & Technology 33(3), 307-315.

Napolitano, A., Costabile, A., Martin-Pelaez, S., Vitaglione, P., Klinder, A., Gibson, G.R., Fogliano, V. 2009. Potential prebiotic activity of oligosaccharides obtained by enzymatic conversion of durum wheat insoluble dietary fibre into soluble dietary fibre. Nutrition, Metabolism and Cardiovascular Diseases 19(4), 283-290.

Ng, S.C., Hart, A.L., Kamm, M.A., Stagg, A.J., Knight, S.C. 2009. Mechanisms of action of probiotics: Recent advances. Inflammatory Bowel Diseases 15(2), 300-310.

Nilsson, U., Björck, I. 1988. Availability of cereal fructans and inulin in the rat intestinal tract. The Journal of nutrition 118(12), 1482-1486.

O .....

Oku, T., Tokunaga, T., Hosoya, N. 1984. Nondigestibility of a new sweetener," Neosugar," in the rat. The Journal of nutrition 114(9), 1574-1581.

Oliveira, D.L., Costabile, A., Wilbey, R.A., Grandison, A.S., Duarte, L.C., Roseiro, L.B. 2012. In vitro evaluation of the fermentation properties and potential prebiotic activity of caprine cheese whey oligosaccharides in batch culture systems. BioFactors 38(6), 440-449.

Omidiran, A.T., Sobukola, O.P., Sanni, A., Adebowale, A.-R.A., Obadina, O.A., Sanni, L.O., Tomlins, K., Wolfgang, T. 2016. Optimization of some processing parameters and quality attributes of fried snacks from blends of wheat flour and brewers' spent cassava flour. Food Sci Nutr 4(1), 80-88.

Osungbaro, T.O. 2009. Physical and nutritive properties of fermented cereal foods. African Journal of Food Science 3(2), 023-027.

Özvural, E.B., Vural, H., Gökbulut, İ., Özboy-Özbaş, Ö. 2009. Utilization of brewer's spent grain in the production of Frankfurters. International Journal of Food Science & Technology 44(6), 1093-1099.

P .....

Paker, R. 1974. Probiotics, the other half of the antibiotics story. AnimNutr Health 29, 4-8.

Penna, A.L.B., Subbarao, G., Barbosa-Cánovas, G.V. 2007. High hydrostatic pressure processing on microstructure of probiotic low-fat yogurt. Food Research International 40(4), 510-519.

Plessas, S., Trantallidi, M., Bekatorou, A., Kanellaki, M., Nigam, P., Koutinas, A.A. 2007. Immobilization of kefir and Lactobacillus casei on brewery spent grains for use in sourdough wheat bread making. Food Chemistry 105(1), 187-194.

Prado, F.C., Parada, J.L., Pandey, A., Soccol, C.R. 2008. Trends in non-dairy probiotic beverages. Food Research International 41(2), 111-123.

Prentice, N., D'Appolonia, B.L. 1977. High-fiber bread containing brewers' spent grain. Cereal Chem 54(5), 1084-1095.

R .....

Rajagopal, M.V. 1977. Production of beer from Cassava. Journal of Food Science 42(2), 532-533.

Rawel, H.M., Kroll, J. 2003. Die Bedeutung von Cassava (Manihot esculenta Crantz) als Hauptnahrungsmittel in tropischen Ländern. Deutsche Lebensmittel-Rundschau 99(3), 102-111.

Rivas, S., Gullón, B., Gullón, P., Alonso, J.L., Parajó, J.C. 2012. Manufacture and Properties of Bifidogenic Saccharides Derived from Wood Mannan. Journal of Agricultural and Food Chemistry 60(17), 4296-4305.

Rivière, A., Selak, M., Lantin, D., Leroy, F., De Vuyst, L. 2016. Bifidobacteria and Butyrate-Producing Colon Bacteria: Importance and Strategies for Their Stimulation in the Human Gut. Frontiers in Microbiology 7(979).

Roberfroid, M. 2002. Functional food concept and its application to prebiotics. Digestive and Liver Disease 34, S105-S110.

Roberfroid, M. 2007. Prebiotics: the concept revisited. The Journal of nutrition 137(3), 830S-837S.

Roberfroid, M., Gibson, G.R., Hoyles, L., McCartney, A.L., Rastall, R., Rowland, I., Wolvers, D., Watzl, B., Szajewska, H., Stahl, B. 2010a. Prebiotic effects: metabolic and health benefits. British Journal of Nutrition 104(S2), S1-S63.

Roberfroid, M., Gibson, G.R., Hoyles, L., McCartney, A.L., Rastall, R., Rowland, I., Wolvers, D., Watzl, B., Szajewska, H., Stahl, B., Guarner, F., Respondek, F., Whelan, K., Coxam, V., Davicco, M.-J., Léotoing, L., Wittrant, Y., Delzenne, N.M., Cani, P.D., Neyrinck, A.M., Meheust, A., 2010b. Prebiotic effects: metabolic and health benefits. British Journal of Nutrition 104(S2), S1-S63.

Roberfroid, M.B. 2000. Concepts and strategy of functional food science: the European perspective. The American journal of clinical nutrition 71(6), 1660s-1664s.

Roberfroid, M.B. 2005. Introducing inulin-type fructans. British Journal of Nutrition 93(S1), S13-S25.

Rodriguez-Colinas, B., Kolida, S., Baran, M., Ballesteros, A.O., Rastall, R.A., Plou, F.J. 2013. Analysis of fermentation selectivity of purified galacto-oligosaccharides by in vitro human faecal fermentation. Applied Microbiology and Biotechnology 97(13), 5743-5752.

Rosemary, O.C., Enechi, O.C., Ogu, E.O., Okechukwu, U.P.C. 2013. Studies on Beer Production Using Low Cyanide Cassava as Adjunct. Pharmanest - An International Journal of Advances in Pharmaceutical Sciences 4(5), 862-871.

Rycroft, C.E., Jones, M.R., Gibson, G.R., Rastall, R.A. 2001. A comparative in vitro evaluation of the fermentation properties of prebiotic oligosaccharides. Journal of Applied Microbiology 91(5), 878-887.

S .....

Saarela, M., Mogensen, G., Fondén, R., Mättö, J., Mattila-Sandholm, T. 2000. Probiotic bacteria: safety, functional and technological properties. Journal of Biotechnology 84(3), 197-215.

Sacca, C., Adinsi, L., Anihouvi, V., Akissoe, N., Dalode, G., Mestres, C., Jacobs, A., Dlamini, N., Pallet, D., Hounhouigan, D.J. 2012. Production, consumption, and quality attributes of Akpan - a yoghurt-like cereal product from West Africa. Food Chain 2(2), 207-220.

Salazar, N., Ruas-Madiedo, P., Kolida, S., Collins, M., Rastall, R., Gibson, G., de los Reyes-Gavilán, C.G. 2009. Exopolysaccharides produced by Bifidobacterium longum IPLA E44 and Bifidobacterium animalis subsp. lactis IPLA R1 modify the composition and metabolic activity of human faecal microbiota in pH-controlled batch cultures. International Journal of Food Microbiology 135(3), 260-267.

Salminen S. 1996. Uniqueness of probiotic strains. IDF Nutr News Lett, 5, 16–8.

Santos, M., Jiménez, J., Bartolomé, B., Gómez-Cordovés, C., Del Nozal, M. 2003. Variability of brewer's spent grain within a brewery. Food Chemistry 80(1), 17-21.

Sarbini, S.R., Kolida, S., Naeye, T., Einerhand, A., Brison, Y., Remaud-Simeon, M., Monsan, P., Gibson, G.R., Rastall, R.A. 2011. In Vitro Fermentation of Linear and α-1,2-Branched Dextrans by the Human Fecal Microbiota. Applied and environmental microbiology 77(15), 5307-5315.

Sarbini, S.R., Kolida, S., Naeye, T., Einerhand, A.W., Gibson, G.R., Rastall, R.A. 2013. The prebiotic effect of α-1,2 branched, low molecular weight dextran in the batch and continuous faecal fermentation system. Journal of Functional Foods 5(4), 1938-1946.

Schaafsma G. 1996 State of art concerning probiotic strains in milk products. IDF Nutr News Lett, 5, 23–4.

Schrezenmeir, J., de Vrese, M. 2001. Probiotics, prebiotics, and synbiotics - approaching a definition. The American journal of clinical nutrition 73(2), 361s-364s.

Scott, K.P., Martin, J.C., Duncan, S.H., Flint, H.J. 2014. Prebiotic stimulation of human colonic butyrate-producing bacteria and bifidobacteria, in vitro. FEMS Microbiology Ecology 87(1), 30-40.

Shah, N.P. 2007. Functional cultures and health benefits. International Dairy Journal 17(11), 1262-1277.

Sharp, M.D., McMahon, D.J., Broadbent, J.R. 2008. Comparative Evaluation of Yogurt and Low-Fat Cheddar Cheese as Delivery Media for Probiotic Lactobacillus casei. Journal of Food Science 73(7), M375-M377.

Shinde, P.B. 2012. Probiotic: an overview for selection and evaluation. Int J Pharm and Pharm Sci 4, 14-21.

Soccol, C.R., Vandenberghe, L.P.d.S., Spier, M.R., Medeiros, A.B.P., Yamaguishi, C.T., Lindner, J.D.D., Pandey, A., Thomaz-Soccol, V. 2010. The potential of probiotics: a review. Food Technology and Biotechnology 48(4), 413-434.

Sousa, S., Pinto, J., Pereira, C., Xavier Malcata, F., Bertoldo Pacheco, M.T., Gomes, A.M., Pintado, M. 2015. In vitro evaluation of yacon (Smallanthus sonchifolius) tuber flour prebiotic potential. Food and Bioproducts Processing 95, 96-105.

Stanton, C., Fitzgerald, G., Paul Ross, R., Desmond, C., Coakley, M., Kevin Collins, J. 2003. Challenges facing development of probiotic-containing functional foods, *Handbook of fermented functional foods*. CRC Press, pp. 27-58.

Stanton, C., Ross, R.P., Fitzgerald, G.F., Sinderen, D.V. 2005. Fermented functional foods based on probiotics and their biogenic metabolites. Current Opinion in Biotechnology 16(2), 198-203.

Stojceska, V., Ainsworth, P., Plunkett, A., İbanogˇlu, S. 2008. The recycling of brewer's processing by-product into ready-to-eat snacks using extrusion technology. Journal of Cereal Science 47(3), 469-479.

Stojceska, V., Ainsworth, P., Plunkett, A., İbanoğlu, Ş. 2009. The effect of extrusion cooking using different water feed rates on the quality of ready-to-eat snacks made from food by-products. Food Chemistry 114(1), 226-232.

Suau, A., Bonnet, R., Sutren, M., Godon, J.-J., Gibson, G.R., Collins, M.D., Doré, J. 1999. Direct analysis of genes encoding 16S rRNA from complex communities reveals many novel molecular species within the human gut. Applied and environmental microbiology 65(11), 4799-4807.

T .....

Tadayoni, M., Sheikh-Zeinoddin, M., Soleimanian-Zad, S. 2015. Isolation of bioactive polysaccharide from acorn and evaluation of its functional properties. International Journal of Biological Macromolecules 72, 179-184.

Tewe, O., Lutaladio, N. 2004. Cassava for livestock feed in Sub-Saharan African. Plant production and protection division. Food and Agricultural Organization. Rome. Italy.

Thomas, C.M., Hong, T., van Pijkeren, J.P., Hemarajata, P., Trinh, D.V., Hu, W., Britton, R.A., Kalkum, M., Versalovic, J. 2012. Histamine Derived from Probiotic Lactobacillus reuteri Suppresses TNF via Modulation of PKA and ERK Signaling. PLoS ONE 7(2), e31951.

Topping, D.L., Clifton, P.M. 2001. Short-Chain Fatty Acids and Human Colonic Function: Roles of Resistant Starch and Nonstarch Polysaccharides. Physiological Reviews 81(3), 1031-1064.

U .....

Ukwuru, M., Egbonu, S. 2013. Recent development in cassava-based products research. Academia Journal of Food Research 1(1), 001-013.

V .....

Van der Meulen, R., Makras, L., Verbrugghe, K., Adriany, T., De Vuyst, L. 2006. In Vitro Kinetic Analysis of Oligofructose Consumption by Bacteroides and Bifidobacterium spp. Indicates Different Degradation Mechanisms. Applied and environmental microbiology 72(2), 1006-1012.

van Houte, J., Gibbons, R.J. 1966. Studies of the cultivable flora of normal human feces. Antonie van Leeuwenhoek 32(1), 212-222.

van Wijk, J. and Kwakkenbos, H. 2011. Beer multinationals supporting Africa's development? How partnerships include smallholders into sorghum-beer supply chains. In: Van Dijk, M.P. and Trienekens, J. Promoting Sustainable Global Value Chains: The Role of Governance, Amsterdam: Amsterdam University Press, forthcoming.

Venter, C.S. 2007. Prebiotics: an update. Journal of Family Ecology and Consumer Sciences Tydskrif vir Gesinsekologie en Verbruikerswetenskappe 35(1), 17-25.

Viëtor, R., Voragen, A., Angelino, S. 1993. Composition of non-starch polysaccharides in wort and spent grain from brewing trials with malt from a good malting quality barley and a feed barley. Journal of the Institute of Brewing 99(3), 243-248.

W .....

Waites, M. J., Morgan, N. L., Rockey, J. S., & Higton, G. 2001. Industrial Microbiology: An Introduction:, 1st edition. Blackwell Science Ltd, United Kingdom, pp. 179

Waters, D.M., Jacob, F., Titze, J., Arendt, E.K., Zannini, E. 2012. Fibre, protein and mineral fortification of wheat bread through milled and fermented brewer's spent grain enrichment. European Food Research and Technology 235(5), 767-778.

Wilson, K.H., Blitchington, R.B. 1996. Human colonic biota studied by ribosomal DNA sequence analysis. Applied and environmental microbiology 62(7), 2273-2278.

Woodmansey, E.J. 2007. Intestinal bacteria and ageing. Journal of Applied Microbiology 102(5), 1178-1186.

World Health Organization. 2014. *Global status report on alcohol and health 2014 ed*. World Health Organization, 31-32

X .....

Xiros, C., Christakopoulos, P. 2012. Biotechnological Potential of Brewers Spent Grain and its Recent Applications. Waste and Biomass Valorization 3(2), 213-232.

Y .....

Yang, J., Maldonado-Gómez, M.X., Hutkins, R.W., Rose, D.J. 2014. Production and in Vitro Fermentation of Soluble, Non-digestible, Feruloylated Oligo- and Polysaccharides from Maize and Wheat Brans. Journal of Agricultural and Food Chemistry 62(1), 159-166.

Z .....

Ziesenitz, S.C., Siebert, G. 1987. In vitro assessment of nystose as a sugar substitute. The Journal of nutrition 117(5), 846-851.

Zoellner, S., Cruz, A., Faria, J., Bolini, H., Moura, M., Carvalho, L., Sant'ana, A. 2009. Whey beverage with acai pulp as a food carrier of probiotic bacteria. Australian Journal of Dairy Technology 64(2), 177-181.