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DEVELOPMENT AND CHARACTERIZATION OF A DAIRY MATRIX INCORPORATED WITH NATURAL EXTRACTS

by
Ana Margarida Massa Faustino

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DESENVOLVIMENTO E CARACTERIZAÇÃO DE UMA MATRIZ LÁCTEA COM INCORPORAÇÃO DE EXTRATOS NATURAIS

Thesis presented to Escola Superior de Biotecnologia of the Universidade Católica Portuguesa to fulfill the requirements of Master of Science degree in Biotechnology and Innovation

by
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This thesis is dedicated to
my grandfather Ernesto (1934-2014)
and to my grandmother Alzira

Resumo

Atualmente, o interesse e preocupação do consumidor relativamente à dieta e á sua influência na saúde e bem-estar tem vindo a aumentar na última década. Os hábitos alimentares da sociedade foram-se alterando devido ao consumidor procurar soluções mais naturais, dando preferência a alimentos funcionais que promovam a saúde ao invés da utilização de cápsulas ou comprimidos. Neste contexto, o trabalho aqui apresentado tem como objetivo principal desenvolver e caracterizar um alimento funcional através da incorporação de extratos enzimáticos pré-selecionados de cogumelo (*Pholiota nameko* obtido com *Flavourzyme*) e de alga (*Osmundea pinnatifida* obtido com *Viscozyme*) com propriedades biológicas de valor acrescentado numa matriz láctea. Pretende-se no final obter uma ou mais matrizes lácteas funcionalizadas com extratos de alga e cogumelo. Além disso, visa também caracterizar o potencial biológico das matrizes envolvidas. Os extratos foram submetidos a diferentes processos de pasteurização e esterilização, na tentativa de definir um processamento térmico eficaz, mas com o menor impacto na matriz láctea. Deste modo selecionou-se uma pasteurização a 90 ºC durante 30 minutos. No entanto, como os níveis de contaminantes microbiológicos no extrato de cogumelo não diminuíram para níveis aceitáveis, independentemente do processo térmico utilizado, este extrato foi descartado tendo-se incorporado na matriz láctea somente extrato de alga. O processo de pasteurização selecionado permitiu um tempo de vida útil até 21 dias da matriz láctea sob condições de refrigeração. No que diz respeito á avaliação sensorial, a matriz láctea com extrato de *O. pinnatifida* obteve o seu melhor resultado aos 2 dias com uma avaliação positiva de 63%. A incorporação do extrato de *O. pinnatifida* resultou em valores mais elevados de capacidade antioxidante (com percentagens de inibição máxima de 14,3%, 74,7% e 5,32% no que respeita à redução dos radicais $ABTS^{+}$, hidroxilo e DPPH, respectivamente) em comparação com as matrizes sem incorporação de extrato. Maior potencial prebiótico foi também observado nas matrizes lácticas com extrato de *O. pinnatifida* principalmente nas amostras após o seu fabrico, tanto em termos de células viáveis de *Lactobacillus acidophilus* (8.82 log UFC/g $_{\text{por matriz láctea}}$) como de *Bifidobacterium animalis* subsp lactis BB12 (7.71 log UFC/g $_{\text{por matriz láctea}}$). No estudo do impacto do tempo de armazenamento no potencial prebiótico das matrizes lácteas, observou-se maior produção de ácido láctico, após 24h de fermentação pela ação da bactéria *L. acidophilus*, na matriz com extrato de *O. pinnatifida* com 21 d (1.135 mg/ g $_{\text{por matriz láctea}}$); em termos de ácido acético valores mais elevados foram observados, após 24h de fermentação, na matriz láctea com incorporação de frutooligossacarídeos (FOS) e inoculada com *B. animalis* BB12, com 0d de armazenamento (0.158 mg/ g $_{\text{por matriz láctea}}$). Em geral, após digestão *in vitro*, o potencial prebiótico diminuiu principalmente no que diz respeito ao número de células viáveis de *B.*

animalis BB12. Menor impacto pela digestão foi observado para as células viáveis de *L. acidophilus* La-5 na matriz digerida que possuía FOS. A matriz láctea com extrato de *O. pinnatifida* apresentou resultados interessantes de atividade anti-hipertensiva com 50% de inibição da angiotensina convertase I (57,4% - 49.5), valores estes que permaneceram relativamente estáveis ao longo dos 21 dias de armazenamento. Por outro lado, não se observou atividade antidiabética significativa (inibição da enzima α-glucosidase) para as matrizes lácteas desenvolvidas quando comparadas com a acarbose

Palavras chave: Extratos enzimáticos, *Osmundea pinnatifida*, Propriedades biológicas, Alimentos funcionais, Bactérias probióticas

Abstract

Currently, consumer interest and concern about diet and its influence on health and well-being has been increasing in the last decade, the dietary habits of society have been changing as consumers seek more natural solutions giving preference to food that promote health rather than the use of capsules or tablets.

In this context, the main objective of this work is to develop and characterize a functional food product thought the incorporation of the pre-selected mushroom (*Pholiota nameko* obtained using Flavourzyme enzyme mix) and seaweed (*Osmundea pinnatifida* obtained with Viscozyme enzyme mix) extracts with previously reported biological properties that are likely to increase the intrinsic value added of the considered dairy matrix. Moreover, it also aims to provide a characterization of the biological potential of the developed matrixes.

The extracts were submitted to different pasteurization and sterilization processes, in an attempt to define efficient thermal processing but with the smallest impact on dairy matrix. In this way a pasteurization was selected at 90 ºC for 30 minutes. As the mushroom extract's contaminant levels did not drop to acceptable levels, regardless of the process used, this extract was not incorporated into the dairy matrix, only seaweed extract was incorporated into the dairy matrix. The selected pasteurization process allowed a shelf life of up to 21 days under refrigeration conditions. For sensory analysis, dairy matrix with *O. pinnatifida* extract obtained the best result at 2 days in which it demonstrated a positive evaluation of 63%. The incorporation of *O. pinnatifida* extract resulted in higher antioxidant capacity levels than those of the plain dairy matrix (with a maximum inhibition percentage of 14.3%, 74.7% and 5.32% for the radicals $ABTS^{+}$, hydroxyl and DPPH, respectively). Higher prebiotic potential was also observed in lactic matrices with *O. pinnatifida* extract mainly in the samples at 0 days, both in terms of viable *Lactobacillus acidophilus* La-5 cells (8.82 log (CFU/g $_{\text{of spreadable dairy cream}}$)) and *Bifidobacterium animalis* subsp lactis BB12 (7.71 log (CFU/g $_{\text{of spreadable dairy cream}}$)). In the study of the impact of the storage time on the prebiotic potential of the dairy matrices, a higher lactic acid production was observed after 24 h of fermentation by the action of *L. acidophilus* La-5 in the dairy matrix with *O. pinnatifida* extract with 21 d (1.135 mg/g $_{\text{of spreadable dairy cream}}$); in terms of acetic acid, higher values were observed after 24 h of fermentation in the dairy matrix with the incorporation of fructooligosaccharides (FOS) and inoculated with *B. animalis* BB12, with 0 d of storage (0.158 mg/g $_{\text{of spreadable dairy cream}}$). After *in vitro* digestion, the prebiotic potential decreased mainly with respect to the number of viable cells of *B. animalis* BB12. Lower impact by digestion was observed for the viable *L. acidophilus* La-5 cells in the digested matrix containing FOS. The dairy matrix with *O. pinnatifida* extract exhibited interesting antihypertensive activity results with 50% inhibition of angiotensin convertase I (57.4-49.5%),

values that remained relatively stable throughout the 21 days of storage. On the other hand, no significant antidiabetic activity (i.e. inhibition of α-glucosidase) was observed for the incorporated dairy matrices when compared to acarbose.

Keywords: Enzymatic extracts, *Osmundea pinnatifida*, Biological properties, Functional food, Probiotic bacteria

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ABTS	2,2'-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid) diammonium salt
$ABTS^{\bullet+}$	2,2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) radical cation
ACE	Angiotensin Converting Enzyme-I
Ca	Calcium
CFU	Colony Forming Unit
DPPH	2,2-Diphenyl-1-Picryhydrazyl Radical
Fe	Iron
$FeSO_4$	Iron (II) Sulphate
FNB	Institute of Medicine's Food and Nutrition Board
FOS	Fructooligosaccharides
H_2SO_4	Sulfuric acid
HCl	Hydrochloric acid
I	Iodine
IC_{50}	Half Maximal Inhibitory Concentration
ILSI	International Life Science Institute
K	Potassium
$K_2S_2O_8$	Potassium persulphate
LC- PUFA	Long-chain polyunsaturated fatty acids (LC- PUFA)
Mg	Magnesium
Mn	Manganese
MRS	de Man, Rogosa and Sharpe
Na	Sodium
NaCl	Sodium chloride
NaOH	Sodium hydroxide
P	Phosphorus
PCA	Plate Count Agar
PUFAs	Polyunsaturated fatty acids
SGF	Simulated Gastric Fluid
SIF	Simulated Intestinal Fluid
SSF	Simulated Salivary Fluid

Tris-HCl	2-Amino-2-(hydroxymethyl) propane-1,3-diol – hydrochloric acid
VRBGA	Violet Red Bile Glucose Agar
w/w	Weight by weight
w/v	Weight by volume
Zn	Zinc
$ZnCl_2$	Zinc Chloride

Introduction

1.1 Functional Foods

In Europe, the demand for functional foods has increased. This is a likely result, not only of the rise in the awareness of the link between diet and health, but also as a means to improve the quality of life and overall health in the later stages of life, a concern brought upon by the increase in life expectancy (EUFIC, 2006, Plaza, Herrero, Cifuentes & Ibáñez,2009). To meet this demand, food research and the food industry have been attempting to develop new food products that are healthier, nutritionally better balanced or help to prevent the risk of some diseases.

The term "functional food" was first introduced in the early 1980s in Japan. This new concept designates foods that are able to promote health or to reduce the risk of some diseases and as such with positive role on consumer's quality of life increasing average life expectancy (Plaza, Herrero, Cifuentes, & Ibáñez, 2009).

Functional foods are generally considered to be consumed as part of the normal diet but contain biologically active compounds/ingredients that can in turn, improve general health and well-being or reduce the risk of developing some pathologies like cardiovascular diseases, cancer and osteoporosis (Abuajah, Ogbonna, & Osuji, 2015). Regardless of their beneficial potential, the ingestion of these foods should be associated with a healthy lifestyle (EUFIC, 2006). A decade later (1990) a new and more widely definition of functional food was proposed by the International Life Science Institute (ILSI, Belgium) in Europe (Ashwell, 2002). It defines functional foods as: i) unaltered natural foods; ii) a food to which an ingredient has been added or removed by (bio) technological means; iii) a food in which the nature of one or more ingredients has been modified; iv) a food in which one ingredient has been replaced by another with more favourable properties.

Over the last decade, numerous other definitions of functional foods have emerged. According to the Institute of Medicine's Food and Nutrition Board (FNB, USA). Functional food is "*Any food or food constituent with possible benefits in health promotion and disease prevention, independent of the essential nutrient function*". This definition encompasses foods that are made up of healthy or beneficial nutrients (Day, et al., 2009; Diplock, et al., 1999), whose consumption has been associated with several potential health benefit like lowering cholesterol, reduction of obesity, defence against oxidative stress, reduction of blood pressure as well as a promotion of a healthy digestion (Abuajah, Ogbonna, & Osuji, 2015; Plaza, Cifuentes, & Ibáñez, 2008). Functional foods must remain as food and must demonstrate their effectiveness in quantities or doses normally consumed in the diet, these can't be tablets or capsules, but

should form part of a normal dietary pattern (Mendes, 2014). More recently, and according to Vicentini, Liberatore, & Mastrocola, (2016) "*a functional food is a food that positively demonstrate to affect beneficially one or more target functions in the body, beyond adequate nutritional import, improving state of health and wellbeing and/or reduction of risk disease".* According to Crowe & Francis (2013) functional foods are divided in three categories, conventional foods, modified foods and food ingredients. Conventional foods containing natural bioactive food compounds, most vegetables, fruits, grains, dairy, fish, and meats contain bioactive food compounds that provide benefits beyond basic nutrition. Some examples would be the antioxidant vitamins in orange juice, isoflavones in soy-based foods, and prebiotics and probiotics in yogurt. Modified foods containing bioactive food compounds through enrichment or fortification, such as n-3 fatty acids in margarine spreads and eggs. Food ingredients that are extracted such as undigestible carbohydrates, which provide prebiotic benefits like oligosaccharides or resistant starch.

Prebiotics have been suggested to have several beneficial effects, including promotion of beneficial bacterial growth, stimulation of intestinal peristalsis, production of short chain fatty acids, and a shortened orofecal transit time (Cummings, Macfarlane, & Englyst, 2001). Prebiotics were initially defined as non-digestible or low digestible food ingredients during the digestive process that benefit the host organism by selectively stimulating the proliferation and/ or activity of probiotic desirable bacteria colon (Gibson & Roberfroid, 1995). Currently, and according to Gibson et al., (2017) the prebiotic concept has expanded, because of advances in tools for microbiome research, which has led to the proposal of a new definition where a prebiotic is a "*non-digestible compound that, through its metabolization by microorganisms in the gut, modulates the composition and/or activity of the gut microbiota, thus, conferring a beneficial physiological effect on the host"*.

Probiotics are a major group of live microbial dietary active ingredients used in application strategies that target the maintenance or modulation of gut microbiota composition and activities. Probiotics, whose nature and functions are presently a major target of research in biomedicine, are used to recover symbiosis between colonic microbiota and the host or re-establish states of dysbiosis (Gomes, Andrade, & Freitas, 2018). In October 2013, the International Scientific Association for Probiotics and Prebiotics brought together a panel of scientific and clinical experts to discuss the appropriate use and scope of the term probiotic (Hill, et al., 2014). As a result, the definition was reinforced as relevant and sufficiently adjustable to all current and future applications to be use by all probiotic stakeholders including industry, with only a minor grammatical correction as "*live microorganisms that, when*

administered in adequate amounts, confer a health benefit on the host". In industry, probiotic microorganisms are ingredients to be incorporated in food products (fermented or non-fermented dairy or non-dairy based) or as a dietary supplement (direct dose powders, sachets, capsules or tablets). The most common species used in either case include strains from the *Bifidobacterium*: *B. bifidum*, *B. breve*, *B. animalis*, *B. longum* and *Lactobacillus* genera: *L. acidophilus*, *L. casei*, *L. rhamnosus* and *L. reuteri* (Gomes, Andrade, & Freitas, 2018).

Most prebiotics are classified as dietary fibers (edible carbohydrates that are neither affected by the digestive process nor absorbed), therefore they can be found in several vegetable foodstuffs such as chicory, onion, garlic, asparagus, artichoke, leek, banana and tomato, several different grains and even seaweed and mushrooms. Overall the most common prebiotic ingredients are inulin-type fructans (which includes native inulin, enzymatically hydrolysed inulin or oligofructose) and fructooligosaccharides (FOS) (Grajek, Olejnik, & Sip, 2005; European Food Safety, 2015; Holscher, 2017).

Vegetable and fruits, given the evidences of their health benefits have been target of several research works aimed at extracting the bioactive ingredients and using them to produce new functional foods (Slavin & Lloyd, 2012). In addition, many other potential functional ingredients such as pigments, phenolic compounds and polysaccharides can be found in alternative sources (Freitas, et al., 2015). Some examples are ingredients from marine sources that are only not yet fully exploited but several new sources such as seaweed still remain to be studied. Moreover, while the interest gathered by the terrestrial sources has been significant, some reservoirs, such as the mushrooms, still possess a significant leeway for exploitation for functional ingredients (Rodrigues, 2016).

1.2 Seaweeds as a Source of Functional Ingredients and its associated Biological Properties

According to Chojnacka, Saeid, Witkowska, & Tuhy, (2012) there are about 10,000 identified species of algae and about 5% of them are used as food for either humans or animals. More than one hundred seaweed species are used worldwide, especially in Asian countries as sea vegetables (Fleurence, et al., 2012). Seaweed, also known as macroalgae, are large multicellular algae species. All seaweeds can be classified into three groups: brown (Phaeophyta), green (Chlorophyta) and red algae (Rhodophyta) (Horn, 2009).

Additionally, seaweeds also produce a wide range of molecules that can be used by man in the field of biomedical, cosmetic, food or pharmaceutical industries, which makes these marine organisms very interesting from a biotechnological point of view (Barsanti & Gualtieri, 2006).

Seaweeds contain about 80-90% water, and dry weight contains about 50% carbohydrates, although this value is variable, 1-3% lipids and 7-38% minerals (El-Said & El-Sikaily, 2013). In what concerns their protein content, the percentage is quite variable, around 10-47%, with high amounts of essential amino acids (El-Said & El-Sikaily, 2013).

Figure 1.1 – An example of *Osmundea pinnatifida* collected from the Portuguese coast.

Most seaweeds are edible algae that represent a natural source of biologically relevant compounds that can be used in the elaboration of functional foods (Plaza, Cifuentes, & Ibáñez, 2008). Among the different groups, the red seaweed such as *O. pinnatifida* (Figure 1.1) have had a more diverse evolution than the green and the brown counterparts (Plaza, Cifuentes, & Ibáñez, 2008). Red algae exhibit a broad range of morphologies, a simple anatomy and display a wide array of life cycles. Their pigments are chlorophyll a, phycobilins and carotenoids including β-carotene, lutein and zeaxanthin (Pereira, 2015). In the study by Rodrigues et al., (2015a) it is shown that edible algae such as *Sargassum muticum* (brown seaweed), *O. pinnatifida* (red seaweed) and *Codium tomentosum* (green seaweed) are natural sources of compounds with important biological interest. In this case, the red seaweed had a high protein content and a low fat and sugar content. On the other hand, compared to brown and green seaweed, these presented high levels of fats and sugars. These algae are known for their low caloric content and their richness in macro and micro nutrients such as:

- Polysaccharides such as agar, carragenaans and alginates are examples of polysaccharides derived from red and brown seaweed (Rodrigues, 2016). Other less predominant polysaccharides of interest are fucoidans (brown seaweed), xylans (certain red and green seaweed) and cellulose (in all genera). Seaweed also contain storage polysaccharides such as laminarin (brown seaweeds) and floridean starch (red seaweeds) (Herrero, Cifuentes, & Ibanez, 2013);
- Dietary fibres, contributing positively to intestinal transit and cholesterol metabolism (Michael, Rajput, & Patil, 2012);

- Proteins and amino acids: in general, protein content in seaweeds will vary according to the geographical location, harvest season and the species; higher contents are found in green and red seaweeds (10-47% of the dry weight) in comparison to the brown seaweed where contents are two-fold less (5-15% of the dry weight) (Rodrigues, 2016). Most seaweed species are a rich source of amino acids. Brown seaweed are a rich source of threonine, valine, leucine, lysine, glycine, cysteine, methionine, histidine, among others (Holdt & Kraan, 2011);
- Sterols with anti-inflammatory, antibacterial, antifungal, antitumour activities, as well as the ability to reduce cholesterol (Othman & Moghadasian, 2011);
- Lipids such as long chain polyunsaturated fatty acids (PUFAs), particularly in red and brown seaweed (Cabral, Shirahigue, de Arruda, Carpes, & Oetterer, 2011; Lordan, Ross, & Stanton, 2011);
- Algae are still a source of soluble (vitamin B, B2, B3, B5, B12, C) and insoluble (vitamin A, E, D and K) vitamins (Domínguez, 2013; El-Said & El-Sikaily, 2013);
- The mineral profile of seaweeds is also of importance and these are recognized as rich sources of calcium (Ca), magnesium (Mg), sodium (Na), phosphorus (P), and potassium (K) or minerals such as zinc (Zn), iodine (I) or manganese (Mn). This abundance of several elements is of high nutritional importance and is related to its ability to retain inorganic compounds that represent up to 36% of dry matter in some species (Lordan, Ross, & Stanton, 2011).

Food stocks of brown algae are characteristically complex polysaccharides, including fucans and cellulose, as well as higher alcohols, many bioactive metabolites with different pharmacological activities such as antioxidants, anti-inflammatory, antitumour, antifungal and cytotoxic compounds were isolated from these seaweed (Gamal, 2010; Je, et al., 2009).

The seaweed of the species *Sargassum* (brown seaweed) has been shown to be a good source of dietary fibre and carotenoids with antioxidant activity and playing an important role in the prevention of neurodegenerative diseases (Kadam & Prabhasankar, 2010; Je, et al., 2009).

Green algae owe their colour to the dominant presence of chlorophyll a and b, and the main polysaccharides present are usually ulvans (Gamal, 2010). They have phycoerythrin and phycocyanin as the main pigments and primary polysaccharides are agar and carrageenans.

In addition to nutritional value, seaweeds are also natural sources of organic compounds with associated biological properties. Seaweeds have revealed a potential for incorporation into the diet as foods because they induce cholesterol reduction, fight obesity, lower blood pressure, counteract free radicals and promote a healthy digestion. (Plaza, Cifuentes, & Ibáñez, 2008). In

addition, some of them have demonstrated biological properties such as antibacterial, antioxidant, anti-inflammatory, anticoagulant, antiviral and/or apoptotic and prebiotic activities in addition to playing important roles in the prevention of neurodegenerative diseases (Ibañez & Cifuentes, 2013; Plaza, Cifuentes, & Ibáñez, 2008), as listed in Table 1.1. In the study by Rodrigues et al., (2015a) it was demonstrated that edible seaweed such as *O. pinnatifida* (red seaweed) are a natural source of compounds with important biological interest having been emphasized the prebiotic (Rodrigues, et al., 2016a; Rodrigues, et al., 2015a), antioxidant (Rodrigues, et al., 2015a), antidiabetic (Rodrigues, et al., 2015a) and anti- hypertensive potential (Rodrigues, 2016).

Table 1.1 - Main functional ingredients present in algae, associated beneficial properties and potential food applications (Rodrigues, 2016; Freitas et al., 2014).

Marine Algae		
Functional Ingredients	**Functions**	**Applicability**
Pigments: Carotenoids (astaxanthin, lutein, fucoxanthin), phycobilins and chlorophylls Phenolic compounds: Phlorotannins and flavonoids	- Precursors of vitamins - Anti-carcinogenic activity - Anti-inflammatory activity - Anti -Aging activity - Reducing the risk of type 2 diabetes - Antiallergic activity - Reducing obesity	- Food colourings - Food antioxidants
Polysaccharides: agar, fucans and exopolysaccharides	- Anticoagulant activity - Antimicrobial, antiviral and antifungal activity - Anti-inflammatory activity - Antidiabetic activity - Prebiotic activity - Antithrombotic activity	- Gelling agents - Stabilizing, thickening and emulsifying agents - Gums
Proteins: lectins, phycobiliproteins Bioactive Peptides (3-20 amino acids) Amino acids: Leucine, Valine and Proline	- Antimicrobial, antiviral activity - Anti-inflammatory activity - Antioxidant activity - Antidiabetic activity - Antihypertensive activity	- Stabilization and thickening agents - Protein replacements - Gelling agents
Lipids: phospholipids; glycolipids; PUFAs (omega-3 and omega-6)	- Reduction of cardiovascular diseases - Anti-inflammatory activity - Regulation of blood pressure and blood coagulation	- Used in confectionery and bread making

1.2.1 Antioxidant activity

Antioxidants can retard or prevent oxidation even at low concentrations (Borguini & Torres, 2006), natural antioxidants include phenolic acids, flavonoids and tocopherols (Silva, Costa, Santana, & Koblitz, 2010). Antioxidants have the ability to retard lipid oxidation through

a competitive bond to oxygen, bond to free radicals and stabilizing the hydroperoxides. The active forms of oxygen involved in the first steps of the oxidation process can be "sequestered" by breaking the entire process, forming a stable antioxidant-radical linkage which can't enter into the following steps of the lipid peroxidation process, thereby preventing the formation of undesirable products (Basu, Temple, & Garg, 1999). In most peptide sequences hydrophobic amino acids such as leucine, valine and proline are present, the latter being the most common, these antioxidant peptides contribute to the antioxidant potential (Mendis, Rajanakse, Byun, & Kim, 2005) and has a relevant role in the inhibition of lipid peroxidation (Kumar, Nazeer, & Jaiganesh, 2011). In what concerns the extracts of the species *O. pinnatifida,* these present a higher production of agar and a higher concentration of sulphate groups (39-60 $\mu g_{Na2SO4\ acid\ equiv}/g_{lyoph\ extract}$), according to Rodrigues, et al.,(2015a). In Rodrigues, et al., (2015a) study, the extract of *O. pinnatifida* showed high a total antioxidant activity of 19% and a low free radical DPPH elimination activity (4%). When compared to the extracts of *S. muticum* and *C. tomentosum* species, the *O. pinnatifida* extract had a higher hydroxyl radical elimination capacity (42-45%), particularly of the superoxide radical (28%).

1.2.2 Antihypertensive activity

According to Rodrigues (2016), there are inhibitors of ACE (angiotensin converting -I enzyme) that have been widely used in the treatment of hypertension and heart failure in humans. The drugs commercially used as ACE inhibitors are known to cause side effects, including skin rashes, dizziness, nausea, shortness of breath and other symptoms (Berlin-Chemie AG, 2011). Thus, the demand for ACE inhibitors from natural resources increases in relevance today. Among the natural resources are seaweed and mushrooms. Thus, according to Rodrigues (2016), the extract of *O. pinnatifida* obtained through the enzyme Viscozyme presented a stronger of ACE inhibition capacity. Value of IC_{50} (concentrations necessary to inhibit 50% of ACE activity) for the ACE inhibitory activities of this extract was 111.2 µg/mL. The results of this study indicated that *O. pinnatida* extract contains important amounts of active substances capable of inhibiting ACE activity.

1.2.3 Antidiabetic activity

Diabetes mellitus is a disease characterized as a congenital or acquired incapacity to transport glucose from the blood stream to cells (Gunawan - Puteri & Kawabata, 2010). For the treatment of diabetes, it is usually used drugs like insulin, a hormone responsible for maintaining adequate glucose levels in the blood stream and facilitating cellular glucose uptake

by regulating the metabolism (Wilcox, 2005), or other compounds with inhibitory effect on α-amylase and α-glucosidase (e.g. acarbose); in this latter case the digestion of carbohydrates along the digestive tract are delayed and the rate of absorption of glucose is negatively affected (Bhandari, Jong-Anurakkun, Hong, & Kawabata, 2008; Rabasa-Lhoret & Chiasson, 2004).

Overall, antidiabetic compounds extracted from seaweed may act using one of the following mechanisms: inhibition of glucose transporters, inhibition of the α-glucosidase enzyme responsible for its absorption or stimulation of the insulin receptors (Khan & Tania, 2012). According to Rodrigues et al. (2015a), the extract of the species *O. pinnatifida* obtained by enzymatic action (Cellulase and Viscozyme) had a high antidiabetic activity, i.e. about 38-40% (α-glucosidase inhibition activity).

1.2.4 Prebiotic activity

According to Zaporozhets et al. (2014), the prebiotic activity of extracts or of polysaccharides from marine seaweeds, combined with a broad spectrum of biological properties, evidences great potential for their use as functional nutrition ingredients enabling modulation of intestinal microbiota and of gastrointestinal tract (GIT) inflammation as well as normalization of the immune system.

Low molecular weight polysaccharides from the red seaweed *Gelidium sesquipidale* caused a significant increase in populations of *Bifidobacterium* sp., as well as an increase in acetate and propionate (Ramnani, et al., 2012). The fermentation of seaweed components by beneficial bacteria has also been shown to generate beneficial metabolites such as short chain fatty acids, particularly butyrate, acetate, and propionate (de Jesus Raposo et al., 2016). The beneficial effects of SCFA on host health include protection from obesity, chronic respiratory disease or asthma, cancer, and inflammatory bowel, as well as modulation of immunity, glucose homeostasis, lipid metabolism, and appetite regulation (Bultman, 2016; Dwivedi et al., 2016; Koh et al., 2016; Morrison et al., 2016).

On Rodrigues (2016a), the extracts of seaweed species such as those from *S. muticum* and *O. pinnatifida* revealed a potential prebiotic effect in comparison to FOS, a known and recognized prebiotic compound. Two pure cultures of *Lactobacillus acidophilus* La-5 and *Bifidobacterium animalis* subsp *lactis* BB12 were selected for this study, demonstrating the prebiotic effect with the use of these seaweed extracts

Another study (Charoensiddhi et al. 2016) has shown that different extraction processes have a significant impact on the prebiotic potential of seaweed extracts. Enzyme-assisted extraction is an effective technique to improve the components in brown seaweed *Ecklonia radiata* that

stimulate a capacity to promote the growth of beneficial microbes such as *Bifidobacterium* sp. and *Lactobacillus* sp., suggests the components of this seaweed could potentially be consumed by humans as prebiotics.

There are several studies that have tested various species of seaweed to prove their properties of biological interest, as shown in Table 1.2.

Table 1.2 - Biological properties of enzymatic extracts of some species of marine seaweed.

Activity	Species	Proteolytic Enzymes	Reference
Antihypertensive (inhibition of ACE enzyme)	*Undaria pinnatifida*	Pepsin	(Suetsuna & Nakano, 2000);
	Porphyra yezoensis	Protease S Alcalase	(Sato, et al., 2002) (Qu, Pan, Luo, Wang, & He, 2010);
	Hizikia fusiformis	Alcalase Flavourzyme	(Suetsuna, 1998)
	Palmaria palmata	Corolase PP	(Harnedy, 2013)
	Costaria costata	Pepsin	(Lee, You, & Kim, 2005)
Antioxidant (DPPH, $OH^{\bullet}$ e $O_2^{\bullet -}$)	*Sargassum fullvelum* *Ecklonia cava* *Ishige okamurae* *Sargassum horneri* *Sargassum thunbergii* *Scytosipon lomentaria*	Alcalase Protamex Kojizyme Flavourzyme Alcalase	(Heo, Park, Lee, & Jeon, 2005)
Antitumour (MTT)	*Costaria costata* *Enteromorpha prorifera*	Pepsin	(Lee, You, & Kim, 2005)
Antidiabetic (DPP IV)	*Palmaria palmata*	Alcalase Flavourzyme Corolase PP	(Harnedy, 2013)

1.3 Seaweeds Based Functional Foods

Fortification of foods with functional ingredients that may impact on specific functions or systems in the human body, providing health benefits beyond nutritional value an increasingly popular method and objective of many studies in the last years (Kadam & Prabhasankar, 2010; Prabhasankar, et al., 2009; Rodrigues, 2016). The growth is promoting by an increase number of health-conscious consumers interested in products that improve life quality. The development of food that promote general health and wellbeing has been a research priority for food industry (Rodrigues, 2016).

In the study by Rodrigues, (2015), comparing the macronutrient composition of the *Fucus vesiculosus* seaweed and the beefsteak, it was shown that the seaweed have a higher concentration of calcium, potassium, magnesium and sodium than cow steak, instead, the beefsteak presents more concentration of phosphorus.

Comparatively, meat and meat products are a good source of proteins and vitamins but have a deficit in fibres and generally contain excess sodium, which makes them good candidates to be supplemented with algae that overcome the technological problem associated with meat products with low salt content (Cofrades, López-López, & Jiménez- Colmenero, 2011; Grupta & Abu-Ghannam, 2011). According to Cofrades, López-López, & Jiménez- Colmenero (2011) due to the consumer's perception of the negative characteristics of meat in terms of general fat content, in particular, saturated fatty acids, cholesterol and sodium, the meat industry should use new ingredients to develop functional foods. Studies have presented as main objective the development of meat products with low salt content with improved nutritional profile, adding different species of marine seaweed (*Himanthalia elongata*) (Cofrades, López-López, Ruiz-Capillas, Triki, & Juménez- Colmenero, 2011; López-López, Cofrades, Ruiz-Capillas, & Jimónez-Colmenero, 2009; López-López, Cofrades, Yakan, Solas, & Jiménez-Colmenero, 2010). The main results reported in these studies are encouraging and according to Cofrades et al., (2011), the incorporation of seaweeds allows reformulation strategies to design and develop functional meat products with lower sodium, fat and cholesterol contents and simultaneously with relevant contribution to the ingestion of dietary fibre, polyphenols, minerals and unsaturated fatty acids. Technologically, the incorporation of seaweeds can overcome some of the negative sensory impacts caused by low sodium levels related to the water-binding and fat-binding properties.

New types of healthier breads, especially gluten-free bread, a rising market due to the increasing diagnoses of celiac disease among adult individuals (Houben & Becker, 2012), has been a challenge to bakers and scientists. Agar-agar and carrageenan are two hydrocolloids that can be used as thickening, swelling, stabilizing, or humectant agents which can be used in gluten-free baking (Houben & Becker, 2012). Different types of bread with the incorporation of sodium alginate and carrageenan have been attempted and investigated in terms of baking properties (Guarda, Rosell, Benedito, & Galotto, 2004; Mikuš, et al., 2013). In general, marine derived polysaccharides are able to impart acceptable textural and sensorial characteristics. However, none of these studies carried out any nutritional evaluation that could enhance some healthy aspects due to the incorporation of marine-derived polysaccharides.

In fact, the design of functional foods based on incorporation of marine ingredients such as seaweeds, has been more successful in bakery and pasta products and was reviewed by Kadam & Prabhasankar (2010). For example, the incorporation of 10-20% edible seaweed wakame (*Undaria pinnatifida*) in pasta has not only received sensorial acceptance but has also resulted in a product with improved amino acid and fatty acid profiles, an increase of antioxidant activity, and a higher content of fucoxanthin (concentration and activity were not affected by processing) and fucosterol (Prabhasankar, et al., 2009).

According to Grupta & Abu-Ghannam (2011) seaweed are currently considered as a potential ingredient for nutritional enrichment of meat, bakery and pasta products because, as previously demonstrated, the addition of seaweed or seaweed extracts not only can improve the quality of the product but may even increase the safety in high doses of natural and antimicrobial antioxidants.

In summary, in the following Table 1.3, there are several species of seaweeds that can be included in different foods.

Table 1.3. – Added-value of foods incorporating seaweeds in their formulation.

Product	Marine Algae	Contribution	Reference
Sausages Frankfurt	*Himanthalia Elongata*	Calcium and sodium enrichment	(López-López, Cofrades, Ruiz-Capillas, & Jimónez-Colmenero, 2009)
Meat Pies	*Undaria Pinnatifida*	Increased sodium / potassium ratio	(López-López, Cofrades, Yakan, Solas, & Jiménez-Colmenero, 2010)
Noodles	*Monostroma Nitidum*	Increase in fibre content	(Chang & Wu, 2008)
Pasta	*Undaria Pinnatifida*	Nutritional increase	(Prabbasankar, et al., 2009)
Chocolate	*Porphyra umbilicalis/ Pyropia yezoensis*	Increase of vitamins, minerals, proteins and flavour	(Wells, et al., 2017)

1.4 Mushrooms as a Source of Functional Ingredients and their Biological Properties

From the terrestrial point of view, mushrooms are also of great interest for the search of new ingredients with functional properties, in addition to being able to provide a set of nutritional benefits. Edible mushrooms are usually appreciated because of their sensory

characteristics and culinary suitability and are recognized for their nutritional benefits and health benefits (Sabaratnam, Kah-hui, Naidu, & David, 2011). In China, there are records with 2000 years in which mushrooms have been used to supplement diets (Barros, Cruz, Baptista, & Estevinho, 2008; Ferrador, 2015; Guillamón, et al., 2010; Kalač, 2009).

Over the past 10 years, edible mushrooms have received increased attention because they are a great source of proteins (20-25%), polysaccharides (37-48%), fibres (12-24%), vitamins, minerals (Alam, et al., 2008; Sabaratnam, Kah-hui, Naidu, & David, 2011) and some secondary metabolites including phenolic, polyketic, terpene and sterol compounds (Cheung, Cheung, & Ooi, 2003). Actually, mushrooms are currently of great interest worldwide for their associated chemical and nutritional properties. Both have been much explored, and, in this sense, the mushrooms have been indicated as foods that help to prevent some diseases, such as hypertension, hypercholesterolemia and cancer (Khan & Tania, 2012).

More recently the compounds that can be extracted from the mushrooms (carotenoids, phenolic compounds, organic acids, oligo and polysaccharides) (Kalač, 2009) are taking great care due to their biological potential. These are promising compounds and can be incorporated into other food matrices or nutraceuticals with the perspective of improving biological functions in humans (Aida, Shuhaini, Yazid, & Maaruf, 2009).

Mushrooms are a good source of high-quality protein, higher than in vegetables and fruits, and of superior quality. They are rich in essential amino acids like lysine and tryptophan, which are deficient in cereals. The protein content of the mushrooms depends on the composition of the substrate, the size of the piles, the time of harvest and the species of mushrooms (Yilmaz, Solamaz, & El mastas, 2006). There are in nature several hundred species of edible mushrooms, 20 species are widely used as food and only 8 to 10 species are regularly grown on a large scale. The most commonly consumed species are *Agaricus bisporus* (Paris Mushroom) and *Lentinula edodes* (Shiitake Mushroom; Figure 1.2), which is the second most cultivated mushroom in the world (Ghoral, et al., 2009), followed by the species *Pleurotus* spp., *Auricula auricula*, *Flamulina velutipes* and *Volvariella volvácea* (Aida, Shuhaini, Yazid, & Maaruf, 2009).

Figure 1.2 – *Lentinula edodes* (Source: UniProp).

The species *A. bisporus* (Figure 1.3) was identified as a natural antimicrobial source and as having antioxidant activity (Abah & Abah, 2010). The "Shiitake mushroom" protein has essential amino acids such as lysine, leucine and tryptophan, so it can be widely used in the vegetarian diet. The bioactive ingredient, lentinan (a polysaccharide), has been shown to reduce the onset of cancer and cholesterol (Ghoral *et al*., 2009).

Figure 1.3– *Agaricus bisporus* (Source: Ganeshpurkar, Rai, & Jain, 2010).

Edible mushrooms generally have a low lipid level, its general content is hardly greater than 4-8% of the dry weight regardless of the species. Long chain fatty acids, among which linoleic (C18:2), oleic (C18:1) and palmitic (C16:0) are the main fatty acids. The presence of polyunsaturated fatty acids (PUFAs) explains their contribution to the reduction of cholesterol (Valverde, Hernández-Pérez, & Paredes-López, 2015). Previous studies have shown that cultivated mushrooms such as *Pholiota nameko* (Figure 1.4) are a good source of minerals such as calcium (Ca), magnesium (Mg), sodium (Na), potassium (K), phosphorus (P), iron (Fe), manganese (Mn) and zinc (Zn); in fact, in this study it was shown that *Ph. nameko* had a higher concentration of K and Mg than *Hericium erinaceus* (Cheung, 2008; Rodrigues, 2015).

Figure 1.4 – *Pholiota nameko* (Source CEMBN, 2014).

Rodrigues et al., (2015b) demonstrated that the species of mushrooms analyzed (*Pleurotus citrinopileatus var. cornucopiae, Pleurotus salmoneo stramineus, Pleurotus eryngii, Hericum erinaceus and Ph. nameko*) were found to be a good source of macroelements such as K, Mg, and P, where K stood out as the predominant macroelement. In the species, *Ph. nameko* in comparison with the species *H. erinaceus* presents the highest percentage of Mg and K. In this same study, it is demonstrated that the species such as *Ph. nameko* and *P. salmoneon stramineus* contain the highest percentage of the micronutrient Iron.

In the following Table 1.4, it is synthesized which functional ingredients are present in the mushrooms that cause them to possess several beneficial properties, such as described in the next section.

Table 1.4 - Main functional ingredients with potential food applications (Rodrigues, 2016).

Mushrooms		
Functional Ingredients	**Functions**	**Potential applications**
Polysaccharides: glycogen, dietary fibres, cellulose, chitin and glucans	- Decreased cholesterol - Immunomodulatory activity - Antitumour activity - Cytotoxic effects - Antioxidant activity	- Gelling agents - Stabilizing agents, thickness and emulsifying agents - Gums processing
Bioactive Compounds	- Antimicrobial activity (bacteria, viruses and parasites) - Antitumour activity - Immunomodual activity	- Stabilizers and thickeners - Protein Replacement - Gelling agents
Phenolic compounds and flavonoids	- Antioxidant activity - Prevention of cancer	- Antioxidants

From a health perspective, mushrooms have been recognized for their antifungal, antibacterial, antioxidant, antimicrobial and antiviral properties and are thus easily contemplated in the development of functional foods (Wani, Bodha, & Wani, 2010). These are

also used in various therapeutic applications, including antitumour treatments, immunomodulation (substances can modulate the immune system by conferring an organic response against pathogens), as antidiabetics (Cheng, Liu, Tao, Lu, & Wu, 2012) and they have a preventive role in some cardiovascular diseases (Guillamón, et al., 2010) because they have dietary fibre. This is recognized as food component that confers protection against cardiovascular diseases, colon and rectum cancer, obesity and diabetes (Chaplin, 2003), however, also have technological properties that can be used in the formulation of products, causing a change in texture, increased the stability of food during its production and storage. For these reasons, the addition of dietary fibres to food products confers several benefits to the body and nutritional value, which motivates the consumer to their intake in order to ensure an optimum concentration of fibres according to the recommendations of the nutritionists (Fraga, 2013). Also have anti-inflammatory and analgesic properties (Smiderle, et al., 2008).

1.4.1 Antioxidant Activity

It has been established that mushroom antioxidants can demonstrate their protective properties at different stages of the oxidation process and by different mechanisms (Brewer, 2011; Ferreira, Barros, & Abreu, 2009; Kozarski, et al., 2014). There are two main types of mushroom antioxidants, namely, primary (chain breaking, free radical scavengers) and secondary. The secondary antioxidants are the consequence of deactivation of metals, inhibition or breakdown of lipid hydroperoxides and regeneration of primary antioxidants. Some mushroom substances that exhibit antioxidant activity function as inducers and/or cell signals, leading to changes in gene expression, which result in the activation of enzymes that eliminate radicals (Kozarski, et al., 2015). In the study by Rodrigues et al. (2016b), the *Ph. nameko* extract obtained by different extraction methods and with different enzymes obtained a total antioxidant capacity of 35-44%. However, the extract of *Ph. nameko* obtained with Viscozyme had the highest antioxidant capacity of 55%.

1.4.2 Antidiabetic Activity

Small molecules with α-glucosidase or α-amylase inhibition potential are of pharmaceutical interest as antidiabetic drugs since by acting as competitive inhibitors of the enzyme they inhibit the hydrolysis of oligosaccharides, trisaccharides and disaccharides to glucose and other monosaccharides in the small intestine and thereby delay postprandial glucose absorption lowering blood glucose (Ali, Houghton, & Soumtanath, 2006).

In the study by Rodrigues et al., (2016b), the extract of *Ph. nameko* presented an α-glucosidase inhibitory activity with the use of enzymes Cellulase and Flavourzyme, which consequently suggests an antidiabetic potential. The antidiabetic potential is attributed to the presence of β-glucans, these are a soluble polysaccharides constituent of the cell wall of fungi such as mushrooms and cereal (Ribeiro, Umemura, Sousa, & Oliveira, 2009). According to Wasser (2002), the polysaccharides present in mushrooms are majority glucans with different types of bonds, such as (1→3), (1→6) e (1→3) - in β or α-glucans, but also heteroglycans. In this case, the lateral chains contain glucuronic acid, xylose, galactose, mannose, arabinose or ribose as a main component or different combination. The β-glucans presented several beneficial activities such as antitumour activity, immunomodulatory, antiviral, antimicrobial, antiparasitic, anticoagulant, healing and hypocholesterolemic activity (Acevedo, Pedroso, & Miranda, 2001; Park, Ikegaki, Alencar, & Aguiar, 2003) increasing insulin sensitivity and glucose tolerance. This glucose tolerance has been related to the property of these polysaccharides to induce high viscosity when in solution, forming a gelatinous layer that decreases the absorption of sugar by the intestine (Ribeiro, Umemura, Sousa, & Oliveira, 2009).

1.4.3 Anti-hypertensive Activity

ACE inhibitory activity *in vitro* is not always directly related to an antihypertensive effect *in vivo* (Herregods, et al. 2011). Thus, demonstration of an *in vivo* antihypertensive effect is of paramount importance, and there have been many investigations on the antihypertensive effect of edible mushrooms and other foodstuff. successfully purified from edible mushrooms, such as *Grifola frondosa* (Choi, et al. 2001), *Pholiota adiposa* (Koo, et al. 2006), *Pleurotus cornucopiae* (Jang, et al. 2011), *Pleurotus cystidiosus* (Lau, Adbullah & Shuib, 2013), *Tricholoma giganteum* (Lee, et al. 2004), *Hypsizygus marmoreus* (Kang, et al. 2013), *A. bisporus* (Lau, Abdullah, Shuib & Aminudin, 2014), *Leucopaxillus tricolor* and *Ganoderma lucidum* (Mohamad, Abdullah & Aminudin, 2013) demonstrated ACE inhibitory peptides and proteins. Moreover, in Lee, Cheng, Enomoto & Nakano (2006) the amino acids proline and lysine or aromatic amino acid residues presents in mushrooms reveals a capacity of ACE-I inhibiton.

The ACE-I inhibitory activity of the crude protein extract of the edible *mushroom Lentinus polychrous Lèv*. was reported for the first time on Krokram, Khammunag & Sarnthima (2016) presenting an IC_{50} value of 1.066 mg protein/ml. In what concerns ACE-inhibitory activity by mushroom derived extracts, D-mannitol, a major phytochemical of *P. cornucopiae* was found to inhibit an ACE and lower the blood pressure (Hagiwara, et al., 2005). Another

study by Rodrigues (2016) revealed that the extract of *Ph. nameko* obtained with Cellulase compared to the extract of *O. pinnatifida* obtained with *Viscozyme* showed to have antihypertensive capacity with an IC_{50} value of 130.4 µg/mL and 111.2 µg/mL, respectively.

1.4.4 Prebiotic Activity

Mushrooms are a potential source of prebiotics because they are rich in non-digestible dietary fibres such as glucans, chitin and heteropolysaccharides (Bhakta & Kumar, 2013) which could play beneficial and prebiotic roles.

According to Rodrigues, et al. (2016a), the majority of the extracts obtained from *Ph. nameko* possess compounds that can be metabolized by *L. acidophilus* La-5 and *B. animalis* subsp *lactis* BB12. Higher prebiotic potential was observed in extracts resulted from hot water extraction (HWE), ultrasound-assisted extraction (UAE), and enzyme-assisted extraction (EAE) with Viscozyme and Cellulase. Given the reported results in terms of growth promotion of *L. acidophilus* La-5 and *B. animalis* subsp *lactis* BB12 and considering the prebiotic potential of polysaccharides and extracts from mushrooms reported by literature concerning other species, further tests (non-digestibility and selective fermentation capacity in model systems) must be performed with these specific extracts. In the studies realized by Rodrigues (2016) and Rodrigues, et al, (2016b), it was found that the digested extract of *Ph. nameko* obtained with the enzyme Flavourzyme hinders the growth of species such as *Clostridium histolyticum* and *Clostridium cocoides/Eubacterium rectale* and promotes the growth of *Bifidobacterium* spp. It was also revealed that the species *Lactobacillus* spp was not affected. This selective increase of the species of *Bifidobacterium* spp. associated with the constant increase in the total production of short chain fatty acids and lactic acid, demonstrated the prebiotic potential of these extracts during the *in vitro* feacal fermentation.

The following Table 1.5 shows more examples of mushrooms species with another biological activities beyond those previously described.

Table 1.5 - List of some edible and inedible mushrooms and their activities.

Mushrooms Species	Activity	Reference
Pleurotus spp.	- Elimination of free radicals	(Chye, Wong, & Lee, 2008)
Hygrocybe spp.	- Chelating effect of iron and calcium compounds	(Chye, Wong, & Lee, 2008)
Pleurotus ostreatus	- Reducing cholesterol levels - Antibacterial activity	(Khatun, Mahtab, Khanam, Sayeed, & Khan, 2007); (Bobek & Galbavy, 1999); (Miguel, Garcia, Espinosa, & Ogura, 1997)
Lentinula edodes	- Anti-mutagenic effects	(de Lima Alves, Delmanto, Sugui, & da Eira, 2001); (Sugui, de Lima Alves, Delmanto, & da Eira, 2003)
Grifola frondosa	- Anticancer activity and hypoglycemic effects	(Konno, et al., 2002)
Sparassis crispa	- Antitumour activity - Immunomodulatory activity	(Ohno, et al., 2002); (Ohno, et al., 2003)
Hypszigus marmoreus	- Antifungal activity - Antiproliferative activity	(Lam & Ng TB, 2001)
Lactarius vellereus	- Genotoxic activity	(Mlinarič, Kac, Fatur, & Filipič, 2004)
Ganoderma lucidum	- Activity elimination of superoxide radical and hydroxyl - Antiallergic - Antihypertensive (ACE inhibitor) - Antioxidant activity (elimination of free radicals) - Genotoxic activity	(Tasaka, Mio, Izushi, Akagi, & Makino, 1988) (Lin, 2004)
Agaricus brasiliensis	- Antiviral activity	(Faccin, Benati, Rincão, & Mantovani, 2007)
Hericium erinaceus	- Cognition improvement properties	(Mori, Inatomi, Ouchi, Azumi, & Tuchida, 2009)
Piptoporus betulinus	- Anti-inflammatory activity	(Wangun, Berg, Hertel, Nkengfack, & Hertweck, 2004)

1.5 Mushrooms Based Functional Foods

From a commercial point of view, this entails the possibility of obtaining new foods with new flavours and which are functionalized. From the standpoint of food itself, it is indispensable to know all possible interactions that could promote or inhibit the bioactivity allocated to each extract/compound. It is well known that the bioactivity associated with an extract/ compound may be affected by the processing activities or by other components present

in food (Giavais, 2014). In the context of bakery products some studies document the incorporation of mushroom polysaccharides in foods containing cereals (Foschia & Brennan, 2013; Ktenioudaki & Gallagher, 2012). In an attempt to produce a novel high-fibre and low-calorie functional food, Kim, et al. (2011) swapped part of the wheat flour in baked foods for glucans from *L. edodes*. These glucans (included in butter at a concentration of 1 g of β-glucan per serving) improved the pasting properties of wheat flour and increased batter viscosity and shear-thinning elasticity without any adverse effect on air holding capacity or hardness. *Lentinula edodes* paste added to wheat flour for making noodles was also studied. This "recipe" resulted in a higher quality, fibre-rich functional food with antioxidant and hypocholesterolemic properties (Kim, Kang, & Kim, 2008; Kim, Chung, Nam, & Kang, 2009).

According to Ulzijargal, Yang, Lin, Chen, & Mau (2013), the incorporation of different mushroom mycelia into bread as a substitute of 5% wheat flour didn't negatively affect the texture profile of the bread and after cooking; bread supplemented with the mycelium also contained substantial amounts of α- aminobutyric acid and ergotonin (0.23-0.86 and 0.79-2.10 mg/ g drying matter, respectively). Mushroom powder has also been incorporated into other bakery products with matching quality for wheat flour counterparts. A healthy version of muffins and biscuits was produced with powders of the species *L. edodes* and *P. eryngii* respectively (Ulzijargal, Yang, Lin, Chen, & Mau, 2013). To develop a novel snack food, *Pleurotus sajor-caju* (Fr.) powder was incorporated in the preparation of "papad", a food adjunct or typical Indian snack. The papad enriched with 20% mushroom powder presented increased values of protein, minerals and crude fibre, giving rise to a new product with improved nutritional value and fibre contents (Parab, Dhalagade, Sahoo, & Ranveer, 2012). *Agaricus bisporus* extracts were used to produce snacks with high antioxidant potential and free radical scavenging capacity. This work aimed to show that mushroom snacks may serve as a good alternative for currently existing snack foods since they retain a significant quantity of polyphenolics and antioxidants, which constitute beneficial health effects to consumers. Moreover, the production process is quite simple and economically viable (Singla, Ghosh, & Ganguli, 2009).

On this thesis, mushroom also can be incorporated in dairy matrices such as cheese and cheese-related products. Okamura-Matsui et al. (2001) produced a functional cheese-like food incorporated with *Schizophyllum. commun*e, which was shown to have preventive effects against cancer and thrombosis. The following Table 1.6 shows some examples of the applicability of mushroom species to food.

Table 1.6 - Application of mushrooms in food (Moon & Lo, 2013).

Product	Mushrooms Species	Characteristics	Reference
Bread	*Pleurotus plumonarius*	Increased protein and nutritional concentration	(Okafor, Okafor, Ozumba, & Elemo, 2012)
Meat sauce	*Agaricus bisporus*	Texture conference	(Kim, et al., 2011)
Beverages like beer	*Ganoderma lucidum*	Increased biological activities	(Leskosek, et al., 2010)

Comparatively, in terms of an amount of protein, the mushrooms are below the meat of animals, but above most other foods, such as milk (Chang, 1980). It is true that animal protein is richer in protein and essential amino acids, but people who consume high amounts of animal protein in their diet are more likely to get a cardiac disease, cancer and diabetes which could in part be related to the fat that meat (animal protein) contains few vitamins, minerals and any antioxidants (Qua, 2015). Or contrary to what happens with the ingestion of mushrooms, rich in minerals, vitamins such as vitamin B (Mattilda, et al., 2000) and antioxidant compounds as mentioned previously in Table 1.6. Despite the studies already developed in this field of mushroom incorporation in foods, these products aren't yet available in the market. As mentioned before, although some works with phenolic compounds have been carried out, there is still a lack of information regarding the bioaccessibility/bioavailability of the compounds and possible interactions with the food matrix. Therefore, most of the mushrooms and their compounds are mainly consumed in their natural form or in dietary supplements (Reis, Martins, Vasconcelos, Morales, & Ferreira, 2017).

1.6 Extraction methods of Bioactive Compounds from Seaweed and Mushroom

In general, the bioactive compounds may be extracted and/or concentrated and used in different food or nutraceutical applications. Many extraction techniques are available for such purpose yet are not always of favourable application due to associated environmental pollution and costs (Rodrigues, et al., 2015a). As a consequence of the abovementioned studies there is a need to resort to extraction methods that may efficiently extract and concentrate the compounds from mushrooms and seaweed, so their biological properties can be evaluated.

Methods for extraction of bioactive compounds of biological interest are generally divided into two large groups, namely, conventional and non-conventional techniques (Antunes, 2015). Within the conventional techniques, extraction methods may include maceration, infusion, decoction and boiling under reflux steps. On the other hand, in the non-conventional techniques we have the assisted extractions by ultrasound, supercritical fluid extraction, pressurized liquid,

hydrotropic extraction and enzyme-assisted extraction (Brusotti, Cesari, Dentamaro, Caccialanza, & Massolini, 2014). In most cases, aqueous extractions by the action of hot water are complemented with extractions with alcohols to isolate compounds that are insoluble in water (Gonçalves & Lima, 2011). Extraction modes tested on mushrooms in the last years include pressurized water extraction (Palanisamy, et al., 2014), or extraction with different solvents such as methanol (Moro, et al., 2012), ethanol, ethyl acetate (Seephonkai, et al., 2012) and chloroform (Antunes, 2015). The extraction efficiency of compounds from seaweeds is limited due to the presence of complex cell walls with mixtures of sulphated and branched polysaccharides associated with proteins and various bound ions such as Ca+ and K+ (Wijesinghe & Jeon, 2012). Water-based extraction is food compatible, non-expensive and environment friendly, but has a low selectivity with low extraction efficiency. In order to improve this method Rodrigues, et al., (2016b; 2015a) prepared water-based extracts using alternative approaches such as enzyme-assisted extraction and ultrasound-assisted extraction. It was demonstrated that this method results for both mushrooms and seaweeds and enables a more efficient extraction.

In the case of mushrooms, this method resulted in a higher extraction of compounds which in the case of *Ph. nameko* revealed an increase in prebiotic and antidiabetic potential (Rodrigues, et al., 2016b). In the case of seaweeds, the enzyme-assisted extraction method with its hydrolytic action on seaweeds weakened or disrupted cell wall structure and also broke down complex interior storage compounds, releasing intracellular compounds (peptides, amino-acids, among others). This process of water-based extraction coupled to enzymes enables the release of secondary plant metabolites and the maintenance of the bioactive properties of the extracts. In case of *O. pinnatifida* this extraction method revealed an increase in antioxidant activity (Rodrigues, et al., 2015a). It is therefore necessary to combine different approaches as a function of the species used in order to obtain targeted compounds with specific biological properties (Gonçalves & Lima, 2011).

1.7 Objectives

Based on this *state-of-art* framework, certain seaweed and mushrooms are sources of biologically active compounds and these can be incorporated into food matrices giving rise to novel functional foods that promote a beneficial impact on health and well-being. In this case taking into consideration the nutritional and biological properties previously identified in the enzymatic extracts of *O. pinnatifida* and mushroom *Ph. nameko* (Rodrigues, et al., 2015a, 2015b; 2016a; 2016b; Rodrigues, 2016), these can be further exploited as functional ingredients through their incorporation into food matrices.

Hence, taking into account the biological properties identified and considering that dairy products are very promising food vectors for the incorporation of functional ingredients in terms of biological stability, the main goal of this research project is to incorporate in a spreadable dairy matrix the natural extracts of *O. pinnatifida* and *Ph. nameko* to obtain new potential functional foods with beneficial biological properties including prebiotic, antioxidant, antidiabetic and antihypertensive activities.

In order to achieve this main goal a set of specific objectives were established:

i) Microbiological quality assessment of the formulated dairy matrices and its evolution over storage period under controlled refrigeration conditions;

ii) Physico-chemical characterization (pH, sugars and organic acids) of the formulated dairy matrices;

iii) Sensory characterization (flavour, aroma, acceptability) of the formulated dairy matrices;

iv) *In vitro* evaluation of the previously identified biological properties in the formulated dairy matrices: (a) prebiotic activity (evaluation of stability and viability bioavailability), (b) antioxidant capacity (DPPH, ABTS and $OH^{\bullet}$ radical), (c) antihypertensive activity (angiotensin-I converting enzyme inhibitory activity*)* and (d) antidiabetic activity (α-glucosidase inhibitory activity).

It is intended to obtain at the end one or more dairy creams functionalized with extracts of *O. pinnatifida* and *Ph. nameko*.

2. Materials and Methods

2.1 Development of a spreadable dairy cream

2.1.1 Seaweed and mushroom extracts' production

The extraction of seaweed and mushrooms was performed according to a method described by Rodrigues et al. (2015a; 2016b). In this thesis, extracts of *O. pinnatifida* obtained with Viscozyme (Sigma-Aldrich, St. Louis, Missouri, USA) and of *Ph. nameko* obtained with Flavourzyme (Sigma-Aldrich, St. Louis, Missouri, USA) were selected, respectively. For each extract, 1 g. dried mushroom, or 2 g. in the case of dried seaweed, were added to 50 ml of deionized water and were incubated in a water bath at 50 ºC for 10 min. After adjusting pH to specific enzyme optimum conditions (Flavourzyme: pH= 7.0 at 50 ºC; Viscozyme® L: pH= 4.5 at 50 ºC) 100 mg of enzyme was added and enzymatic hydrolysis ran at 50ºC for 24 h. The enzymatic reaction was then stopped by heating the sample at 90-100 ºC for 10 min followed by immediate cooling in an ice bath. The enzymatic aqueous solutions were then centrifuged at 4000 rpm, 10 min (Universal 320R, Hettich, Germany), filtered in vacuum with a filter 0.22 µm, frozen at -80 ºC and lyophilized. The pH of all extracts was adjusted to pH 7.0 with 1M HCl and/or NaOH before freezing at -80 ºC. The extracts were then lyophilized (Armfield SB4 model, UK).

2.1.2 Spreadable dairy cream

The rationale behind the development of a spreadable dairy cream was based on a very preliminary work developed by Rodrigues (2016) which showed the potential of this product. Hence, this thesis explored deeper the development of a versatile and healthy dairy matrix based on whey cheese (*requeijão*) and greek yoghurt, thus producing a new spreadable dairy cream. Pasteurized whey cheese from cow's milk and a Greek-type yoghurt (without added sugar) were obtained from a local supermarket (Porto, Portugal). The recipe for the spreadable dairy cream was based on 75% (w/w) of pasteurized whey cheese, 22% (w/w) of Greek type yoghurt and 3% (w/w) of *O. pinnatifida* or *Ph. nameko* lyophilized extract according to previous work of Rodrigues (2016).

2.2 Thermal and non-thermal treatments of extracts and dairy creams

2.2.1 Pasteurization (thermal) and Ultraviolet radiation (non-thermal) treatments

In order to seek the safest microbiological standards as well as proper stability of the spreadable dairy cream throughout shelf-life to make a dairy product suitable for possible commercialization, several experiments were performed targeting the microbial load of the

different ingredients in the dairy cream (i.e. whey cheese, yoghurt and extract) especially of the whey cheese, known to be a perishable food with a limited shelf-life.

Taking into account the preliminary results obtained by Rodrigues (2016), an initial thermal treatment was applied using a hot water bath at 90° C for 10 min. Several variations, including the non-thermal treatment (ultraviolet radiation - UV) of the extracts, were also tested according to the description listed in Table 2.1.

Table 2.1 – Schematic description of thermal and non-thermal treatments tested on dairy creams and extracts.

Experiment 1	**Experiment 2**	**Experiment 3**	**Experiment 4**
Whey cheese 75% (w/w)	Whey cheese 75% (w/w)	Whey cheese 75% (w/w)	Whey cheese 75% (w/w)
Yoghurt 22% (w/w)	Yoghurt 22% (w/w)	Extract 3% (w/w)	Yoghurt 22% (w/w)
Thermal treatment 10 min. at 90ºC	Extract 3% (w/w)	Thermal treatment 10 min. at 90ºC	Thermal treatment 10 min. at 90ºC
Extract 3% (w/w)	Thermal treatment 10 min. at 90ºC	Yoghurt 22% (w/w)	UV radiation, 15 min (non-thermal treatment) Extract 3% (w/w)

After thermal or non-thermal treatment, all samples were evaluated in terms of microbiological quality: total viable cell numbers of aerobic mesophilic bacteria, enterobacteria and lactic acid bacteria were monitored. These analyses were performed according to the methods described in 2.3.2.

2.2.2 UV treatment of mushroom extracts

Considering the microbiological load observed for the mushroom extract, additional UV treatments were performed according to the description in Table 2.2. The extracts, either in the form of extract powder or incorporated in different matrices (yoghurt or water), were exposed to 13,44 watt/m^2 of UV radiation for 10 or 20 min.

Table 2.2 - Schematic description of UV treatments applied to mushroom extract powder, in simple form or incorporated in different matrices.

	Mushroom extract powder	**Mushroom extract in yoghurt**	**Mushroom extract in sterile water**
13,44 watt/m^2 of UV radiation[1]	10 min	10 min	10 min
	20 min	20 min	20 min

[1]UV radiation was carried out directly in the 1g powdered extract or in 0,9 g of extract incorporated in 6,6 g yoghurt or in 3 g of extract with 10 mL of sterile water.

After UV treatment, the total viable cell numbers of mesophilic bacteria were determined according to the methods described in 2.3.2.

2.2.3 Pasteurization versus sterilization of extracts

Both mushroom and seaweed extracts were submitted to new thermal treatments in order to achieve the maximum microbiological quality. Samples of 5 g of the mushroom or seaweed extracts were dissolved in 4 mL of sterilized water and half of this solution was submitted to 90 ºC for 30 min in a water bath and the other half was submitted to 100-120 ºC in a pressure cooker for 10 min. After thermal processing (0d), treated samples were placed under refrigeration at 4º C for 7 days. Samples were analyzed at 0 and 7d of storage for total viable cell numbers of mesophilic bacteria according to the methods described in 2.3.2.

2.3 Spreadable dairy cream: Production and microbiological evaluation throughout storage

2.3.1 Production of spreadable dairy creams

The formulations of the spreadable dairy creams produced, and the respective thermal treatment applied, are listed in Table 2.3.

Table 2.3 – Composition of spreadable dairy creams and thermal treatment applied.

Spreadable dairy cream with *O. pinnatifida* extract (SPExtract)	**Spreadable dairy cream with FOS[1,2] (SPFos)**	**Spreadable dairy cream[3] (SP)**
Whey cheese 75% (w/w)	Whey cheese 75% (w/w)	Whey cheese 75% (w/w)
Yoghurt 22% (w/w)	Yoghurt 22% (w/w)	Yoghurt 22% (w/w)
Extract 3% (w/w)	FOS 3% (w/w)	_____
Thermal treatment - 30 min at 90ºC		

[1]Frutooligosaccharide (Orafti® P95; Orafti, Belgium). [2]positive control; [3]negative control.

The preparation and the distribution of each formulation of the spreadable dairy creams (SPExtract, SPFos and SP) produced for microbiological and sensorial evaluation over 21 days of storage at 4±1 ºC with sampling points at 0, 7, 14 and 21 days, as well as its biological characterization (prebiotic activity, antidiabetic activity, antihypertensive activity and antioxidant activity) was performed as schematized in Figure 2.1.

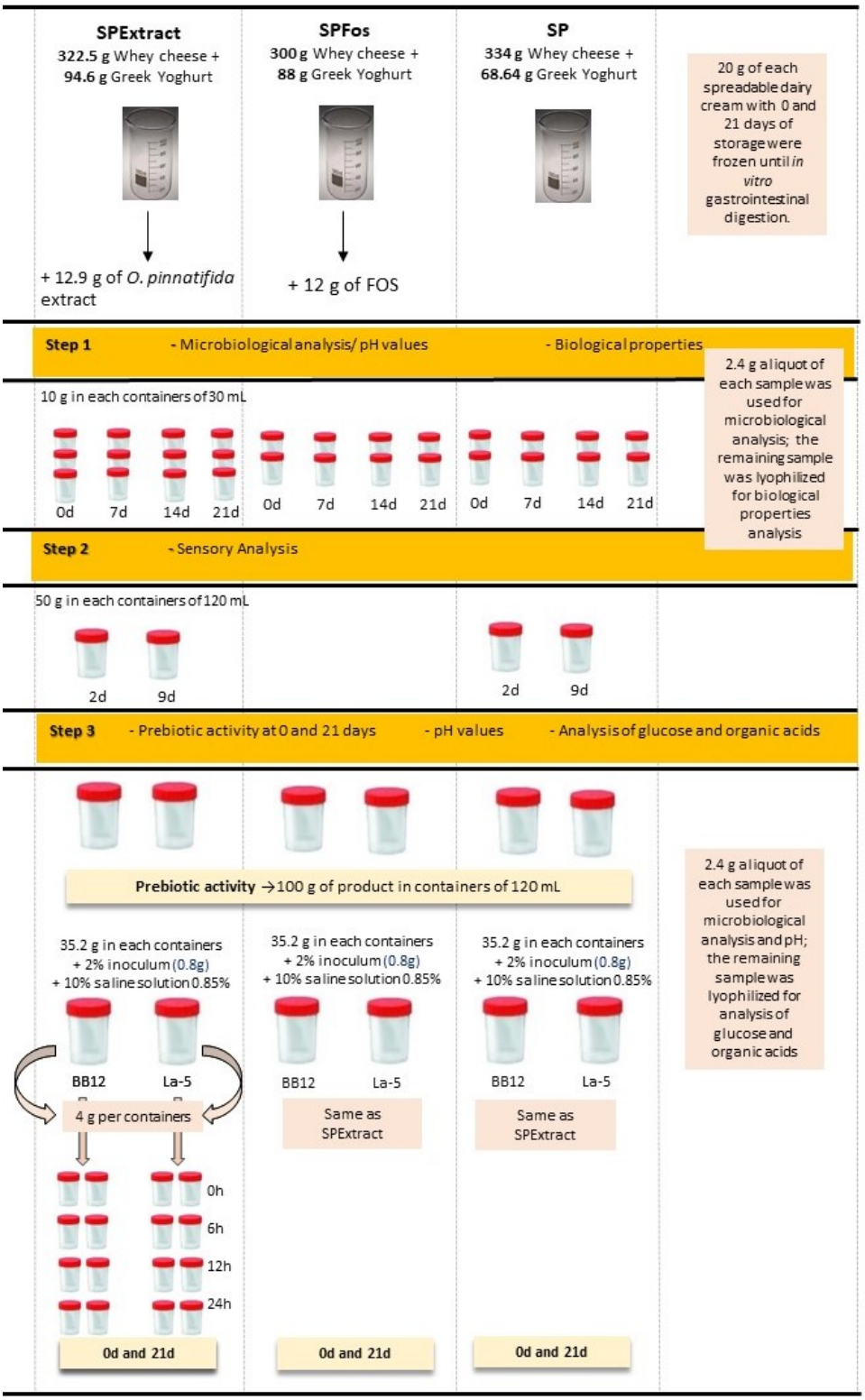


Figure 2.1 - Schematic description of the preparation and distribution of each spreadable dairy cream for analysis.

2.3.2 Microbiological analysis

The microbiological evaluation after production (0d) and throughout storage under refrigeration was determined according to the method described by Rodrigues (2016).

For each spreadable dairy cream at each sampling time, a 2.4 g aliquot of each sample was homogenized in 24 mL of sterile 0,1% (w/v) peptone water (Sigma-Aldrich, St. Louis, Missouri, USA) for 3 min, in a Stomacher blender (Model 400 circulator, from Seward Laboratory Systems, UK). Sequential dilutions of spreadable dairy cream homogenates were made with sterile 0,1% (w/v) peptone water and 20 µL aliquots were plated (Miles & Misra, 1938), in duplicate, on Petri dishes of:

- de Man, Rogosa and Sharpe agar (MRS agar, Biokar Diagnostics, France) incubated for 48 h at 37ºC in aerobic environment for total viable cell numbers of lactic acid bacteria;
- Plate Count Agar (PCA, Sigma- Aldrich, St. Louis, Missouri, USA) incubated aerobically at 30 ºC for 2 to 5 d for total viable cell numbers of aerobic mesophilic bacteria;
- Violet Red Bile Glucose Agar (VRBGA, Biokar Diagnostics) incubated aerobically at 37 ºC for 48 h for total viable cell numbers of enterobacteria.

2.4 Sensory Analysis

For the evaluation of the global acceptance of each developed dairy cream, the spreadable dairy cream with O. *pinnatifida* extract and the spreadable dairy cream without addition of carbon source, at 2 and 9 days of storage, were subjected to a sensory analysis by specifically trained panel members.

The panel consisted of 16 members with an average age of 30±8, a gender distribution of female (62.5%) and male (37.5%), and among which 90% indicated regular consumption of whey cheese several times in a month. The spreadable dairy creams were placed in tight plastic containers and conditioned at room temperature for 15 minutes before evaluation. Between analyses, the panel took water and toasts, to eliminate the taste of the previous analysis.

Consumer acceptance was measured by overall liking ratings, provided on a 9-point hedonic scale, (Peryam & Girardot, 1952; Peryam & Pilgrim, 1957), whereas the consumption intent was evaluated by using a probability scale ranging from 0 to 9 based on Juster (1966). During the assay, the intensity of aspect, odour, flavour, creaminess and consistency sensory attributes were measured by ratings provided on a just-about-right scale (Moskowitz, 1972). Just-about-right (JAR) scales were designed as continuous line scales with three descriptive anchors, low intensity (i.e. “Much Too Weak”) on the left end, “Just About Right” at the center, and high intensity (i.e. “Much Too Strong”) on the right end. All the values were converted to 0 to 1 scale.

2.5 Biological Properties

The biological properties evaluated included those that were previously observed for these extracts (Rodrigues, et al., 2015a, 2015b; 2016a; 2016b; Rodrigues, 2016), namely prebiotic, antidiabetic, anti-hypertensive and antioxidant activities.

For prebiotic activity, the different spreadable dairy creams were evaluated after 0 and 21 days of storage (Figure 2.1). Prebiotic activity was also evaluated on lyophilized samples with 0 and 21 days storage and exposed to *in vitro* simulated gastrointestinal digestion.

For the evaluation of antioxidant, anti-hypertensive and antidiabetic activities, samples of different spreadable dairy creams with different times of storage (Table 2.4) were previously lyophilized.

Table 2.4 – Description of samples of spreadable dairy creams analyzed for each bioactivity.

Bioactivity	**Storage time (days)**			
	0	**7**	**14**	**21**
Prebiotic	X			X
Antidiabetic	X			X
Anti-hypertensive	X	X	X	X
Antioxidant	X	X	X	X

2.5.1 Prebiotic Activity

In order to evaluate the biological potential of the spreadable dairy cream incorporating selected seaweed (*O. pinnatifida*) extract, assays with pure cultures of *Lactobacillus acidophilus* La-5 and *Bifidobacterium animalis* subsp *lactis* BB12 were performed in order to monitor the potential prebiotic activity. The assessment was performed as described by Rodrigues (2016) in spreadable dairy creams after production (0d) and after 21 days of refrigerated storage.

2.5.1.1 Non-digested spreadable dairy creams

2.5.1.1.1 Microbiological analysis

2.5.1.1.1.1 Inocula preparation

To obtain each probiotic inoculum used to inoculate each spreadable dairy cream, freeze-dried bacteria of *B. animalis* subsp *lactis* BB12 or of *L. acidophilus* La-5 (CHR-Hansen, Denmark) were inoculated in MRS broth (Biokar Diagnostics, France); 2% (v/v) of a 24 h

probiotic culture was then inoculated in MRS broth supplemented with 0.5 g/L of L-cysteine-HCl (Panreac, Spain) in 50 mL flat-bottomed glass flasks, and incubated for another 24 h.

2.5.1.1.1.2 Spreadable cheese inoculation and microbiological analysis

The prebiotic potential of three spreadable dairy cream formulations was evaluated immediately after production (0 d) and after 21 days of storage at -4 ºC, by evaluating the viable cells of *L. acidophilus* La-5 and *Bifidobacterium animalis* subsp *lactis* BB12 over 24h incubation at 37 ºC. The preparation and distribution of each spreadable dairy cream in replicas was described on section 2.3.1 in Figure 2.1.

Samples were collected in the beginning of incubation period (0 h) and after 6, 12 and 24 h (2 separate containers per spreadable dairy cream and microorganism; Figure 2.1). For each sample a 2.4 g aliquot was homogenized in 24 mL of sterile 2% (w/v) peptone water (Sigma-Aldrich, St. Louis, Missouri, USA) for 3 min using a Stomacher blender (Model 400 circulator, from Seward Laboratory Systems, UK). Sequential dilutions of spreadable dairy cream homogenates were made with sterile 0.1% (w/v) peptone water (Sigma-Aldrich, St. Louis, Missouri, USA) and 20 µL aliquots were plated, in duplicate, on Petri dishes of MRS agar (Biokar Diagnostics), plain for lactobacilli or supplemented with 0.5 g/L L-cysteine- HCl for bifidobacteria (Miles and Misra, 1938). The resulting plates were then incubated at 37 ºC under aerobic or anaerobic conditions for *Lactobacillus* and *Bifidobacterium*, respectively.

2.5.1.1.2 Glucose, organic acids and pH analysis

Variation in glucose, short-chain fatty acids and pH were determined according to the methods described by Rodrigues (2016) in all samples after 0, 6, 12 and 24 h of incubation at 37 ºC. The pH of the spreadable dairy creams with *O. pinnatifida* extract, FOS and without addition of a carbon source (SPExt, SPFos and SP) was measured directly with a pH meter (Micro pH 2002, Crison, Spain). Duplicate samples of each spreadable dairy cream were assessed for glucose and organic acids (lactate and acetate) by HPLC in a single run and their quantification were based on calibration curves previously prepared with appropriate chromatographic standards, using an apparatus from Merck LaChrom (Fullerton CA, USA), with an Aminex HPX-87X cation exchange column from BioRad (Richmond CA, USA); the flow rate was 0.8 mL/min; 13 mM H_2SO_4 (Merck, USA) was used as eluent; and detection was by refractive index at 65 ºC for glucose, and UV absorbance at 220 nm for organic acids. Prior to analysis, all samples were pre-treated as follows: 1 g of spreadable dairy cream was homogenized with 5 mL of 13 mM H_2SO_4 in an Ultra-Turrax (IKA®, Canada) at 4000 rpm,

allowed to stand for 3 min in an ice-bath, centrifuged at 14 000 rpm for 10 min at 4 ºC, and then filtered through a 0.22 µm membrane filter (Millipore, USA). The detection limit and quantification limit values for acetic and lactic acids are 0.05 g/L and 0.1 g/L, respectively.

2.5.1.2 Prebiotic Activity of digested spreadable dairy creams

2.5.1.2.1 *In Vitro* gastrointestinal digestion

To simulate the digestion through the gastrointestinal tract, samples of SPExt, SPFos and SP with 0 and 21 days of storage, were treated according to the procedures described by Minekus et al. (2014) with slight modifications. The composition of the simulated fluids as well as the pH values are described in Table 2.5.

Table 2.5 – Composition, concentration and pH values of each simulated fluid based on Minekus et al, (2014).

Fluids composition and stock			SSF[1] pH 7		SGF[2] pH 4		SIF[3] pH 7	
Compound	Stock (g/L)	Conc. (mol/L)	Vol (mL)	Conc. (mmol/L)	Vol. (mL)	Conc. (mmol/L)	Vol. (mL)	Conc. (mmol/L)
KCl	37.3	0.5	15.1	15.1	6.9	6.9	6.8	6.8
KH_2PO_4	68	0.5	3.7	3.7	0.9	0.9	0.8	0.8
$NaHCO_3$	84	1	6.8	13.6	12.5	25	42.5	85
NaCl	117	2	-	-	11.8	47.2	9.6	38.4
$MgCl_2(H_2O)_6$	30.5	0.15	0.5	0.15	0.4	0.1	1.1	0.33
$(NH_4)_2CO_3$	48	0.5	0.06	0.06	0.5	0.5	-	-
			For pH adjustment					
	(mol/L)		(mL)	(mmol/L)	(mL)	(mmol/L)	(mL)	(mmol/L)
NaOH	1		-	-	-	-	-	-
HCl	6		0.09	1.1	1.3	15.6	0.7	8.4
$CaCl_2(H_2O)_2$ is not added to the simulated digestion fluids, see details in legend								
	(g/L)	(mol/L)		(mmol/L)		(mmol/L)		(mmol/L)
$CaCl_2(H_2O)_2$	44.1	0.3		1.5 (0.75*)		0.15 (0.075*)		0.6 (0.3*)

* In brackets is the corresponding Ca^{2+} concentration in the final digestion mixture

[1]Simulated salivary fluid; [2]Simulated gastric fluid; [3]Simulated intestinal fluid

To 2 g of lyophilized SPExt, SPFos and SP was added 1.4 mL of simulated salivary fluid (SSF), 10 µl of 0.3 mM of $CaCl_2$ and α-amylase at 75 U/mL (Sigma-Aldrich, St. Louis, Missouri, USA) and finally 0.390 mL of water and the mixture was incubated for 2 min at 37 ºC in a shaker at 130 rpm (Bench Top Shaking Incubator, Wiggen Hauser, Malaysia). Afterwards, the pH was adjusted to 2.0 with 6 M HCl and pepsin at 2000 U/mL dissolved in simulated gastric fluid (SGF) (6 mL) was added as well as 19 µL of $CaCl_2$ and 0.271 mL of water before its incubation at 37 ºC for 2 h in a shaker at 130 rpm. After this period, the pH was increased to 7 with 6 M NaOH and 4.4 mL of simulated intestinal fluid (SIF), 2 mL of trypsin at 7.0 U/mg of pancreatin power, 1 mL of bile (B 8631, Sigma; 12 g/L), 16 µL of $CaCl_2$, 0.584 mL of water and 0.1 mL of aminoglycoside at 204 U/35 mL (Sigma; 37 mg in SIF) were added. The sample was incubated at 37ºC for 3 hours in a shaker at 130 rpm (Bench Top Shaking Incubator, Wiggen Hauser).

Digested samples were submitted to dialysis according to Mills, et al. (2008). The spreadable dairy creams were transferred to 1 kDa molecular weight cut-off regenerated cellulose dialysis tubing (Spectra/Por® 6, Spectrum Europe, Netherlands) and dialysed against NaCl 0.01 M at 5 ºC, to remove low molecular mass digestion products. After 15 h the NaCl dialysis fluid was changed and dialysis continued for an additional 2 h. Afterwards the digested samples were frozen at -80 ºC and lyophilized in a freeze dryer (Armfield SB4 model, Ringwood, UK). All assays were performed in duplicate.

2.5.1.2.2 Microbiological analysis

The evaluation of potential prebiotic activity of digested samples was performed according to the procedures described above in section 2.4.1 with modifications. *Lactobacillus acidophilus* La-5 and *B.animalis* subsp *lactis* BB12 overnight cultures at 2% were inoculated in MRS broth without conventional carbon source [(10 g/L peptone (Sigma-Aldrich, USA), 10 g/L meat extract (Merck, Germany), 5 g/L yeast extract (Biokar, Diagnostics, France), 1.08 g/L tween 80 (Merck), 2 g/L potassium dihydrogen phosphate (Merck), 5 g/L sodium acetate (Merck), 2 g / L ammonium citrate (Sigma-Aldrich), 0.2 g/L sulfate of magnesium (Merck), 0.04 g/L of manganese sulphate (Sigma-Aldrich)] containing digested samples of SPExtract, SPFos or SP (2%) and incubated for 24 h at 37 ºC, according to Rodrigues (2016). Besides MRS with digested spreadable dairy creams (SPExtract, SPFos and SP), it was also prepared MRS without carbon source (negative control), MRS with 2% (w/v) glucose and MRS with 2% (w/v) FOS (positive controls) which were also inoculated with the two probiotic strains under

study. The 2% concentration of the carbon source/digested samples was chosen based on the composition of the commercial MRS medium containing 2% (w/v) glucose.

The probiotic cultures *B. animalis* subsp *lactis* BB12 and *L. acidophilus* La-5 were prepared according to the procedures described above in section 2.5.1.

From each MRS medium inoculated with *L. acidophilus* La -5, 300 µL were transferred, in triplicate, to a 96-well microplate (Nunc, Denmark), whereas from each MRS medium inoculated with *B. animalis* subsp *lactis* BB12 only 250 µL were transferred, in triplicate, and then were covered with 50 µL of paraffin to ensure anaerobic conditions. The microplates were incubated for 24 h at 37 ºC and in a microplate reader (FLUOstar Optima, BMG Labtech, Offenburg, Germany) programmed to measure absorbance (Abs) at 660 nm every hour (using the software Fluostar Control version 1.32 R2).

2.5.2 Antidiabetic, Antihypertensive, Antioxidant Activities

2.5.2.1 Sample preparation

Lyophilized dairy cream samples were prepared according to the method described by Apostolidis, Kwon, & Shetty, (2007) with modifications. For the evaluation of antioxidant activity, 10 mg of each freeze dried spreadable dairy creams (SPExt, SPFos and SP) were dissolved on 1 ml of deionized water. For the evaluation of antidiabetic and anti-hypertensive activities, 30 mg of each freeze dried spreadable dairy creams were dissolved on 1 ml of 13 Mm Sulfuric acid or 1 ml of deionized water, respectively. Homogenized freeze-dried samples were centrifuged at 14 000 rpm for 10 min at 4 ºC (Universal 320R, Hettich, Germany).

2.5.2.2 Antidiabetic Activity: α-Glucosidase inhibitory activity

The α-Glucosidase inhibitory activity was determined in 96 well plates according to the method described by Kwon, Apostolidis, & Shetty (2008). The homogenized spreadable dairy creams (SPExt, SPFos and SP) (50µL), were mixed with 100 µL of 0.1 M phosphate buffer (pH 6.9) containing an α-Glucosidase solution (1.0 U/mL) (Sigma-Aldrich, St. Louis, Missouri, USA), and pre-incubated at 25ºC for 10 min. Then, 50 µL of 5 mM p-nitrophenyl-α-D-glucopyranoside (Sigma-Aldrich, St. Louis, Missouri, USA) solution in 0.1 M phosphate buffer (pH 6.9) was added to each well at 5s intervals. The reaction mixtures were incubated at 25 ºC for 5 min, and the absorbance readings were recorded at 405 nm by a multiscan microplate fluorimeter (FLUOstar Optima, BMG Labtech, Offenburg, Germany) and compared to a control which had 50 µL of buffer solution. Acarbose (Sigma) was used as positive control at

the concentration of 10 mg/mL. The α-Glucosidase inhibitory activity was expressed as inhibition (%) and was calculated as follows:

$$\text{Scavenging \%} = \left(\frac{\Delta Abs\ Control - \Delta Abs\ sample}{\Delta Abs\ Control}\right) \times 100 \qquad \text{Equation (2.1)}$$

Where $\Delta Abs_{control}$ is the variation of absorbance of the control and ΔAbs_{sample} is the variation of absorbance of the samples. All assays were performed in triplicate.

2.5.2.3 Antihypertensive Activity: Angiotensin-I converting enzyme (ACE)

The assay of the angiotensin-I converting enzyme (ACE) inhibitory activity was based on a method by Tavares et al. (2011). To each microtiter-plate well (Nunc, Denmark), 160 µL of fluorescent substrate Abz-Gly-Phe(NO_2)-Pro (Bachem Feinchemikalien, Germany) and 40 µL of homogenized spreadable dairy creams (SPExt, SPFos and SP) after 0, 7, 14 and 21 days of storage, with different concentrations, were added. The enzyme reaction was initiated by the addition of 2 mU of ACE (peptidyl-dipeptidase A, EC 3.4.15.1 Sigma Chemical, St. Louis, MO, EUA), dissolved in glycerol (50%) and prepared in buffer solution Tris-HCl (150 mM) with 0.1 mM de $ZnCl_2$ pH 8.3 that are immediately mixed and incubated at 37 ºC. The generated fluorescence was measured after 30 min by a multiscan microplate fluorimeter. Excitation and emission wavelengths were 350 and 420 nm, respectively. All assays were performed in triplicate.

In order to determine the IC_{50} values (half maximal inhibitory concentration necessary to inhibit 50% of ACE activity) it was necessary to know the total protein content in the *O. pinnatifida* extract. The total protein content was determined via Kjeldahl method according to (IDF, 1985). All assays were performed in duplicate.

2.5.2.4 Antioxidant Activities

2.5.2.4.1 ABTS radical scavenging activity

The total antioxidant capacity of the homogenized spreadable dairy creams (SPExt, SPFos and SP) was measured according to the method described by Gião et al. (2007). This method is able to quantify both water and lipid-soluble antioxidants, as pure compounds or in crude extracts via direct production of the 2,2'-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid) diammonium salt radical cation ($A_{ABTS\bullet+}$) chromophore (blue/green) by reaction of 2,2'-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid) diammonium salt (ABTS) (Sigma-Aldrich, St. Louis, Missouri, USA) and potassium persulphate ($K_2S_2O_8$) (Merck, Darmstadt, Germany).

To 2 mL of a diluted $ABTS^{\bullet+}$ solution, 120 µL of each homogenized spreadable dairy creams was added and left to react for 6 min; after that its absorbance at 734 nm (A_{sample}) in the UV-VIS spectrophotometer (Shimadzu, Brasil). Three replicates were performed. Using ascorbic acid as standard (0 to 1 mg/mL), the results were expressed as an equivalent concentration of ascorbic acid ($mg_{ascorbic\ acid\ equiv}$/mL). The percentage of scavenging activity was also determined using the following formula:

$$\text{Scavenging \%} = \left(\frac{A_{ABTS\bullet+} - A_{sample}}{A_{ABTS\bullet+}}\right) \times 100 \qquad \text{Equation (2.2)}$$

For each sample, the initial absorbance of 2 mL of diluted $A_{ABTS+}$was measured ($A_{ABTS\bullet+}$).

2.5.2.4.2 DPPH radical scavenging activity

The 2,2-Diphenyl-1-picrylhydrazyl (DPPH)-free-radical scavenging activity was measured according to the method described by Suresh *et al*., (2013) with modifications. An aliquot (0.1 mL) of each homogenized spreadable dairy creams (SPExt, SPFos and SP) was added to 3.0 mL of 0.1 mM ethanolic DPPH solution (Sigma-Aldrich, St. Louis, Missouri, USA) and absorbance was measured at 515 nm after incubation for 30 min at 30 ºC in the dark (A_{sample}) using a UV-VIS Spectrophotometer (Shimadzu, Brasil). Three replicates were performed. Using Trolox (Sigma-Aldrich, St. Louis, Missouri, USA) as standard (0 to 0.05 mg/mL), the results were expressed as equivalent concentration of trolox ($mg_{trolox\ equiv}$ /mL) whereas the percentage of scavenging activity was also calculated using the following formula:

$$\text{Scavenging \%} = \left(1 - \frac{Asample - Ablank}{Acontrol}\right) \times 100 \qquad \text{Equation (2.3)}$$

For each sample, the absorbance of 3 mL of DPPH plus 0.1 mL ethanol was measured as control ($A_{control}$) whereas the absorbance of 3 mL of ethanol plus 0.1mL of homogenized spreadable dairy cream was measured as the blank (A_{blank}).

2.5.2.4.3 Hydroxyl-radical scavenging activity ($OH^{\bullet}$)

Hydroxyl-radical scavenging activity was measured according to the method described by Sudha, Priya, Shree, & Vadivukkarasi (2011). An aliquot (1 mL) of each homogenized spreadable dairy creams (SPExt, SPFos and SP) was added to 2 mL of reaction mixture containing 1 mL of 1.5 mM $FeSO_4$ (Merck, Darmstadt, Germany), 0.7 mL of 6 mM hydrogen peroxide (Merck, Darmstadt, Germany). and 0.3 mL of 20 mM of sodium salicylate (VWR, Leuven, Belgium). After incubation for 1 hour at 37°C, the absorbance of the hydroxylated salicylate complex was measured at 562 nm (A_{sample}) using a UV-VIS

spectrophotometer (Shimadzu, Brasil). Three replicates were performed. Using ascorbic acid as standard (0 to 1 mg/mL), results were expressed as the equivalent concentration of ascorbic acid equivalent ($mg_{ascorbic\ acid\ equiv}$/mL) whereas the percentage of scavenging activity of hydroxyl radical was also calculated using the following formula:

$$\text{Scavenging \%} = \left(1 - \frac{Asample - Ablank}{Acontrol}\right) \times 100 \qquad \text{Equation (2.4)}$$

For each sample, the absorbance of 2 mL reaction mixture plus 1 mL of deionized water was measured as the control ($A_{control}$) whereas 2 mL of reaction mixture, with sodium salicylate substituted by water plus 1 mL of extract was measured as blank (A_{blank}).

2.6 Statistical analysis

Data are expressed as the mean plus standard deviation of replicates. To analyse the differences between the different spreadable dairy creams (SPExt, SPFos and SP), when a normal distribution was observed, one-way ANOVA was carried out in association with Tukey's Multiple Comparison Test with a 95% confidence interval. The normality of the distributions was evaluated via the Shapiro-Wilk's Test.

All tests were realized with a significance level of 5% and using the Statistical Package for Social Sciences software (version 21, SPSS, Chicago, IL, USA).

3. Results and Discussion

3.1 Thermal and non-thermal treatments of extracts and dairy creams

3.1.1 Pasteurization (thermal) and Ultraviolet radiation (non-thermal) treatments

The dairy cream with the selected extracts was produced with the ingredients as described in section 2.1.2. To extend its shelf life, it was pasteurized as mentioned in section 2.2.1, in which all the ingredients that make up the dairy cream were subjected to heat treatment and subsequently stored under refrigerated conditions of 4 ± 1 ºC, for 21 days.

Taking into account the microbiological stability of the whey cheese (perishable food), the Greek type yoghurt (lactic acid bacteria) and the extracts used, a thermal processing was required yet the process to be selected should not impart detrimental changes in texture and colour. A thermal processing in a water bath at 90 ºC for 10 min was therefore selected. The processing of experiments 1, 2 and 4 (Table 2.1) were those that appeared to be more effective to ensure microbiological quality in the dairy cream. However, throughout storage between 0 and 21 days, different levels of microbiological contamination were obtained depending on the seaweed or mushroom extract added (Table 3.1). In the dairy creams resulting from experiments 1, 2 and 4 but containing *O. pinnatifida* extract, no viable cells above limit of detection (LOD = 2.7 log CFU/g of $_{\text{spreadable dairy cream}}$) were detected over the 21 days of storage. For those dairy creams containing *Ph. nameko* extract, after processing the number of viable cells ranged between 3 and 8 log CFU/g $_{\text{of spreadable dairy cream}}$ reaching high levels after 7 days in some dairy creams (Table 3.1).

Table 3.1 - Mean and standard deviation of the viable cell numbers [log (CFU/ g $_{\text{of spreadable dairy cream}}$)] grown on PCA and MRS culture media found in the dairy cream with extract of *O. pinnatifida* or with *Ph. nameko* up to 21 days of storage.

Pasteurization	Days	log (CFU/g $_{\text{of spreadable dairy cream}}$) *O. pinnatifida* extract		log (CFU/g $_{\text{of spreadable dairy cream}}$) *Ph. nameko* extract	
		PCA	MRS	PCA	MRS
Experiment 1	0	< 2.7	< 2.7	3.05±0.12	5.59±2.04
	7	< 2.7	< 2.7	5.38±2.95	5.58±4.08
	14	< 2.7	< 2.7	8.48±0.00	8.48±0.00
	21	< 2.7	< 2.7	8.80±0.70	5.58±2.04
Experiment 2	0	< 2.7	< 2.7	8.48±0.00	8.48±0.00
	7	< 2.7	< 2.7	7.97±0.50	8.48±0.00
	14	< 2.7	< 2.7	____*	____*
	21	< 2.7	< 2.7	____*	____*
Experiment 3	0	8.46±0.03	8.50±0.17	8.48±0.09	8.78±0.43
	7	7.41±0.03	7.44±0.15	7.27±1.00	6.89±0.53
	14	8.44±0.12	8.01±0.15	____*	____*
	21	8.54±0.13	8.13±0.32	____*	____*
Experiment 4	0	< 2.7	< 2.7	3.63±0.02	8.48±0.00
	7	< 2.7	< 2.7	3.42±0.06	3.47±0.00
	14	< 2.7	< 2.7	____*	____*
	21	< 2.7	< 2.7	____*	____*

___* due to the existing contaminations, the experiment was not carried out at 14 and 21 days

Based on the results obtained, the application of the thermal treatment (90 ºC for 10 min) to the dairy cream including all the ingredients: whey cheese, yoghurt and extract, in particular for those incorporating the seaweed extract, was the option selected (experiment 2) for further studies. However, in terms of the mushroom extract, additional approaches had to be explored because evidences of bacteria resistant to 90 ºC for 10 min over storage were observed, hence microbiological quality and stability over storage had to be improved.

3.1.2 UV treatment on mushroom extracts

Non-thermal processing based on UV radiation was tested through direct exposure of extract powder or when incorporated in different matrices (yoghurt or water) for 10 min and 20 min at 13,44 watt/m^2.This method has been used to sterilize packet juices as demonstrated by the study of Guerrero-Beltran & Barbosa-Canovas, (2004). UV-C irradiation is commonly used in surface sterilization of fruits and vegetables as well as the treatment of drinking water. Ultraviolet (UV) irradiation is classified as a non-thermal disinfection method; UV is electromagnetic radiation and subdivided by wavelength into UV-A (320–400 nm), UV-B

(280–320 nm), UV-C (200–280 nm) and Vacuum-UV (100–200 nm). UV-C has the highest germicidal effect, specifically between 250 and 270 nm, and is capable of destroying bacteria, viruses, protozoa, yeasts, moulds and algae (Christen, Lai, Hartmann, Hartmann, & Geddes, 2013). According to Christen, Lai, Hartmann, Hartmann, & Geddes, (2013) UV-C irradiation not only facilitates the required reduction of vegetative bacteria contamination to a safe level but also preserves the activity of heat sensitive enzymes.

As shown in Table 3.2 it is possible to observe that this method was not effective in eliminating the contamination present in the mushroom extract. Viable cell numbers above 8 log CFU/g were observed in all three tested combinations. Gram staining performed to colonies grown on PCA medium indicated the possible presence of Gram-positive bacilli. Therefore, the non-thermal method using UV radiation at 13,44 watt/m^2 was not sufficiently effective to ensure safe extracts with adequate microbiological quality.

Table 3.2 - Mean and standard deviation of the number of viable cells [log (CFU/g)] grown on PCA and MRS media from *Ph. nameko* extract powder or incorporated in water of yoghurt after 10 min and 20 min of UV radiation.

UV radiation	Time (min)	log (CFU/g)	
		MRS	PCA
Ph. nameko extract powder	10	< 2.7	> 8.5
	20	< 2.7	> 8.5
Ph. nameko extract with water	10	< 2.7	> 8.5
	20	< 2.7	> 8.5
Ph. nameko extract with yoghurt	10	8.46±0.03	8.50±0.17
	20	7.41±0.03	7.44±0.15

In Figure 3.1 it is possible to observe visual characteristics of the products after UV radiation processing. It can be observed that after 20 min (maximum time that the food can be in contact with UV radiation) the extracts in water or in yoghurt had a darker colour in comparison to 10 min of processing.

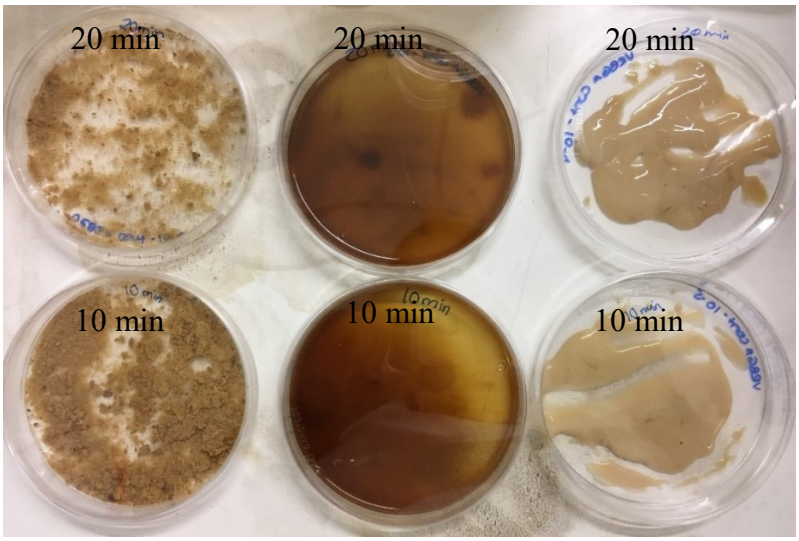


Figure 3.1 – Visual characteristics of *Ph. nameko* extract powder (left) or when incorporated in water (middle) or yoghurt (right), after 10 min (bottom) and 20 min (top) of UV radiation.

In a study of Christen, Lai, Hartmann, Hartmann, & Geddes (2013), exponential reductions of *Escherichia coli*, *Staphylococcus epidermidis*, *Enterobacter cloacae* and *Bacillus cereus* in human milk were observed after irradiation with UV-C light. But in this study, it was observed that UV-C irradiation was not able to eliminate the viable microbiological load in the mushroom extract, being able to only reduce slightly the viable cell numbers in the extract when incorporated in yoghurt after 20 minutes of processing.

Taking into account that the UV treatment applied to the mushroom extract both in powder form or when incorporated in yoghurt or water revealed to be inefficient to eliminate its microbiological load further attempts resorting to a more aggressive thermal treatment (higher temperatures through longer periods of time) were implemented.

3.1.3 Pasteurization versus sterilization of extracts

Due to the need to obtain a larger quantity of extracts from both seaweed and mushroom origin and due to some evidences of higher microbiological load inclusive in *O. pinnatifda* extract, it was decided to test both extracts with the new thermal processing.

Attempting to pasteurize or sterilize the extracts and taking into account pros and cons of each process in food (Prevention 2017; Sthelik, 2008) it was decided upon to submit mushroom or seaweed extracts in water to 90 ºC for 30 min in a water bath or to 100-120 ºC in a pressure cooker for 10 min.

From data displayed in Table 3.4, it is possible to observe that both thermal processing treatments were able to reduce the number of viable cells in *O. pinnatifida* extract to numbers below the limit of detection over 7 days of storage but not for the *Ph. nameko* extract.

Table 3.3– Mean and standard deviation of the viable cell numbers [log (CFU/g)] in PCA from the *O. pinnatifida* and *Ph. nameko* extracts over 7 days storage after thermal processing treatment.

Days	30 min at 90ºC		10 min at 100-120ºC	
	log (CFU/g) *O. pinnatifida* extract	log (CFU/g) *Ph. nameko* extract	log (CFU/g) *O. pinnatifida* extract	log (CFU/g) *Ph. nameko* extract
	PCA	PCA	PCA	PCA
0	< 2.7	5.61±0.02	< 2.7	< 2.7
7	< 2.7	> 8.5	< 2.7	2.7±0.00

In fact, the sterilization process was the more effective method for the elimination of microbiological contamination in the seaweed extract, as opposed to the *Ph. nameko* extract that presented traces of microbiological contamination at 7 days of storage. One of the drawbacks, in addition to those mentioned above, was that the sterilized extract had a grainy and pasty texture, which unabled its mixture with the other ingredients that make up the novel food.

Based on these observations and constraints, it was decided to use the pasteurized *O. pinnatifida* extract for further incorporation into the functional food to be developed. It is expected that with this type of thermal treatment, the sensory characteristics and nutritional value of the *O. pinnatifida* extract are minimally affected (Nunes, 2014) since the exposure time was also short. Furthermore, and according to Marques, et al., (2015), thermal processing at very high temperatures has been reported to significantly decrease the antioxidant activity of derived peptides and some researchers reported that heat processing cause irreversible modifications on the original structures of polysaccharides (Bonvehi & Coll, 1993; Femenia, Garcia-Pascual, Simal, & Rosselló, 2003). In addition, pasteurization is a method in which there is no total denaturation of proteins as opposed to sterilization. On the other hand, sterilization eliminates completely any contaminant but sacrifices proteins of interest with bioactive properties. According to Daliri, Oh, & Lee (2017) and Li & Shah (2016) food processing and thermal degradation methods can significantly affect the biological activity of bioactive peptides and properties of polysaccharides presents in the *Ph. nameko* extract being possible reasons for the significant decrease of antioxidant activities. Physical processing methods such as ultrasound, heat and irradiation may affect the protein structure and functions (Li & Shah 2016).

3.2 Spreadable dairy cream and microbiological evaluation throughout storage in a refrigerated environment

3.2.1 Microbiological analysis/shelf life in a refrigerated environment

Upon selection of the more favourable thermal process to ensure microbiological quality, the plain spreadable dairy creams (SP) or those incorporating the *O. pinnatifida* extract (SPExtract) or FOS (SPFos) were carefully prepared by mixing the whey cheese, the yoghurt and the extract together, and subsequently heat treating the dairy cream in an agitated water bath at 90ºC for 30 min. The spreadable dairy cream containing FOS instead of the extract was also prepared to be used as a positive control to test prebiotic activity.

Figure 3.2 – Samples of spreadable dairy cream incorporating enzymatic extract of *O. pinnatifida* obtained with Viscozyme (a) and plain spreadable dairy cream (b).

Microbiological control of plain spreadable dairy cream (without addition of carbon source, SP) or with incorporated seaweed extract or FOS (SPExtract or SPFos) was made weekly up to 21 days of storage (Table 3.5). The results showed that after 21 days no microbiological contamination was able to be detected (log < 2.7, below limit of detection of the technique). The pH values increased slightly over time, but such variation was not of statistical significance (Table 3.5). These results are an indication that, currently, the newly developed spreadable dairy cream has a shelf-life of at least 21 days. It may be interesting to test if shelf-life may be extended in further studies which would increase the potential economic interest of the product if commercialized.

Table 3.4 – Evolution of the viable cell numbers [mean log (CFU/g $_{\text{of spreadable dairy cream}}$)] grown on PCA, VRBGA and MRS agar media in the spreadable dairy creams without addition of carbon source (SP) and with extract of *O. pinnatifida* obtained with Viscozyme (SPExtract) and FOS (SPFos) over 21 days of storage under refrigerated conditions

Spreadable cream	Time (Days)	log (CFU/g $_{\text{of spreadable dairy cream}}$)			pH values
		PCA	VRBGA	MRS	
Without carbon source (SP)	0	< 2.7	< 2.7	< 2.7	5.95±0.00
	7	< 2.7	< 2.7	< 2.7	5.96±0.06
	14	< 2.7	< 2.7	< 2.7	6.09±0.07
	21	< 2.7	< 2.7	< 2.7	6.05±0.04
With FOS (SPFos)	0	< 2.7	< 2.7	< 2.7	5.46±0.00
	7	< 2.7	< 2.7	< 2.7	5.98±0.09
	14	< 2.7	< 2.7	< 2.7	6.00±0.07
	21	< 2.7	< 2.7	< 2.7	6.04±0.07
O. pinnatifida obtained Viscozyme (SPExtract)	0	< 2.7	< 2.7	< 2.7	5.61±0.00
	7	< 2.7	< 2.7	< 2.7	5.68±0.12
	14	< 2.7	< 2.7	< 2.7	5.70±0.26
	21	< 2.7	< 2.7	< 2.7	5.77±0.24

Upon microbiological control validation, all three spreadable dairy cream types (SP, SPFos, SPExtract) were evaluated for prebiotic activity immediately upon their production, whereas spreadable creams SP and SPExtract were evaluated in terms of sensorial attributes upon two and nine days of storage, as schematized in Figure 2.1.

3.3 Sensory Analysis

As previously mentioned, the spreadable dairy creams SP and SPExt were submitted to sensorial evaluation in terms of overall acceptance, consumption intention and sensorial attributes intensity. The results obtained are displayed in Table 3.6. Product assessment to identify specific sensory attributes that drive product acceptance is vital to the introduction of new functional dairy products in the market. Application of an adequate sensory methodology enables one to obtain important results on the formulated dairy foods, providing prior knowledge with respect to its acceptance and/or specific characteristics or a descriptive sensory profile, serving as the foundation for making an alteration, when required.

Table 3.5 - Sensorial evaluation of spreadable dairy cream without any carbon source (SP) and with *O. pinnatifida* extract (SPExtract) upon 2 and 9 days of manufacture and storage under refrigerated conditions.

Sensorial Test	Spreadable cream with *O. pinnatifida* extract		Spreadable cream without any carbon source	
	2 d	**9 d**	**2 d**	**9 d**
Overall acceptance	6.1 ± 1.7	5.6 ± 1.7	7.5 ± 1.0	6.9 ± 1.2
Positive evaluation frequency	63%	56%	100%	88%
Negative evaluation frequency	25%	19%	0%	13%
Indifferent evaluation frequency	13%	25%	0%	0%
Consumption intention	6.3 ± 3.6	5.3 ± 3.1	9.4 ± 1.9	7.3 ±3.0
Attributes intensity				
Aspect	0.6	0.4	1	1
Odour	0.8	0.7	0.9	0.9
Flavour	0.6	0.5	0.9	0.8
Creaminess	0.8	0.8	0.9	0.8
Consistency	0.7	0.8	0.9	0.8

The spreadable dairy cream containing the seaweed extract was less favoured both in terms of overall consumer acceptance (Positive evaluation) and consumption intention, despite the wider variability in response. The panel classified the spreadable cream as a “like it lightly” matrix with an average chance to be consumed. The flavour and general aspect were the sensorial attributes that least pleased the panel. The spreadable dairy cream without addition of carbon source, SP, reported a much more stable response among panel members and all sensorial scores were comparably higher than those reported for the spreadable dairy cream with seaweed extract. It is important to note that this dairy food in itself is a new product, thus the “like extremely” and “like moderately” classifications for consumption intention and overall acceptance, respectively, are most welcomed. Unfortunately, the seaweed extract lowers sensory attributes scores slightly; nevertheless, such impact may be minimised by addition of flavour masking agents.

As time evolved the sensory attributes of the spreadable dairy creams declined and at 9 days of storage sensory scores were significantly lower overall. The order of magnitude in consumer acceptance declined equally (ca. 8%) for both spreadable dairy creams (SP and SPExtract), whereas decrease in consumption intention was slightly higher for the SP (ca. 16%) than for SPExtract (ca. 22%).

In terms of scores, the spreadable dairy cream SPExtract remained the less appreciated registering a lower frequency of positive evaluation by panel members. According to the 1 to 9 hedonic scale SPExtract was negatively classified as “not like it” and “do not like it” for

consumption intention and consumer acceptance, respectively. Once again, the aspect and flavour were the sensorial attributes that least pleased the panel. Unlike to scores reported at 2 days storage, the consistency increased one-point, but the odour decreased accordingly. Similarly, to trends observed by 2 days of storage, the spreadable dairy cream without the addition of carbon source (SP) had higher sensorial scores. Even though consumption intention and overall acceptance decreased over time, SP was still classified positively as a "like it lightly" and "like moderately", respectively.

Regardless of the fact that the control spreadable dairy cream SP was, in general, better classified in terms of sensorial evaluation the scores obtained for spreadable dairy cream with *O. pinnatifida* extract are still promising up to 9 days of storage. Moreover, if spreadable dairy cream with *O. pinnatifida* extract was associated to functional properties that could meanwhile be highlighted, consumers may expectedly increase their degree of acceptance ad intention to purchase.

3.4 Biological Properties

3.4.1 Prebiotic potential of non-digested spreadable dairy creams

According to Zaporozhets et al. (2014), some research has been done in identifying potential prebiotic compounds in marine resources, such as seaweed. Seaweeds have a rich profile in polysaccharides present in the cells walls that may be potential available sources of prebiotic polysaccharides. Kim, Lee & Lee (2012) demonstrated that, in general, fermentation of seaweed polysaccharides led to the stimulation of the growth of healthful intestinal microbiota; furthermore, prebiotic properties of polysaccharides from marine seaweed were combined with a hypolipidemic effect.

The evaluation of potential prebiotic properties of the three spreadable dairy creams (SPExtract, SPFos and SP), upon 0 and 21 days of storage, was tested with pure cultures of probiotic bacteria including *L. acidophilus* La-5 and *B. animalis* subsp *lactis* BB12 over 24 h of incubation at 37 ºC (Figures 3.3 and 3.4 a, b). The variations in pH in the three spreadable matrices were also recorded over the 24 h incubation and are displayed in Figures 3.3 and 3.4 (c, d) the three spreadable matrices after 0 and 21 days storage, respectively.

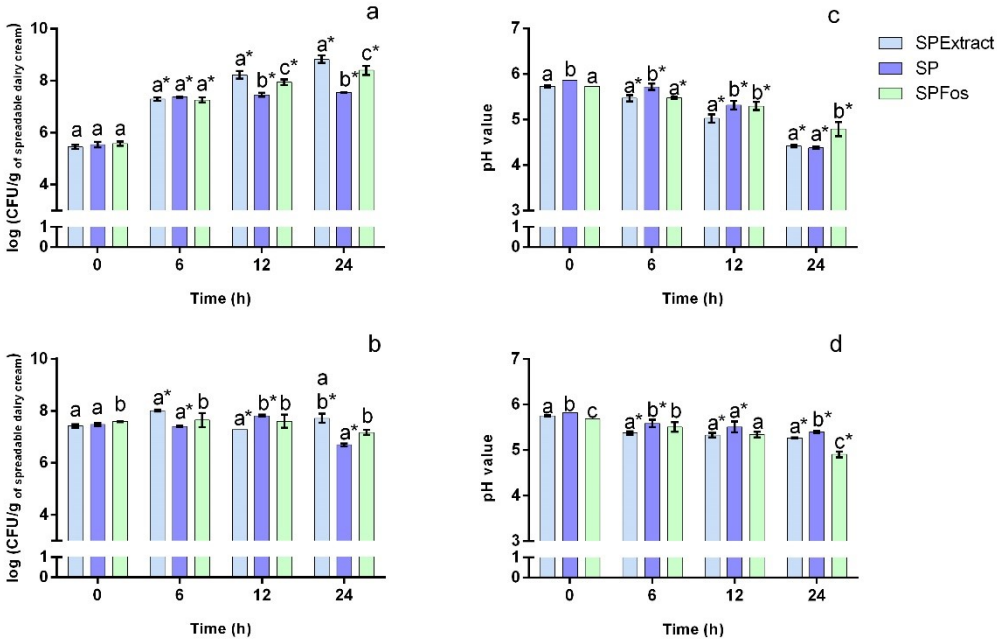


Figure 3.3- Evolution of viable cell numbers of *Lactobacillus acidophilus* La-5 (a) and of *Bifidobacterium animalis* subsp *lactis* BB12 (b) and associated pH values (c and d, respectively) in spreadable dairy cream incorporated with *O. pinnatifida* obtained with Viscozyme (■), spreadable cream without any source of carbon (■) and spreadable dairy cream with FOS (■) upon manufacture (0 days), incubated over 24 h at 37ºC. Different letters indicate statistically significant differences ($p < 0.05$) between the three dairy spreadable creams at each sampling time while bars marked with * means statistically significant differences ($p < 0.05$) in comparison to data obtained at 0h.

The results obtained were able to confirm that the spreadable dairy cream SPExtract, upon manufacture, has a prebiotic potential as previously identified by Rodrigues (2016). Viable cell numbers, in particular those of *L. acidophilus* La-5, in SPExtract increased steadily by 6 h (Figure 3.3a). On 12 h the SPExtract reveal a 7.29 log (CFU/g $_{\text{of spreadable dairy cream}}$) of viable cells number, in this same sampling point statistically significant differences ($p < 0.05$) were demonstrated between SPFos (positive control) or in SP (negative control - without any additional carbon source).

Indeed, interesting viable cell numbers were observed for *L. acidophilus* La-5 in SPExtract, which increased around 2 log cycles between 0 and 12 h of incubation reaching values of 8.22 log (CFU/g $_{\text{of spreadable dairy cream}}$) and increasing to 8.82 log (CFU/g $_{\text{of spreadable dairy cream}}$) by 24 h incubation. The SPFOs did not reach such high numbers reporting viable cell numbers 0.29 and 0.43 log cycles lower at both 10 and 24 h, respectively.

In what concerns *B. animalis* subsp. *lactis* BB12, growth promotion by SPExtract was not as effective as for *L. acidophilus* La-5 (Figure 3.3b). A very modest non-significant ($p > 0.05$) increase in viable cell numbers was registered up to 6 h incubation, and then numbers fell

slightly by 12 h to recover original values by 24 h of incubation. In fact, no significant increase in viable cell numbers was observed over the full 24 h time framework, denoting a lower prebiotic potential of SPExtract. Despite, the absence of a significant prebiotic effect by SPExtract, it must be highlighted that its protective capacity as a simple probiotic carrier was better than that achieved by SPFos where viable cell numbers of *B. animalis* subsp *lactis* BB12 decreased at 6 and 24 h. In SP, no significant growth was reported and a decrease in original viable cell numbers was observed at 24 h.

Our results are in agreement with those reported by Rodrigues et al. (2015a) in what concerns the seaweed extracts (*O. pinnatifida*, *S. muticum* and *C. tomentosum*) impact on *B. animalis* subsp *lactis* BB12 growth; it was found that, in general, such extracts have a lower prebiotic potential. Nonetheless viable cell numbers of *B. animalis* subsp *lactis* BB12 in all *S. muticum* and *C. tomentosum* extracts after 48 h of incubation were significantly higher than those obtained with prebiotic FOS and glucose.

According to Ramnani, et al., (2012), the lower prebiotic potential observed for *O. pinnatifida* extracts, may be related with the large amount of sulphated sugars observed in these extracts. Red seaweed *O. pinnatifida* has been mostly characterized as an agar producer and it is known that microorganisms are not able to hydrolyse and metabolize this polysaccharide which is used as solidifying agent of culture media. On the other hand, it should not be totally overlooked since it was shown recently that some low molecular weight polysaccharide fractions derived from seaweed agar and alginate by acid hydrolysis or degradation with hydrogen peroxide, were fermented by gut microbiota, namely bifidobacterial populations, exhibiting potential to be used as a novel source of prebiotics (Rodrigues, et al., 2015a).

In relation to the pH values observed in the spreadable dairy creams inoculated with *L. acidophilus* La-5 (Figure 3.3 c) or with *B. animalis* subsp. *lactis* BB12 (Figure 3.3 d), these were inversely correlated with growth capacity; a higher increase in viable cell numbers (*L. acidophilus* La-5) led to a higher decrease in pH values (figure 3.3 c). These pH values are certainly related with the higher content of lactic acid in the spreadable dairy creams at the end of the 24 h (Table 3.6).

Table 3.6 – Mean and standard deviation of glucose, lactic acid and acetic acid contents (mg/$g_{spreadable\ dairy\ cream}$) in spreadable dairy creams, without addition of carbon source, with *O. pinnatifida* extract and with FOS, inoculated with *Bifidobacterium animalis* subsp *lactis* BB12 and *Lactobacillus acidophilus* La-5 and incubated for 24 h at 37ºC for evaluation of prebiotic activity at 0 days.

	Time (h)	Spreadable dairy cream with *O. pinnatifida* extract (SPExtract)			Spreadable dairy cream with FOS (SPFos)			Spreadable dairy cream without any carbon source (SP)		
		Glucose	Lactic Acid	Acetic Acid	Glucose	Lactic Acid	Acetic Acid	Glucose	Lactic Acid	Acetic Acid
Lactobacillus acidophilus La-5	0	0.718±0.844	0.020±0.003	< 0.0001	0.217±0.088	0.028±0.014	< 0.0001	0.236±0.048	0.018±0.0097	< 0.0001
	6	1.036±0.598	0.039±0.017	< 0.0001	1.065±1.012	0.058±0.003	< 0.0001	1.489±0.975	0.052±0.022	< 0.0001
	12	0.059±0.044	0.076±0.044	< 0.0001	0.275±0.116	0.091±0.037	< 0.0001	0.291±0.212	0.094±0.0565	< 0.0001
	24	0.484±0.051	1.066±0.100	< 0.0001	0.355±0.026	0.407±0.014	< 0.0001	0.487±0.057	0.852±0.1772	< 0.0001
Bifidobacterium animalis subsp *lactis* BB12	0	0.117±0.071	0.013±0.013	< 0.0001	0.175±0.062	0.016±0.013	< 0.0001	0.239±0.044	0.021±0.0070	< 0.0001
	6	1.785±1.391	0.046±0.016	< 0.0001	2.457±1.286	0.049±0.018	< 0.0001	0.631±0.550	0.049±0.0132	< 0.0001
	12	0.026±0.019	0.030±0.018	< 0.0001	0.300±0.331	0.033±0.030	< 0.0001	0.226±0.088	0.040±0.0217	< 0.0001
	24	0.705±0.520	0.045±0.011	0.117±0.022	0.275±0.042	0.063±0.008	0.158±0.064	0.924±0.876	0.060±0.0080	0.137±0.013

It is possible to verify from Table 3.6 that there is an increase in the lactic acid concentrations over time in both samples and for both bacteria tested. Acetic acid was only detected after 24 h in *B. animalis* subsp *lactis* BB12 up holding the lower growth and metabolic activities previously observed (Figure 3.3 b, d). Given the fact that *B. animalis* subsp *lactis* BB12 is heterofermentative, the earlier production of more acetic acid would be expected, independently of the growth condition (Table 3.6) and not only at 24 h (Pokusaeva, Fitzgerald, & Sinderen, 2011). Nevertheless, this phenomenon also occurred for Rodrigues, (2016) where acetic acid was only detected at 24 h in spreadable dairy creams incorporated with *B. animalis* subsp *lactis* BB12. The absence of appreciable production of acetic acid by *L. acidophilus* La-5 (Table 3.6) over 24 hours was expected given its homofermentative nature (Ibrahim, 2016).

In case of glucose, contents varied erratically over the 24 h incubation period; between 0 and 6 h glucose concentration increased, between 6 and 12 h glucose concentration decreased and between 12 and 24 h an increase in concentration was once again observed. This fact may be due to the lactase enzyme present in both strains *L. acidophilus* La-5 and *B. animalis* subsp *lactis* BB12. The enzyme lactase or β – galactosidase hydrolyses lactose into glucose and galactose (Mahalakshmi, Kumar, Hameeda, & Reddy, 2013).

Due to this phenomenon it can be seen that there is a consumption of glucose over time by these microorganisms, but at the same time there is production of enzymes such as lactase which breaks the lactose into glucose thereby increasing glucose over time.

At 21 days upon storage, prebiotic activity was once again tested in order to assess whether bioactivity was stable and maintained throughout storage. The spreadable dairy cream with *O. pinnatifida* extract, SPExtract, confirmed its prebiotic activity for both *L. acidophilus* La-5 (Figure 3.4a) and *B. animalis* subsp *lactis* BB12 (Figure 3.4b) promoting statistically significant increments ($p < 0.05$) in viable cell numbers by 24 hours, unlike the other spreadable dairy creams with FOS, SPFos, or without any carbon source, SP, where viable cell numbers were not statistically different (in case of *B. animalis* subsp *lactis* BB12). Moreover, it is important to note that the initial viable counts of *B. animalis* subsp *lactis* BB12 were significantly higher than those observed in the other assays carried out throughout this work and, therefore the results ascertained for this variable do not allow for an inference on whether the bacteria still have the same growth behaviour as that of day 0, matrices upon manufacture.

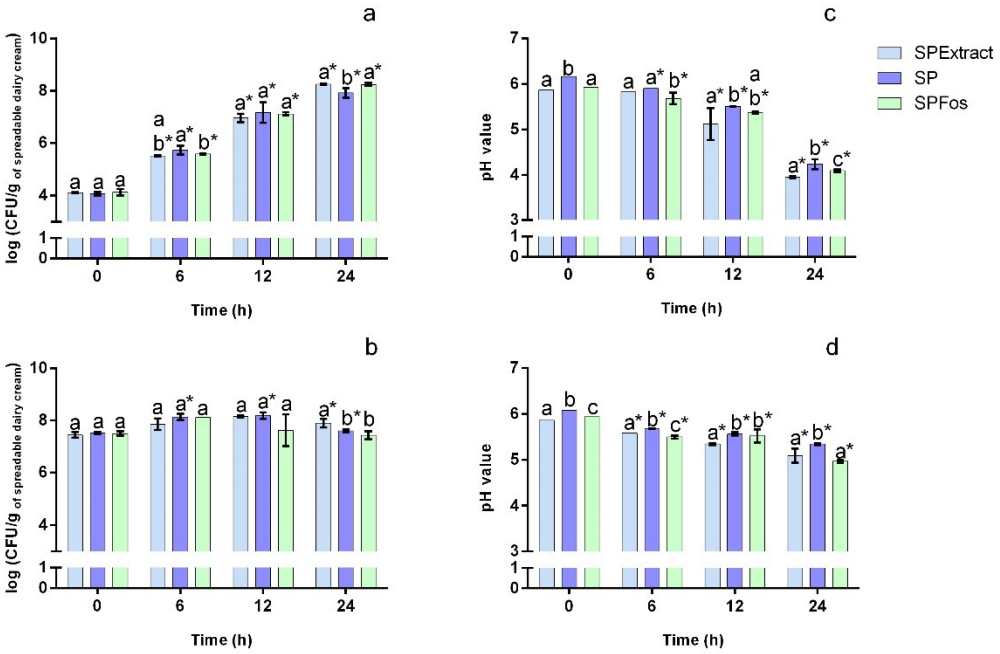


Figure 3.4 - Evolution of viable cell numbers of *Lactobacillus acidophilus* La-5 (a) and of *Bifidobacterium animalis* subsp *lactis* BB12 (b) and associated pH values (c and d, respectively) in spreadable dairy cream incorporated with *O. pinnatifida* obtained with Viscozyme (■), spreadable cream without any source of carbon (■) and spreadable dairy cream with FOS (■) upon manufacture (21 days), incubated over 24 h at 37ºC. . Different letters indicate statistically significant differences ($p < 0.05$) between the three dairy spreadable creams at each sampling time while bars marked with * means statistically significant differences ($p < 0.05$) in comparison to data obtained at 0h.

Once again, a more significant activity was reported for *L. acidophilus* La-5 in comparison to *B. animalis* BB12. Interesting values of viable cell numbers were observed for *L. acidophilus* La-5, which registered increases around 3 log cycles between 0 and 24 hours of incubation from values of 4.11 log (CFU/g $_{\text{of spreadable dairy cream}}$) to 8.25 log (CFU/g $_{\text{of spreadable dairy cream}}$) in SPFExtract (Figure 3.4a).

Over time within SPExtract exist differences statistically significantly ($p < 0.05$) prosing the growth of viable cell numbers of both probiotic bacteria, in particular for *L. acidophilus* La-5 where a significant continuous increase of viable cells until 24h is observed (Figure 3.4 a). For *B. animalis* Bb12 (Figure 3.4 b), slightly lower values are observed after 24h of incubation (7.89 log CFU/g $_{\text{of spreadable dairy cream}}$) in comparison to 12h (8.15 log CFU/g $_{\text{of spreadable dairy cream}}$), but statistically higher in SPExtract than in SPFos and SP ($p < 0.05$) and in comparison, to 0h ($p < 0.05$).

Taking into consideration the differences observed at 24h incubation for both probiotic bacteria in SPExtract with 0 and with 21 d of storage, in comparison to FOS, a confirmed prebiotic

compound (Gibson, et al., 2017), evidence of some prebiotic activity could be impart to the composition of *O. pinnatifida* extract obtained with Viscozyme.

In relation to the pH values observed in the spreadable dairy creams with 21 d of storage and inoculated with *L. acidophilus* La-5 (Figure 3.4 c) or with *B. animalis* subsp. *lactis* BB12 (Figure 3.4 d), it was observed for all a decrease of the values of pH, in particular for those inoculated with *L. acidophilus* La-5 and after 24h of incubation. This trend is certainly related with the higher content of lactic acid in produced by *L. acidophilus* La-5 in the spreadable dairy creams (Table 3.7).

Table 3.7 – Mean and standard deviation of glucose, lactic acid and acetic acid contents (mg/$g_{spreadable\ dairy\ cream}$) in spreadable dairy creams, without addition of carbon source, with *O. pinnatifida* extract and with FOS, inoculated with *Bifidobacterium animalis* subsp *lactis* BB12 and *Lactobacillus acidophilus* La-5 and incubated for 24 h at 37ºC for evaluation of prebiotic activity at 21 days.

	Time (h)	Spreadable dairy cream with *O. pinnatifida* extract (SPExtract)			Spreadable dairy cream with FOS (SPFos)			Spreadable dairy cream without any source of carbon (SP)		
		Glucose	Lactic Acid	Acetic Acid	Glucose	Lactic Acid	Acetic Acid	Glucose	Lactic Acid	Acetic Acid
Lactobacillus acidophilus La-5	0	2.338±0.051	0.134±0.175	< 0.0001	2.441±1.450	0.037±0.006	< 0.0001	3.373±0.183	0.040±0.008	< 0.0001
	6	2.534±0.012	0.057±0.021	< 0.0001	2.518±1.572	0.041±0.015	< 0.0001	3.087±1.018	0.058±0.008	< 0.0001
	12	0.263±0.183	0.095±0.020	< 0.0001	0.442±0.171	0.090±0.040	< 0.0001	0.699±0.622	0.070±0.027	< 0.0001
	24	0.670±0.371	1.135±0.210	< 0.0001	1.223±0.478	0.909±0.147	< 0.0001	3.263±1.245	0.962±0.088	< 0.0001
Bifidobacterium animalis subsp *lactis* BB12	0	2.126±0.644	0.042±0.005	< 0.0001	0.609±0.079	0.340±0.199	< 0.0001	2.881±0.325	0.140±0.205	< 0.0001
	6	2.830±0.265	0.075±0.013	< 0.0001	3.334±0.742	0.046±0.023	< 0.0001	3.145±0.110	0.059±0.013	< 0.0001
	12	0.427±0.339	0.063±0.040	< 0.0001	3.225±0.288	0.080±0.004	< 0.0001	2.431±0.667	0.070±0.011	< 0.0001
	24	3.284±0.319	0.062±0.018	0.171±0.049	3.427±0.623	0.075±0.029	0.198±0.066	2.299±0.089	0.060±0.029	0.096±0.056

Similar profile and trends were observed in general in terms of lactic acid and acetic acid production over 24 h incubation in the spreadable creams with 21 d of storage in comparison to those upon manufacture (0d) with some exceptions.

Contrary to what occurred in spreadable creams with 0 d, SPExtract inoculated with *L. acidophilus* La-5 as well as SPFos and SP inoculated with *B. animalis* subsp *lactis* BB12 present higher content of lactic acid at 0h incubation, decreasing until 6h incubation and then increasing until 24 h (Table 3.7). Since no contamination was observed in spreadable creams with 21 days of storage under refrigeration, (Table 3.4), this content of lactic acid could be provided from the inoculum itself. However, this phenomenon was not observed in the other spreadable creams at 0h.

3.4.2 Screening of prebiotic potential of digested spreadable dairy creams

Today, the prebiotic concept has expanded, in part, because of advances in tools for microbiome research, which have improved our knowledge of the composition of the microbiota and enabled identification of additional substances influencing colonization (Gibson, et al., 2017).

In order to evaluate the prebiotic potential of digested samples, growth curves, obtained by optical density (OD), of the two probiotic strains *L. acidophilus* La-5 and *B. animalis* subsp *lactis* BB12 were performed in microplates with media supplemented with digested samples of SPExtract, SP and SPFos with 0 and 21 days of storage, respectively (Figures 3.5-3.6).

Analysis of the growth curves controls for *L. acidophilus* La-5 controls (Figure 3.5 a) revealed that the bacteria controls showed, as expected a very good growth when basal medium (MRS without sugar registering a maximum value of only 3.36 log N/Ni) was supplemented with glucose (6.44 log N/Ni), and boosted growth when MRS medium was supplemented with FOS (12.49 log N/Ni).

When the prebiotic potential of the spreadable dairy cream for *L. acidophilus* La-5 was assessed (Figure 3.5b), it was observed that the spreadable dairy cream with FOS, SPFos, enabled the highest increase of log N/Ni absorbance over time reaching maximum values of 4.84-4.95 log N/Ni at 24 h incubation with SPFos with 0 and 21 days of storage, respectively (Figure 3.5 b). The spreadable creams containing *O. pinnatifida* extract, SPExtract, were not able to reach as high numbers having achieved a two-fold lower maximum value of 2.25 log N/Ni at 24 h at 0 d manufacture. Noticeably, the prebiotic potential of the SPExtract was not comparable to that of SPFos, and upon 21 days of refrigeration, the prebiotic potential of the extract was almost entirely lost (0.40 log N/Ni) (Figure 3.5b)

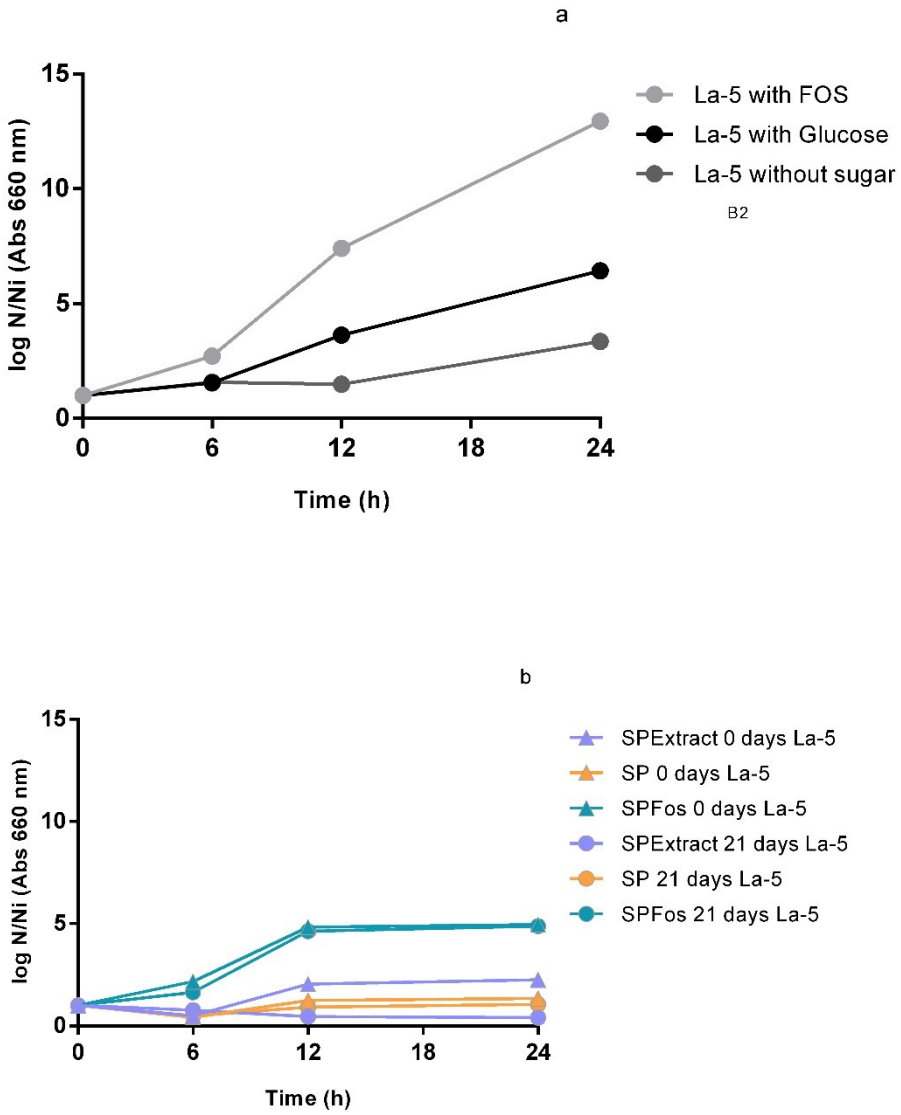


Figure 3.5 – Growth curves of *Lactobacillus acidophilus* La-5 in MRS broth supplemented with FOS, glucose or without carbon source (a) or with digested spreadable creams (SPExtract, SPFos or SP) with 0 and 21 days of storage under refrigeration, over 24 h incubation at 37 ºC:

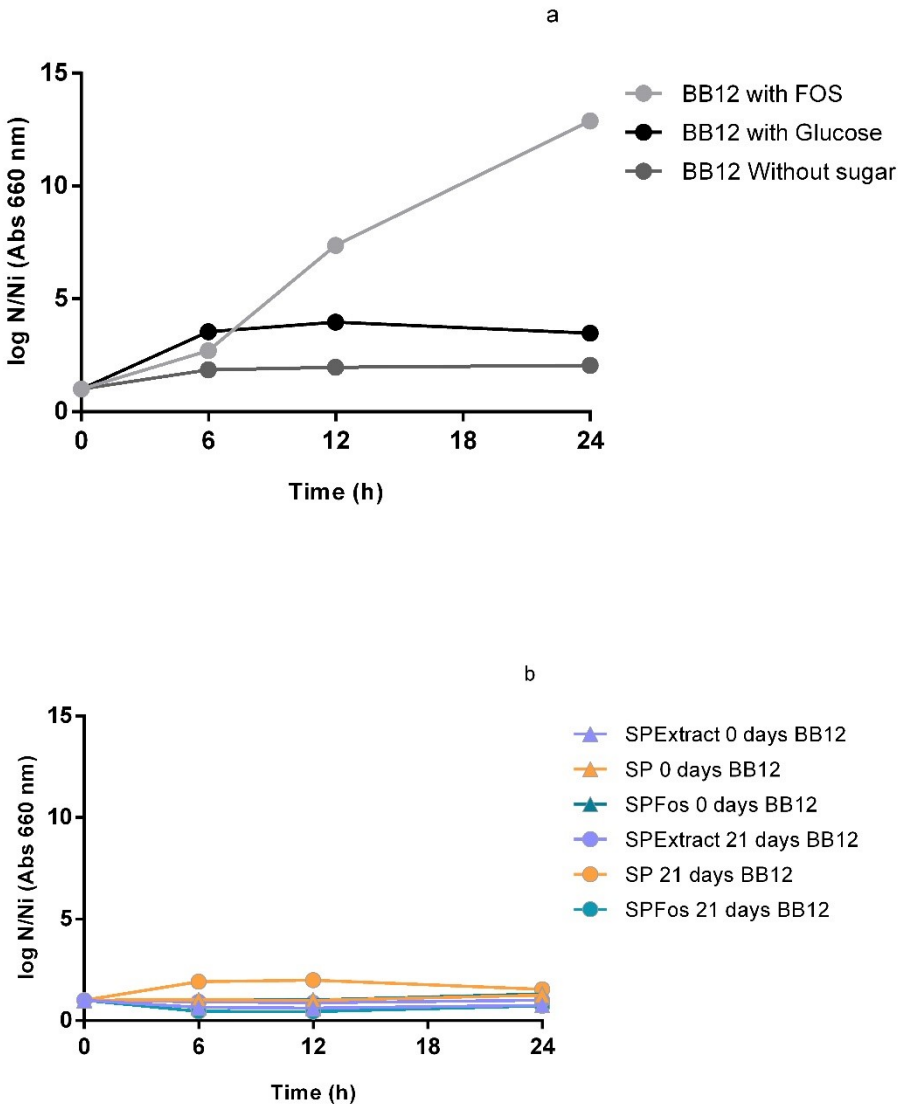


Figure 3.6 – Growth curves of *Bifidobacterium animalis* BB12in MRS broth supplemented with FOS, glucose or without carbon source (a) or with digested spreadable creams (SPExtract, SPFos or SP) with 0 and 21 days of storage under refrigeration, over 24 h incubation at 37 ºC:

A similar behaviour was reported for *B. animalis* BB12 control in basal MRS medium (Figure 3.6a). The basal MRS medium (without carbon source) supplemented with FOS revealed the highest growth (12.8 log N/Ni) in comparison to basal MRS medium supplemented with glucose (3.48 log N/Ni) and MRS medium without any source of sugar (2.06 log N/Ni). Surprisingly, growth was not significantly different between MRS-glucose and basal MRS medium – such observation may be accounted for by traces of oxygen that may have influenced viable cell numbers, bearing in mind that with glucose the bacteria would always grow more than without sugar.

The addition of spreadable dairy cream alone or supplemented with FOS or with *O. pinnatifida* extract did not influence growth of *B. animalis* subsp *lactis* BB12 significantly (Figure 3.6b), except for digested SP with 21 d of storage; where a significant increase by 6 hours was observed – nonetheless, OD values dropped to values achieved by the other matrices by 24 h of incubation.

This absence of growth may be due to the fact that this assay was performed on a microplate, which may have impaired the results of *B. animalis* subsp *lactis* BB12; due to its susceptibility to oxygen it while sterile paraffin was added to the top of each well, it might have not been enough to ensure anaerobic conditions which presents a limitation of the procedure. These smaller amounts could be a limitation of the procedure. However, is important to note that the OD decrease between 0 and 6 h incubation was more pronounced in media supplemented with digested SPExtract with 0 d and SPFos with 21 d and therefore no particular trend is perceived for the different digested spreadable creams.

According to Rodrigues (2016), the prebiotic potential of *O. pinnatifida* extract was validated, in a dairy matrix, for both *L. acidophilus* La-5 and *B. animalis* subsp *lactis* BB12, however after gastrointestinal simulation, the growth promotion of *B. animalis* subsp *lactis* BB12 was partially lost.

In conclusion, these results indicate a possible partial degradation of the bioactive compounds in the *O. pinnatifida* extract by the simulated digestion leading to a loss in prebiotic activity. To confirm these results, it would be necessary to perform the macro-scale prebiotic potential study in order to obtain more precise conclusions by monitoring actual growth of viable cell numbers.

3.5 Antidiabetic Activity: α-Glucosidase inhibitory activity

Diabetes has been associated with the development of micro and macro vascular diabetic complications (Brownlee & al, 1988). Inhibition of the enzyme activity involved in the hydrolytic metabolism of carbohydrates is one of the passible therapeutic approaches for reducing postprandial hyperglycemia (Ortiz & al, 2006). The key enzyme in carbohydrate digestion is a α-glucosidase, it catalyzes the hydrolysis of 1,4 α-glucosidic bonds within carbohydrates resulting in release of α- D-glucose and the subsequent increase of blood glucose levels after a meal (Murugesan, Bhuvaneswari, & Sivamurugan, 2016). With the objective to evaluate the inhibitory potential of the α-glucosidase enzyme of the different types of spreadable dairy creams (SPExtract, SPFos, SP) a positive control (acarbose, used as a drug to reduce glucose levels) was used for comparison purposes. In general, α-glucosidase inhibitory

activity was highest for SPExtract both at 0 and at 21 days (Figure 3.7) compared to SP and SPFos.

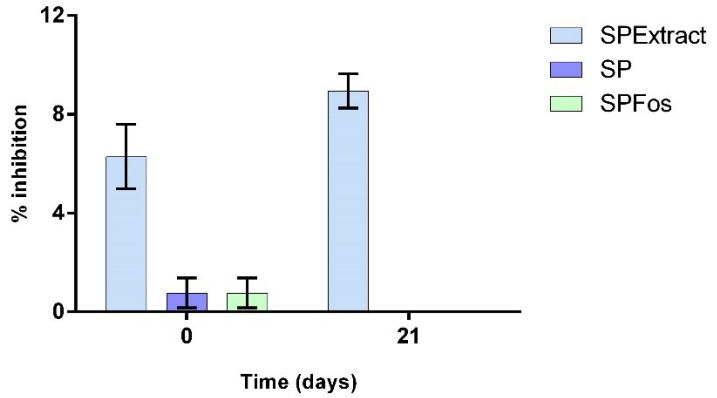


Figure 3.7 – α – Glucosidase inhibitory activity, expressed as inhibition percent, of spreadable dairy cream incorporated with *O. pinnatifida* extract obtained with Viscozyme ( ), spreadable cream without any carbon source ( ) and spreadable dairy cream with FOS ( ) at 0 and 21 days of storage

According to the results obtained, the antidiabetic activity was very variable between the spreadable dairy creams. The SPFos and SP dairy creams showed similar and ca. six-fold lower inhibition activity potential (0.77%) than the SPExtract. In opposite, for SPExtract, it was possible to observe a higher activity after 21 days (8.95%) than in time.

As the goal of this prospect was to develop a nutraceutical (*O. pinnatifida* extract) and/or functional food that may be used in the maintenance of healthy blood glucose levels, the use of this extract could prove interesting

According to Rodrigues, et al., (2015a) EA extracts from *O. pinnatifida* and *C. tomentosum* showed promising inhibitory potential (38-40% for *O. pinnatifida* and 45-49% for *C. tomentosum*) against α-glucosidase and, although further *in vivo* studies are required to test for effective hypoglycemic activity, these may prove to be an important strategy in the prevention (functional food) or management (oral therapeutic agent) of blood glucose level in type 2 diabetic and pre-diabetic patients.

However, in this study, the high antidiabetic activity potential previously reported Rodrigues, et al., (2015a) was not achieved, probably due to the high heat treatment *O. pinnatifida* extract underwent, which did not occur in the study of Rodrigues, et al., (2015a). It is well established that food processing methods may affect the nutritional and biological value of foods. For example, Daliri, Oh, & Lee, (2017) have discussed how food processing methods can significantly affect the biological activity of bioactive peptides. Methods such as ultrasound, heat and irradiation may affect the protein structure and functions. However,

processing methods that may reduce the activity of one peptide may enhance the activities of others. For example, Murugesan, Bhuvaneswari, & Sivamurugan (2016) reported the mechanism by which *Portieria hornemannii* and *Spyridia fusiformis* may exert their action pinpointing that it could be related with their action on carbohydrate binding regions of α-glucosidase enzyme, α- amylase, endoglucanases that catalyse hydrolysis of internal α-1,4 glucosidic linkages in starch and other related polysaccharides that have also been targeted for the suppression of postprandial hyperglycemia.. Moreover, the inhibitory effects of these extracts upon α- amylase and α- glucosidase activities, was also associated with the presence of phytochemicals such as flavonoids, tannins and saponins.

3.6 Antihypertensive Activity: Angiotensin-I converting enzyme (ACE) inhibitory activity

A few angiotensin-converting-enzyme (ACE) inhibitors have been systematically used in the treatment of essential hypertension and heart failure in humans. An example is captopril against which novel natural sources of antihypertensive agents are compared in terms of enzyme inhibition (Rodrigues, et al., 2016a). The commercial ACE inhibitory drugs have been known to cause side effects such as dermatological, pulmonary, metabolic, central nervous system and cardiovascular effects (Husserl & Messerli, 1981). Therefore, the search for ACE inhibitors from natural resources has become of great relevance (Rodrigues, et al., 2016a).

The ACE-inhibitory activity of foods has been studied, with works showing that some ACE-inhibitory peptides may be produced by enzymatic digestion of various food proteins including casein, zein, soybean protein, dried-salted fish, ovalbumin, fish sauce, and fish water-soluble protein (Suetsuna, Maekawa, & Chen, 2004).

The results obtained for the IC_{50} protein, in μg/mL amount of compound extract, which inhibits 50% of the angiotensin-converting-enzyme (ACE) activity, obtained for the different spreadable dairy creams, SP, SPFos and SPExtract (with *O. pinnatifida* extract), by 0, 7, 14 and 21 d of storage, in comparison with *O. pinnatifida* extract are described in Table 3.8.

Table 3.8 - IC_{50} values (µg/mL) determined for *O. pinnatifida* extract, Spreadable dairy cream with *O. pinnatifida* extract, Spreadable dairy cream with FOS and Spreadable dairy cream without any carbon source.

Samples	% Inhibition	IC_{50} (µg/mL)	Activity
O. pinnatifida extract obtained with Viscozyme	68.04	117.6	Good activity
Spreadable dairy cream with *O. pinnatifida* extract (0 d)	57.40	282.1	Intermediate activity
Spreadable dairy cream with *O. pinnatifida* extract (7 d)	49.51	250.9	Intermediate activity
Spreadable dairy cream with *O. pinnatifida* extract (14 d)	50.33	209.9	Intermediate activity
Spreadable dairy cream with *O. pinnatifida* extract (21 d)	50.50	232.6	Intermediate activity
Spreadable dairy cream with FOS (0 to 21 d)	-	> 1000	-
Spreadable dairy cream without any source of carbon (0 to 21 d)	-	> 1000	-

According to Antunes (2015), antihypertensive peptides identified or applied in foods with IC_{50} values below 50 are indicators of an excellent antihypertensive activity, values between 50 and 150 are considered good, values between 150 and 400 are indicators of an intermediate activity and above 400 of low activity.

As observed in Table 3.8, it is possible to conclude that the capacity to inhibit the angiotensin-converting enzyme (ACE) by the SPExtract in the spreadable dairy cream remained fairly constant over time - about 50% (49%-57%). In comparison with the *O. pinnatifida* extract alone, it can be concluded that the SPExtract has a promising inhibitory activity and based on the amount of spreadable dairy cream portion to be ingested (50 g) it will be able to promote its antihypertensive activity in the consumer.

According to Rodrigues, et al., (2016a), the *O. pinnatifida* extract obtained with Viscozyme exhibited the strongest ACE inhibitory activity (111.2 µg/mL). The results of this study are in line with those previously reported and evidence the fact that the *O. pinnatifida* extract may contain important amounts of compounds capable of inhibiting the ACE activity.

According to Yamori, Nara, Tsubouchi & Horie (1988), the alginate presents in seaweed had a hypotensive effect in spontaneously hypertensive rats. In fact, the extract of *O. pinnatifida* incorporated in the developed spreadable dairy cream has some peptides with potent ACE-inhibitory activity that may be effective for maintaining blood pressure at a healthy level.

3.7 Antioxidant Activities

3.7.1.1 ABTS radical scavenging activity

Antioxidant peptides derived from diverse sources can be used as ingredients either to control oxidative induced deterioration of foods or as ingredients for functional foods, nutraceuticals and potentially pharmaceutical or cosmetic products (Samarakoon & Jeon, 2012; Ngo, Wijesekara, Vo, Ta, & Kim, 2011).

In literature, polyphenols or phenolic compounds have been described as found, in high levels, in seaweeds and have been strongly associated with a high antioxidant capacity of seaweed extracts, for species such as *Porphyra* sp. (Rodophyta, red algae) being considered as good sources of polyphenolic compounds (Lordan, Ross, & Stanton, 2011; Rodrigues, 2016).

To assess the impact of the addition of the seaweed extract to the antioxidant capacity of the spreadable dairy creams, the quenching of the $ABTS^{+}$, DPPH and hydroxyl radical was tested for the different SP and the results can be seen in Figures 3.8, 3.9 and 3.10.

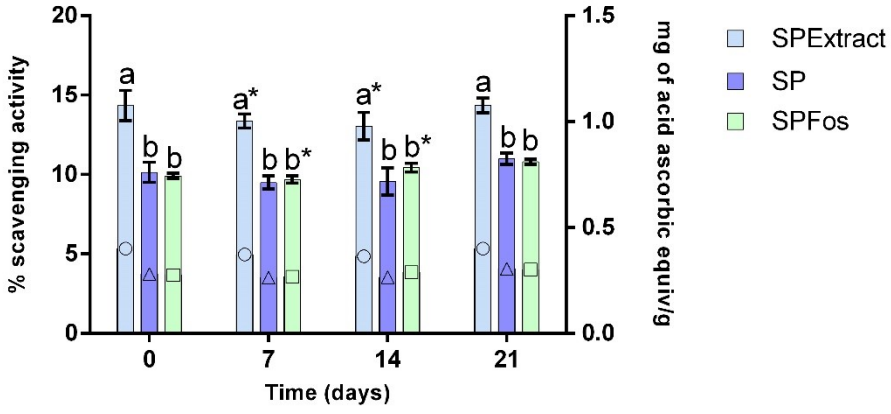


Figure 3.8 – ABTS radical Scavenging activity expressed as % scavenging activity (% scavenging activity) and total phenolic content (mg of phenolic compounds equivalent/ g of sample) (mg of phenolic compounds equivalent/g) in Spreadable dairy cream with *O. pinnatifida* (), Spreadable dairy cream without any source of carbon () and Spreadable dairy with FOS () during the storage time 0,7,14 and 21 d. Different letters indicate statistically significant differences ($p < 0.05$) between the three dairy spreadable creams at each storage time while bars marked with * means statistically significant differences ($p < 0.05$) in comparison to data obtained at 0 d.

Rodrigues et al. (2015a) reported that the *O. pinnatifida* extract had a scavenging activity of 15%. So, it's possible to conclude that the low antioxidant capacity observed is in line with the literature. Bearing in mind that SPExtract had a scavenging activity of 14.34% (0.401 mg $_{\text{of acid ascorbic equiv}}$/g) at 0 d and that, over time, no significant decrease was observed it can be conclude that while the product has a low antioxidant capacity, it is somewhat stable.
The analysis of the *O. pinnatifida* extract used in the spreadable dairy cream demonstrated that it had a scavenging activity of 21.3%, so the incorporation of 3% of *O. pinnatifida* extract into spreadable dairy cream resulted in a decrease of activity comparatively to the extract used.

As illustrated in Figure 3.8, can be seen that over the 21 d of storage the product quenching ABTS $^{\bullet+}$ capacity did not suffer significant variations.
Statistically it can be stated that, during storage, the negative and positive controls exhibited similar antioxidant capacities ($p > 0.05$) while the spreadable dairy cream with *O. pinnatifida* (SPExtract), when compared with the controls negative and positive (SP and SPFos), exhibited systematically significant higher levels of antioxidant capacity ($p < 0.05$). During storage in SPExtract at 21 d is similar to ($p > 0.05$) 0 d, unlike 7 and 14 d which were statistically significantly different ($p < 0.05$) than at 0 d. This phenomenon was observed for SPFos also. On the other hand, SP (negative control) exhibited no significant variation in antioxidant capacity ($p < 0.05$) of the extract and/or matrix.
According to Marques et al., (2015), thermal processing has been reported to significantly decrease the antioxidant activity of derived peptides and phenolic compounds. Therefore, is possible that the temperature of pasteurization may have affected antioxidants capacities.

In relation to controls (SPFos and SP), the antioxidant capacity observed is likely associated with whey and casein proteins intrinsic antioxidant capacity (Athira et al., 2015). This may be one of the reasons upholding the negative control, SP, and positive control, SPFos, antioxidant activity. The exact mechanism underlying the antioxidant activity of peptides has not fully been understood, yet various studies have displayed that these are inhibitors of lipid peroxidation, scavengers of free radicals and chelators of transition metal ions (Moure, Dominguez, & Parajo, 2006; Rajapakse, Mendis, Jung, Je, & Kim, 2005).
Overall, there was no antioxidant capacity higher than 14.3% for, with both the controls (SP and SPFos) and SPExtract exhibiting a low antioxidant capacity in regard to quenching of the $ABTS^{+}$ radical with the overall activity observed likely being related to the proteins/peptides/phenolic compounds presents not only in the seaweed extract but also in the whey cheese, matrix used.

3.7.1.2 DPPH radical scavenging activity

Since synthetic antioxidants can cause adverse health effects their use should be minimal and controlled, the need and search for natural antioxidants (perceived as safer alternatives to synthetic compounds) is of utmost importance in the food industry. In fact, the use of natural antioxidative peptides to control oxidation processes in food products is an application of the utmost interest especially because natural antioxidants present in foods are presumed safe, nutritious, and have a potential therapeutic value (Freitas et al. 2013). According to Ngo, Wijesekara, Vo, Ta, & Kim (2011) among the marine fauna and flora, marine algae are considered a rich source of natural antioxidants.

The DPPH method is a stable free radical at room temperature and accepts an electron or hydrogen radical to become stable diamagnetic molecules. This method has been extensively used for screening antioxidants, such as polyphenols, which scavenge the DPPH radical through the donation of hydrogen, forming the reduced form of DPPH (Soares, Dinis, Cunha, & Almeida, 1997).

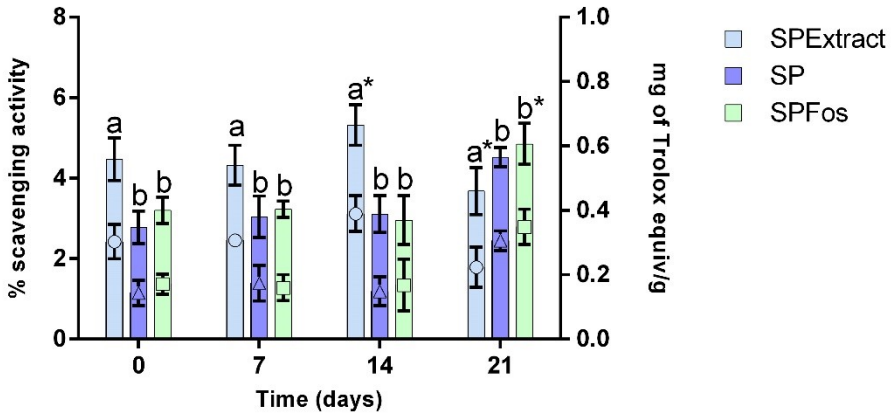


Figure 3.9 – DPPH free radical scavenging activity expressed as scavenging activity (% scavenging activity) and mg of trolox equivalent/ g of sample (mg of phenolic compounds equivalent/g) in spreadable dairy cream with *O. pinnatifida* extract ( ), spreadable dairy cream without any source of carbon ( ) and spreadable dairy with FOS ( ) throughout the storage time (0, 7, 14 and 21 d). Different letters indicate statistically significant differences ($p < 0.05$) between the three dairy spreadable creams at each storage time while bars marked with * means statistically significant differences ($p < 0.05$) in comparison to data obtained at 0 d.

The decrease in absorbance of DPPH radical is caused by antioxidants because as the reaction between antioxidant molecule and radical progresses the scavenging of the radical (by hydrogen donation). Results in a visible discoloration from purple to yellow.

According to Rodrigues, et al., (2015a) in *O. pinnatifida* extract exhibited a lower total antioxidant capacity and DPPH-free-radical scavenging activity (4%) than any of the two other seaweeds considered such as *S. muticum* (14%) and *C. tomentosum* (18%). Moreover, these

results fall in line with those observed in this work where the *O. pinnatifida* extract used in the spreadable dairy cream had a scavenging activity of the 8.75%.

Once again, the SPExtract has the best activity in comparison to the SP and SPFos ($p<0.05$) and this is maintained, although to a less extent, decreasing slightly by 21 days ($p<0.05$). The spreadable dairy cream with *O. pinnatifida* extract, SPExtract, reaches a maximum of scavenging activity at 14 days (in comparison to 0 days, $p<0.05$) with 5.32% (0.390 mg $_{\text{of Trolox equiv}}$/g) so the spreadable dairy cream with 3% of *O. pinnatifida* extract was able to maintain practically the same activity as the extract (8.75%). On the other hand, the controls (SPFos and SP) do not reveal statistically significant differences ($p > 0.05$) between samples and over time ($p > 0.05$).

The radical scavenging activity of phenolic compounds is dependent on the phenolic structure and number and location of hydroxyl groups (Balboa et al., 2013) and therefore the radical scavenging potential of the extracts is dependent of qualitative and quantitative phenolic combination. Recall that according to Rodrigues et al. (2015a), the *O. pinnatifida* extract has 109.2 $\mu g_{\text{cathecol equiv/g}_{\text{lyoph extract}}}$ of total phenolic content. In relation to the percentage of inhibition of the extract in the DPPH method, those authors were able to register values between 1-7% depending on the extraction method and the enzymes used in their study (Rodrigues et al. (2015a). Indeed, these values corroborate a low antioxidant activity associated with the extract of *O. pinnatifida* obtained with Viscozyme.

3.7.1.3 Hydroxyl-radical scavenging activity ($OH^{\bullet}$)

The hydroxyl radical is an extremely reactive free radical formed in biological systems from superoxide anions and hydrogen peroxide in presence of metal ions (such as copper and iron). It has been implicated as a highly damaging species in free radical pathology as it is capable of damaging almost every biologically molecule relevant like proteins, DNA, unsaturated fatty acids and other lipids found in almost every cell membranes (Aruoma, 1998; Hochestein & Atallah, 1988; Rollet-Labelle, et al., 1998; Trease & Evans, 1983).

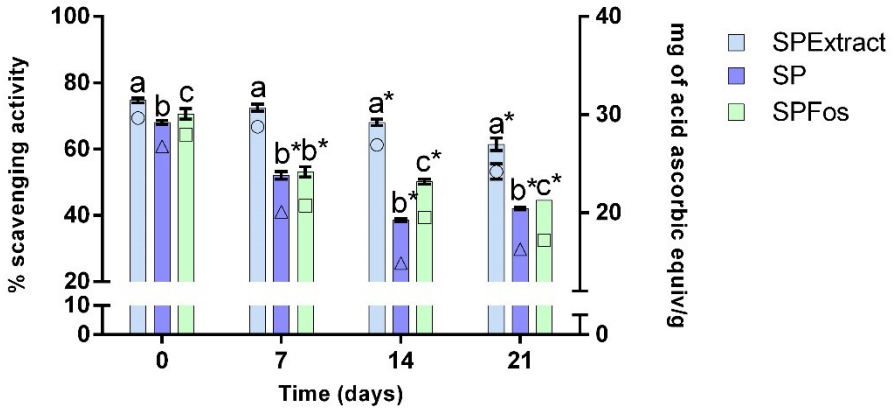


Figure 3.10 – Hydroxyl radical scavenging activity expressed as % scavenging activity (% scavenging activity) and mg of phenolic compounds equivalent/ g of sample (mg of phenolic compounds equivalent/g) in spreadable dairy cream with *O. pinnatifida* extract ( ), spreadable dairy cream without any source of carbon ( ) and spreadable dairy with FOS ( ) throughout the storage time (0,7,14 and 21 d). Different letters indicate statistically significant differences ($p < 0.05$) between the three dairy spreadable creams at each storage time while bars marked with * means statistically significant differences ($p < 0.05$) in comparison to data obtained at 0 d.

In Figure 3.10, it is possible to observe that SPExtract revealed the highest hydroxyl radical scavenging activity ($p<0.05$) in comparison to SP controls (SPFos and SP), independently of storage time. In comparison with the previous two antioxidant activities (ABTS and DPPH) tested, this one reveals the highest order of magnitude in all samples (SPExtract, SP and SPFos). However, the scavenging activity decreased significantly ($p<0.05$) over time reporting ca. 20% decrease by 21 days storage, from 74.7% to 61.5%. This decreasing trend over time was also observed for SPFos and SP; SPFOs reported slightly higher scavenging activity, yet statistically significant ($p<0.05$), than SP independently of storage time.

According to Rodrigues, et al. (2015a) the hydroxyl radical, EAE (Enzyme-Assisted Extractions) extract (extracts were capable of 40–42% scavenging activity (corresponding to 70–79 $\mu g_{ascorbic\ acid\ equiv}$/mL) for *O. pinnatifida* EAE extracts prepared with Cellulase and that *O. pinnatifida* EAE with Viscozyme exhibited a 40-45% scavenging activity (corresponding to 75–79 $\mu g_{ascorbic\ acid\ equiv}$/mL). Compared with that obtained in Figure 3.10 and considering that

the spreadable dairy cream has only 3% of the extract of *O. pinnatifida,* a maximum value of scavenging activity was 74.7% (29.65 mg $_{\text{of acid ascorbic equiv}}$/g) at 0 days; values at 21 days of 61.5% (24.14 mg $_{\text{of acid ascorbic equiv}}$/g) for SPExtract, remained higher than those reported earlier by Rodrigues et al. (2015a) and were greater than 50%. The analysis of the *O. pinnatifida* extract used in the spreadable dairy cream had a % scavenging activity of 80.65%, so the spreadable dairy cream with 3% of *O. pinnatifida* extract was able to maintain practically the same activity as the extract (variation of 5%); synergistic effects between the extract and the matrix are naturally present.

This value of scavenging activity observed can be associated with the highly effective action upon hydroxyl radicals of the abundant -OH groups present in the polysaccharides of algal cell walls. The hydrogen-bond network in algal cellulose is the bottleneck of seaweed enzymolysis with the improvement in algal biomass biodegradability (after the hydroxyl radical-aided thermal pre-treatment) suggesting that the pre-treatment effectively deconstructed the algal cellulose hydrogen-bond network and crystalline structure and therefore supporting the hypothesis of its interaction in the scavenging observed (Yu, Chen, Men, & Hwang, 2009).

4. Conclusions

Dairy products are being explored, more and more, by industry and by scientific researchers because of their nutritional quality and versatility as vectors for bioactive compounds or compounds of added-value for those who consume them. Therefore, this new developed product prototype, formulated and exploited by the incorporation of *O. pinnatifida* extract obtained with Viscozyme, has an advantage in being a multifunctional spreadable dairy cream combining whey cheese and greek yoghurt and offering a high quality nutritional profile, good organoleptic quality and multi biological activities that may successfully contribute to the health and well-being of citizens.

Product formulation and characterization of this innovative functional food went from thermal treatment of ingredients and extracts, assessing microbial stability of the product, establishment of shelf life, monitorisation of functional properties (antioxidant, antidiabetic, antihypertensive and prebiotic) and stability thereof over time to the sensory analysis and consumer acceptance studies, completing the full pipe-line in successful functional food development.

After completing this work, whose main objective was to develop a novel functional food with the incorporation of bioactive extracts it is possible to conclude that:

- Pre-treatment of dairy ingredients and extract at 90 ºC for 30 min was identified as the best heat treatment to ensure microbiological stability of the product at least for 21 days;
- The combination of plain greek yoghurt and whey cheese in a 22% and 75% proportion was revealed as an excellent vector to incorporate the *O. pinnatifida* (seaweed) extract, both in terms of organoleptic balance as well as overall preservation of biological activities of extract;
- Given the formulation, and the storage conditions, it was possible to establish a final spreadable dairy cream with functional properties with a shelf life of up to 21 days;
- Consumer acceptance and degree of preference for this new product was quite satisfactory, being accepted by almost two thirds of the panel members. The evaluation of the product at the sensorial level is critical, because without these data there is no knowledge of the product acceptability by the target community. The evaluated product showed very promising overall results;

- The spreadable dairy cream with *O. pinnatifida* extract was able to convey several biological activities, namely prebiotic, antidiabetic, anti-hypertensive and antioxidant capacities, although with different orders of magnitude;
- The SPExtract boosted the growth of *L. acidophilus* La-5 at both 0 and 21 d but potentiated the survive of the *B. animalis* subsp *lactis* BB12 at 0 and 21 d; however, after the presentation of simulation of the gastrointestinal tract, the growth promotion of *B. animalis* subsp *lactis* BB12 was partially lost in the digested spreadable dairy cream with *O. pinnatifida* extract, while for *L. acidophilus* La-5 an increase in growth was observed;
- The spreadable dairy cream with *O. pinnatifida* extract showed a low antidiabetic activity (8.56%) compared to the positive control; nevertheless, if portion size is considered ingestion of this spreadable dairy cream may aid in controlling post-prandial glucose uptake by hindering carbohydrate breakdown;
- In what concerns antihypertensive activity, the spreadable dairy cream with *O. pinnatifida* extract revealed a 50% inhibition capacity of the ACE-I enzyme and revealed an IC_{50} of 209.9 μg/mL, which according to the literature is understood to be an intermediate antihypertensive activity capacity;
- The developed spreadable dairy cream with *O. pinnatifida* extract showed best activity in the scavenging of the hydroxyl radical (74.7%), followed by some activity in the capturing of the $ABTS^{+}$ radical (21.3%), but with little DPPH activity (8.8%).

Thus, it was verified that the *O. pinnatifida* extract with bioactive properties could be a possible solution when consumers more natural additive solutions capable to promoting health and wellbeing. This extract proved to be a source of bioactive compounds with nutritional, nutraceutical and cosmetic applications.

5. Future Work

The results obtained in this research endeavour, although quite enlightening, require validation at model studies level in some cases and complimentary studies in others, in order to complement or explain the results obtained so far.

It will be important to evaluate the prebiotic potential of the digested samples at macro-scale (spreadable dairy cream with *O. pinnatifida* extract, spreadable dairy cream with FOS and spreadable dairy cream without any carbon source) registering viable cell numbers to obtain more precise conclusions; *ex vivo* fermentation studies using donor faecal samples will also be important for prebiotic potential validation. Further monitorization of sugar and organic acids profiles in these fermentation studies, particularly of glucose, will be important in order to infer and verify the phenomenon occurred.

It will also be relevant to re-test all the biological activities with the digested samples and if loss in activity is very significant, studying protection mechanisms for the *O. pinnatifida* extract, such as immobilization or encapsulation, need to be explored.

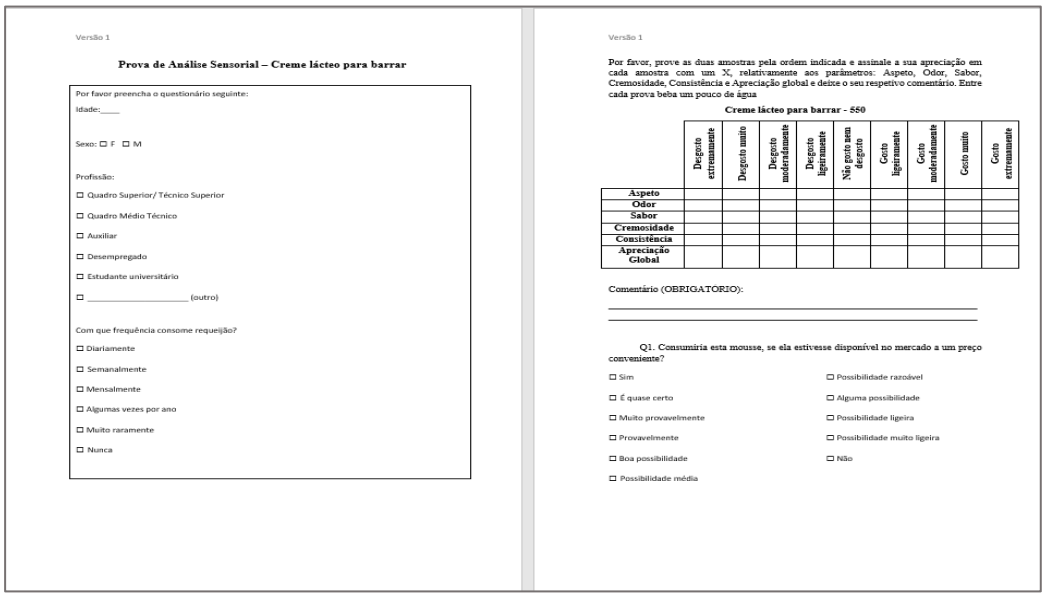
Another interesting continuation for this research line would be to consider the impact of the spreadable dairy cream with seaweed extract upon the human gut microbiota through faecal fermentations or even by screening the variation in microbiota composition after continued product ingestion of the product.

Non-thermal processing technologies such as High Pressure Processing (HPP) may be explored to increase microbial stability of developed products but without compromising product characteristics and biological properties of extracts.

Given the current trends, organoleptically interesting dairy products, with a clean-label and capable of promoting well-being and health, such as the ones developed herein will be very recurrent and desired by consumers.

6. Appendixes

6.1 Appendix I -Sensory Analysis Questionnaire



Versão 1

Prova de Análise Sensorial – Creme lácteo para barrar

Por favor preencha o questionário seguinte:

Idade:____

Sexo: ☐ F ☐ M

Profissão:

☐ Quadro Superior/ Técnico Superior

☐ Quadro Médio Técnico

☐ Auxiliar

☐ Desempregado

☐ Estudante universitário

☐ _____ (outro)

Com que frequência consome requeijão?

☐ Diariamente

☐ Semanalmente

☐ Mensalmente

☐ Algumas vezes por ano

☐ Muito raramente

☐ Nunca

Versão 1

Por favor, prove as duas amostras pela ordem indicada e assinale a sua apreciação em cada amostra com um X, relativamente aos parâmetros: Aspeto, Odor, Sabor, Cremosidade, Consistência e Apreciação global e deixe o seu respetivo comentário. Entre cada prova beba um pouco de água

Creme lácteo para barrar - 550

	Desgosto extremamente	Desgosto muito	Desgosto moderadamente	Desgosto ligeiramente	Não gosto nem desgosto	Gosto ligeiramente	Gosto moderadamente	Gosto muito	Gosto extremamente
Aspeto									
Odor									
Sabor									
Cremosidade									
Consistência									
Apreciação Global									

Comentário (OBRIGATORIO):

_____

_____

Q1. Consumiria esta mousse, se ela estivesse disponível no mercado a um preço conveniente?

☐ Sim

☐ É quase certo

☐ Muito provavelmente

☐ Provavelmente

☐ Boa possibilidade

☐ Possibilidade média

☐ Possibilidade razoável

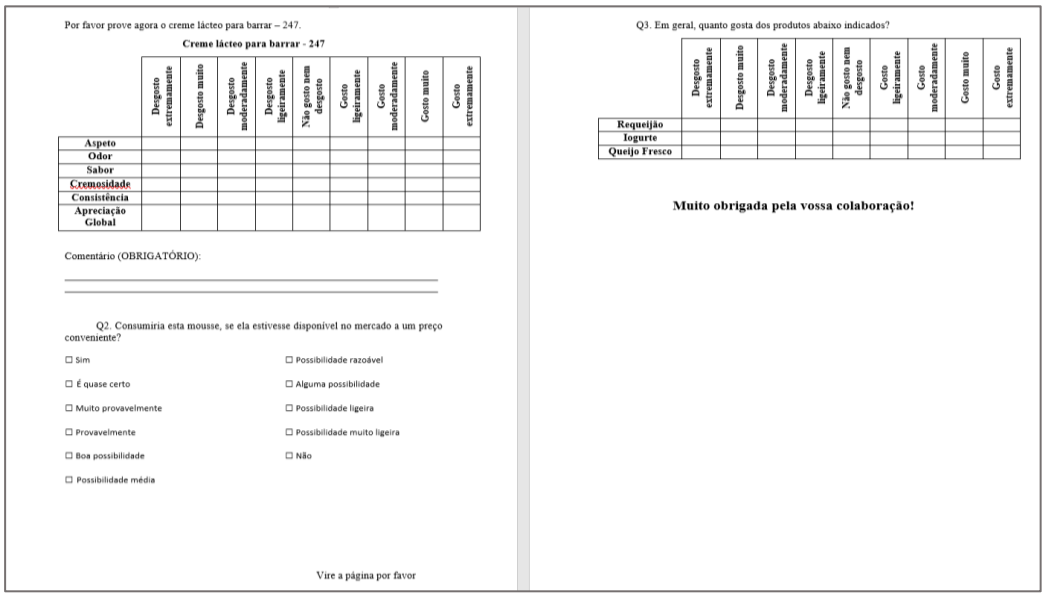
☐ Alguma possibilidade

☐ Possibilidade ligeira

☐ Possibilidade muito ligeira

☐ Não

Figure 6.1 First part of the sensory analysis questionnaire.



Por favor prove agora o creme lácteo para barrar – 247.

Creme lácteo para barrar - 247

	Desgosto extremamente	Desgosto muito	Desgosto moderadamente	Desgosto ligeiramente	Não gosto nem desgosto	Gosto ligeiramente	Gosto moderadamente	Gosto muito	Gosto extremamente
Aspeto									
Odor									
Sabor									
Cremosidade									
Consistência									
Apreciação Global									

Comentário (OBRIGATÓRIO):

_____

_____

Q2. Consumiria esta mousse, se ela estivesse disponível no mercado a um preço conveniente?

☐ Sim

☐ É quase certo

☐ Muito provavelmente

☐ Provavelmente

☐ Boa possibilidade

☐ Possibilidade média

☐ Possibilidade razoável

☐ Alguma possibilidade

☐ Possibilidade ligeira

☐ Possibilidade muito ligeira

☐ Não

Vire a página por favor

Q3. Em geral, quanto gosta dos produtos abaixo indicados?

	Desgosto extremamente	Desgosto muito	Desgosto moderadamente	Desgosto ligeiramente	Não gosto nem desgosto	Gosto ligeiramente	Gosto moderadamente	Gosto muito	Gosto extremamente
Requeijão									
Iogurte									
Queijo Fresco									

Muito obrigada pela vossa colaboração!

Figure 6.2 Second part of the sensory analysis questionnaire.

6.2 Appendix II – Calibration Curves

6.2.1 Ascorbic acid equivalent calibration curve

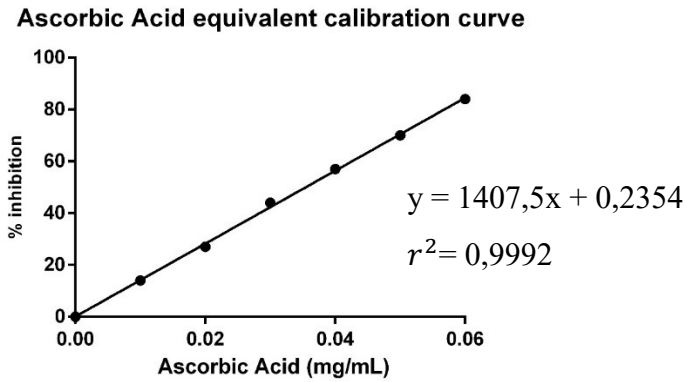


Figure 6.3 Calibration curve obtained, using standard ascorbic acid solutions to calculate the result obtained in the ABTS radical cation assay.

6.2.2 Trolox equivalent calibration curve

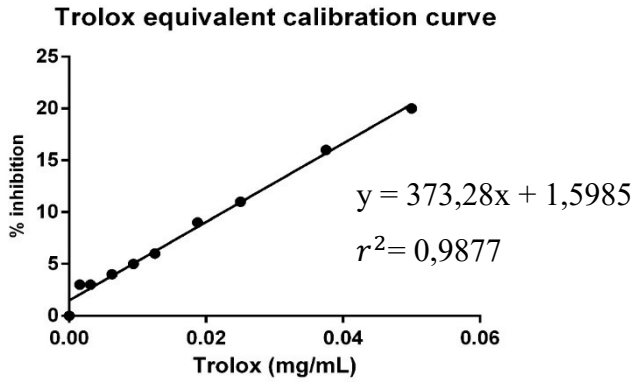


Figure 6.4 Calibration curve obtained, using standard trolox solutions to calculate the result obtained in the DPPH.

6.2.3 Ascorbic Acid equivalent calibration curve

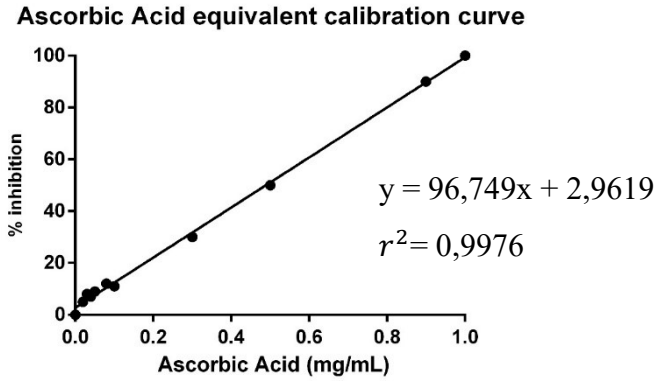


Figure 6.5 Calibration curve obtained, using standard ascorbic acid solutions to calculate the result obtained in the Hydroxyl-radical Scavenging Activity

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