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OKARA (BY-PRODUCT OF SOYA BEVERAGE): POTENTIAL APPLICATION IN FOOD AND AQUAFEED

Thesis submitted to the Universidade Católica Portuguesa to attain the degree of PhD in Biotechnology - with specialization in Food Science and Engineering

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Supervisor: **Prof Dr. Maria Manuela Estevez Pintado**
Co-supervisor: **Prof. Dr. Luísa Maria Pinheiro Valente**

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Resumo

A soja é uma das oleaginosas mais consumidas no mundo. Entre os principais subprodutos da transformação industrial da soja destaca-se o okara. Este subproduto, resultante da produção de bebidas de soja, representa ainda um problema ambiental devido às altas quantidades produzidas. Adicionalmente, o seu elevado teor de humidade (~ 80%) dificulta sua conservação e, consequentemente, a sua posterior utilização. No entanto, apesar da sua perecibilidade, o okara possui uma composição nutricional rica em proteínas, lípidos e fibras bem como elevados teores de compostos com potencial bioativo como as isoflavonas. Neste contexto, o principal objetivo deste programa de doutoramento foi encontrar alternativas para aumentar a estabilidade do okara, através de processos térmicos ou biotecnológicos (nomeadamente hidrólise enzimática e fermentação com bactérias probióticas), por forma a melhorar as propriedades biológicas do okara tendo em vista a sua utilização na alimentação humana bem como na animal, mais especificamente na aquacultura.

A primeira parte do trabalho incidiu no estudo do impacto de diferentes temperaturas na secagem do okara (80 ºC/5 h e 200 ºC/1 h). De um ponto de vista microbiológico, em ambas as amostras foi verificada uma redução na contaminação microbiana no tempo inicial. Adicionalmente, no okara seco a 200 ºC foi observada uma redução da atividade dos inibidores de tripsina, no entanto, esta amostra também apresentou uma maior oxidação lipídica durante o armazenamento. De seguida, estudou-se a influência de um processo de autoclavagem (1 atm, 121 ºC por 15 min) prévio à secagem a 65 ºC (AOK) em comparação com o okara seco não autoclavado (NAOK), tendo-se observado que o processo de autoclavagem, além de reduzir a atividade dos inibidores de tripsina (0.86 mg TUI/ mg de amostra seca), manteve as características da farinha de okara durante o armazenamento.

Na segunda parte do trabalho foram estudadas diferentes condições (tempo e concentração de enzima) na hidrólise enzimática das farinhas de okara (NAOK e AOK) utilizando duas enzimas, Alcalase (AL) e protéases de *Cynara cardunculus* (CY). Os resultados mostraram que o processo térmico facilitou o ataque enzimático resultando numa elevada atividade anti-hipertensiva para ambos os hidrolisados (9.97 e 54.30 μg de proteína/mL, AL e CY, respetivamente) bem como antioxidante, em particular para a AL. Este estudo também permitiu a identificação de novas sequências peptídicas para ambas as enzimas. Com base nos resultados anteriores, nomeadamente a atividade anti-hipertensiva, iniciou-se a terceira componente deste

trabalho, i.e. o desenvolvimento de bebidas fermentadas utilizando okara fresco previamente hidrolisado com CY. Para elaborar as bases da bebida, aos hidrolisados de okara foi adicionada frutose (6% m/v) e frutoligossacarídeos (FOS) (2% m/v), sendo a mistura resultante fermentada utilizando duas bactérias probióticas *Bifidobacterium animalis* Bb-12 e *Lactobacillus rhamnosus* R11, individualmente e em mistura. As bebidas fermentadas de okara foram analisadas ao longo de 28 dias de armazenamento com os níveis de células viáveis mantendo-se estáveis durante esse período. Adicionalmente, quando as bebidas de okara foram submetidas ao sistema gastrointestinal (*in vitro*), verificou-se um aumento das atividades antioxidante e anti-hipertensivas (IC_{50}), o que se poderá traduzir numa maior bioacessibilidade. No entanto, sensorialmente as bebidas fermentadas não foram bem aceites pelos participantes, que indicaram uma acidez elevada e doçura insuficiente.

A última parte deste trabalho consistiu na incorporação das farinhas, previamente descritas, em dietas para uma espécie de peixe herbívora, a Tilápia do Nilo (*Oreochromis niloticus*). Começou por se avaliar *in vivo* a digestibilidade de seis farinhas: NAOK, AOK, okara hidrolisado com AL e autoclavado (ALOK), okara hidrolisado com CY e autoclavado (CYOK), CYOK fermentado com *L. rhamnosus* R11 (CYR11OK) e CYOK fermentado com *B. animalis* Bb-12 (CYB12OK). Seguidamente, as farinhas de okara que apresentaram digestibilidades mais elevadas (AOK e CYOK) foram incorporadas em dietas para Tilápia do Nilo (a 10 e 20%) à custa de ingredientes vegetais (farinhas de soja, milho e trigo) de forma a avaliar o seu impacto no crescimento e utilização de nutrientes ao longo de um ensaio de crescimento de 10 semanas. De um modo geral, os resultados mostraram que a incorporação das farinhas de okara selecionadas (até 20%) não alteram significativamente a performance de crescimento nem a utilização de nutrientes da Tilápia do Nilo.

Considerando todos os resultados obtidos, é possível concluir que, com um tratamento térmico adequado é possível melhorar a qualidade nutricional do okara e mantê-lo estável durante o armazenamento. Adicionalmente, a hidrólise enzimática e a fermentação podem contribuir para melhorar as características nutricionais e funcionais da okara, quando se consideram aplicações na nutrição humana. Relativamente ao uso de okara na aquacultura, este pode ser incorporado até 20% em dietas para a Tilápia do Nilo, substituindo outros ingredientes de origem vegetal e diminuindo os custos de produção, sem que se verifique qualquer efeito adverso no crescimento dos peixes nem na sua composição corporal.

Abstract

Soybean is one of the most consumed oilseeds in the world. Among the main industrial by-products resulting from soya processing, okara can be highlighted.

This by-product, resulting from the production of soy beverages, is still an environmental problem due to the high quantities produced. In addition, its high moisture content (~ 80%) makes it difficult to preserve and, consequently, its subsequent use

However, despite its high instability, okara has an interesting nutritional composition (it is rich in fiber, protein and lipids) as well as significant levels of antioxidant compounds, mainly isoflavones. In this context, the general objective of this PhD programme was to increase the stability of okara (through thermal and/or biotechnological processes such as enzymatic hydrolysis and fermentation with probiotic bacteria) and improve its biological properties towards the development of new food and aquafeed solutions.

Firstly, the impact of different drying temperatures (80 ºC/5 h (D80) and 200 ºC/1 h (D200)) was studied, with all samples showing a reduction of microbial contamination afterwards. D200 exhibited a reduction of trypsin inhibitors' activity, however it also showed a stronger increase in lipid oxidation than D80 during storage. Afterwards, the influence of autoclaving (1 atm, 121 ºC and 15 min) was evaluated with the autoclaved okara flours (AOK) exhibiting reduced trypsin inhibitors' activity (0.86 mg TUI/ mg dry sample) and maintaining their characteristics during the storage better than the non-autoclaved flours (NAOK).

The second part of the hereby proposed work focused on the study of different hydrolysis conditions (time and enzyme concentration) upon the okara flours (NAOK and AOK) using two enzymes, alcalase (AL) and proteases from *Cynara cardunculus* (CY).

The results showed that the thermal process facilitated enzymatic attack resulting in high antihypertensive activity for both hydrolysates (9.97 and 54.30 µg protein / mL, AL and CY respectively) as well as antioxidant, in particular for AL. This study also allowed the identification of new peptide sequences for both enzymes. Based on the previous results, namely the antihypertensive activity, the third stage focused on the development of fermented beverages using fresh okara previously hydrolyzed with CY. The hydrolysates, supplemented with fructose (6% w/v) and FOS (2% w/v), were fermented with two probiotic bacteria: *Bifidobacterium animalis* Bb-12, *Lactobacillus rhamnosus* R11 and a combination of the two. The resulting beverages were analyzed over 28 days of storage with the bacteria remaining viable during this

period. Moreover, when subjected to a simulated gastrointestinal system, the antioxidant and antihypertensive activities (IC_{50}) of the beverages increased, hinting at an improvement of the bioaccessibility of bioactive components. However, despite the interesting bioactivity results, sensory wise the fermented beverages were not so well accepted with the participants of the study remarking on the fermented beverages' high acidity and low sweetness.

The last part of this PhD program focused on the incorporation of the different okara flours, obtained using the different processes described above, in Nile Tilapia (*Oreochromis niloticus*) diets. Firstly, a digestibility trial was performed *in vivo* with six different okara flours: NAOK, AOK, okara hydrolyzed with AL and autoclaved (ALOK), okara hydrolyzed with CY and autoclaved (CYOK), CYOK fermented with *L. rhamnosus* R11 (CYR11OK) and CYOK fermented with *B. animalis* Bb-12 (CYB12OK). Afterwards, the ingredients with the highest apparent digestibility coefficients values (AOK and CYOK) were included in diets for Nile Tilapia (at 10 and 20%), at the expense of other vegetable sources (soybean, wheat and corn meals), in a 10 weeks growth trial. In general, results showed that the dietary inclusion of okara up to 20% did not significantly affect the growth performance or nutrient utilization of Nile tilapia.

Overall, considering all the results obtained, it can be concluded that a correct heat treatment can result not only in an increase of the nutritional quality of okara but also improve the storage time of this by-product. In addition, the biotechnological processes studied (enzyme mediated hydrolysis and fermentation by probiotic microorganisms) may also contribute to the improvement of the nutritional and functional characteristics of okara when considering food applications for human consumption. Regarding the potential use of okara in aquaculture, the hereby described results hint that it can be successfully incorporated into aquafeed formulations for Nile Tilapia, replacing other, more expensive ingredients of plant origin, therefore reducing production costs without having any adverse effects on fish growth or whole body composition.

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“Experience is not what happens to you;
it's what you do with what happens to you”
Aldous Huxley.

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List of Abbreviations

ABTS– 2,2'-Azino-Bis (3-ethylbenzThiazoline-6-Sulphonic acid)

ACE- Angiotensin-Converting Enzyme

AL- Alcalase enzyme

ALOK- Hydrolyzed okara by Alcalase

ALOKd- Diet with ALOK

AOK- Dried okara previously autoclaved

AOK10- Diet with 10 % of autoclaved okara meal

AOK20 – Diet with 20 % of autoclaved okara meal

AOKd – Diet with AOK

CY- Proteases from *Cynara cardunculus* extract

CYB12OK- Okara hydrolyzed using proteases from *Cynara cardunculus* and fermented by *B. animalis* ssp. *lactis* Bb12

CYB12OKd- Diet with CYB12OK

CYOK –Hydrolyzed okara by proteases from *Cynara cardunculus*

CYOK10- Diet with 20 % of okara hydrolyzed by protease from *Cynara cardunculus* meal

CYOK20 - Diet with 20 % of okara hydrolyzed by protease from *Cynara cardunculus* meal

CYOKd- Diet with CYOK

CYR11OK- Okara hydrolyzed using proteases from *Cynara cardunculus* and fermented by *L. rhamnosus* R11

CYR11OKd – Diet with CYR11OK

D200- Dried okara at 200 ºC for 1 h

D80- Dried okara at 80 ºC for 5 h

DGI- Daily growth index

DM- Dry matter

DPPH- 1,1-diphenyl-2-picrylhydrazy

FA- Fatty acid

FAA– Free Amino Acids

FAOK- Fresh okara autoclaved

FCR- Feed Conversion Ratio

FNAOK- Fresh okara not autoclaved

FPLC- Fast Protein Liquid Chromatography

HIS- Hepatosomatic Index

HPLC– High Performance Liquid Chromatography

IC_{50}- Antihypertensive activity

K- Condition factor

MW – Molecular Weight

NAOK- Dried okara not autoclaved

NAOKd- Diet with NAOK

OkaBb- Fermented okara by *B. animalis* ssp. *lactis* Bb12 and no carbohydrates added

OkaBb3- Fermented by *B. animalis* ssp. *lactis* Bb12 with fructose (3%) and FOS (2%)

OkaBb6- Fermented okara by *B. animalis* ssp. *lactis* Bb12 with fructose (6%) and FOS (2%)

OkaLR- Fermented okara by *L. rhamnosus* R11 and no carbohydrates added

OkaLR3- Fermented okara by *L. rhamnosus* R11 with fructose (3%) and FOS (2%)

OkaLR6- Fermented okara by *L. rhamnosus* R11 with fructose (6%) and FOS (2%)

OkaMix- Fermented okara by *B. animalis* ssp. *lactis* Bb12 and *L. rhamnosus* R11 combined and no carbohydrates added

OkaMix3- Fermented okara by *B. animalis* ssp. *lactis* Bb12 and *L. rhamnosus* R11 combined with fructose (3%) and FOS (2%)

OkaMix6- Fermented okara by *B. animalis* ssp. *lactis* Bb12 and *L. rhamnosus* R11 combined with fructose (6%) and FOS (2%)

OkaNF- Okara beverage unfermented

PER- Protein Efficiency Ratio

REF – Reference diet

ROS– Reactive Oxygen Species

SBM- Soybean meal

SEM– Scanning Electronic Microscope

SGR- Specific Growth Ratio

VSI- Viscerosomatic Index

Keywords

Alcalase

Amino acids

Antihypertensive activity

Antioxidant activity

Aquaculture

Aquafeed

Beverage

Bifidobacterium animalis ssp. *lactis* Bb12

Bifidocbacteria

Bioactivity

By-product

Circular economy

Digestibility

Digestibility

Drying

Enzymatic hydrolysis

Fatty acids

Fermentation

Fish nutrition

Gastrointestinal digestion

Isoflavones

Lactic acid bacteria

Lactobacillus rhamnosus R11

Nile Tilapia

Okara

Organic acid

Peptide

Prebiotic

Probiotic

Part I- General Introduction

Scope and outline

This thesis is organized in six parts, including 9 chapters, which describe how the research/project progressed along time.

The general objective was to obtain a stable of okara flour, to be submitted to enzymatic hydrolysis to obtain a bioactive ingredient enriched in bioactive peptides. These hydrolyzed okara beverage was tested as a matrix towards the development of synbiotic beverages using probiotic bacteria (*Lactobacillus rhamnosu*s R11 and *Bifidobacterium animalis* ssp. *lactis* Bb12) and prebiotic (FOS) in their composition, as well to evaluate the stability of these beverages through the storage time and *in vitro* gastrointestinal digestion and comprehend their bioavailability. Moreover, the different okara flours obtained were incorporated in diet of Nile tilapia to study the digestibility and two demonstrating the best digestibility were studied in tilapia nutrition and growth trials as replacers of vegetal dietary proteins (up to 20%) of the common aquafeed diets.

Part I includes Chapter 1 that is a general literature review about soybeans, properties and composition of okara, as well their generation and respective use. Furthermore, this chapter also present the importance of processes, such as enzymatic hydrolysis and fermentation, as well as relevance of probiotics products and bioactive peptides to promote health benefits in particular diseases prevention. Additionally, the current aquaculture scenario was addressed, as well as the ingredients used for fish diets and the importance of finding new ingredients for aquaculture application and cost savings.

Part II: Chapters 2 and 3 describe the effect of different thermal process to obtain okara with different moisture contents. The microbiological stability, chemical composition and bioactive compounds were analyzed throughout storage time

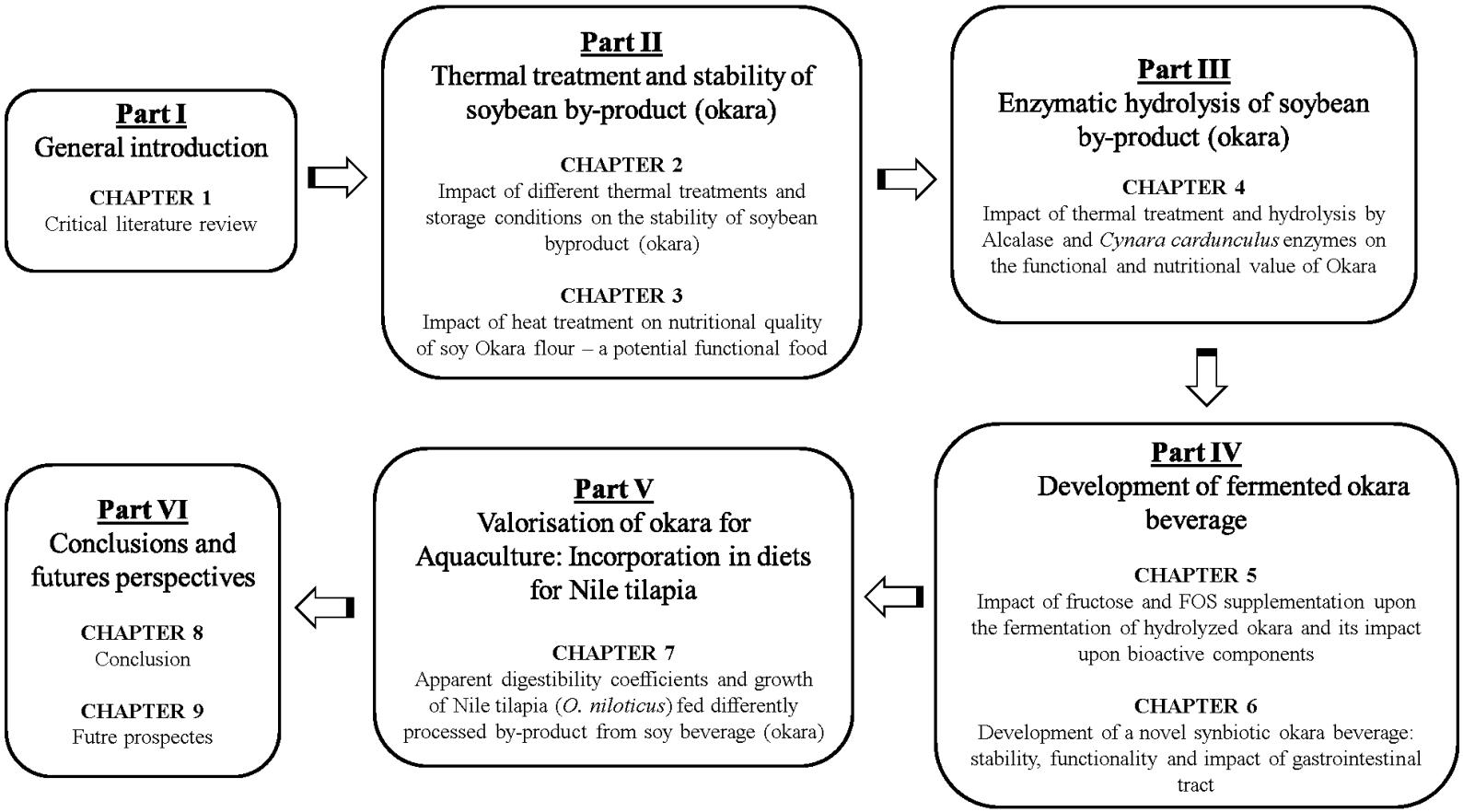
Part III: Chapter 4 focus on the enzymatic hydrolyses of okara flour using two enzymes: proteases from *Cynara cardunculus* and alcalase. The antioxidant and antihypertensive activities were evaluated, as well the peptides profile and peptides composition.

Part IV includes chapter 5 and 6. Chapter 5 reports the study of the fermentation of okara previously hydrolyzed by proteases from *Cynara cardunculus,* using two different bacteria (*Lactobacillus rhamnosu*s R11 and *Bifidobacterium animalis* ssp. *lactis* Bb12) and different concentration of fructose (3 and 6%), and their impact on viable cells of probiotic strains, isoflavones and acid organic content generated during the process. In addition, the Chapter 6

describes the study of stability of the four okara beverages added of the fructose (6%) and FOS (2%): (1) beverage fermented by *Lactobacillus rhamnosus* R11, (2) beverage fermented by *Bifidobacterium animalis* ssp. *lactis* Bb12, (3) beverage fermented by a mix of both bacteria and (4) beverage without fermentation. In addition, the viable cells of probiotics, microbiological stability, antioxidant activity and isoflavones profile of beverages throughout 28 days of storage at 4 ºC were evaluated, as well as, the sensory acceptability of these products. Furthermore, the total phenolics, antioxidant and antihypertensive activities were evaluated throughout simulated gastrointestinal tract in order to better understand their bioavailability.

Part V: Chapter 7 focuses on the apparent digestibility coefficients and growth of Nile fed with differently processed okara, and this chapter present two main objective: 1) to assess the *in vivo* apparent digestibility coefficients (ADCs) of different okara meals (autoclaved, fermented and hydrolyzed) in Nile tilapia; and 2) to further evaluate the effects of the dietary incorporation (10 and 20%) of the most digestible okara meals (okara autoclaved and okara previously hydrolyzed by proteases from *Cynara cardunculus*), as alternative protein sources, in Nile Tilapia growth performance and nutrient utilization after a 10 weeks growth trial.

Finally, the general conclusions and the future perspectives are presented in Part VI, respectively, chapter 8 and Chapter 9.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Impact of different thermal treatments and storage conditions on the stability of soybean byproduct (okara)
CHAPTER 3
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Conclusion
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Part of the work presented in the 9 chapters has been already published or submitted to international journals, or submitted to patent application according to the following list:

Chapter 2

Voss, G. B., Rodríguez-Alcalá, L. M., Valente, L. M. P., Pintado, M. M. (2018). Impact of different thermal treatments and storage conditions on the stability of soybean by-product (okara). *Journal of Food Measurement and Characterization*, 1–34. http://doi.org/10.1007/s11694-018-9813-5.

Chapter 3

Voss, G. B., Valente, L. M. P., Pintado, M. M.E.. Impact of heat treatment on nutritional quality of soy Okara flour – a potential functional food. Submitted *to LWT- Food Science and Technology*.

Chapter 4

Voss, G. B., Osorio, H., Valente, L. M. P., Pintado, M. M. E. Impact of thermal treatment and hydrolysis by Alcalase and *Cynara cardunculus* enzymes on the functional and nutritional value of Okara. Submitted to *Process Biochemistry*.

Chapter 5

Voss, G. B., Valente, L. M. P., Pintado, M. E. Impact of fructose and FOS supplementation upon the fermentation of hydrolyzed okara and its impact upon bioactive components. Subimitted to *Food Research International*.

Chapter 6

Voss, G. B., Monteiro, M. J. P., Jauregi, P., Valente, L. M. P., Pintado, M. M. E. Development of a novel synbiotic fermented beverage from hydrolyzed okara: nutritional, functional characterization and impact of gastrointestinal tract passage. Submitted to *Food Microbiology*.

Chapter 7

Voss, G. B., Rema, P., Pintado, M. M. E.., Valente, L. M. P. Apparent digestibility coefficients and growth of Nile tilapia (*O. niloticus*) fed differently processed by-product from soy beverage (okara). Submitted to *Aquaculture*.

Patent

Processo de obtenção de peptídeos bioativos e elaboração de bebida hidrolisada, fermentada a base de okara (subproduto de bebida de soja) e formulação de dieta para tilápia com incorporação de farinha de okara. Submitted.

CHAPTER 1- Critical literature review

1.1. Introduction

In recent years, eating habits and lifestyle of the consumers has changed encouraged by finding healthier and more nutritious food habits to envisage health benefits and the prevention of chronic diseases. As a result, a new term "functional food" has been proposed, and among these foods, probiotics may exert positive effects on health, in particular gastrointestinal disorders, but also other gut microbiome related-diseases. In addition, an increase of the demand for non-dairy probiotic products comes from vegetarianism, milk cholesterol content, allergenic and intolerant dairy-consumers, and according Silanikove et al. (2015) it is estimated that about 75% of the adult population had some form of lactose intolerance. These factors also encourage the food industry to invest in innovation for vegetable-based and lactose-free foods.

Additionally, soybeans are considered a great ally for a healthier diet, considering that they have components of proven beneficial action on human (Bedani et al., 2014a; Coward et al., 1993; Friedman and Brandon, 2001). In the composition of soybeans, it can be highlighted: proteins, fibers, lipids, isoflavones and vitamins (Redondo-Cuenca et al., 2006; Villares, Rostagno et al., 2010). Moreover, soy gained functional food status, with preventive action of some diseases. The daily consumption of this oil has been associated with the prevention of heart disease, obesity, hypercholesterolemia, cancer, diabetes, kidney disease and osteoporosis (García et al., 1998). Additionally, several biologically active peptides (bioactive peptides) from soybean have been identified and present an important role in soybean physiological activities, especially to the prevention of chronic diseases (Wang and Mejia, 2005). In parallel, research suggests that the presence of isoflavones in soybean makes it a functional food capable of acting to prevent the symptoms of menopause, while previous studies have suggested that the consumption of soybeans may prevent the incidence of some types of cancer, especially breast and prostate cancer (He and Chen, 2013; Sarkar and Li, 2003).

Through the processing of soybeans, one can get different raw materials such as soy flour, textured protein and water-soluble extracts (soya beverage). However, the development of soya beverage (“soymilk”) generates a huge quantity of by-products to the food industry,

called okara. This factor consists of a large problematic, considering that from 1 kg of soybeans is produced about 1.1 Kg of fresh okara (Khare et al.,1995), which is most often discarded directly into the environment, incinerated or used in animal feed, however due to antinutritional factors and high amount of fiber, its use has been limited to animal feeds (Vong and Liu, 2016).

In addition, the residue of aqueous extract of soybean (okara) still holds many nutrients (on a dry weight basis, approximately 50% carbohydrates, 20-30% proteins and 10-20% lipids, as well as minerals and phytochemicals) (Vong and Liu, 2016), that could be used for human consumption and has been wasted by the food industry. Nevertheless, a major problem associated with this by-product is the fact that present a great instability. Due to its high humidity (ca. 80%) and rich nutritional composition this by-product is quickly degraded.

So, one of the possible alternatives is drying the okara, which would maintain this product stable for longer storage periods, furthermore, according to the conditions applied, the product can maintain the nutritional composition. However, other alternatives, such as application of bioprocesses, as enzymatic hydrolysis and fermentation using bacteria, are interesting processes for the reuse of this by-product (Bedani et al., 2014a; Bedani et al., 2014b Sbroggio et al., 2016). In addition, these processes can increase the bioactivities in the final products. Previous studies have reported that the enzymatic hydrolysates generated bioactive peptides, with antioxidant activity, antihypertensive, antidiabetic among others (Gibbs et al., 2004; Singh et al.., 2014). In parallel, it was also related, that the isoflavones profile of soya beverage can change during the lactic fermentation, improving their bioactivities (Delgado et al., 2018).

On the other hand, aquaculture sector has grown continuously, and several alternative plant protein source ingredients have been studied for aquafeed in recent years. In addition, the development of processes for by-product recovery and utilization, showed to be more economically feasible than discarding the by-products. Thus, based on composition and properties, okara is an interesting alternative as feed ingredient on aquaculture (El-Saidy, 2011; Mo et al., 2018), as well as assisting in circular economy, considering that this by-product can be obtained worldwide. Furthermore, the sustainable development of the aquaculture sector is an important condition to meet future food demand from a world population of 9.6 billion by 2050 (Nadarajah and Flaaten, 2017).

1.2. Soybeans

Soybean (Glycine max L) is an oilseed crop that has been cultivated for over 3000 years in China and in other Asian countries and it is consumed worldwide (Nishinari et al., 2014). Currently, the United States, Brazil and Argentina are the largest producers of soybeans and according the United States Department of Agriculture (USDA), the latest report issued estimated the world soybean production in the year 2017/2018 as 336.7 million tons, in addition the United States and Brazil stood out, with a production of 119 and 117 million tons, respectively (USDA, 2018). This oilseed has great commercial and nutritional interest, which serves as a good source of protein for animals and human beings (Friedman and Brandon, 2001; He and Chen, 2013; Vidal et al., 2015). Furthermore, the consumption of food from soy-based has increased due the proved benefits on human health and nutrition. Previous studies has related that soy induced lowering of cholesterol, protection against intestinal and renal diseases, besides prevention of cancer, obesity and diabetes (Friedman and Brandon, 2001; Guimarães et al., 2018).

Additionally, soy contain high protein content, rich and healthy fat composition, rich in unsaturated fatty acids (especially polyunsaturated linoleic acid) and relevant biologically active compounds, such as phytoestrogens, called isoflavones; these nutritional and chemical characteristics qualify this oilseed such as a functional food (Delgado et al., 2018; García et al., 1998; He and Chen, 2013). This fact has generated great interest in food industry, thus a variety of products from soybeans with properties to the organism, called as "functional foods" were created (Guimarães et al., 2018). This fact, also increase the interest of the consumers for these products, however the flavor of soy-based product is still a limiting factor for the consumption of these products by the western population (Torres-Penaranda and Reitmeier, 2001).

Furthermore, other limitation of soybean use is related with the presence of antinutritional factors (ANFs), since the soybean contains a range of antinutritional substances, that are broadly classified as lipoxygenase, urease, protease trypsin inhibitors, lectin, and others in smaller quantities (Francis et al., 2001; Vagadia et al., 2017; Yuan et al., 2013). In addition, the trypsin inhibitors are the major antinutritional factors in soybeans and this can cause serious adverse effects in humans and animals (Hossain and Becker, 2002). Thus, raw soybeans cannot be used as human food or animal feed and in order to improve the retention and utilization of

soy nutrients, the inactivation of the ANFs is extremely important during food processing (Vagadia et al., 2017). In addition, studies established a direct association between processing and the protein availability (Hossain and Becker, 2002). The presence of trypsin inhibitors reduce the biological activity of the digestive enzymes (trypsin and chymotrypsin) and consequently affect the digestibility of the soybean consumed (Vagadia et al., 2017). However, these inhibitors are heat liable and previous studies showed that processing during soy food preparation improve the protein digestibility (El-Hady and Habiba, 2003).

In contrast, the processing of soybeans originates different raw materials that can be used in the production of other foods, such as soy flour, textured proteins and soy beverage, products that have been part of the eastern diet for a long time. Nonetheless, in recent years, the demand for soy-based foods has increased due to the search for a healthier diet, considering the many health benefits that have been linked to this oilseed (Jenkins et al., 2000; Jiménez-Escrig et al., 2008). However, along with this demand for soy-based products, the number of by-products generated in the food industries has also increased, especially in the case of the soya beverage, one of the main product used actually for people with intolerance to lactose or allergic to cow's milk protein (Guimarães et al., 2018), which generates a large amount of waste, called okara (O´Toole, 1999).

1.3. Okara: by-product from soya beverage

Soya beverage is used as a base in a wide variety of products, including tofu, soy yogurt and cheese. This soybean product is achieved by aqueous extraction of whole soybeans. During this process, a by-product known as okara rich in fiber, protein and fat, is obtained (Mateos-Aparicio et al., 2010; O´Toole, 1999; Redondo-Cuenca et al., 2008; Van der Riet et al., 1989). Okara is produced in high amounts, since for each 1 kg of soybeans about 1.1 kg of okara is produced (Khare et al., 1995). Furthermore, the composition of okara depend of the cultivar of soybean, the method used in the manufacture of soya beverage and the amount of compounds extracted from the ground soybeans. In figure 1.1, the main steps used in the production of soy beverage and tofu, and consecutively the waste generated (okara and whey) are represented.

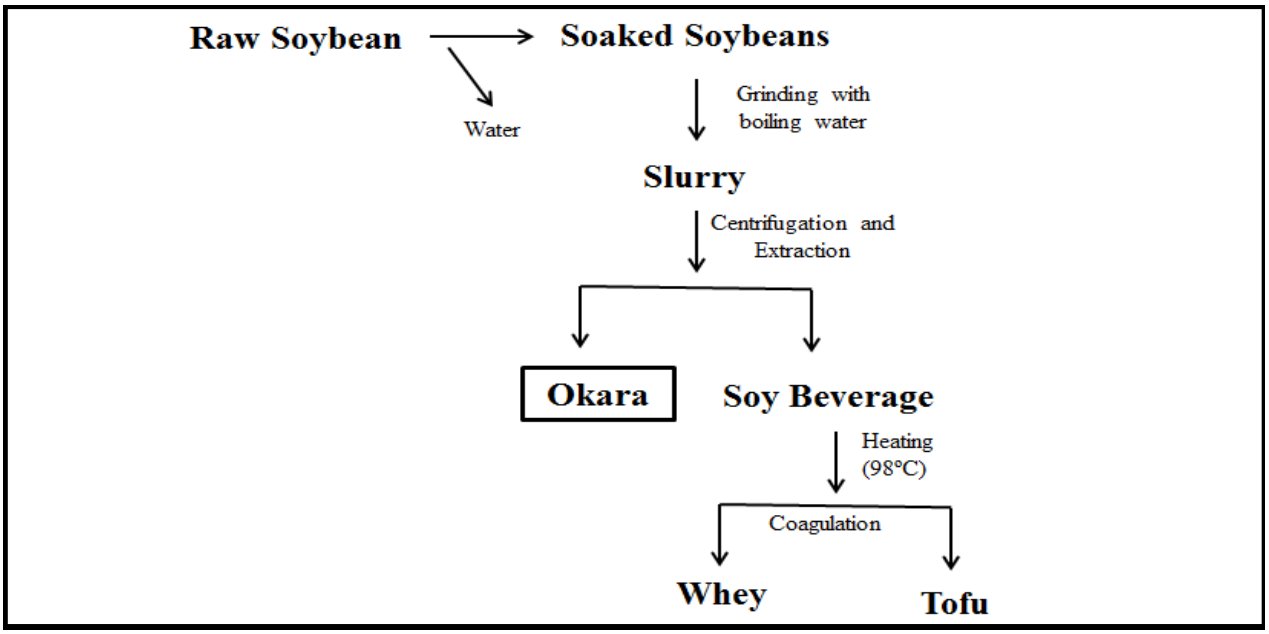


Figure 1.1. The major steps involved in soya beverage production and by-product generation. Adapted from Jackson et al. (2002).

The okara (Fig 1.2) can be used in traditional dishes but when produced in large quantities by the industry, its use is unfeasible, considering its great instability attributed to its high humidity (~ 80%) that makes it susceptible to spoilage. So, it is usually incinerated or used in agriculture (O´Toole, 1999; Redondo-Cuenca et al., 2008). A possible alternative for okara´s use is to dry it, however, this process is still costly and very difficult to be performed and can also degrade some compounds present in okara, reducing its commercial value (Jankowiak et al., 2014; Perussello et al., 2014). Therefore, an alternative for this by-product usage in fresh would be to include it in food and animal feeding, adding value and reducing its negative ambient impact (Redondo-Cuenca et al., 2008).

Figure 1.2. Okara- by-product from soya beverage and tofu.

The okara by-product has a high nutritive value, as is presented in Table 1.1. However, during soymilk manufacturing, this by-product is submitted to a severe heat treatment, which causes a large protein denaturation and the resulting okara protein isolate has poor solubility, which could restrict its direct use in food (Chan and Ma, 1999). However, previous studies reported that the protein from okara has a high quality and show good digestibility, in addition the protein efficiency ratio and the essential amino acid to total amino acid ratio in okara is superior to other soy products (Ma et al., 1997; O´Toole, 1999). Although okara protein isolates showed low solubility, other functional properties as emulsification, foaming and binding properties, were comparable to those of commercial soy isolate, suggesting the potential use of okara protein as a food ingredient (Ma et al., 1997).

Table 1.1. Composition of okara in protein, fat, fiber, carbohydrate and ash (% dry matter) reported in the literature.

Protein	Fat	Fiber	Carbohydrate	Ash	Reference
25.4-28.0	9.3-10.9	52.8-58.1	3.8-5.3	-	Van der Riet et al.(1989)
26.8	12.3	-	52.9	-	Ma et al. (1997)
28.5	9.8	55.5	2.6	3.6	Redondo-Cuenca et al. (2008)
18.37	9.65	55.4	8.72	3.45	Kuan and Liong (2008)
33.4	8.5	54.3	3.9	3.7	Mateos-Aparicio et al. (2010)
34.9	14.3	20.6	-	3.9	El-Saidy (2011)

Other component that stands out in okara are the dietary fiber, which cost is low. It is well known that dietary fiber plays an important role in many physiological processes and in the prevention of diseases of different origin (Jha et al., 2017). Despite of its high total fiber amount, this by-product is not rich in the soluble fiber fraction. Thus, using enzymatic treatment hydrolyses the insoluble fiber into soluble fiber, improving prebiotic benefits of this by-product (Lu et al., 2013; Villanueva-Suárez et al., 2013).

Nakornpanom et al. (2010) showed the importance of sources of dietary fiber digestibility *in vitro*, and the okara has shown potential use as a source of dietary fiber in high calorie emulsions, both hydrolyzed and pectinase-hydrolyzed forms. However, more studies are needed to understand the okara roles in controlling the stability of the emulsion and release of oil during digestion under different physiological conditions. This knowledge is of fundamental importance for the design of processing and formulation of medical foods with desirable digestibility and absorption.

In a previous study, Villanueva-Suárez et al. (2013) showed that the enzyme-treated okara has a higher soluble dietary fiber content when compared to untreated okara. This factor, along with its good physico-chemical properties and its *in vitro* bifidogenic properties, show that the enzymatically treated okara may represent a useful alternative component for the development of functional ingredients.

Furthermore, okara also has a high lipids content, with polyunsaturated fatty acids (PUFA) such as linolenic acid and linoleic acid, 9 and 54% of the total fatty acids content, respectively (Palermo et al., 2012). In addition, in the literature has been reported that PUFA present many advantageous for the consumers' health (Galão et al., 2014; Kerdiles et al., 2017).

1.4. Isoflavones

Isoflavones are an important group into phenolic compounds, that are exclusive in leguminous plants, and soy and its processed products are the main source of isoflavones in the human diet (Manach et al., 2004). According to the chemical structure, the isoflavones are polyphenolic compounds, which belong to the subgroup of flavonoids (Valdés et al., 2015). The amount of soy isoflavones can range from 0,4 mg to 9,5 mg per gram of soybean (Rostagno et al., 2009).

In soybeans twelve isoflavone components have been isolated. Including three types of isoflavones in four chemical forms groups: the aglycones (daidzein, genistein and glycitein), the β-glycosides (daidzin, genistin and glycitin), their 6”- O-acetyl-β- glycosides and their 6”- O-molonyl-β- glycosides conjugates (Jackson et al., 2002; Villares et al., 2010).

The chemical structures of isoflavones present in soybean, derived products and by-products are shown in Figure 1.3.

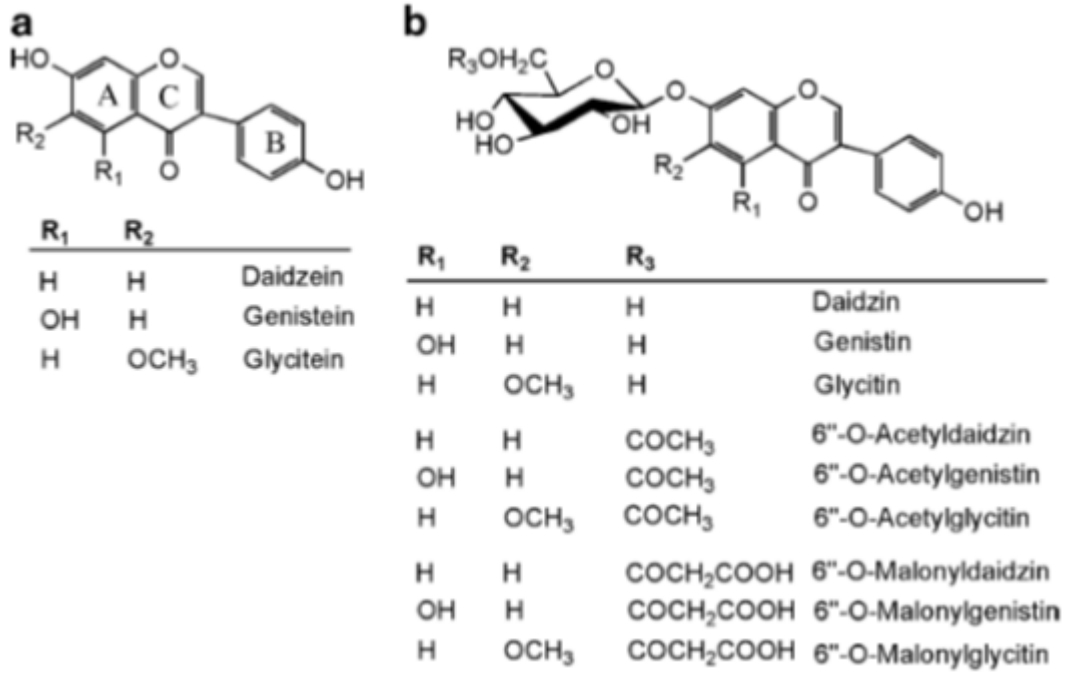


Figure 1.3. Chemical structure of the main isoflavone conjugates: aglycones (a) and glycosides (b) (Villares et al., 2010).

Although the isoflavone profile can change during the soymilk processing conditions, generally this by-product contains the same isoflavones present in original soybeans (Villares et al., 2010). According to Coward et al.(1993) the distribution between forms of isoflavones varies according to soy product and thermal treatments. The hot aqueous extraction, normally used to produce tofu or soymilk, results in the formation of isoflavones β-glycoside conjugates. Also, the use of dry heat, as in soy flour toasting, may generate large amounts of 6-O-acetyl-β-

glycoside conjugates, resulting from the heat-induced decarboxylation of the malonate group to acetate (Coward et al., 1993).

Additionally, previous studies have also related, that during the fermentation process of soya beverage, the isoflavone glycosides are transformed into their corresponding aglycones (daidzein, genistein, glycitein) through the deglycosylation reaction (Delgado et al., 2018; Martinez-Villaluenga et al., 2012). This transformation has an important role for human health, considering that the isoflavones aglycones are absorbed faster and in greater amounts than the isoflavones glycosides (Izumi et al., 2000).

Isoflavones have shown several health benefits attracting a great deal of attention due to their properties (Villares et al., 2010), especially in relation to antioxidant activity (Lee et al., 2005). In last years, these compounds have gained interest due the epidemiological studies and He and Chen (2013) reported a lower incidence of breast and prostate cancer in Asian countries, where the traditional diet is rich in soy products. Besides, Sarkar and Li (2003) indicated that soy isoflavones inhibited the carcinogenesis in animal models, so this compounds have shown an important role in cancer prevention, especially genistein, that is predominant isoflavone in soy.

Other benefits from isoflavones and their metabolites is the inhibition of low-density lipoprotein (LDL) oxidation *in vitro* (Kapiotis et al., 1997), and this was confirmed in study *in vivo* by Jenkins et al. (2000) that incorporated soy in breakfast cereals for twenty-five hyperlipidemic subjects and concluded that LDL oxidative also was reduced in the test breakfast and that the intake of the high amount of isoflavone in soy breakfast cereals may decrease the risk of cardiovascular disease. The isoflavones also can play an important role in the prevention of several diseases, including osteoporosis, and menopausal symptoms (Ahsan and Mallick, 2017; Taku et al., 2011).

1.5. Okara application

Previous studies have suggested different alternatives for the use of okara. In Table 1.2 are shown some research conducted thus far, for okara valorization. It is possible to see that okara by-product was applied for different purposes. The use of okara as a food ingredient and

its effects on the human health were evaluated. On the other hand, the use of okara as a feed ingredient was also evaluated, and promising results were found in farm animal and fish.

Table 1.2. Studies of okara: valorization and food application.

Okara application	Reference
Okara use for producing citric acid by fermentation with *Aspergillus terreus and Aspergillus nigel*	(Khare et al., 1995)
Characterization of okara protein	(Ma et al., 1997)
Peptides from okara protein and study of its antioxidative activity	(Okomizo et al., 2002)
Study of use the okara and obesity prevention	(Matsumoto et al., 2007)
Study of health effects in rats using a concentrate dietary fiber from okara	(Jiménez-Escrig et al., 2008)
Use of soy yogurt with okara in diet of rats, showed lower liver weight and hepatic triglyceride compared to control.	(Kitawaki et al., 2009)
Study of okara protein digestibility *in vitro*	(Nakornpanom et al., 2010)
Flash drying of okara	(Grizotto, 2011)
Incorporation of okara in diet of Nile tilapia and nutritional study	(El-Saidy, 2011)
Dietary fiber and hypoglycemic effect of okara foods	(Lu et al., 2013)
Physicochemical properties and in vitro fermentability of okara polysaccharides extracted by enzymatic hydrolysis	(Villanueva-Suárez et al., 2013)
Synbiotic fermented soy product supplemented with okara on cardiovascular disease risk markers	(Bedani et al.2014 a)
Soymilk fermented (La-5, Bb-12, and *Streptococcus thermophilus*) and supplemented with inulin and okara	(Bedani et al. 2014 b)

Thermophysical properties of okara during drying	(Perussello et al., 2014)
Okara as a biomass for a bioethanol production	(Choi et al., 2015).
Use of fresh okara in cookies formulation	(Park et al., 2015)
Isolation of isoflavones from okara	(Jankowiak et al., 2015, 2014)
Soymilk fermented by *Lactobacillus* incorporated of okara flour	(Moraes Filho et al., 2016)
Influence of enzymatic hydrolysis in antioxidant activity of okara	(Sbroggio et al., 2016)
Okara as a protein supplement for feed of ewes, evaluation of milk composition and lamb growth	(Harthan and Cherney, 2017)
Alterations of isoflavones and color during the drying of okara	(Muliterno et al., 2017)
Production of vegetable paste enriched with okara	(Guimarães et al., 2018)

1.6. Drying process

Drying is the most viable approach of conserving the by-product, due the reduction of moisture content and water activity (a_w) (Kowalski and Mierzwa, 2013). This process increased the value, enhanced shelf life, and also bring savings to storage and transportation costs, due to reduction in bulk and storability without refrigeration conditions. In addition, the priority of the process is not only the efficiency, but also the quality of the product, because the consumer search products with functional effects and this has encouraged the demand for product that after processing maintains its nutritional composition (Sehrawat et al., 2018). Nonetheless, for the application of okara as a food ingredient, in addition to the nutritional aspect, also is essential the technological functional properties, that is non-nutritional properties, which can to influence in the texture and physical behavior of food (Grizotto, 2011).

Furthermore, during the storage of dried products, the stability is not given only by the moisture content of food, but free water (a_w) is related with the main chemical and enzymatic degradation reactions in food, besides being a determinant factor for microbial growth, being that almost all microbial activity is inhibited below aw of 0.6 (Maltini et al., 2003). The water activity (a_w) is defined as 'the ratio of the vapour pressure of water in a food to the saturated vapour pressure of water at the same temperature'(Fellows, 2000) and is an important parameter for quality and stability of the dried food (Fellows, 2000; Maltini et al., 2003).

Previous studies have been using different drying process in okara, as flash drying, spray dryer, air jet impingement and oven-dryer with air circulation and evaluate the drying kinetic, physicochemical parameters, nutritional composition and isoflavones profile (Grizotto, 2011; Muliterno et al., 2016; Perussello et al., 2014). However, more studies, about the microbiological and chemical stability of dried okara are essential for the development of stable okara products for human consumption.

1.7. Enzymatic hydrolysis

Hydrolysis of food proteins have been largely studied and present an important contribution for many industrial applications, especially in protein from vegetable and milk that are widely used in the food industry (Kristinsson and Rasco, 2000). Enzymatic protein

hydrolysis has been used in by-products from agro-industry to generate other food, considering that this process can be used to modify the proteins and thus increase their value, due the functional properties, attributed to bioactive peptides generated (Valencia et al., 2015). These peptides are specific protein fragments, obtained from breakdown of proteins by proteases, that can possess positive impact on body function or influence health (Kitts and Weiler, 2003). In addition, previous studies have been related that they exert various biological roles, i.e. antioxidant, antihypertensive and antidiabetic activities, among other potential physiological activities (Chen and Yamauchi, 1995; Kitts and Weiler, 2003; Sarmadi and Ismail, 2010; Singh et al., 2014). In addition, previous studies have been related many bioactivities in soy protein after enzymatic hydrolysis using different enzymes (Coscueta et al., 2016; Gibbs et al., 2004; Guan et al., 2017)

Additionally, the enzymatic modification of proteins depends of the selected proteolytic enzyme, among the enzymes used in food industry, it is possible to highlight two enzymes: alcalase and cardoon proteases. Alcalase is an enzymatic extract from *Bacillus licheniformis*, with endopeptidase activities used to hydrolyze protein. This alkaline protease presents several proteinases with different specificities in their composition and during hydrolysis this enzyme has the capacity to produce peptides with shorter chain and stronger bioactivities (Osman et al., 2016; Zhang et al., 2018). In previous studies, alcalase has been used extensively to prepare hydrolysates of soy (Zhang et al., 2018; Zhong et al., 2007), whey (Doucet et al.,2003), fish (Rebeca et al., 1991) and sweet potato protein (Zhang and Mu, 2017) with bioactive peptides, including antihypertensive, antioxidant and hypocholesterolemic activities.

Moreover, the aqueous extracts from cardoon (*Cynara cardunculus*) that is a thistle whose flowers can be considered the best-known vegetable coagulant used since ancient times in the farm production of various types of traditional ewe´s milk cheeses in Portugal and Spain (De Sá and Barbosa, 1972; Liburdi et al., 2018). The cardoon extract present three acid proteases and were named cardosins: the forms I and II are similar and thus together are denominated Cardosin A, whereas form III is denominated Cardosin B (Lamas et al., 2011).This proteinases (Cardosin A and Cardosin B), shown different amino acid sequences but both have proteolytic activity and promote a milk clotting (Barros and Malcata, 2002; Liburdi et al., 2018). According to Verissimo et al. (1995) although Cardosin B is less abundant than Cardosin A (~75%) it has a higher proteolytic activity. In addition, Cardosin A is comparable with chymosin, while

Cardosin B is comparable with pepsin. Nonetheless, the Cardosin B present a less specificity than the pepsin and hydrolyses bonds of phenylalanine, tyrosine, leucine or valine residues (Barros and Malcata, 2002). Although the cardosins are well known milk-clotting enzymes, nevertheless to the best of our knowledge, there are no reports of its use in soy or its by-products to date.

1.8. Probiotics, prebiotics and synbiotic food

1.8.1. Probiotic functional food

Probiotics are defined as live microorganisms with beneficial effects on human and animal health, when given in adequate amounts (Gibson et al., 2017; Kerry et al., 2018), but the dose of probiotic microorganisms depends on the strain and the product (Martinez et al., 2015). Currently, most part of the consumers ingest probiotic through food products, and most of these products are categorized as a functional food (Tripathi and Giri, 2014). However, in order to promote a health effect, the oral ingestion of a probiotic will have to satisfy a predetermined threshold of viable cells and according to previous studies a functional product should contain between 10^6 and 10^8 colony forming units per gram (CFU/g) of viable probiotic cells during the shelf-life (Donkor et al., 2007; Martinez et al., 2015; Mondragón-Bernal et al., 2017). This is generally accepted as being sufficient for successful and adequate colonization of the gastrointestinal tract. In addition, the impact of probiotic food on human health are being increasingly studied (Kerry et al., 2018; Liu et al., 2017; Panghal et al., 2018). The oral administration of probiotic food may be beneficial in a multitude of disorders both inside and outside the gastrointestinal tract (Parvez et al., 2006). Previous studies found that the probiotics also can present different properties, such as anti-pathogenicity, anti-allergic, anti-diabetic, anti-obesity, anti-inflammatory, anti-cancer, and angiogenic activities, and their effect on the brain and central nervous system (Kerry et al., 2018). Furthermore, Figure 1.4 show other health benefits of probiotics consumption.

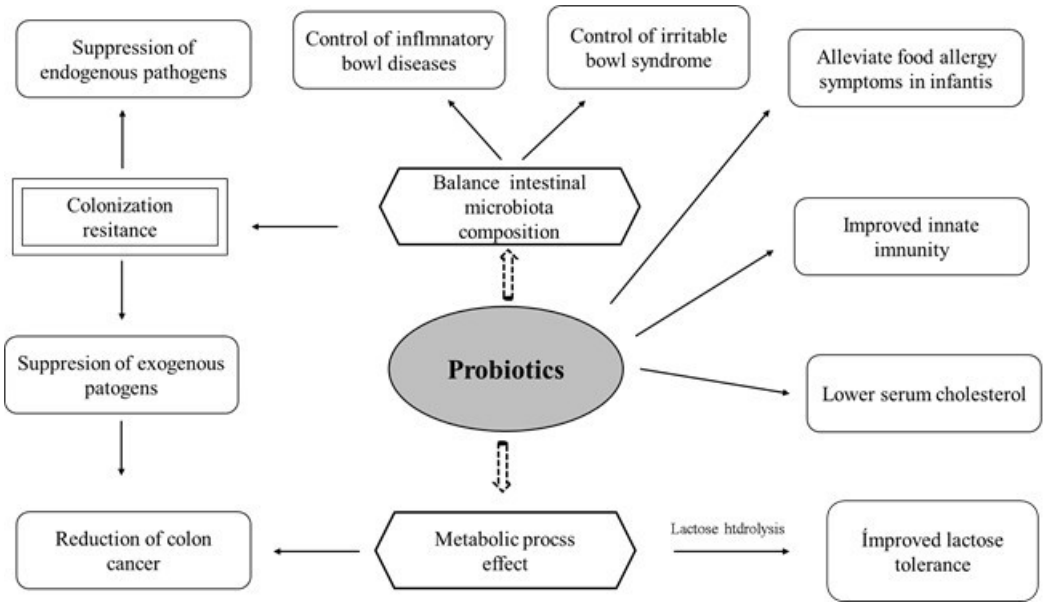


Figure 1.4 Health benefits from probiotics consumption. Adapted from Parvez et al. (2006) and Tripathi and Giri (2014).

The selection of probiotics for the suitable probiotic strains is the first requirement for developing a probiotic food product, as well the viability during processing, remain viable throughout the shelf-life of the product, survival during intestinal transit and association with health benefits for consumers, also is desirable criteria in strains of probiotic bacterial species (Parvez et al., 2006; Tripathi and Giri, 2014). Moreover, other technological and physiological characteristics of probiotic strains are important during the selection of probiotics for application in commercial food products, such as: safety criteria (pathogenicity and infectivity, origin), technological (genetically stable strains, good sensory properties, production in large-scale and viability during process and storage), functional (tolerance to gastric acid and bile, adhesion to mucosal surface) and physiological (cholesterol metabolism, lactose metabolism, properties of antimutagenic and anticarcinogenic) (Tripathi and Giri, 2014). The genera of *Bifidobacterium* and *Lactobacillus* strains are the probiotic microorganisms most extensively studied and widely used, due the beneficial effects attributed (Bedani et al., 2014a). In addition, among the probiotic organisms, the lactobacilli are generally stronger than bifidobacteria, due its adaptation for other

food substrates, non-dairy and are resistant to low pH, furthermore, lactobacilli is found naturally in traditional fermented foods (Tripathi and Giri, 2014).

The viability of probiotics microorganism in different food products is influenced by many factors during production, processing and storage. According (Tripathi and Giri, 2014) the factors include microbiological parameters (strains of probiotics and proportion of inoculation), food ingredient (pH; acidity, water activity, presence of salt, sugar and chemicals), fermentation conditions (dissolved oxygen, incubation temperature), process applied (scale of production, heat treatment, cooling rate of the product, packaging materials and storage condition).

1.8.2. Dietary fiber and prebiotics

Fiber consist in the structural part of a plant, which are found in vegetables, fruits, grains, and legumes (Jha et al., 2017).The dietary fiber is the edible part of plant and analogous carbohydrates, include polysaccharides, oligosaccharides and lignin, which are resistant to digestion, and their absorption occur in the small intestine and are fermented in the large intestine (American Association of Cereal Chemists, 2001).

The dietary fiber is usually classified by chemists in soluble and insoluble dietary fiber. Insoluble fiber is insoluble in water, but show relevant properties, such as hygroscopic and swelling, while the soluble fiber is soluble in water and can form a gel (Chiewchan, 2018). The containing fiber in food commonly is a combination of both (soluble and insoluble) fiber. Furthermore, the dietary fiber exhibits beneficial effect, such laxation, blood cholesterol or glucose attenuation (American Association of Cereal Chemists, 2001). The different constituents of dietary fiber according definition of the American Association of Cereal Chemists is shown in Table 1.3.

On the other hand, inulin and oligosaccharides are dietary fiber, however, these substances are best known as prebiotics. The currently definition of prebiotics was provided by Gibson et al. (2017), as a substrate that is selectively utilized by host microorganisms and promote a health benefit, and although all prebiotics are fiber, not all fibers are considered prebiotic (Slavin, 2013). In addition, according the actual definition of prebiotics, other substances might fit the updated definition, as polyphenols and polyunsaturated fatty acids

converted to respective conjugated fatty acids, considering the benefit on host health (Gibson et al., 2017).

Table 1.3. Constituents of dietary fiber according American Association of Cereal Chemists.

Dietary fiber	**Non-starch polysaccharides and resistant oligosaccharides**	- Hemicellulose ▪ Arabinoxylans ▪ Arabinogalactans - Polyfructoses ▪ Inulin ▪ Oligofructans - Cellulose - Galactooligosaccharides - Gums - Mucilages - Pectins
	Analogous carbohydrates	- Indigestible dextrins ▪ Resistant maltodextrins ▪ Resistant potato dextrins - Synthesized carbohydrate compounds ▪ Polydextrose ▪ Methyl celulose ▪ Hydroxypropylmethyl cellulose - Indigestible (resistant) starches
	Lignin	
	Substances associated with the non-starch polysaccharide and lignin complex in plants	- Waxes - Phytate - Cutin - Saponins - Suberin - Tannins

(American Association of Cereal Chemists, 2001).

Nonetheless, fiber with prebiotics properties differ from most dietary fiber, which stimulate the growth of a wide variety of intestinal microorganisms, in addition these nutrients

can modify the microbial flora of the intestine and are not easily digested by humans and serve for beneficial microorganisms housed in the host (Gibson et al., 2017; Kerry et al., 2018). According Gibson et al. (2017) these compounds provoke a biased metabolism for health promotion broadly rather than being extensively metabolized. While, the functional properties of dietary fiber on human health are related with the chemical structure and microstructural characteristics, and the effect of dietary fiber in gastrointestinal system occurs in both parts of intestine. However, insoluble and soluble dietary fiber expressed different functional properties and action during digestion, besides that is possible to observe changes in their microstructure for different steps of digestion (Chiewchan, 2018). Furthermore, according to Aggett et al. (2003), the ingestion of inadequate amount of fiber can lead to gastrointestinal disorders, such as constipation. Furthermore, the consumption of dietary fiber has been growing in last years, this fact is associated with beneficial health effects, considering that several studies have been related an inverse relation between higher dietary fiber intake and reduction the risk of many chronic diseases, such as diabetes, coronary heart disease, obesity, constipation and even prevention effect for different cancer: colon, stomach, esophageal, breast, prostate and others (SOLER et al., 2001; Gao and Yue, 2012).

Additionally, prebiotic can also be recommended as part of fiber intake, considering that the previous studies have been related several benefits of prebiotic intake to health promotion (Bouhnik et al., 1999; Gibson et al., 2010; Guigoz et al.,2002). Furthermore, the consumption of different fiber sources is important in a healthy diet due to the variability of fiber‘s effects in the body. However, even though fiber consumption has increased in recent years, it still less than half of recommended, so the increase of fiber intake is critical public health goal, due to it´s health promotion and disease prevention (Slavin, 2013).

1.8.3. Potential non-dairy synbiotic and probiotic product

Changing lifestyles, eating habits and health awareness have made consumers more conscious about role of food for a better quality of life, consequently leading to a growing search for healthy and nutritious foods with more benefits broad to health, able to preventing diseases (Panghal et al., 2018). Previous studies have been related several impacts of probiotic and synbiotic food on human health and this has driven probiotic foods market growth. Synbiotic

products combine probiotic microorganism and prebiotic substances; this help the survival and the implantation of live microbial dietary supplements in the gut and might promote a positive interaction *in vivo*, besides that the synbiotic may confer the stability of the final product (Martinez et al., 2015; Mondragón-Bernal et al., 2017). Studies also related that the synbiotic relationship between prebiotics and probiotics has a significant influence on host health and that the synergistic benefits are more efficient when the interaction between probiotics and prebiotics occurs *in vivo* (Kerry et al., 2018). Furthermore, previous studies has shown benefits for gut health and disease prevention, as cardiovascular diseases (Bedani et al., 2014a; Kerry et al., 2018).

These characteristics contributed to the increase of the commercial interest of functional foods, in addition the synbiotic concept offers great potential for explore the connection between probiotics and prebiotics and it´s advantages benefits on the stability of the product during shelf life (Martinez et al., 2015)

Usually, most probiotic or synbiotic food products, use milk as base formulation, but allergenicity or intolerance factors in dairy-food were some of the limiting factors for consumption of dairy probiotics (Panghal et al., 2018). According to Silanikove et al. (2015) up to 75% of the adult human population may be lactose intolerant, since most humans stop producing lactase after weaning.

Additionally, the preference of consumers for vegetarian food have stimulated the use of fruit, vegetables, cereals and legume for the development of probiotics or synbiotic food products (Mondragón-bernal et al., 2017; Nazzaro et al., 2008). Besides, cereals and legumes are most important plant group and shown a rich nutritional composition, responsible to meet nutritional requirement world-wide (Panghal et al., 2018). Although, several probiotic food products can be prepared using legumes, the use of soya beverage for probiotic products is the principal, due to their phytochemicals and nutritional composition (Bedani et al., 2014; Delgado et al., 2018). In recent years, studies also reported the incorporation of okara flour in probiotic and synbiotic products from soya beverage, due the high nutritional quality, phenolic compounds and fiber (Bedani et al., 2014a; Bedani et al., 2014b; Moraes Filho et al., 2016), but the development of beverages using only okara without soya beverage has not been reported so far. Nevertheless, the production of non-dairy probiotics at the industrial level, present

limitations, such as sensory characteristics, overall acceptance and the survival of probiotics during shelf-life, but still the market for non-dairy probiotics is promising (Panghal et al., 2018).

1.9. Digestion and bioavailability of nutrients

Digestion is an important physiological process that is involved in the release of nutrients from food and feed matrix. Methods for determining bioavailability and/or bioaccessibility of nutrients involve human and animal (*in vivo*) or simulated experiments performed in a laboratory (*in vitro*) (Parada and Aguilera, 2007). In general, the animal experiment and clinical trials for study digestibility of nutrients or bioactivity are expensive and time consuming (Bao et al., 2018). On the other hand, the simulation of gastrointestinal system *in vitro* has been broadly used to investigate the stability, bioactivity and bioavailability in several food matrix or bioactive compounds, as peptides and antioxidant compounds (carotenoids, tocopherols, or polyphenols) due to the simple, rapid and reproducible property, and do not have the ethical restrictions of *in vivo* methods (Wang et al., 2018). The *in vitro* digestion process is simulated under controlled conditions using commercial digestive enzymes, such as pepsin, pancreatin, and so on (Parada and Aguilera, 2007).

The gastrointestinal tract has essential functions in the digestive process and absorption of nutrients. As it is shown in Figure 1.5, the gastrointestinal tract includes: mouth, esophagus, stomach, small intestine (duodenum, jejunum, ileum), large intestine (colon), rectum and anus.

The digestion process begins during mastication, a mechanic process, which breakdown of large food particles into small pieces, generating a larger surface area for the digestive enzymes. Still, during mastication the enzymes present in oral secretion (lingual lipase and salivary amylase) mix with food to form a bolus, where is initiated the digestion of carbohydrates and triglycerides (Pedersen et al., 2018). Further, in the stomach the physical processing and mixing (by triturate antral contractions) with the strongly acidic secretions (gastric juice) containing hydrochloric acid (around pH 1) and pepsin, lead to the formation of a semi-solid paste (‘chyme’) which is gradually released, when sufficiently fluid, into the small intestine (Jackson and McLaughlin, 2009; McDonald and MacFarlane, 2018). Although, the stomach is not the essential factor in a digestion, but the low pH of gastric juice provides protection against ingested microorganisms (McDonald and MacFarlane, 2018).

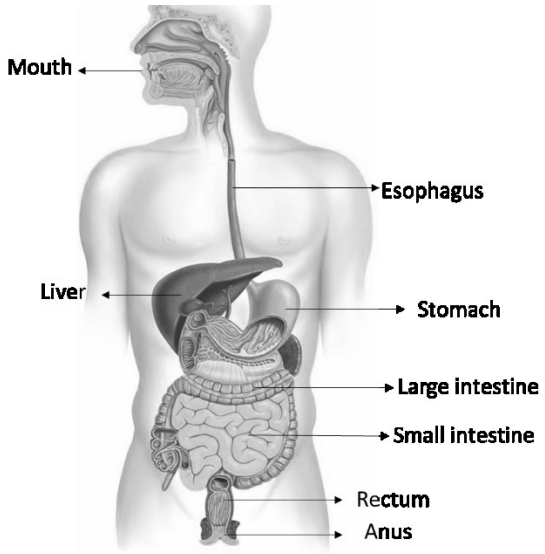


Figure 1.5. Human digestive system. Adapted from Nursey 101 (2018).

In the duodenum, the chyme mix with the alkaline pancreatic juice, and the gastric acid is neutralized by bicarbonate, so the duodenal pH is approximately 6-7 (Jackson and McLaughlin, 2009). The alkaline pancreatic secretions contains powerful enzymes that break down protein (trypsin, chymotrypsin); it also contains amylase and lipase, for carbohydrate and fat digestion, respectively, and enzymes that digest nucleic acids and phospholipids (McDonald and MacFarlane, 2018). The digestion of the liquefied food continues in the small intestine and it is divided in jejunum (ca. 40%) and ileum (60%) (Jackson and McLaughlin, 2009; McDonald and MacFarlane, 2018). However, most of the nutrient absorption occurs in the jejunum (McDonald and MacFarlane, 2018).

Thus, tracking the fate of food nutrients and antioxidants compounds in the digestive tract leads to better understanding of their bioavailability. In addition, epidemiological evidence suggests that diet and nutrition can improve the health and may have a significant effect in the prevention of serious diseases (Wootton-Beard et al., 2011). Nowadays, numerous studies have been conducted to illustrate the influence of digestion on the physicochemical and bioactivity properties of different food matrix and bioactive compounds, this reflect the interest devoted to this issue (Quirós et al., 2009; Rinaldi et al., 2015; Rodríguez-Roque et al., 2013; Wootton-Beard et al., 2011). Moreover, it is also known that the food processing (such as fermentation, grinding and mild heating) may improve bioavailability, probably due to the dissociation of the

nutrient-matrix complexes, disruption of the cell walls of plant tissues, or transformation into more active molecular structures (Parada and Aguilera, 2007). So, these factors may lead to the development of food with healthy benefits for consumers (Sayd et al., 2016).

1.10. Aquaculture

The global aquaculture sector has grown continuously over the last five decades at an average annual growth rate of 3.2 %, between 1961 and 2013. In addition, the aquaculture has been responsible for the impressive growth in the supply of fish for human consumption, in Figure 1.6, it is possible to observe that aquaculture production presents a steadily increase every year, while capture production has almost stabilized since the late 1980´s (FAO, 2016). In 2014, the world fisheries and aquaculture production were estimated 167.2 million tonnes, from which 93.4 million tonnes were from capture and 73.8 million tonnes from aquaculture. Being that 146.3 million tonnes were used for human consumption (FAO, 2016).

Nevertheless, the observed growth in the aquaculture sector also increased the aquafeed production and the requirement for high quality feeds. However, the expansion of aquaculture sector is limited by the availability of sustainable nutritive and inexpensive ingredients for aquafeeds. Many studies have reported the use of vegetable protein sources as ingredients in fish diets (Ngugi et al., 2017; Wang et al., 2006), but the selection of ingredients should be based, not only on nutrient level, digestibility and cost, but also in the need of being evaluated by other factors, such as sustainability and environmental impact of production, and fish-in fish-out ratio (Tacon et al., 2011). Thus, the current challenge for the development of sustainable aquaculture is to find alternative protein ingredients economically viable and with the lowest environmental impact (Gatlin III et al., 2007; Ngugi et al., 2017; Tacon et al., 2011).

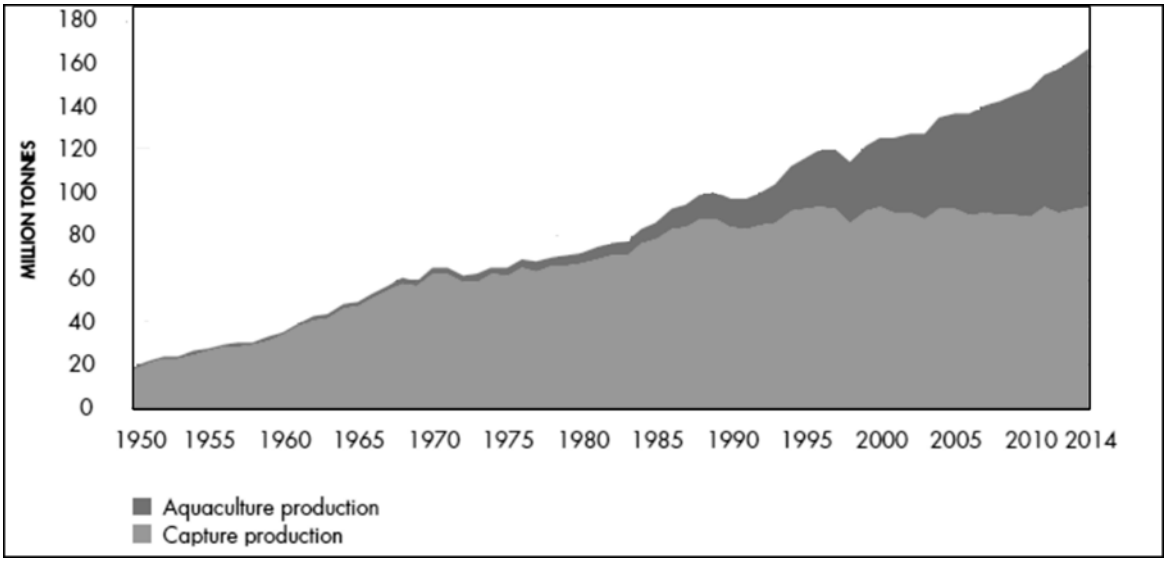


Figure 1.6 World capture fisheries and aquaculture production between 1950 and 2014. Adapted from FAO (2016).

In general, the aquaculture industry presents a great importance in meeting food and nutrition requirements of human population. Furthermore, fish has a better source of high quality protein, micronutrients and essential fatty acids, compared to other farm animals. In addition, the sustainable development of the aquaculture sector and its contribution to food security, is an important condition to meet future food demand from a world population (Nadarajah and Flaaten, 2017). On the other hand, the evaluation of the environmental impact of the aquaculture sector is extremely important and largely depend on aquafeeds. The reduction of waste in aquaculture could be achieved through improvement of feed formulation, which affect the palatability, nutrient digestibility and retention. Nonetheless, the use of plant ingredients in aquafeeds can affect the waste production in aquaculture, due the presence of indigestible components and antinutrients in plants (Kokou and Fountoulaki, 2018).

1.10.1. Plant nutrient sources for aquafeed

In aquaculture production, feeds have a high importance and present a huge part of costs. According to Sun et al. (2007), fish feeds to meet nutritional and energy needs, however the feed intake is negatively correlated with protein level and energy of feed in most aquatic animals. So, nutritionally balanced diets are essential for aquaculture development. In addition, with the nutritionally adequate diets, it is possible to obtain products with high end quality for

consumers. Moreover, in intensive aquaculture, the fish feeding represents half of the operating expenses, being the protein the most expensive dietary source (Pereira et al., 2012).

Fish meal is the protein source most used in aquafeeds, due to many reasons, as high protein content and nutrient digestibility, balanced amino acid profile, absence of antinutrients, availability and low price (until recently) (Gatlin III et al., 2007). On the other hand, most of the vegetable protein sources showed disadvantages compared to fish meal, as protein content, amino acid imbalances, low palatability, presence of antinutritional factors, and large amounts of carbohydrates (Gatlin III et al., 2007). In addition, plant protein meals often present a lower dry matter digestibility in relation to fish meal and, this fact contributes to an increase in waste production (Kokou and Fountoulaki, 2018). However, due the fish meal limitation for aquafeeds, due its decreasing availability and increasing price, it was crucial to replace it by more sustainable sources, as plant feedstuffs.

Several oilseeds, legumes and cereal grains have been used for protein or energy concentrates for fish feeding (Gatlin III et al., 2007; Hassaan et al., 2015; Kokou and Fountoulaki, 2018; Ngugi et al., 2017). Additionally, several studies are being conducted to improve the characteristics of plant protein in order to adequately respond to fish nutritional needs (Dossou et al., 2018; Hassaan et al., 2018, 2015; Vidal et al., 2015; Zaminhan et al., 2017). Nevertheless, the presence of high levels of antinutritional factors (ANFs) in plant source, can affect the availability, digestibility and retention of nutrient. Besides, the increase of the aquaculture production costs can also arises from environment problems, such as increased waste production from nutrients not retained in biomass and its fecal or non-fecal losses in the environment (Kokou and Fountoulaki, 2018). However, according Francis et al. (2001) the thermal processing can eliminate the heat labile antinutrients factors, such as protease and phytic acid inhibitors, improving plant nutritional value.

1.10.2 Use of by-product in aquaculture

With an estimated one-third of the edible parts of food produced for human consumption, gets lost or wasted globally, which generates about 1.3 billion ton per year (Jenny Gustavsson and Meybeck, 2011). In addition, the food by-products have been generated environmental problems, as they are often discarded in the environment and end up causing contamination.

Moreover, food by-products show a rich nutritional composition, and their sustainable use is important. In parallel, the aquaculture sector is looking for alternative ways to use industrial by-products in fish feed, making diets more efficient and less costly (Kokou and Fountoulaki, 2018). These factors combined with the continually growth of the aquaculture sector and need to adopt sustainable practices became key factors for remaining competitive and maintaining long-term profitability.

Plant by-products have been regarded as promising sources of protein for aquafeeds. Among the plant by-products with greatest potential for use in fish diets are the oilseeds. Previous studies have reported the use of by-product from soybean (Biswas et al., 2017; El-Saidy, 2011; Hassaan et al., 2015; Sun et al., 2007), sunflower (Hassaan et al., 2018) and rapeseed (Dossou et al., 2018) in fish diets.

Furthermore, in recent years, several processing techniques have been reported in attempts to improve the quality of plant by-products for utilization in aquafeeds, however some methods presented disadvantages, as protein loss, cost, commercial feasibility and environmental sustainability. However, other methods, as fermentation have shown several advantages, as nutritional quality, antioxidant properties and ant nutrients reduction (Dossou et al., 2018). Subsequently, studies showed that the use of fermented meal, as soybean (Hassaan et al., 2015), sunflower (Hassaan et al., 2018) and rapeseed (Dossou et al., 2018) was possible in aquafeed, without affecting growth performance and feed utilization. In general, the use of by-product as ingredient in aquafeed might be economic feasible, furthermore, some treatments or process used in by-products can improve the nutrition and properties of product, becomes the diet more digestible.

1.10.3. Fish digestibility

The nutritional value of a diet is not only related to the chemical composition of the ingredients, having direct relation with the amount of nutrient or energy to be absorbed or used by the animal organism. The chemical composition of a feed may give the impression that it is excellent as a source of nutrients but will be of little actual value unless it can be digested and absorbed in the gastrointestinal tract of the target species (Köprücü and Özdemir, 2005). Moreover, the fish species due to physiological and morphological characteristics have different

abilities to use the nutrients and energy contained in ingested food and the knowledge of ingredients digestibility coefficients is an factor important for the formulation of balanced diets with lower cost and environmental impact (Irvin and Tabrett, 2005). Digestibility tests are one of the best criteria used to assess the nutritional quality of any feedstuff and to measure the efficiency of complete diets. The methods for determination of the digestibility coefficients include the direct method, which involves the total collection of feces and measurement of consumed nutrient quantities and the indirect method in which the collection of feces is partial, using inert markers as reference (Choubert et al., 1979). The collection of fecal material in fish is a difficult process and can influence in the apparent digestibility coefficients of nutrients in aquafeed. Several methods have been used in aquaculture to collect feces for the determination of apparent digestibility, including manual stripping, filtration, suctioning of fecal material, intestinal dissection, settling columns (Irvin and Tabrett, 2005) and netting system (Choubert et al., 1979). In the netting method (Choubert system) the fecal matter is sieved continuously by screens present at the water outlet of tank. The collection of feces using Choubert system present an important advantage compared to other feces collection systems, due the brief contact of feces with water, so their composition is similar to that of feces at the moment of ejection (Choubert et al., 1982).

The indirect methods with inert markers are the most used in aquaculture, the use of markers is advantageous as it allows maintenance of the fish in normal culture conditions (Austreng, 1978). However, to be effective, markers must be indigestible, non-toxic to the fish, unaltered chemically, should move through the gut uniformly and not alter the passage of nutrients through the gastro-intestinal tract or the metabolism of the fish (Ward, et al., 2005). Previous studies related a variety of markers, however, the chromic oxide (Cr203) have been used in the majority of studies (Carter et al., 2003). The use of indirect determinations of ADC present a several advantages compared to other methods, such as the use of a large number of fish in the experiment and the fact that the experimental fish do not have to be killed to obtain results (Austreng, 1978). Thus, the apparent digestibility coefficients (ADC) are estimated by the difference in marker and nutrient concentration in feed and feces (NRC, 2011).

The evaluate of digestibility of single ingredients in aquatic animals is rarely possible, as fish do not accept these ingredients separately, in some cases the voluntary feed intake and nutrient utilization are reduced, due to nutrient unbalances (Booth et al., 2001). However,

ingredient digestibility can be obtained by the substitution method. In this method, the test ingredient replaces part of a reference diet to obtain the test diet, usually it is obtained using 30% of the test ingredient and 70% of the test diet. This method provides several advantages, once the difference of nutrient content among feed ingredients is reduced (Sugiura et al., 1998). Currently, this method has been widely used to study the apparent digestibility coefficients of different ingredients for the improvement or replacement of feedstuffs in different fish species (Booth et al., 2001; Campos et al., 2017; Dias et al., 2010; Köprücü and Özdemir, 2005; Pereira et al., 2012; Vidal et al., 2015).

1.10.4. General aspects of Tilapia

Tilapia is the *most* common finfish species farmed worldwide, although the significant worldwide distribution of tilapias has only occurred during the 1940s and 1950s, with primarily *Oreochromis mossambicus*. However, Nile tilapia (*Oreochromis niloticus*) farming dates back over 4000 years in Egyptian times, which showed the fish held in ornamental ponds (FAO, 2018). Total global tilapia production in 2015 was 5.67 million tonnes and is expected to reach 7.3 million tonnes by 2030 (FAO, 2015).

Nile tilapia is an herbivorous fish species that naturally feeds from natural fauna and flora. However, for an intensification of tilapia farming it is necessary to develop acceptable feeds for complete and supplementary feeding to assure fast growth (Köprücü and Özdemir, 2005). In addition, prepared feeds able to provide a complete diet (adequate protein, lipids, carbohydrates, vitamins and minerals levels) are already available in both developed or developing countries, which has an export market of tilapia products with high quality. However, these feeds are often too expensive to the developing countries that use manures and by-products to lower production costs of fish sold for domestic markets (FAO, 2018). Generally, the commercial production of tilapia requires the use of male monosex populations. According FAO (2018), the presence of female tilapia leads to uncontrolled reproduction, in addition, the males grow about twice faster than the females, causing a large size disparity among harvested fish and affecting marketability. The production cycle of Nile tilapia is shown in Figure 1.7.

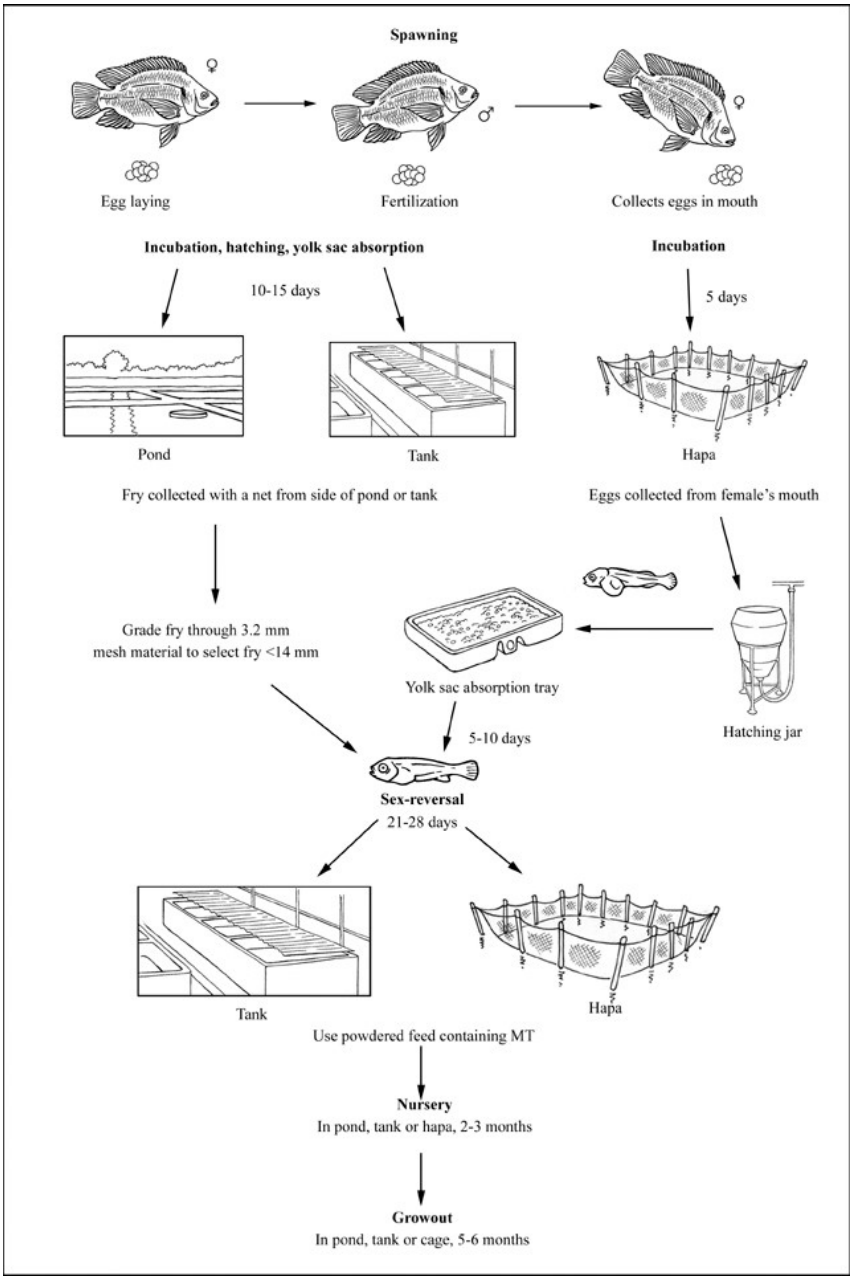


Figure 1.7. Production cycle of Nile tilapia (*Oreochromis niloticus*). (FAO, 2018)

Tilapia is considered one of the species that best adapts, within its limits of tolerance to different conditions of water quality, being also more resistance to diseases than other commonly cultured fish (Popma and Lovshin, 1995). Also present hardiness and adaptability to a wide range of culture system and is commercialized in more than 100 countries and will expand further in the coming years (FAO, 2018).

1.11. Objectives

Okara is the soybean residue that remains after the manufacture of soya beverage or soybean curd and large amounts of this by-product are produced annually worldwide. In addition, the high moisture content makes it very perishable, thus okara sometimes is used as animal feed but most is dumped and burned as waste. Nevertheless, okara still holds many nutrients, such as fibers, proteins, lipids, minerals and phytochemicals. So, considering the previous arguments, the general objective of this work was to develop new food and aquafeed added-value solutions from okara, through the application of thermal or biotechnological processes, in order to increase its stability and/or improve okara's biological properties. Two accomplish this, several specific goals were established:

i) select a stabilization process in order to increase of okara's storage time and understand the impact of each processing factor upon okara's characteristics.

ii) produce and characterize peptides extracts with improved biological properties through enzymatic hydrolysis of okara.

iii) produce fermented beverages, using okara previously hydrolyzed, with bioactive properties, and understand their potential as probiotics carrier

iv) understand the impact of gastrointestinal tract on stability of each bioactive component to understand their bioavailability.

v) evaluate the potential of okara meal obtained by different processes as an ingredient for tilapia diets.

Part II- Thermal treatment and stability of soybean by-product (okara)

CHAPTER 2- Impact of different thermal treatments and storage conditions on the stability of soybean by-product (okara)

2.1. Abstract

Okara is the by-product obtained from tofu or soymilk production process. It has a rich nutritional composition, especially in fibers, proteins and lipids. Stabilization processes are required to assure its efficient and safe use, because there are few studies on okara. The main objective of this study was to evaluate the chemical composition (protein, fiber, lipids, ash and isoflavones), microbiological stability, antioxidant capacity and antinutritional factors in fresh okara stored at 4 ºC and -18 ºC and dried okara (80 ºC/5 h and 200 ºC/1 h) stored at room temperature for 15 days. Okara showed a rich nutritional composition − ca. 35% of fiber, 30% of protein and 11% of lipids. The okara's lipid profile showed high and valuable level of PUFA (ca. 56 %) followed by MUFA (ca. 21%) and SFA (ca. 23%). The Fresh okara showed the highest antioxidant activity and total phenolics, however for isoflavones (genistin, genistein, daidzin, daidzein) the dried Okara at 200 ºC exhibited higher content compared to dried Okara at 80 ºC and fresh Okara. Okara samples submitted to thermal treatment showed a decrease in antinutritional factors in relation with fresh okara, whereas the treatment of dried okara at 200 ºC was the most efficient inducing a decrease of ca. 6 times. In conclusion, the nutritional richness of this by-product suggests okara as a valuable nutritional ingredient for further inclusion in food and feed, but considering the limited studies carried out to date, further studies are warranted to better stabilize okara.

2.2. Introduction

Soy is an oilseed crop that has been cultivated for over 3000 years in China and other Asian countries, being heavily consumed in Asia and other continents (Nishinari et al., 2014). Currently Brazil, Argentina and China are the largest soybeans producers, with Brazil occupying the first position with an overall grain production of 87.5 million tons in the 2013/2014 harvest (USDA, 2014). This oilseed has great commercial interest especially due to the soybean-derived

products namely soy oil, soy proteins and soymilk. The soymilk, in particular, has high economic value since it is used as a base in a wide variety of food products, including tofu, soy yogurt and cheese based on soybean (O´Toole, 1999; Van der Riet et al., 1989). Soymilk is obtained by aqueous extraction of whole soybeans using temperatures of 80-95 ºC for 10-45 min (Vishwanathan et al., 2011). During this processing, a by-product rich in fiber, protein and fat, known as okara is obtained (Van der Riet et al., 1989; O'Toole, 1999; Redondo-Cuenca et al., 2008; Mateos-Aparicio et al., 2010). In average, for each 1 kg of soybeans processed, ca. 1.1 kg of okara is produced (Khare et al., 1995).

The okara can be directly used in traditional dishes (tempeh), however, its large-scale industrial application is limited by the high humidity (ca. 80%) and great instability of this product. So, okara is frequently used in agriculture as a biofertilizer (O´Toole, 1999; Redondo-Cuenca et al., 2008) or simply discarded or incinerated causing severe environmental contamination. So, it is crucial to find a useful application for these by-products. Okara has high nutritional value especially due to its richness in protein and fiber, which is mainly rich in arabinose, galactose, xylose and galacturonic acid; it is also a good source of antioxidant compounds, especially isoflavones (Chan and Ma, 1999; O´Toole, 1999; Redondo-Cuenca et al., 2008). Moreover, diverse bioactivities have also been related to okara, namely hypocholesterolemic and hypolipidemic effects (Villanueva et al., 2011), as well as chronic diseases prevention such as type 2 diabetes by lowering glycemic index when ingested (Lu et al., 2013). Okara can hence be considered a feasible way of adding value to food and animal feed (Lu et al., 2013). According to He and Chen (2013) the okara can be used as an ingredient for making extruded snacks.

Okara high moisture content is a major factor limiting its large-scale use, being extremely important to find feasible stabilization solutions for easier and more efficient use of such by-product. Nevertheless, the impact of drying and storage conditions has been poorly investigated. Drying is the most frequent way used to stabilize okara, however, depending on the selected temperature, this process can also degrade valuable nutritional compounds and consequently reduces its commercial value (Jankowiak et al., 2014; Perussello et al., 2014). It is hence essential to evaluate okara stability during storage, either in fresh format and dried using different methods, with particular focus on the consequent impact on its final nutritional and functional value.

So, this study aimed to assess the impact of two storage conditions (4 and -18 ºC) and two thermal drying treatments (to reduce moisture content) at low temperature for long periods (80 ºC for 5 h) vs high temperatures for short periods (200 ºC for 1 h), on the chemical composition and microbiological quality of okara. The antioxidant capacity and related stability throughout storage was also evaluated. Results will help select the most appropriated and feasible treatment to preserve and stabilize the quality of this soybean by-product, for longer periods, promoting its further inclusion in dietary products.

2.3. Material and methods

2.3.1. Design of experiment of processing and storage of okara

The okara used in this study was kindly provided by a soymilk industry NUTRE (Aveiro, Portugal). The okara sample was collected and rapidly transported (max. 2 h) to the laboratory where it was either submitted to two thermal treatments, both samples were dried in an oven with air circulation (one at 200 ºC for 1 h and another at 80 ºC for 5 h) or simply stored fresh at low temperatures (4 and -18 ºC). Dried samples were further stored at room temperature (20 ºC). All samples were stored in plastic bags sealed for 15 days and sampling were performed at 0, 3, 8 and 15 days. The flowchart (Figure 2.1) represents the main conditions considered in experimental design.

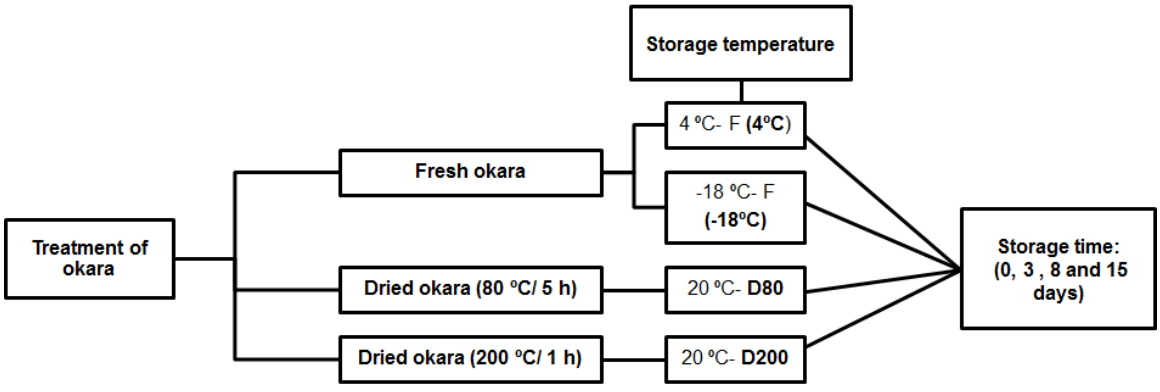


Figure 2.1. Flowchart of the experimental design including thermal processing and storage of okara throughout 15 days.

2.3.2. Analytical methods

Analyses were done on homogenized samples in triplicate. Results were recorded as the mean and standard deviation.

2.3.3. Proximate composition analysis

Moisture and ash of okara were determined by official methods of analysis (AOAC, 1995). The crude protein was determined using a nitrogen analyser (N × 6.25, Leco N analyzer, Model FP-528, Leco Corporation, St. Joseph, USA) and crude lipid content by petroleum ether extraction (Soxtec™ 2055 Fat Extraction System, Foss, Hilleroed, Denmark). The contents of total dietary fiber (TDF), insoluble dietary fiber (IDF) and soluble dietary fiber (SDF) in okara were determined by the method of Goering and Soest (1970).

2.3.4. Physical and chemical quality analysis

Thiobarbituric acid-reactive substances (TBA-RS) were assessed in okara using the method adapted by Rosmini et al. (1996). The incubation was performed under heating in water bath at 100 ºC for 35 min. Quantification was done in a Spectrophotometer (UV mini 1240, Shimadzu, Tokyo, Japan) at 532 nm. The 1,1,3,3 tetraethoxypropane was used as standard in the range 1x10-6 – 14x10-6 mol/L. Results were expressed as mg malondialdehyde/kg okara. Analysis was performed in triplicate.

The pH values of the okara were measured in triplicate with a pH meter equipped with a glass electrode (Basic 20- Crison). Certified buffers (pH 7.00 and pH 4.00) were used to calibrate the electrode. The acidity determination was carried out using the method of acid-soluble alcohol; 2.5 g of sample was weighed in an Erlenmeyer with lids and added 50 ml of ethanol (99.5%). The vials were then shaken and maintained for 24 h. After that time, 20 mL of supernatant were collected and submitted to titration with 0.01 M NaOH, until the pink phenolphthalein end-point. The blank was made using 20 ml of ethanol (Adolfo Lutz, 2008). The water activity (a_w) was measured in triplicate at 25 ºC in an Aqualab Series 3 (Dacagon Devices, Pullmam, USA).

2.3.5. Microbiological analysis

Analysis of *Listeria monocytogenes* and *Salmonella spp*. were made using VIDAS *Listeria monocytogenes* (LMO2) BIOMÉRIEUX (Marcy L'Étoile, France). To determine the positives the EN ISO 290-1 1996/AMD.1: 2004 for *Listeria monocytogenes* and EN ISO 6579-2002 for *Salmonella* method were used. For the other microbiological analysis 10 g of sample was homogenized in 100 ml sterile peptone water 0.1% (Peptone - Sigma Aldrich) on sterile bags for 2 min at 250 rpm with the aid of a stomacher (Lab Blender 400, London, UK). Then decimal dilutions were prepared using the sterile peptone water 0.1% and inoculated in a particular culture media for each group of microorganisms: total mesophilic bacteria were determined in PCA (Plate Count Agar diagnostics provided by Biokar) incubated aerobically at 30 °C for 48 h. Yeasts and molds were counted in PDA (Potato Dextrose Agar provided by Biokar diagnostics), incubated aerobically at 30 °C for 5 d and *Bacillus cereus* in BCA (*Bacillus Cereus* Agar supplied by Biokar Diagnostics, Portugal with addition of polymyxin B and egg yolk) incubated aerobically at 30 °C for 12-30 h. Inoculation was performed using the drop method as described by Miles and Misra (1938), using 20 µL. *Enterobacteriaceae* was determined on Violet Red Bile Glucose Agar (VRBGA, Oxoid, Basingstoke, United Kingdom) using the pour plate method and were incubated aerobically at 37 °C for 24 h.

2.3.6. Extracts preparation

The extracts were made at a concentration of 10% (weight/volume) using methanol: water (80:20) as solvent and the extraction was performed in an orbital shaker (SI-100C, Wiggenhauser, Germany) at 10 ºC, 150 rpm for 1 h. The extracts were then centrifuged at 1677 x g, at 4 ºC for 30 min and filtrated by filter paper Whatman nº 1. The extracts were stored at -80 ºC until further analysis. For the analysis of isoflavones the supernatant was filtered by a 0.45 µm polytetrafluoroethylene (PTFE) membrane.

2.3.7. Scavenging activity (ABTS)

The scavenging activity was measured in the methanolic extracts using ABTS radical according the method described by Gião et al. (2007). The quantification was performed at 734 nm (mini UV 1240, Shimadzu, Tokyo, Japan) using 50 µl of samples in 1 ml ABTS with absorbance of 0.7 (±0.02). Trolox was used as a standard to prepare a calibration curve in the range 0.01 - 0.12 mg/ml. The results were expressed in as milligrams of Trolox per 100 grams of dried okara.

2.3.8. Scavenging activity (DPPH)

The scavenging activity of DPPH (2.2- Diphenyl-1-picrylhydrazyl) radicals was measured in the methanolic extracts, according the method described by Bondet et al. (1997). Quantification was done in a spectrophotometer (mini UV 1240, Shimadzu, Tokyo, Japan) at 515 nm. Briefly, 0.5 ml of the sample extract was added to 3.5 ml methanol solution of DPPH (60 µM). Trolox was used as a standard to prepare a calibration curve in the range 0.005 - 0.08 mg/ml. The results were expressed in as milligrams of Trolox per 100 grams of dried okara.

2.3.9. Determination of total phenolics content

The concentration of total phenolic compounds was determined calorimetrically in the methanolic extracts by the Folin-Ciocalteu method (Singleton and Rossi, 1965) and the quantification was done at 750 nm (UV mini 1240, Shimadzu, Tokyo, Japan). Total phenolic content in each sample was determined using a standard curve prepared by gallic acid in the range 0.025 - 0.5 mg/ mL. The results were expressed in as milligrams of gallic acid per 100 grams of dried okara.

2.3.10. Microstructural analysis

The morphology of okara samples was evaluated by Scanning Electron Microscopy (SEM) using a JEOL-5600 Lv microscope (Tokyo, Japan). Briefly, a small amount of okara was

placed on metallic stubs with carbon tape and was dried in a desiccator during 24 h. Subsequently, samples were coated with gold/palladium using a Sputter Coater (Polaron, Bad Schwalbach, Germany). SEM was operated at the high vacuum mode, using a spot size of 36–37 and a potential of 10–15 kV. All analyses were performed at room temperature (20 °C). The visualization was made using a magnification of 250 times.

2.3.11. Analysis of fatty acids

For the total fatty acid (FA) analysis, 200 mg of sample were accurately weighed and prepared according to Castro-Gomez et al. (2014). For quantitation purposes samples were added 100 µL of glyceryl tritridecanoate (1.28 mg/mL) prior to derivatization. FAME were analyzed in a gas chromatograph HP6890A (Hewlett-Packard, Avondale, PA, USA), equipped with a flame-ionization detector (GLC-FID) and a BPX70 capillary column (50m x 0.32 mm x 0.25 µm; SGE Europe Ltd, Courtaboeuf, France) according to the conditions described by Vingering and Ledoux (2009). Supelco 37 and CRM-164 were used for identification of fatty acids. GLC-Nestlé36 was assayed for calculation of response factors and detection and quantification limits (LOD: 0.15 µg/mL in the FAME extract; LOQ: 0.46 µg/mL in the FAME extract).

2.3.12. Trypsin inhibitor activity

The trypsin inhibitor activity was measured in okara using a method adapted from Kakade et al. (1974)with slight modifications. So, 1.00 g of okara was weighed and defatted with 50 mL of 0.01 M NaOH under slowly agitation, for 3 h at room temperature. At the end of this time period, the solution was centrifuged for 20 min at 1800 x g and the supernatant was analyzed. Two conditions were measured: control (trypsin activity without inhibitor) and sample (trypsin activity in the presence of inhibitor). Both measurements were made against a blank with the substrate, prepared with Tris buffer pH 8.20, 0.050 M and BAPNA (1.3 mL). The results are expressed in trypsin inhibitor units (TIU) per gram of dry okara, according to the Equation 2.1:

$$TIU/g = \frac{100 \times 10\left(m_{Control} - m_{Sample}\right)}{0.020 \times 4} D \times 50$$

Equation (2.1)

Where 100 is the factor to convert 0.01 u. Abs in TIU units; $m_{Control}$-m_{Sample}, the difference between the slopes of progress curves in absence and presence of TI respectively; D, the dilution factor of supernatant, calculated as the final volume divided by the amount of aliquot taken to dilute the extract; 50 is the extraction volume (mL) of 0.01 M NaOH used per gram of soybean flour; 0.020, aliquot (mL) used in the current assay, 10, the final reaction time in discontinuous method, and 4, factor of decrease in the final reaction volume from 10 to 2.5 mL.

2.3.13. Analysis of isoflavones

Isoflavones were analyzed using an adaptation of the method described by Wang and Murphy (1996). Briefly, twenty microliters of each extract (obtained as explained in 2.3.6.) were analyzed by HPLC (Waters Series 2695, Mildford, MA, USA) coupled to a diode array detector, using a Kromasil C-18 column (250 x 4.6 mm) to separate the isoflavones at 25 ºC. The mobile phase consisted of 0.1% acetic acid in water (v/v) (solvent A) and 0.1% acetic acid in acetonitrile (v/v) (solvent B). Elution started with 20% B, and then increased to 100% in 20 min and to 20% in the next 12 min. The flow rate was 0.8 ml/min. Spectral data from peaks were accumulated in the 200–400 nm range. The data were processed on Empower 3 software. Isoflavones quantification was achieved by measuring the absorbance recorded in the chromatograms at 255 nm relative to external standards. The standards were dissolved in analytical grade DMSO (Sigma-Aldrich, St Quentin Fallavier, France) and diluted in methanol. Concentrations of the calibration curve were: 0.7- 40 µg/mL (Genistin), 0.23 - 13 µg/mL (Genistein), 1.4 – 80 µg/mL (Daidzin) and 0.3 – 16.7 µg/mL (Daidzein) (Sigma-Aldrich, St. Louis, USA). This procedure was performed in triplicate.

2.3.14. Statistical analysis

Statistical analysis was performed using GraphPad Prism version 5.00 for Windows. Statistical differences were assessed by a Two-Way analysis of variance with thermal treatment and storage time as main factors. When a significant effect was noted within a factor, a

Bonferroni post hoc test was applied. In all cases, significant differences were considered when $P < 0.05$.

2.4. Results and discussion

2.4.1. Proximate composition analysis

The composition of the okara dried at 80 ºC for 5 h (D80) and 200 ºC for 1 h (D200) and stored at 20 ºC, and of fresh okara stored at (F (4 ºC) and (-18 ºC)) during 15 d are presented in Table 2.1. This by-product has a rich chemical composition mainly fibers and proteins, also including a high content in polyunsaturated fatty acids. As the proteins are of high nutritional value, okara may be considered a potential source of vegetable protein for human consumption (Ma et al., 1997). However, during soymilk manufacturing, this by-product is submitted to a severe heat treatment which causes protein denaturation, resulting in a low solubility okara protein isolate, which restricts its direct use in food (Chan and Ma, 1999). Chemical composition of fresh okara samples was general similar to that reported by Ma et al. (1997) and Van der Riet et al. (1989) in terms of protein (29.43% ± 0.42), ash (3.87% ± 0.06), lipids (11.65% ± 0.04) and moisture (80.2% ± 0.03) content. The moisture of okara samples submitted to thermal treatments was significantly reduced to 48.19% ± 0.23 and 44.55% ± 0.45, respectively for samples dried at 80 ºC - D80 and at 200 ºC - D200, compared to the fresh ones; those values were maintained over 15 days with slight but significant variation ($P < 0.05$) between treatments (Table 2.1).

As can be seen in Table 2.1 dietary fibers are the main constituent of the okara. Several healthy benefits have been attributed to the fibers, namely its cholesterol reduction capacity (Bedani et al., 2014a), reduction of glycemic levels in diabetic subjects (Lu et al., 2013), improvement of the immune system and prevention of colon cancer (Repo-Carrasco-Valencia et al., 2009) leading to increasingly fiber intake among consumers. The present results showed that the total fibers are generally present in great amounts in okara, irrespectively of the thermal treatment and storage conditions. In fact, non-significant differences ($P > 0.05$) were observed between the treatments applied before storage, with small fiber reductions in heat treated okara (34.67 ± 2.91 to 34.14 ± 0.88 and 33.43 ± 1.63 in D80 and D200). It was also noted that with

the heat treatment, the amount of total fibers decreased and the D200 showed the lowest content. This may be due to fiber solubilization, breakage of the insoluble fibers or even formation of reducing sugars due to the temperature used during drying (Repo-Carrasco-Valencia et al., 2009). Previous studies reported higher values (41.93% ± 1.70) for insoluble fiber and lower values (13.90% ± 1.12) than those obtained in this study for soluble fiber in freeze-dried okara, in three different soybean cultivars (Van der Riet et al., 1989). Already for insoluble fiber O'Toole (1999) found values between 40.2 to 43.6% for okara obtained from different species of soybeans, whereas Kuan and Liong (2008b) found 49.81% of insoluble fiber in okara. These differences in chemical composition may be due to different agronomic practices, genotype and local environmental conditions of the soybean productions (Boydak et al., 2002; Tyug et al., 2010). Moreover, the industrial conditions selected to generate the soymilk may also have contributed to different results, since higher temperature for longer time may promote solubilization of fiber, increasing soluble fraction (Margareta and Nyman, 2003).

The insoluble dietary fibers are mainly derived from structural parts of plants, such as cell walls, which include cellulose, hemicellulose and lignin (Grosvenor and Smolin, 2002). Several studies have previously reported the ability of these fibers to absorb water and act as bulk agent, thus shortening the transit time through the intestinal tract (Periago et al., 1995).

Table 2.1. Water activity and chemical composition (% dry matter) of okara submitted to distinct treatments (fresh, F (4 ºC and -18 ºC), or dried at 80 ºC (D80) or 200 ºC (D200), before (T0) and after 15 days (T15) of storage at different temperatures.

Storage time	Sample treatment	Lipids (% DM)	Proteins (% DM)	Ash (% DM)	Total fiber (% DM)	Insoluble fiber (% DM)	Moisture (%)	Water activity (a_w)
T0	F (4ºC) and F (-18 ºC)	11.65 ± 0.04^{aA}	29.43 ± 0.42^{aA}	3.87 ± 0.06^{aA}	34.67 ± 2.91^{aA}	17.79 ± 0.61^{aA}	80.17 ± 0.03^{aA}	0.997 ± 0,001^{aA}
	D80	10.92 ± 0.15^{aB}	28.14 ± 0.21^{aB}	3.53 ± 0.22^{aB}	34.14 ± 0.88^{aA}	20.60 ± 0.64^{aB}	48.19 ± 0.23^{aB}	0.951 ± 0.002^{aB}
	D200	10.85 ± 0.11^{aB}	27.59± 0.26^{aB}	3.41 ± 0.52^{aB}	33.43± 1.63^{aA}	22.97± 1.21^{aC}	44,55 ± 0.45^{aC}	0.937 ± 0.003^{aC}
T15d	F (4 ºC)	13.05 ± 0.13^{bA}	31.25 ± 0.06^{bA}	4.00± 0.33^{aA}	32.90 ± 2.18^{aA}	23.12 ± 1.40^{bA}	81.67 ± 0.19^{bA}	0.995 ± 0.001^{aA}
	F (-18 ºC)	11.49 ± 0.17^{aB}	28.94 ± 0.08^{aB}	3.47 ± 0.42^{aB}	35.69 ± 2.40^{aA}	21.96 ± 1.06^{bA}	80.00 ± 0.11^{aB}	0.996 ± 0,001^{aA}
	D80 (20 ºC)	11.71 ± 0.09^{bC}	29.62± 0.03^{bC}	3.14 ± 0.18^{aC}	31.96 ± 1.02^{aA}	22.36 ± 0.31^{aA}	48.08± 0.60^{bC}	0.939 ± 0.004^{bB}
	D200 (20 ºC)	13.37 ± 0.02^{bD}	29.76 ± 0.27^{bC}	3.61 ± 0.79^{aD}	32.77 ± 0.69^{aA}	22.00 ± 0.60^{aA}	46.74 ± 0.85^{bD}	0.913 ± 0.002^{bC}

Results are expressed as mean ± standard deviation; lipids, protein (n=2); ash, moisture, neutral and acid fiber (n=3). F (4 ºC): fresh okara stored at 4 ºC, F(-18 ºC): Fresh okara stored at -18 ºC, D80:dried okara at 80 ºC for 5h; D200: dried okara at 200 ºC for 1 h. Different lowercase letters (difference between times) and uppercase letters (difference between treatments) in the same column of the group are statistically different ($P < 0.05$).

The total fibers remained stable over the 15 days of storage as there were no significant differences ($P > 0.05$) in its content compared to initial values. In the case of insoluble fiber content, significant differences ($P < 0.05$) can be observed between different treatments (Table 2.1). At initial time (T0), the amount of insoluble fiber increased concomitantly with increasing drying temperature. Higher temperatures may break bonds of the polysaccharide chains in the cell wall and more small particles can be released (Repo-Carrasco-Valencia et al., 2009). Yet, during storage it can be seen that the samples submitted to heat treatment D80 and D200 remained stable over the 15 days of storage, while for refrigerated and frozen samples, there was a small fiber increase ($P < 0.05$) reaching 23.12 ± 1.40 and 21.96 ± 1.06% of insoluble fiber, in samples stored at 4 and -18 ºC, respectively. This is a direct consequence of the high stress induced by cooling and freezing plant tissue affecting the cell structure, mainly causing the damage or break of cell walls and the release of components influencing the final composition of food (Paciulli et al., 2014). Moreover, after 15 days at 4 ºC there were already visible signs of okara degradation, which may have driven the greater solubility of the fibers. Similarly, for storage at -18 ºC the insoluble fibers also increased after storage. However, the relationship between the increase of the insoluble fibers and cooling is still not well understood, considering the very few studies about stability okara, but merits further evaluation.

In general, the treatment F (-18 ºC) induced the smallest soluble variations, preserving the original characteristics of the okara. Previous studies reported several benefits associated with soluble fiber which are able to form a soft surface layer on the small intestine mucosa and form a barrier to the absorption of some nutrients. This tends to delay the metabolism of sugars and fats, thus controlling of the glycemic levels and decreasing total cholesterol and LDL rates (Bedani et al., 2014a; Gunness and Gidley, 2010; Lu et al., 2013). It is possible to observe that heat treatments affected okara composition. The application of heat treatment on fresh okara led to small but significant reductions ($P < 0.05$) in lipid content, from 11.65 to 10.92 and 10.85 %, in D80 and D200, respectively. Grizotto (2011) also observed a reduction in the oil content for the okara after drying in at 300 ºC over 4.75 min in a spray drier. The lipid content of the heat treated okara were similar to those found by Van der Riet et al. (1989) that reported values between 9.3-10.9, in okara from different variants of soybeans, however fresh okara showed higher values (11.65%).

Concerning the storage conditions, there was a significant ($P < 0.05$) increase in the lipid content of all samples after 15 days, with the exception of fresh okara stored -18 ºC (F

(-18 ºC)) that remained constant. The highest lipid increase was observed with the D200. These variations may be related to the drying temperature used, given that the D200 showed the greatest lipid decrease prior storage and also the greatest variation with storage time. This might be related to fat oxidation, however, as studies on okara are very scarce, it is still difficult to completely understand the modifications of total fat over time.

Fresh okara has a high protein content (29.43 ± 0.42 (%)). The application of a heat treatment led to a slight protein decrease to 28.14 and 27.59, in D80 and D200, respectively. These values were similar to those found in other studies (Ma et al., 1997; O´Toole, 1999; Van der Riet et al., 1989; Villanueva et al., 2011). Okara ash showed no differences among treatments prior storage (Table 2.1), whereas the heat treatment reduced okara ash from 3.87 ± 0.06 to 3.53 ± 0.22 and 3.41 ± 0.52, D80 and D200, respectively. In relation to ash content during storage time, all samples remained fairly constant after the 15 days of storage, but significant differences ($P < 0.05$) were observed among treatments. The values found in this study correspond well to previous studies by Lu et al. (2013).and by Van der Riet et al.(1989) reporting values of 3.85% for dry okara flour and 3-3.7% in okara from different soybean species, respectively. According to Mateos-Aparicio et al.(2010b) the main microelement in okara is iron (ca. 1.20%), but usually its bioavailability is low.

Water activity (a_w) defines the free water (water not bound to food molecules) in a product (Vesterlund et al., 2012). According to Maltini et al.(2003), a_w is important for the quality and stability of the total amount of water present in food. It is also known that water activity is related to the chemical and enzymatic reactions in the food, besides being a determinant factor for microbial growth. In general, food has an a_w level varying from 0.2 in very dry foods to 0.99 in moist fresh foods. As can be seen in Table 2.1, okara samples moisture was reduced from 80.17 to 48.19% and 44.55% in samples D80 and D200, respectively. Accordingly, water activity of the fresh okara decreased from 0.997 ± 0.001 to 0.951 ± 0.002 and 0.937 ± 0.003, in D80 and D200, respectively. The still high water activity is related to the moisture content of the samples that was fixed around 50%. This means that not all water has been removed during the drying process, especially remaining that more strongly connected.

All samples were kept in sealed packaging over the 15 days of storage. The samples of fresh okara, refrigerated F (4 ºC) and frozen F (-18 ºC), had a constant water activity throughout storage time and, as expected, showed no significant differences between treatments ($P > 0.05$). However, water activity showed small reductions in samples D80 and

D200 ($P < 0.05$) compared to the initial time, that were more pronounced in D200 samples probably due to ambient relative humidity. In literature there are no studies on a_w in okara, therefore the okara drying conditions should be further studied since the a_w of the dried samples was still high, thus promoting the microbial growth.

2.4.2. pH, acidity and lipid oxidation (TBARS)

The pH, acidity and lipid oxidation changes in the different thermal treatment and storage conditions are shown in Fig. 2.2. The initial pH of fresh (F (4 ºC) and F(-18 ºC)) and heat treated (D80 and D200) okara was 7.1, which is a suitable pH for most bacteria growth. According to Coton and Leguerinel (2014) at this pH, the following pathogenic bacteria, with an optimal pH around 7.0, may be present in food: *Bacillus cereus, Listeria monocytogenes, Staphylococcus aureus, Clostridium perfringens and Enterohemorrhagic Escherichia coli*. With storage time, pH did not significantly decrease until 8 days of storage for D80, and until 3 days of storage for F (4 ºC) and D200, and decreased significantly until the end of storage period. This reduction may be associated with fermentation of the present carbohydrates (Cai et al., 2014). There was a slight pH increase in okara samples stored at -18 ºC (Fig. 2.2b) that can be related with the release of amino acids due to cell walls disruption during freezing. It is well known that slow freezing can result in cell rupture, due to the size of the crystals formed (Lili et al., 2013). These pH changes are commonly reported, especially when there is an initial microbial charge and storage occurs at refrigerated, and particularly at room temperature, and are usually associated with the metabolic activity of microorganisms present in the matrix (Maltini et al., 2003). In D80 and D200, although there was a moisture reduction (ca. 32 - 36%), the moisture and water activity values did not restrict the microbial growth at room temperature, leading to an increasing acidification due to microbial growth and consequent metabolism. After 8 days of storage, the D200 has remained constant, while the D80 and F (4 ºC) still showed a significant decrease in pH.

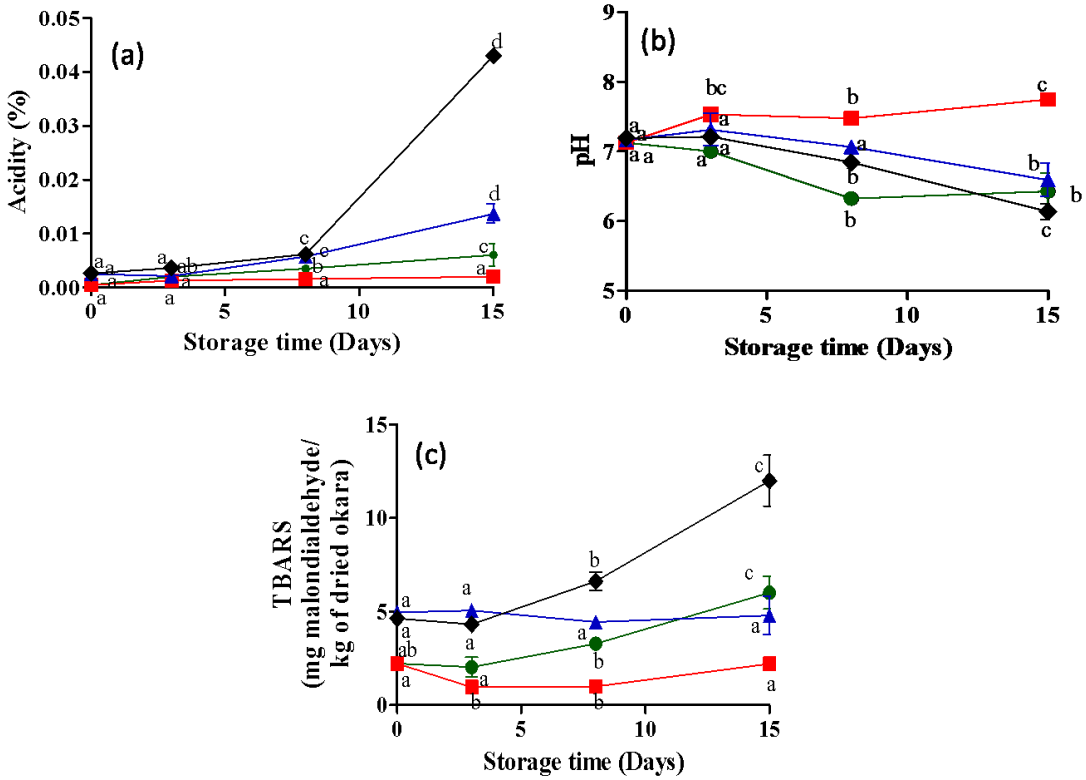


Figure 2.2. Profiles of acidity (a), pH (b) and TBA-RS (c) throughout storage time (-●- F (4 ºC); -■- F (-18 ºC), -▲- D80 (20 ºC), -♦- D200 (20 ºC)).

Regarding the storage time, the drying temperature interferes with sample acidity, as can be seen in Fig. 2.2a. The D200, with 15 days of storage, showed the largest increase, about 17 times higher than at baseline. For F (-18 ºC), there was no significant difference ($P < 0.05$) in acidity during the storage time as expected. On the other hand, for the F (4 ºC) and D80 a significant increase occurred, with D80 showing the highest increase since it was stored at ambient temperature.

Acidity differences ($P < 0.05$) were observed between the fresh okara and heat treated. Based on the foregoing, it is evident that the heat treatment temperature used can increase the acidity by promoting a series of oxidation reactions. For the okara F (4 ºC), it was possible to observe a significant increase of acidity during storage, while F (-18 ºC) remained stable until the end of the storage. For D80 sample the acidity increase was gradual and significant ($P < 0.05$) over time and doubled between 8 and 15th day of storage. The D200 sample presented a similar behavior until the 8th day, but 15 days after storage showed a sharp increase in acidity, reaching an almost 10 times higher value than that found 8 days

after storage. There are no previous studies on the titratable acidity of okara. The influence of the heat treatment in the okara was also evaluated by the analysis of TBARS, a sensitive method to quantify the malondialdehyde (MDA), which is one of the main decomposition products of polyunsaturated fatty acid hydroperoxides formed during the oxidative process. For D200, the increase in TBARS over time was significantly higher ($P < 0.05$) than that observed for D80. Similar results were reported by Wachiraphansakul and Devahastin (2007) in fresh okara samples compared to heat-treated ones. For F (4 ºC) at the end of storage time TBARS exceeded the oxidation levels acceptable for food products, which according to Cadun et al.(2005) should be less than 5.0 mg of malondialdehyde/kg of wet sample. Interestingly, although there was a large oxidation in the last storage stages for D200, the oxidation level in D80 was low and did not change up to 15 days of storage at room temperature. These results show that drying at low temperatures may assure a better product stability by limiting the oxidation processes. In previous studies Lee and Yoon (2013) and Wachiraphansakul and Devahastin (2007) also observed increased lipid oxidation with increasing heating time and temperature, in commercial soybean oil and soybean powder. This increase in lipid oxidation is associated to thermal treatment at high temperatures, where the decomposition of hydroperoxides may occur. Besides, autoxidation still continues to occur due to free radicals (peroxides, hydroperoxides) formed by reaction of free fatty acids with the oxygen present in air.

2.4.3. Microbiological analysis

The results for *Salmonella* and *Listeria monocytogenes* (<1.0 x 10^{-1}/g) were negative in fresh okara. The evolution of bacterial counts during the 15 days of storage are reported in Fig. 2.3. As a result of okara heat treatment a decrease of *Enterobactereacea* (Fig. 2.3a) was observed, in the beginning, from 6.5 in fresh okara to 3.1 and 2.9-log, in D80 and D200, respectively However, *Enterobactereacea* increased during storage in the heat-treated samples D80 and D200. At the end of the 15 days, a significant *Enterobactereacea* increase of 6 and 4.4 log cycles, respectively ($P < 0.05$) was observed in those samples, compared to the fresh okara at initial time. In F (-18 ºC) a decrease of 2.5 log cycles was observed compared to initial fresh okara.

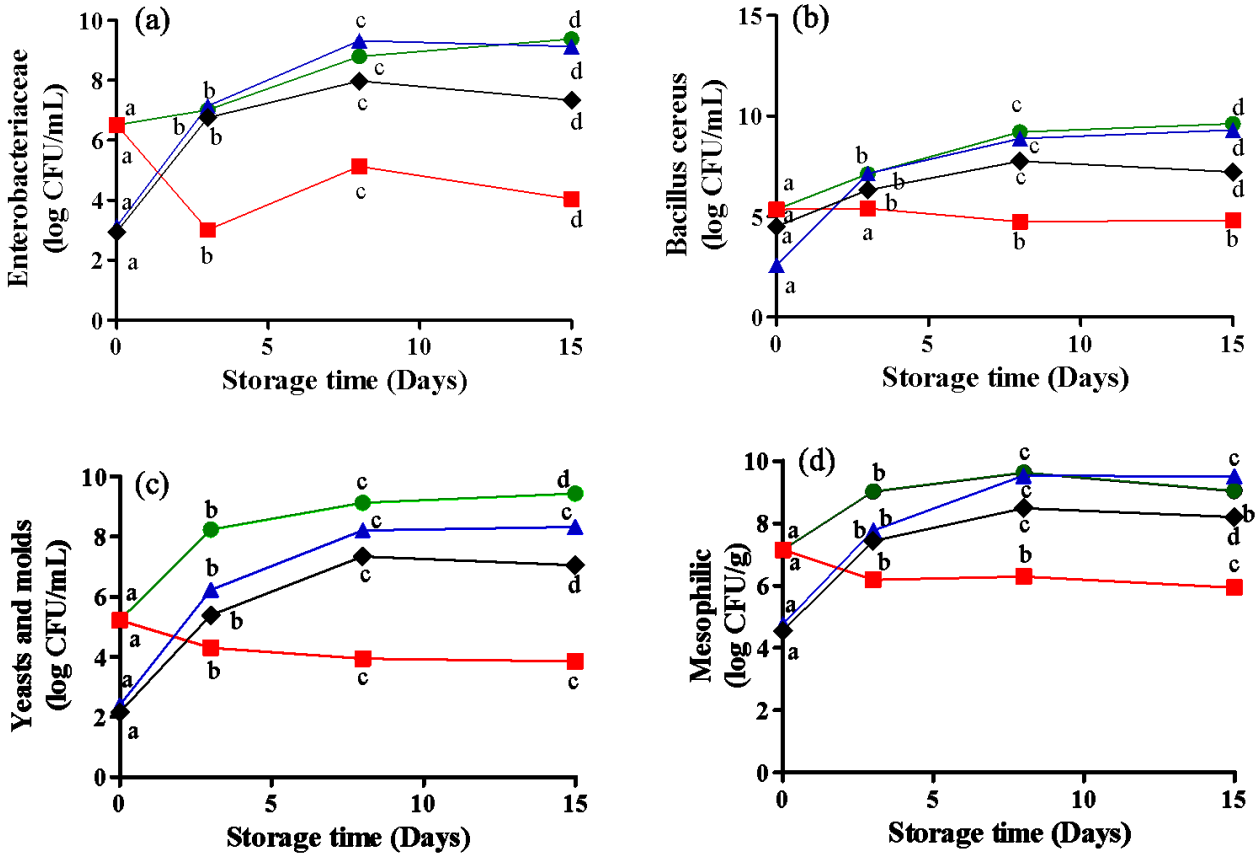


Figure 2.3. Microbiological analyses Enterobactereacea (a), Bacillus cereus (b), mesophilic (c) and molds and yeasts (d), of okara throughout storage time (-●- F (4 ºC); -■- F (-18 ºC), -▲- D80 (20 ºC), -♦- D200 (20 ºC)).

Regarding *Bacillus cereus* counts (Fig. 2.3b) it was observed that D80 had the greatest decrease (2 log cycles) at initial time, but increased throughout storage time. The initial microbial reduction in D200 sample was lower than that observed in D80, tendency maintained throughout time. This is due to the fact that the heat treatment intensity differently affects the spores. Moderate heat treatments (65 ºC and 75 ºC) can activate the germination of *Bacillus cereus*, whereas the most severe heating conditions can inactivate or damage its spores (Warda et al., 2015). Thus, all treatments, with the exception of F (-18 ºC), showed increasing *Bacillus cereus* ($P < 0.05$) levels throughout storage time. The high levels of *Bacillus cereus* in food indicates a potential risk to consumers, because *Bacillus cereus* can produce enterotoxins that cause food poisoning with low infectious dose. Han et al. (2001) have previously detected *Bacillus cereus* counts in a fermented food soybean product, the Sufu.

The mesophilic bacteria count (Fig. 2.3d) were similar to those registered for *Bacillus cereus*, because these were also growing in the same media and conditions. Whereas the heat treatment promoted reduction ($P < 0.05$) of 1.5 log cycles at baseline, counts of D200 were lower compared to D80, and F (4 ºC).

Yeasts and molds were detected at levels of 5.22 log CFU/g in the fresh okara. However, at the end of storage F (4 ºC) their levels reached 9.44 log CFU/g, while for F (-18 ºC) these values decreased to 3.22 log CFU/g. Also, for the dried samples, at the end of storage, D80 and D200 reached the highest values, 8.33 and 7.05 log CFU/g, respectively.

Although the heat treatment reduced the initial microbial contamination of all samples, throughout storage, the final counts were still relevant due to the significant moisture content in the dried samples. It is hence important to further decrease water to better stabilize the microbiological growth. Comparing storage temperatures, the storage under freezing F (-18 ºC) showed the best stability, however the initial microbial counts were still maintained. In the literature there are not results concerning microbiological quality of okara, so that more studies are still necessary.

2.4.4. Determination of the antioxidant activity and total phenolics

The antioxidant activity (ABTS and DPPH) and total phenolics of okara extracts over the 15 days of storage are depicted in Figure 2.4.

Concerning total phenolics, it can be seen that at the initial time the thermally treated samples (D80 and D200) showed a significant reduction ($P < 0.05$) compared with fresh okara. However, the samples dried at 80 ºC remained constant during storage, whereas D200, showed a significant increase ($P < 0.05$), from 52.09 to 237.07 (mg gallic acid/100g of dried okara) between 8 and 15 days. This was probably due to the formation of compounds from the Maillard reaction that can be quantified by the Folin-Ciocalteu method. Palermo et al. (2012) suggested that the amount of dietary fiber present in the okara (ca. 35 %) could be responsible for the Maillard reaction in cookies prepared with this by-product, as insoluble fibers can reduce the water activity during the cooking process and increase the compounds formed in the Maillard reaction.

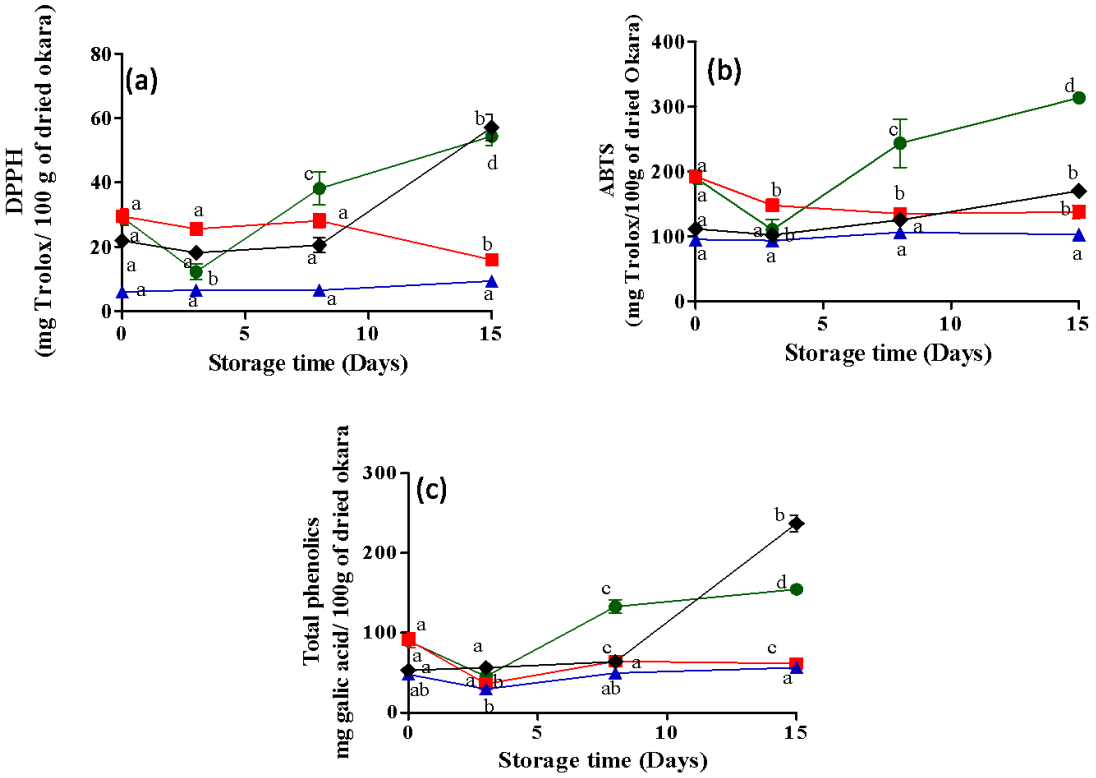


Figure 2.4. Antioxidant activity ABTS (a) and DPPH (b) and total phenolics (c) over storage time (-●- F (4 ºC); -■- F (-18 ºC), -▲- D80 (20 ºC), -♦- D200 (20 ºC)).

For F (4 ºC) there was a reduction during the first storage days, but from day 8 onwards these values were significantly ($P < 0.05$) increased reaching 153.56 mg gallic acid/100g of dried okara. In a study by Barbosa et al. (2006) the concentration of total phenols in soybeans were similar to the presently found in the okara sample stored under refrigeration or frozen, without heat treatment.

The antioxidant activity decreased with the heat treatment. In D80 it remained low during storage, but in D200 there was an increase, especially after 8 days, ($P < 0.05$) for both tests (DPPH and ABTS). A similar behavior was observed in fresh okara stored at 4 ºC that showed a significant decrease ($P < 0.05$) until 3 days of storage, followed by a double-fold increase after 8 days and a 3-fold increase after 15 days of storage. But compared to the D200, the antioxidant activity of the samples refrigerated were still higher, differing from results previously reported by Niamnuy et al. (2011) that observed an increase in free radicals capacity in dry soybeans compared to crude soy. In this study it was possible to

relate the antioxidant capacity of okara with its isoflavone contents; the effects of drying methods and temperature on the β-glycosides and soy antioxidant activity followed the very same trend Lee and Yoon, (2013) have also reported that the β-glycosides have about three times greater antioxidant activity when compared to malonyl-β-compound glycosides and aglycones. Moreover, according to Ruiz-Larrea et al. (1997) the antioxidant capacity of isoflavones follows this order: genistein> daidzein> genistin> daidzin, or it is associated with the number of free hydroxyls of the aromatic ring. The increased antioxidant capacity found were lower for DPPH values than for ABTS values, probably due to the lower steric accessibility of these radicals when the active sites are in the middle of the structure; thus, the antioxidants can access the sites of radical DPPH to a lesser extent (Suvarnakuta et al., 2011)

The F (-18 ºC) antioxidant activity varied between DPPH and ABTS tests. A significant decrease in ABTS was registered by 3 days of storage and remained constant thereafter. In the case of DPPH, the antioxidant activity remained fairly constant over the 8 days of storage, while a decrease was observed between 8 and 15 days. D80 also showed lower values ($P < 0.05$) compared with fresh frozen okara (F (-18 ºC)), so the heat treatment promoted initial degradation of some antioxidants.

It is also worth mentioning that the antioxidant activity determined by DPPH radical capture method resulted in lower values than those obtained for the ABTS method, which may be due to the fact that some antioxidants capture faster the ABTS then the DPPH radicals.

2.4.5. Isoflavones

Isoflavones are important secondary metabolites present in soybeans. In okara samples four main isoflavones were identified (Table 2.2), including two aglycones (daidzein and genistein) and their glycosides (daidzin and genistin, respectively). The results showed that okara is rich in isoflavones mainly present in the form of glycosides (daidzin and genistin). The HPLC analysis revealed that genistin was the most abundant okara glycoside at the initial time of storage: 0.3343; 0.3288 and 0.4720 (mg/g of the dried okara) for F (4 and -18 ºC), D80 and D200, respectively. After 15 d of storage F (4 ºC) and D200 samples there was a significant ($P < 0.05$) reduction in the content of daidzin and genistin, and consequently an increase in daidzein and genistein content. Grün et al.(2001) observed

that thermal processing in tofu can influence the profile of the major isoflavones, showing that daidzein and its various forms might be heat labile and that the thermal processing degrades malonylglycosides conjugates of genistein and daidzein. In this study, although malonylglycosides were not analyzed, the daidzein showed an increase ($P < 0.05$) of about 23 times for F (4 ºC) and 13.5 times for D200, after 15 d of storage. The samples F (4 ºC) and D200 also showed an increase in the concentration of genistein with the storage time.

Previous studies reported similar or higher total isoflavones in okara compared to the present results, with values ranging between 0.528 and 0.849 mg/g of okara (Genovese et al., 2006; Jankowiak et al., 2014). This can be related to the fact that these studies were able to identify more groups of isoflavones. Moreover, Silva et al. (2013) have also observed differences in the isoflavones profile of sprouts from different soybean, reporting total isoflavones between 0.278 and 0.814 mg/g of soy, with a predomination of daidzin.

The content of daidzein increased with thermal treatment for D200. According to Izumi et al. (2000), the aglycones (genistein and daidzein) are absorbed faster and in greater amounts than their glycosides in humans. Therefore, isoflavone aglycones may be more effective than glycoside for preventing chronic diseases. During a 15 days storage period, daidzin and genistin content was maintained in D80 and F (-18 ºC), but not in D200 and F (4 ºC). In the case of aglycones (daidzein and genistein), although there was a significant increase ($P < 0.05$) over storage time, the values found in this study are lower than those found by Jankowiak et al.(2014) and Jackson et al. (2002). Therefore, it is important to better study the behavior of isoflavones by-products as affected by thermal treatments.

Table 2.2. Isoflavones concentration in okara with different thermal treatments throughout the storage (mg per g of dried okara).

	Retention time (min)	**Daidzin** 5,7		**Genistin** 7,1		**Daidzein** 9,9		**Genistein** 11,9		
	Sample	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Σ
T0	F (4 and -18 ºC)	0.2828^{Aa}	0.0005	0.3343^{Aa}	0.0017	0.0209^{Aa}	0.0016	0.0308^{Aa}	0.0010	0.6688
	D80	0.2566^{Ba}	0.0047	0.3288^{Ba}	0.0019	0.0209^{Aa}	0.0011	0.0217^{Ba}	0.0004	0.6280
	D200	0.3477^{Ca}	0.0065	0.4720^{Ca}	0.0019	0.0260^{Ba}	0.0008	0.0249^{Ca}	0.0001	0.8706
T 15d	F (4 ºC)	0.0711^{Ab}	0.0014	0.0444^{Ab}	0.0011	0.4846^{Ab}	0.0023	0.4642^{Ab}	0.0048	1.0643
	F (-18 ºC)	0.2578^{Bb}	0.0027	0.3082^{Bb}	0.0030	0.0156^{Bb}	0.0003	0.0276^{Bb}	0.0005	0.6093
	D80	0.2506^{Ca}	0.0026	0.4006^{Cb}	0.0014	0.0370^{Cb}	0.0009	0.0312^{Bb}	0.0003	0.7194
	D200	0.0633^{Db}	0.0007	0.0834^{Db}	0.0012	0.3506^{Db}	0.0020	0.2599^{Cb}	0.0004	0.7571

*Results are expressed as mean ± standard deviation (n=3). F (4 ºC): fresh okara stored at 4 ºC, F(-18 ºC): Fresh okara stored at -18 ºC, D80:dried okara at 80 ºC for 5h; D200: dried okara at 200 ºC for 1 h. T0: initial time, T15d: 15 days of storage. Different lowercase letters (difference between times) and uppercase letters (difference between treatments) in the same line of the group are statistically different ($P < 0.05$).

2.4.6. Fatty acids profile of okara

Okara is obtained from soybean seeds that have a high percentage of fat (18-22%), and still can be considered a fat-rich by-product (Redondo-Cuenca et al., 2008, 2006). All okara samples were composed of polyunsaturated fatty acid (PUFA), mainly linoleic acid followed by linolenic acid in all samples (Table 2.3), likewise soy oil (Galão et al., 2014). Linoleic acid reached maximum values in D80 at the initial time.

The okara also presented representative amounts of monounsaturated fatty acids (MUFA), about 21 g/100 g fat, although more than 19 g/100 g fat were constituted by oleic acid (C18:1 n-9). The predominant saturated fatty acids (SFA Table 2.4) were stearic acid (C18: 0) and palmitic acid (C16: 0), with values ca. 6 to 14 g/100 g fat, respectively. These were also the prevalent ones in a previous study with transgenic soybeans and non-GM, conducted by Galão et al. (2014). The profile of the fatty acids analyzed in the soybean milk by-product was not affected by the heat treatment applied (Tables 2.3 and 2.4). Therefore, the observed oxidation suggested by the TBARS and acidity analysis may be due to other compounds oxidation, such as proteins, as mentioned above.

Table 2.3. Unsaturated fatty acid composition (g FA/100 g) and total fatty acid concentration (µg FA/mg sample) in the samples of okara.

Temperature (Storage)	F(4ºC) and F(-18ºC)						D80				D200			
	T0 okara (n=2)		Tf F (4ºC) (n=2)		Tf F (-18ºC) (n=2)		T0 (n=2)		Tf (n=2)		T0 (n=2)		Tf (n=2)	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
MUFA	20.680 Aa	0.772	21.534 Aa	0.100	21.350 Aa	0.095	21.202 Aa	0.195	21.369 Aa	0.397	21.265 Aa	0.260	21.442 Aa	0.174
C16:1 c7	0.022	0.005	0.074	0.073	0.020	0.001	0.020	0.001	0.019	<0.001	0.019	0.001	0.023	0.002
C16:1 c9	0.067	0.005	0.058	0.001	0.075	0.009	0.075	0.005	0.079	0.001	0.070	0.002	0.083	0.004
C16:1 c11	0.014	0.005	0.015	0.001	0.011	0.001	0.012	0.001	0.015	0.002	0.010	<0.001	0.012	0.001
C17:1 c9	0.003	<0.001	0.003	<0.001	0.003	<0.001	0.005	0.002	0.003	0.001	0.005	0.001	0.004	0.001
C17:1 c10	0.051	<0.001	0.052	0.001	0.053	<0.001	0.052	0.003	0.054	0.004	0.050	0.001	0.054	0.002
C18:1 trans	0.036	0.002	0.035	0.003	0.038	0.005	0.052	0.013	0.046	0.012	0.043	0.005	0.046	0.003
C18:1 c9	18.906	0.701	19.420	0.179	19.509	0.101	19.375	0.137	19.508	0.372	19.437	0.224	19.594	0.170
C18:1 c11	1.291	0.049	1.561	0.002	1.332	0.002	1.314	0.020	1.340	0.021	1.316	0.006	1.322	0.009
C18:1 c13	0.046	0.003	0.048	<0.001	0.047	<0.001	0.047	0.001	0.046	0.002	0.048	<0.001	0.046	<0.001
C20:1 c9	0.027	0.003	0.035	0.001	0.030	0.001	0.028	0.001	0.029	0.003	0.030	0.004	0.029	0.002
C20:1 c11	0.204	0.020	0.219	0.002	0.221	0.004	0.208	0.009	0.217	0.014	0.219	0.014	0.216	0.004
C24:1	0.012	0.002	0.013	<0.001	0.010	0.007	0.013	0.002	0.012	<0.001	0.018	0.008	0.013	0.001
PUFA	56.483Aa	0.549	55.278 Aa	0.253	55.796 Aa	0.346	56.610 Aa	1.071	55.809 Aa	1.226	56.221 Aa	1.038	55.769 Aa	0.251
C16:2 c9t12	0.004	<0.001	0.004	<0.001	0.004	<0.001	0.004	<0.001	0.005	0.001	0.003	<0.001	0.004	<0.001
C18:2 c9t12	0.061	0.010	0.068	0.009	0.059	0.009	0.070	0.008	0.074	0.019	0.077	0.001	0.077	0.001

C18:2 c9c12	48.863	0.291	48.030	0.145	48.488	0.241	49.087	0.805	48.469	0.852	48.811	0.825	48.491	0.134
C18:2 cc	0.018	<0.001	0.017	0.001	0.016	<0.001	0.017	0.002	0.017	0.002	0.018	0.001	0.017	0.001
C18:3 t9t12c15	0.017	0.001	0.020	0.001	0.017	<0.001	0.019	0.001	0.017	0.003	0.022	0.006	0.019	0.002
C18:3 c6c9c12	0.026	0.002	0.023	0.001	0.027	<0.001	0.027	0.001	0.029	0.003	0.031	0.006	0.027	0.001
C18:3 t9c9c12	0.031	0.002	0.030	0.002	0.028	0.002	0.031	0.002	0.032	0.004	0.036	0.001	0.032	0.001
C18:3 c9c12c15	7.344	0.263	6.955	0.103	7.024	0.100	7.220	0.261	7.009	0.367	7.093	0.232	6.955	0.123
C20:2 c11c14	0.043	0.002	0.046	<0.001	0.045	0.001	0.044	0.001	0.045	0.001	0.045	0.001	0.050	0.001
C20:3 c8c11c14	0.010	0.001	0.011	0.003	0.012	<0.001	0.013	0.001	0.010	<0.001	0.014	<0.001	0.007	0.002
C20:5 c5c8c11c14c17	0.024	<0.001	0.026	0.002	0.028	<0.001	0.039	0.002	0.056	0.002	0.020	0.002	0.044	0.001
C22:5 n3	0.041	0.011	0.049	0.003	0.048	0.008	0.040	0.003	0.047	0.007	0.051	0.007	0.046	0.001
µg FA/mg simple	146.339	14.191	156.152	7.607	153.275	1.539	149.095	4.987	153.487	9.795	155.554	6.331	155.847	1.591

F (4 ºC): fresh okara stored at 4 ºC, F(-18 ºC): Fresh okara stored at -18 ºC, D80:dried okara at 80 ºC for 5h; D200: dried okara at 200 ºC for 1 h. MUFA: Total monounsaturated fatty acids; c/t: *cis/trans* double bond; PUFA: Total polyunsaturated fatty acids; n3: omega 3 fatty acid. Results are expressed as mean ± standard deviation (n=2). Different lowercase letters (difference between times) and uppercase letters (difference between treatments) in the same line of the group are statistically different ($P < 0.05$).

Table 2.4. Saturated fatty acid composition (g FA/100 g) in the samples of okara.

Temperature (Storage)	F(4ºC) and F(-18ºC)						D80				D200			
	T0 okara(n=2)		Tf F (4 ºC) (n=2)		Tf F (-18 ºC) (n=2)		T0 (n=2)		Tf (n=2)		T0 (n=2)		Tf (n=2)	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
SFA	22.838 ^{Aa}	0.223	23.188 ^{Aa}	0.153	22.854 ^{Aa}	0.250	22.188 ^{Aa}	0.876	22.822 ^{Aa}	0.829	22.514 ^{Aa}	0.777	22.788 ^{Aa}	0.077
C14	0.087	0.003	0.114	0.001	0.091	0.011	0.107	0.024	0.095	0.008	0.085	0.001	0.108	0.008
C15	0.032	0.001	0.035	<0.001	0.033	0.001	0.033	0.004	0.034	0.001	0.046	0.021	0.044	0.003
C16i	0.025	<0.001	0.025	<0.001	0.025	<0.001	0.025	0.001	0.025	0.002	0.024	0.001	0.027	<0.001
C16	14.730	1.170	14.270	0.036	13.974	0.023	13.846	0.276	14.062	0.251	13.876	0.198	14.035	0.056
C17	0.164	0.008	0.172	<0.001	0.169	<0.001	0.165	0.009	0.170	0.003	0.165	0.005	0.172	0.003
C18	6.134	0.609	6.676	0.096	6.630	0.129	6.340	0.386	6.575	0.348	6.485	0.300	6.588	0.063
C20	0.536	0.084	0.597	0.020	0.606	0.024	0.547	0.047	0.588	0.057	0.583	0.055	0.585	0.013
C21	0.056	0.010	0.063	0.002	0.063	0.005	0.056	0.006	0.061	0.008	0.063	0.010	0.060	0.002
C22	0.640	0.131	0.729	0.035	0.750	0.062	0.640	0.074	0.714	0.099	0.709	0.099	0.704	0.012
C23	0.124	0.026	0.141	0.009	0.144	0.015	0.127	0.013	0.148	0.017	0.133	0.022	0.093	0.067
C24	0.275	0.072	0.318	0.020	0.328	0.046	0.269	0.035	0.311	0.050	0.305	0.058	0.335	0.010
C26	0.034	0.008	0.048	0.009	0.041	0.005	0.033	0.004	0.038	0.006	0.038	0.009	0.037	0.001

F (4 ºC): fresh okara stored at 4 ºC, F(-18 ºC): Fresh okara stored at -18 ºC, D80:dried okara at 80 ºC for 5h; D200: dried okara at 200 ºC for 1 h. SFA: total saturated fatty acids. Results are expressed as mean ± standard deviation (n=2). Different lowercase letters (difference between times) and uppercase letters (difference between treatments) in the same line of the group are statistically different ($P < 0.05$).

2.4.7. Trypsin inhibitor activity (TIA)

The trypsin inhibitor activity (TIA) for okara is shown in the Table 2.5. The trypsin inhibitors can cause serious adverse effects in humans and animals and this is the major antinutritional factor present in soybeans (Hossain and Becker, 2001). In the case of non-ruminant animals such inhibitors may interfere with the physiological process of digestion because they inhibit the operation of the pancreatic proteolytic enzymes. In ruminant animals, when these inhibitors are ingested in large quantities they can pass through the rumen and exert harmful effects (El-Hady and Habiba, 2003; Hossain and Becker, 2001).

Few studies have addressed various ways to minimize these damaging effects by reducing the activity of trypsin inhibitors (TIA) in legumes, as these inhibitors are labile term. Siddhuraju et al. (1996) studied different heat treatments which achieved major reductions of activity of trypsin inhibitors, reducing ca. 93% with dry heat and 96% by autoclaving, without succeeding in totally inactivating TIA. The same occurred in this study, where trypsin inhibitors could still be detected in soy by-product (okara) submitted to different heat treatments: 80 and 200 ºC (Table 2.5).

Table 2.5. Trypsin inhibitor activity obtained for soy protein and okara with thermal different treatments (F (4 ºC and -18 ºC), D80 and D200) at initial time of storage.

Sample	TUI / mg dry sample
F (4 ºC and -18 ºC)	8.75 ±0.65 [a]
D80	7.06 ± 0.085 [a]
D200	1.52 ± 0.66 [b]
Soy protein	1.91 ± 0.57 [b]

TUI: trypsin inhibitor activity units. F: fresh okara, F (4 ºC): fresh okara stored at 4 ºC, F(-18 ºC): Fresh okara stored at -18 ºC, D80:dried okara at 80 ºC for 5h; D200: dried okara at 200 ºC for 1 h. Different lowercase letters in are statistically different ($P < 0.05$).

However, in D200 (1.52 ± 0.66) values were about 6 times lower than those found in fresh okara (8.75 ± 0.65). The values found for fresh okara are lower than those found by Olguin et al. (2003), which were in the order of 27.2 TUI/ mg dry grain raw soybeans. The main reason for the resistance of these inhibitors is probably the heat resistance proteins mainly located in the seed coat and associated with tannins (De Lumen and Salamat, 1980;

Elias et al., 1979). Moreover, Hafez and Mohamed (1983) found that ca. 4 to 15% of the total heat resistant TIA in winged bean.

2.4.8. Morphological properties

The scanning electron micrographs of okara (Fig.2.5) showed little differences in the surface morphology between fresh and dried okara (D80 and D200). The okara contained irregular clumps of fiber with smooth surfaces, similar to "honeycomb" as previously observed by Fung et al.(2010) and Kuan and Liong (2008) in similar samples. The main difference among samples was related to the moisture content, which was lower in both D80 and D200 than in fresh okara. This difference can be noted in okara structure, as the water removal resulted in a greater porosity specially noticed in D200.

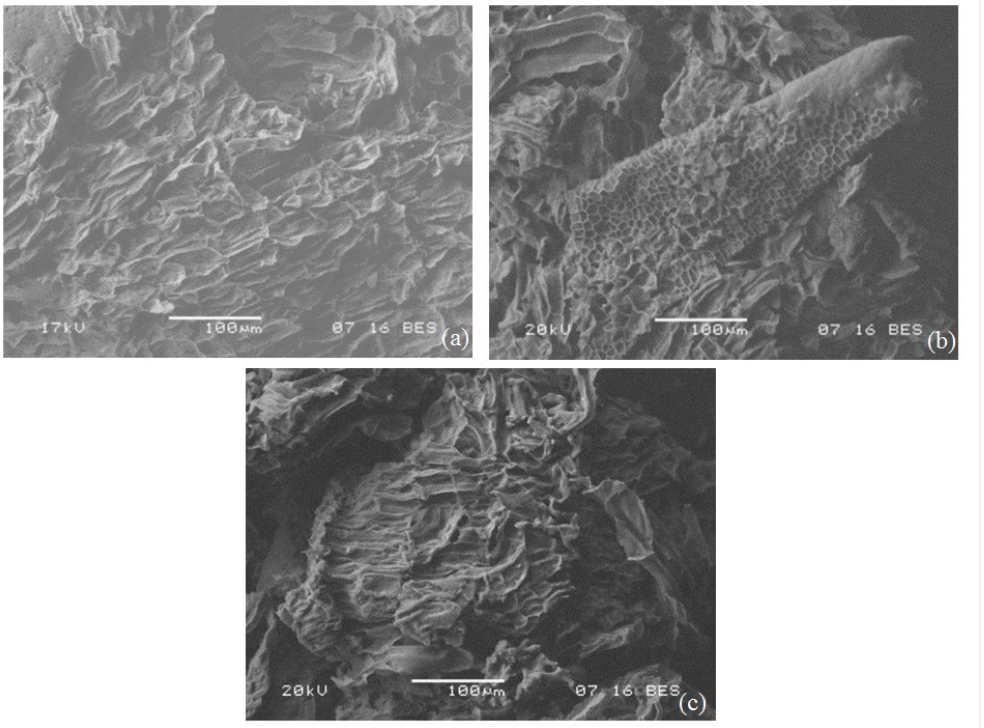


Figure 2.5.1 SEM micrographs of thermal denaturation on the microstructure of okara (a) fresh okara, (b) D80 and (c) D200 with a magnification of 250 times.

2.5. Conclusions

The okara is a by-product with high nutritional value, in particular with high fiber, protein, and lipids content. The high initial microbial charge makes okara a very unstable product, requiring heat treatments, not only to reduce its microbial contamination, but also to reduce water content avoiding microbial growth. Although the two drying methods evaluated in this study assured a reduction of okara initial microbial contamination, the reduction of moisture to ca. 45% was not enough to allow microbial stabilization. So, it is important to further decrease okara water content to better stabilize the product and prolong storage time, whilst preserving its maximum nutritional value and avoid chemical and microbiological degradation. It can be concluded that storage of fresh okara under freezing F (-18 ºC) can stabilize all reactions and consequently maintain the original nutritional quality of okara, but without eliminating initial microbial counts. Dried okara samples showed a decrease in microbial contamination compared to the fresh ones, but high temperatures (200 ºC) may promote secondary reactions that lead to oxidations and acidifications, mainly after 10 days storage at ambient temperature. Therefore, the best stabilization seems to require heat treatment at lower temperature (80 ºC) and for longer periods. Okara is rich in PUFA and MUFA with high stability, since they remained unchanged during storage, and for both thermal treatments applied. Regarding anti-nutritional factors in soybeans, the okara samples submitted to thermal treatment showed a significant positive decrease in TIA in relation with fresh okara. The thermal treatment at 200 ºC (D200) was the most efficient, with similar values to those reported for soy protein. The sample D200 had a 5.7times decrease in relation the fresh okara. Still, it must be taken into consideration that it is not possible to completely eliminate TIA in legumes, because ca. 4 to15% of them are heat resistant.

In conclusion, the nutritional richness of this by-product with a high quantity of vegetable protein (ca. about 30%) and mainly fiber (ca. 35%), combined with the considerable amount of linoleic and oleic acids suggests that okara can be considered a valuable nutritional ingredient for further inclusion in food and feed. Considering the limited studies carried out to date, further studies are warranted to better stabilize okara, increasing its storage potential and future application.

CHAPTER 3- Impact of heat treatment on nutritional quality of soy Okara flour – a potential functional food

3.1. Abstract

Okara is a by-product obtained from soy beverage presenting a rich nutritional composition. There are few studies focused on okara processing and stabilization to generate a nutritional and functional flour. Thus, the main objective of this work was to evaluate the impact of thermal treatment (autoclaving process) and subsequent drying at low temperature, on the nutritional, microbiological quality and biological properties of okara throughout 60 days of storage. Okara flour showed a rich nutritional composition− ca. 30% of total dietary fiber, 27% of protein and 5-10% of lipids. The okara´s lipid profile presented a high level of PUFA, MUFA and SFA. The fresh okara showed the highest antioxidant activity, although the autoclaved dried okara flour could still maintain relevant antioxidant activity throughout the storage. Besides, the highest concentration of isoflavones was observed for autoclaved okara (83.21 mg/ 100 g). The autoclaving process also produces a flour with reduced content of antinutritional factors and lower lipid oxidation than the fresh one. In conclusion, the autoclaving sterilization process maintained or improved nutritional and functional characteristics of okara assuring a stable product throughout the storage, suggesting that okara flour can be a valuable nutritional ingredient for further inclusion in new food products.

3.2. Introduction

In recent years, the search of alternative use and value addition to by-products generated in food processing has been outstanding, taking into account the fact that the discarding of by-products by industries leads to environmental problems and economic loss (Vong and Liu, 2016). Soybean is an important oilseed with great commercial interest especially due to its derived products namely soy oil, soy proteins and soy beverage. In particular, the beverage produced from soybean has high economic value and has been used as a base in a wide variety of food products (tofu, soy yogurt and cheese) (O'Toole, 1999; Van der Riet et al. 1989). However, the processing used for soy beverage production is not

able to extract the total content of proteins and other nutrients present in the soybean, thus the by-product (okara) generated from this process still possesses a high nutritional value (Mateos-Aparicio et al., 2010b). Soya beverage industry produce a large amount of okara, considering that from 1 Kg of soybean 1.2 Kg of fresh okara is produced (Vong and Liu, 2016). Moreover, this by-product is highly perishable, due to its high moisture content (~80%) and rich nutritional composition that are determinant factors for rapid degradation by chemical reactions and action of microorganisms. Some studies have been investigating different technologies for drying okara considering the impact on the thermophysical properties and isoflavones content of the dried product (Grizotto, 2011; Muliterno et al., 2016; Perussello et al., 2014; Wachiraphansakul and Devahastin, 2007). Nevertheless, considering the high potential of okara to be directly used as a flour or indirectly as an ingredient to produce diversified functional foods, there is a lack of information about the okara, with scarce studies on the influence of the thermal process and storage conditions on the nutritional profile and stability of dried okara.

Okara is currently used in agriculture as a biofertilizer (Redondo-Cuenca et al., 2008) and in animal feed (Rinaldi et al.,2000), but often, it is still simply discarded causing severe environmental problems. Detailed studies are hence required to optimize the preservation conditions of okara, considering its growing interest in recent years for application in human food. Recent studies have shown the feasibility and potential of okara, in particular fermented, to produce a variety of bioactive substances and processed products for consumer (Vong and Liu, 2016). The composition of okara may vary according to cultivar of soybean, extraction method and amount of water soluble components extracted from soybeans. In general, okara present a rich composition, especially concerning the content of protein and fiber, as well as polyunsaturated fatty acids (PUFA) (Mateos-Aparicio et al., 2010c). Many antioxidant compounds are also present in okara, especially isoflavones, although in lower levels than in other soy-based foods. The heat processing and other processes like hydrolysis and fermentation might, however, alter the composition of those isoflavones (Jackson et al., 2002; Wang and Murphy, 1994). Drying can then be a very interesting alternative to preserve the okara, and after milling it is possible to obtain a flour of good nutritional quality at low cost, that can be further used as raw material in several products. In addition, when subjected to thermal processing, it becomes free of antinutritional factors, however the consequent impact on its final nutritional and functional value remains poorly evaluated.

In the present work, it was aimed to study the impact of thermal treatment (sterilization in autoclave under standard conditions, 121 ºC and 1 atm) and subsequent drying at low temperature, on the nutritional and microbiological quality and biological properties of okara throughout 60 days of storage. The isoflavones were also monitored in this by-product throughout storage. This study will provide information of dry okara nutritional value relevant for its application in food products or feed.

3.3. Material and methods

3.3.1. Drying of okara

The okara used in this study was kindly provided by a soy food processor in Portugal (NUTRE Industry). After collection, the okara sample was divided in two lots: one was immediately dried at 65 ºC for 68 h, to constant weight, whereas the other was firstly autoclaved (121 ºC and 1 atm for 15 minutes) and only after dried under the same conditions used for the non-autoclaved lot. After, both samples were ground in a mill (Retsch, Haan, Germany) with 1 mm sieve and packaged in sealed bags and stored at room temperature (23 ºC) during 60 days. The fresh okara (autoclaved and non-autoclaved) was also packaged in sealed bags and stored under refrigeration (4 º C) over 40 days, due the deterioration.

3.3.2. Analytical methods

3.3.2.1. Proximate composition analysis

The proximate composition in okara was performed according to the official methods of analysis Association of Official Analytical Chemists, 1995. Moisture was determined at 105 ºC for 24 h and ash content was determined by incineration at 550 ºC in a muffle furnace. The crude protein was determined using a nitrogen analyser (N × 6.25), Leco N analyzer, Model FP-528, Leco Corporation, St. Joseph, USA) and crude lipid content by petroleum ether extraction (Soxtec™ 2055 Fat Extraction System, Foss, Hilleroed, Denmark). The contents of total dietary fiber (TDF) and insoluble dietary fiber (IDF) in okara were determined by the method of (Goering and Soest, 1970). The analyses were done on homogenized samples in duplicate or triplicate.

3.3.2.2. Physical and chemical quality analyses

Thiobarbituric acid-reactive substances (TBA-RS) were assessed in okara using the method adapted by Rosmini et al. (1996). The incubation was performed under heating in water bath at 100 ºC for 35 minutes. Quantification was done in a Spectrophotometer (UV mini 1240, Shimadzu, Tokyo, Japan) at 532 nm. The 1,1,3,3 tetraethoxypropane was used as standard. Results were expressed as mg malondialdehyde/ kg dry okara. The water activity (a_w) was measured in triplicate at 25 °C in an Aqualab Series 3 (Aqualab Series 3, Decagon Devices Inc., Pullmam, Washington, USA). The pH values of the okara were measured in triplicate with a pH meter equipped with a glass electrode (Basic 20- Crison), properly calibrated. The acidity determination was carried out using the method of acid-soluble alcohol; 2.5 g of sample was weighed in an Erlenmeyer with lids and added 50 ml of ethanol (99.5%). The vials were then shaken and maintained for 24 h. After that time, 20 ml of supernatant were collected and submitted to titration with 0.01 M NaOH, until the pink phenolphthalein end-point. The blank was made using 20 ml of ethanol (Adolfo Lutz, 2008). All analysis were performed in triplicate.

3.3.3. Microbiological analysis

Microbiological analysis was performed using 10 g of sample homogenized in 100 ml sterile peptone water 0.1% (Peptone - Sigma Aldrich) on sterile bags for 2 minutes at 250 rpm with the aid of a stomacher (Lab Blender 400, London, UK). Then decimal dilutions were prepared using the sterile peptone water 0.1% and inoculated in a particular culture media for each group of microorganisms: total mesophilic bacteria were determined in PCA (Plate Count Agar diagnostics provided by Biokar) incubated aerobically at 30 °C for 48 h. Yeasts and molds were counted in PDA (Potato Dextrose Agar provided by Biokar diagnostics), incubated aerobically at 30 ºC for 5 d and *Bacillus cereus* in BCA (*Bacillus Cereus* Agar supplied by Biokar Diagnostics, Portugal with addition of polymyxin B and egg yolk) incubated aerobically at 30 °C for 12-30 h. Inoculation was performed using the drop method as described by Miles and Misra (1938), using 20 µL. *Enterobacteriaceae* were determined on Violet Red Bile Glucose Agar (VRBGA, Oxoid, Basingstoke, United Kingdom) using the pour plate method and were incubated aerobically at 37 °C for 24 h.

3.3.4. Polyphenols and antioxidant for determination

3.3.4.1. Extracts preparation

The extracts were made at a concentration of 10% (weight/volume) using methanol: water (80:20) as solvent and the extraction was performed in an orbital shaker (SI-100C, Wiggenhauser, Germany) at 10 ºC, 150 rpm for 1 h. The extracts were then centrifuged at 1677 x g, at 4 ºC for 30 min and filtrated by filter paper Whatman nº 1. The extracts were stored at -80 ºC until further analysis. For the analysis of isoflavones the supernatant was filtered through a 0.45 µm filter (Orange Scientific, Braine-l'Alleud, Belgium) before chromatographic analysis.

3.3.4.2. Scavenging activity (ABTS)

The scavenging activity was measured in the methanolic extracts using ABTS radical according the method described by Gião et al. (2007). The quantification was performed at 734 nm (mini UV 1240, Shimadzu, Tokyo, Japan) with 50 µl of sample extract in 1 ml ABTS with absorbance of 0.7 (± 0.02). Trolox was used as a standard to prepare a calibration curve in the range 0.01 - 0.12 mg / ml. The results were expressed in as milligrams of Trolox per 100 g of dried okara.

3.3.4.3 Scavenging activity (DPPH)

The scavenging activity of DPPH (2.2-Diphenyl-1-picrylhydrazyl) radicals was measured in the methanol extracts, according the method described by Bondet, Berset, and Chimie, (1997). Quantification was done in a spectrophotometer (mini UV 1240, Shimadzu, Tokyo, Japan) at 515 nm. Briefly, 0.5 ml of the sample extract was added to 3.5 ml methanol solution of DPPH (60 µM). Trolox was used as a standard to prepare a calibration curve in the range 0.005 - 0.08 mg/ml. The results were expressed in as milligrams of Trolox per 100 g of dried okara.

3.3.4.4. Determination of total phenolic content

The concentration of total phenolic compounds was determined calorimetrically in the methanol extracts by the Folin-Ciocalteu method (Singleton and Rossi, 1965) and the quantification was done at 750 nm (UV mini 1240, Shimadzu, Tokyo, Japan). Total phenolic content in each sample was determined using a standard curve prepared by gallic acid in the range 0.025 - 0.5 mg/ mL. The results were expressed in as milligrams of gallic acid per 100 g of dried okara.

3.3.4.5. Analysis of isoflavones

Isoflavones were analyzed by a modification of a method described by (Wang and Murphy, 1996). Twenty microliters of each extract (obtained as explained in 3.3.4.1) were analyzed by HPLC (Waters Series 2695, Milda ford, MA, USA) coupled to a diode array detector, using a Kromasil C-18 column (250 x 4,6 mm) to separate the isoflavones at 25 ºC. The mobile phase consisted of 0.1% acetic acid in water (v/v) (solvent A) and 0.1% acetic acid in acetonitrile (v/v) (solvent B). Elution started with 20% B, and then increased to 100% in 20 min and to 20 % in the next 12 min. The flow rate was 0.8 ml/min. Spectral data from peaks were accumulated in the 200–400 nm range. The data were processed on Empower 3 software. Isoflavones quantification was achieved by measuring the absorbance recorded in the chromatograms at 255 nm relative to external standards. The standards were dissolved in analytical grade DMSO (Sigma-Aldrich, St Quentin Fallavier, France) and diluted in methanol. The calibration curve was performed with Genistin, Genistein, Daidzin and Daidzein (Sigma-Aldrich, St. Louis, USA). This procedure was done in triplicate and the results were expressed in mg of isoflavone per 100 g dry okara.

3.3.5. Analysis of fatty acids

Total fatty acid (FA) analysis were performed according ISO 12966-2:2011 using gas chromatography.

3.3.6. Amino acid analysis

The dry okara autoclaved and non-autoclaved were hydrolyzed with solution of 6 M HCl at 116 ºC over 24 h in nitrogen-flushed glass vials, before total amino acid analyses. All

samples were then pre-column derivatized with Waters AccQ Fluor Reagent (6-aminoquinolyl-N-hydroxysuccinimidyl carbamate) using the AccQ Tag method (Waters, USA). Analyses were done by ultra-high-performance liquid chromatography (UPLC) in a Waters Reversed-Phase Amino Acid Analysis System, using norvaline as an internal standard. The resultant peaks were analyzed with EMPOWER software (Waters, USA).

3.3.7. Trypsin inhibitor activity

The trypsin inhibitor activity was measured in okara using a method adapted from Kakade et al. (1974) with slight modifications. So, 1.0 g of okara was weighed and defatted with 50 mL of 0.01 M NaOH under slowly agitation, for 3 h at room temperature. At the end of this time period, the solution was centrifuged for 20 min at 1800 x g and the supernatant was analyzed. Two conditions were measured: control (trypsin activity without inhibitor) and sample (trypsin activity in the presence of inhibitor). Both measurements were made against a blank with the substrate, prepared with Tris buffer pH 8.2, 0.05 M and BAPNA (1.3 mL). The results are expressed in trypsin inhibitor units (TIU) per gram of dry okara, according to the Equation 3.1 below:

$$TIU/g = \frac{100 \times 10\left(m_{Control} - m_{Sample}\right)}{0.020 \times 4} D \times 50$$

Equation (3.1)

Where 100 is the factor to convert 0.01 u. Abs in TIU units; $m_{Control}$-m_{Sample}, the difference between the slopes of progress curves in absence and presence of TI respectively; D, the dilution factor of supernatant, calculated as the final volume divided by the amount of aliquot taken to dilute the extract; 50 is the extraction volume (mL) of 0.01 M NaOH used per gram of soybean flour; 0.020, aliquot (mL) used in the current assay, 10, the final reaction time in discontinuous method, and 4, factor of decrease in the final reaction volume from 10 to 2.5 mL.

3.3.8. Statistical analysis

The results of chemical composition were expressed as mean ± CI and other were expressed as mean ± SD. Initially, data were checked regarding homogeneity of variances using the Levene test. Statistical significance of differences among groups means that followed the conditions was evaluated by Student´s T-test and ANOVA (analysis of variance) with Tukey post hoc test. For data did not follow normality was used non-parametric test (Kruskal-Wallis and Mann-Whitney). The correlation of each isoflavone

content, total phenolics and antioxidant capacity was analyzed using Pearson correlation. For all tests were used a significance level of $P < 0.05$. Statistical analysis was performed using SPSS version 23.00 for Windows.

3.4. Results and discussion

3.4.1 Proximate composition analysis

The composition of the dry okara previously autoclaved (AOK) and dry okara non-autoclaved (NAOK) in initial time (T0) and after storage at 23 ºC during 60 days, and fresh okara autoclaved (FAOK) and fresh okara non-autoclaved (FNAOK) in T0 and after storage at 4 ºC during 40 days are shown in Table 3.1. This by-product has a rich composition mainly fiber, proteins and lipids. The protein content of okara after different treatments ranged between 26.84 to 28.34 %, and FNAOK showed a higher content in initial time and after storage. It is well known that the protein from okara has a high nutritional value and may be considered a potential vegetable source of protein for human consumption. However, its direct use in food can be restricted due the severe heat treatment that this by-product is submitted to for extraction of soy beverage, which causes protein denaturation and reduces solubility of okara protein isolate (Ma et al., 1997).

The drying treatment (65 ºC) affected the content of lipids in okara (Table 3.1), inducing a significant reduction ($P < 0.05$) from 9.12 % (AOK) to 5.92 % (NAOK). Similarly, in a previous study by Grizotto (2011) it was also observed a reduction of the fat content in okara after drying using spray drier. Nevertheless, the lipids content of dry okara previously autoclaved (AOK) did not vary and showed similar values to those previously reported in raw okara (Ma et al., 1997; Van der Riet et al., 1989). Therefore, the decrease in content lipids in NAOK may be related to lipid oxidation, as shown in Fig. 3.1 (A and C) since the TBARS and acidity was high in NAOK. While AOK showed value of total lipids similar to those obtained in previous studies of okara (Van der Riet et al., 1989) and maintained the value of acidity and TBARS during storage. Nevertheless, there are few studies about thermal process and stability of okara in literature and it is difficult to completely understand this modification in lipids after drying of okara. In addition, the drying temperature not affected the lipids in okara previously autoclaved.

Okara presents a higher quantity of insoluble fiber, derived from structural parts of plants, such as cell walls and include cellulose, hemicellulose and lignin. The range of total dietary fiber for different treatments in okara was 29.27- 32.52%. Previous studies reported that these fibers possess the ability to absorb water and increase bulk, thus shortening the transit time through the intestinal tract (Kuan and Liong, 2008). In literature many other benefits have been associated with the fibers, including improvement of the immune system, prevention of colon cancer (Jansen et al., 1999), hypoglycemic effect among others (Bedani et al., 2014b). It was noted that with the autoclaving process the insoluble fiber decreased. This may be due to fiber solubilization and breakage of the insoluble fibers during this thermal process (Repo-Carrasco-Valencia et al., 2009).

Okara total fiber showed non-significant differences ($P > 0.05$) between the treatments applied before storage. The value obtained for total fiber and insoluble fiber was lower than previous studies, but this difference may be associated with the method used for quantification (Kuan and Liong, 2008; Van der Riet et al., 1989) as several studies used the enzymatic methods; other important factors that can justify such differences are the agronomic practices, genotype and local environmental conditions of the soybean productions (Boydak et al., 2002). Previous studies also have reported benefits associated with soluble fiber which are able to form a soft surface layer on the small intestine mucosa and form a barrier to the absorption of some nutrients. This tends to delay the metabolism of sugars and fats, thus controlling the glycemic levels and decreasing total cholesterol and LDL rates (Gunness and Gidley, 2010; Bedani et al., 2014a; Lu et al., 2013).

Table 3.1. Chemical composition for okara with different thermal treatment and storage time.

Time (days)	Sample	Lipids (% DM)	Proteins (% DM)	Ash (% DM)	Insoluble fiber (% DM)	Total dietary fiber (% DM)	Moisture (%)
T0	FAOK	9.29 ± 0.38^{Aa}	27.04 ± 0.25^{ABa}	3.51 ± 0.39^{Aa}	21.99 ± 3.65^{Aba}	29.27 ± 13.91^{Aa}	86.23 ± 0.34^{Aa}
	FNAOK	9.12 ± 2.29^{Aa}	27.87 ± 3.18^{Aa}	3.48 ± 0.76^{Aa}	22.69 ± 1.05^{Aa}	32.84 ± 2.92^{Aa}	84.91 ± 0.60^{Ba}
	AOK	10.39 ± 1.46^{Ba}	27.03 ± 2.22^{ABa}	$3,35 \pm 0.22^{Aa}$	18.82 ± 4.06^{BCa}	30.66 ± 5.53^{Aa}	3.39 ± 0.34^{Ca}
	NAOK	5.92 ± 0.83^{Ca}	26.84 ± 0.44^{Ba}	3.41 ± 0.19^{Aa}	16.37 ± 3.32^{Ca}	30.06 ± 5.35^{Aa}	3.80 ± 0.27^{Ca}
TF	FAOK	11.28 ± 1.02^{Db}	28.86 ± 0.32^{Db}	3.90 ± 0.44^{Db}	20.41 ± 2.80^{Da}	31.64 ± 1.84^{Da}	87.15 ± 0.23^{Db}
	FNAOK	10.26 ± 0.88^{Eb}	29.34 ± 0.38^{Eb}	3.67 ± 0.76^{Ea}	17.72 ± 1.40^{Eb}	26.01 ± 12.20^{Eb}	84.93 ± 0.71^{Ea}
	AOK	10.76 ± 0.76^{Aa}	27.14 ± 0.95^{Aa}	3.53 ± 0.06^{Aa}	17.35 ± 1.72^{Aa}	36.15 ± 2.67^{Aa}	6.68 ± 0.32^{Ab}
	NAOK	4.29 ± 0.14^{Bb}	27.18 ± 5.65^{Aa}	3.47 ± 0.06^{Aa}	17.03 ± 2.14^{Aa}	33.57 ± 2.67^{Ba}	6.21 ± 0.13^{Bb}

Results were expressed in average ± confidence interval at 95%, protein and lipids (n=2) and ash, moisture, insoluble fiber and total dietary fiber (n=3). Lipids, protein, ash, insoluble fiber and dietary fiber is expressed % dry matter and moisture in %. Different lowercase letters (difference between times for the same treatment) and uppercase letters (difference between treatments in the same storage time) in the same column of the group are statistically different ($P < 0.05$), the TF was divided in two groups for statistical analysis, fresh (D and E) and dried (A and B)
FAOK T0: Fresh okara autoclaved (0 day)
FNAOK T0: Fresh okara not autoclaved (0 day)
AOK T0: Dry okara autoclaved (0 day)
NAOK T0: Dry okara not autoclaved (0 day)
FAOK TF: Fresh okara autoclaved and stored at 4 ºC (40 days)
FNAOK TF: Fresh okara not autoclaved and stored at 4 ºC (40 days)
AOK TF: Dry okara autoclaved and stored at 23 ºC (60 days)
NAOK TF: Dry okara not autoclaved and stored at 23 ºC (60 days)

The values found for ashes in okara were similar to those found in literature (Lu et al., 2013; Van der Riet et al., 1989) ranging from 3.35-3.51 % in initial time. The calcium, potassium and sodium are the main microelement constituents in okara (Van der Riet et al., 1989). Although iron is the microelement with higher amount (ca. 1.20%) its bioavailability is low (Mateos-Aparicio et al., 2010). No significant difference ($P > 0.05$) was observed for ash between treatments applied in initial time. However, the ashes in FAOK had a significant increase ($P \leq 0.05$) from 3.51 to 3.90 % after 40 days of storage. The moisture in fresh okara was 84.91% in initial time, while for samples submitted to drying at 65 ºC the moisture was significantly ($P \leq 0.05$) reduced with value around 3.39 and 3.80%, respectively for AOK and NAOK. The moisture in dried okara showed slightly significantly increase ($P < 0.05$) over 60 days of storage at ambient temperature (23 ºC) in sealed packages. Furthermore, the AOK (6.68%) had a slight but significant increase of moisture in relation to NAOK (6.21%).

3.4.2. Fatty acid composition

The results of fatty acid (FA) composition are given in Table 3.2. A total of nine different fatty acids were detected in the present study. It reveals that the okara lipids are rich in unsaturated fatty acids, such oleic acid (C18:1) and linoleic acid (18:2) that were identified as the major fatty acids in both samples of dry okara, followed by linolenic acid (C18:3) and saturated acids, mainly palmitic acid (C16) and stearic acid (C18).

The sum of mono and polyunsaturated fatty acids (PUFA) account for 84.80 and 73.65 %, in AOK and NAOK, respectively. The presence of high levels of the unsaturated fatty acids in okara is a positive characteristic assuming the health benefits usually reported in human nutrition studies (Delgado et al., 2017; Kerdiles et al., 2017). Furthermore, the profile of the fatty acids herein reported for AOK was similar to results previously reported by Mateos-Aparicio et al. (2010), although they used okara processed by Japanese guidelines. NAOK showed a different fatty acid profile, as shown in Table 3.2. The total PUFA decreased 1.7 times, while MUFA and SFA increased 1.8-fold and 1.7-fold, compared to AOK. It is well known that during food heat processing different reactions may occurs leading to changes on FA composition, such lipid oxidation. In Table 3.2 it is possible to observe that fatty acid composition did not change after 60 days of storage at 23 ºC for AOK,

on the other hand the NAOK showed an increase in total SFA while PUFA decrease 1.6-fold. According (Cui et al., 2017) this decrease of PUFA on FA composition in oils was a marker of oxidation process, and this can be confirmed by the TBA-RS and acidity analysis; in Fig. 3.2 it is possible to observe that NAOK has a higher value of TBA-RS ca.10- fold more than AOK.

Table 3.2. Main saturated and unsaturated fatty acid composition (g FA per 100 g).

Fatty Acid	AOK T0[a]	AOK TF[b]	NAOK T0[c]	NAOK TF[d]
C16	10.15 ± 0.07	10.50 ± 0.00	14.55 ± 0.35	25.40 ± 0.00
C18	4.60 ± 0.14	4.70 ± 0.00	11.00 ± 0.28	12.60 ± 0.00
C18:1	20.15 ± 0.07	20.20 ± 0.00	36.70 ± 1.84	37.20 ± 0.14
C18:2	56.25 ± 0.21	55.60 ± 0.00	34.95 ± 0.21	22.00 ± 0.00
C20	0.11 ± 0.13	0.20 ± 0.00	ND	0.50 ± 0.00
C20:1	0.06 ± 0.00	ND	0.46 ± 0.63	ND
C18:3	8.40 ± 0.00	8.50 ± 0.00	2.00 ± 0.28	1.50 ± 0.00
C22	0.20 ± 0.00	0.20 ± 0.00	Nd	0.50 ± 0.00
C24	ND[e]	ND	Nd	0.30 ± 0.00
SFA (%)	15.06	15.60	25.55	39.30
MUFA (%)	20.21	20.20	37.16	37.20
PUFA (%)	64.65	64.10	36.95	23.50

The results were expressed in average ± SD (n=2).
[a] AOK T0: Dry okara autoclaved (0 day).
[b] AOK TF: Dry okara autoclaved and stored at 23 ºC (60 days).
[c] NAOK T0: Dry okara not autoclaved (0 day).
[d] NAOK TF: Dry okara not autoclaved and stored at 23 ºC (60 days).
[e] ND: not detected.

Furthermore, the oxidative decomposition of fatty acid is generally accelerated by heating process, however in this work it is possible to observe that autoclaving process maintained polyunsaturated fatty acids in AOK. This fact may be related with the inactivation of lipoxygenase by autoclaving process. Endogenous lipoxygenases can influence the flavor, color and other nutritive properties. Furthermore this enzyme may catalyze the hydroperoxidation of unsaturated fatty acids and failure of inhibition of the lipoxygenases

leads to formation of compound responsible for the beany flavor of soybean products, such ketones, aldehydes and short-carbon-chain acids (Zhu et al., 1996).

3.4.3. Total amino acid

The amino acids found in plant foods can be divided into two classes, namely essential and nonessential. The essential amino acids can´t be synthesized by humans, but they need to be obtained from animal protein or alternatively from vegetable sources such vegetables, pulses, nuts, oilseed and fruits (Egydio at al., 2013). The data reported in Table 3.3 shows the compositions of total amino acids in dry okara autoclaved and not autoclaved. Okara showed 8 essential amino acids (histidine, lysine, threonine, isoleucine, leucine, valine, methionine and phenylalanine) in their composition and the total amino acids in okara samples ranged from 286.7 to 265.8 mg/ g dry matter, for AOK and NAOK, respectively. Wang and Cavins (1989) found a similar composition of total amino acids in okara, tofu and soy beverage. According Table 3.3, AOK showed a higher total amino acid than NAOK, however in previous study of (Khalil and Mansour, 2016) in faba beans the autoclaving process did not change the composition of total amino acid. On the other hand, Dajanta et al. (2011) applied different thermal processes in soybeans and observed that the soybeans autoclaved had 2 times more free amino acids than boiled soybeans, probably due the severe thermal condition that autoclave present (121 ºC).

Furthermore, previous studies reported that amino acids from soybeans, besides the high nutritive value, also provide several health benefits such antiobesity potential (Gibbs et al., 2004) and reduction of blood cholesterol (Fontaine et al., 2003; Gibbs et al., 2004). However, more studies about composition of total amino acids in okara are necessary, considering that most studies only evaluate free amino acids and not total amino acids present in this by-product.

Table 3.3. Composition of total amino acid (mg amino acid g^{-1} dry matter) in okara autoclaved and not autoclaved at initial time of storage.

Amino acid	NAOK[a]	AOK[b]
Arginine	19.96	21.25
Histidine	7.68	8.17
Lysine	17.79	19.07
Threonine	11.27	12.09
Isoleucine	12.01	12.79
Leucine	19.39	20.73
Valine	15.19	16.28
Methionine	3.60	4.01
Phenylalanine	15.30	16.36
Cystine	1.47	1.60
Tyrosine	8.64	9.29
Aspartic acid + Asparagine	32.80	35.55
Glutamic acid + Glutamine	48.33	51.88
Alanine	12.46	14.78
Glycine	11.43	12.34
Proline	13.82	14.90
Serine	14.65	15.60
Total amino acid	265.80	286.69

The results were expressed in average ± SD (n=2).
[a] AOK: Dry okara autoclaved.
[b] NAOK: Dry okara not autoclaved.

3.4.4. Physical and chemical analyses

The moisture, water activity (a_w), acidity and lipid oxidation for different thermal treatment in okara over different storage conditions are shown in Figure 3.1. The moisture for both fresh okara (FAOK and FNAOK) was similar to previous studies (O´Toole, 1999; Van der Riet et al., 1989; Voss et al., 2018), moreover, the moisture and the rich composition of okara are the main reasons for the perishability of this by-product, thus correctly drying

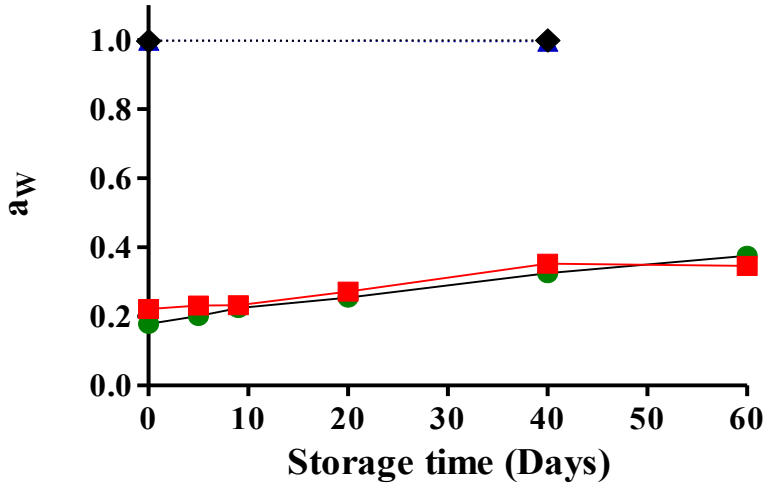
this by-product is of great importance to guarantee its stability for longer storage periods. The moisture content showed a significant ($P < 0.05$) decrease for both okara samples (autoclaved and not autoclaved) after drying at 65 ºC and the moisture content ranged between 3.39-3.80 %, for AOK and NAOK, respectively.

Water activity is the water not bound to food molecules and this is important for the quality and stability of food, because it is related to the chemical and enzymatic reactions in the food, besides being a determinant factor for microbial growth. As is shown in Figure 3.1b the a_w in dried okara was 0.178 and 0.221 for AOK and NAOK, respectively, while the fresh okara (FAOK and FNAOK) showed high value of 0.998. It is also possible to observe a slight and significant ($P < 0.05$) increase of a_w for both dried okara over storage (Fig. 3.1b) and this can be related with the increase of moisture from ca. 3 to 6%. The increase in moisture and a_w throughout the storage can be related to the lower microbiological stability, considering that the water activity presented the highest increase after 40 days of storage and the microbiological counts were also higher for the same time of storage, as shown in Table 3.4 and Fig 3.2.

Furthermore, the influence of thermal treatment in the okara was also evaluated by the analysis of TBA-RS, a sensitive method to quantify the malondialdehyde (MDA), which is the main product of decomposition obtained during the oxidative process from polyunsaturated fatty acid. The oxidation values of fresh okara were low (0.61 ± 0.08 to 0.86 ± 0.13 mg of MDA Kg^{-1} of dry matter), in initial time of storage for FAOK and FNAOK, respectively, while for dried okara TBA-RS were 5.10 ± 0.16 and 45.47 ± 0.16 mg of MDA Kg^{-1} of dry matter, respectively for AOK and NAOK as shown in Figure 3.1d.

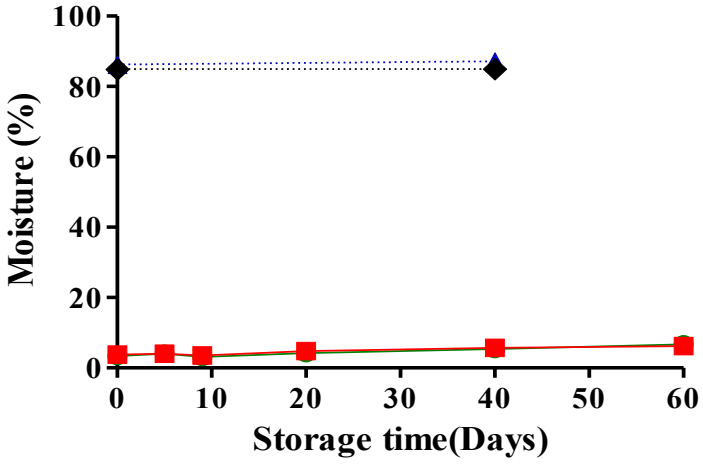
The level of lipid oxidation was lower in okara previously autoclaved, besides it is possible to observe that the drying process applied in okara increased significantly ($P < 0.05$) the TBA-RS, especially for NAOK. Similar results were found by Wachiraphansakul and Devahastin (2007) that related the increase in lipid oxidation (TBARS) in okara dried in a jet spouted bed of sorbent particles, presenting values from 0.86 to 2.28 mg of MDA Kg^{-1} of wet sample, lower than TBARS observed for our samples.

(a)

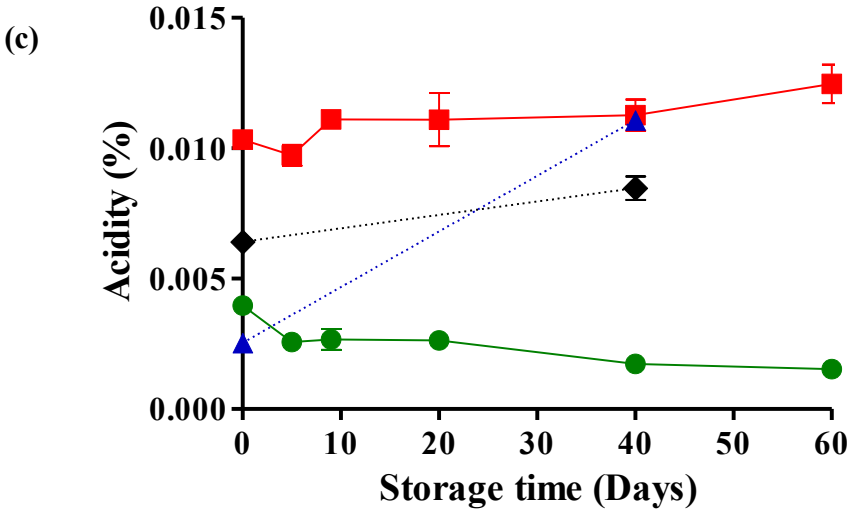


Days	0	5	9	20	40	60
AOK	Aa	Ab	Ac	Ad	Ae	Af
NAOK	Ba	Ba	Aa	Bc	Bd	Bd
FAOK	Ca				Ca	
FNAOK	Ca				Ca	

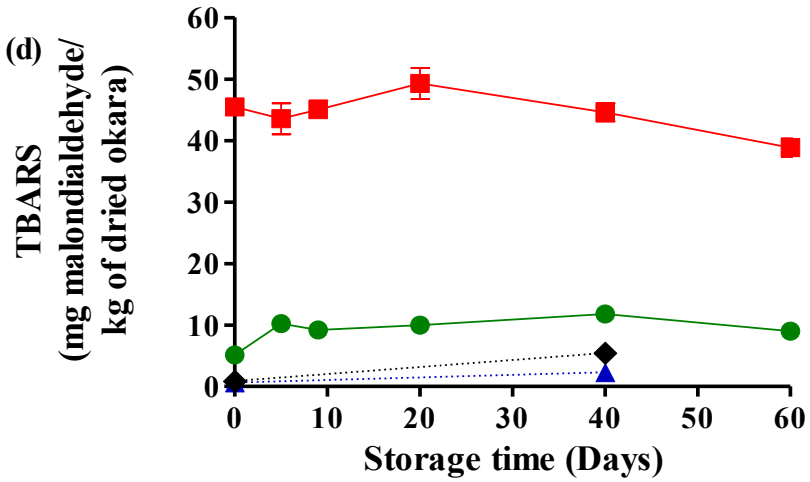
(b)



Days	0	5	9	20	40	60
AOK	Aa	Aa	Aa	Aa	Aa	Aa
NAOK	Ba	Aa	Ba	Ba	Aa	Ba
FAOK	Ca				Bb	
FNAOK	Da				Ca	



Days	0	5	9	20	40	60
AOK	Aa	Ab	Ab	Ab	Ac	Ac
NAOK	Ba	Ba	Bab	Bab	Cab	Bb
FAOK	Ca				Cb	
FNAOK	Da				Bb	



Days	0	5	9	20	40	60
AOK	Aa	Abc	Ab	Ab	Ac	Ab
NAOK	Bac	Bab	Bac	Bc	Bac	Bb
FAOK	Ca				Cb	
FNAOK	Ca				Db	

Figure 3.1. Profiles of (a) water activity, (b) moisture, (c) acidity and (d) TBARS of dried and fresh okara (autoclaved and not autoclaved) throughout the storage (-●- AOK; -■- NAOK, -▲-FAOK, -♦- FNAOK). Different lowercase letters in the same row and different uppercase letters in the same column are statistically different ($P < 0.05$).

Although there were slight changes of oxidation over storage for AOK and NAOK, the values were maintained. FAOK and FNAOK showed a significant ($P < 0.05$) increase of lipid oxidation after 40 days of storage, however it was still lower ($P < 0.05$) than that found for dry okara. Furthermore, in this work there was a clear influence of autoclaving on okara lipid oxidation, considering that both samples were dried in same conditions, AOK showed a lower (ca. 10 times) lipid oxidation for initial storage compared with NAOK.

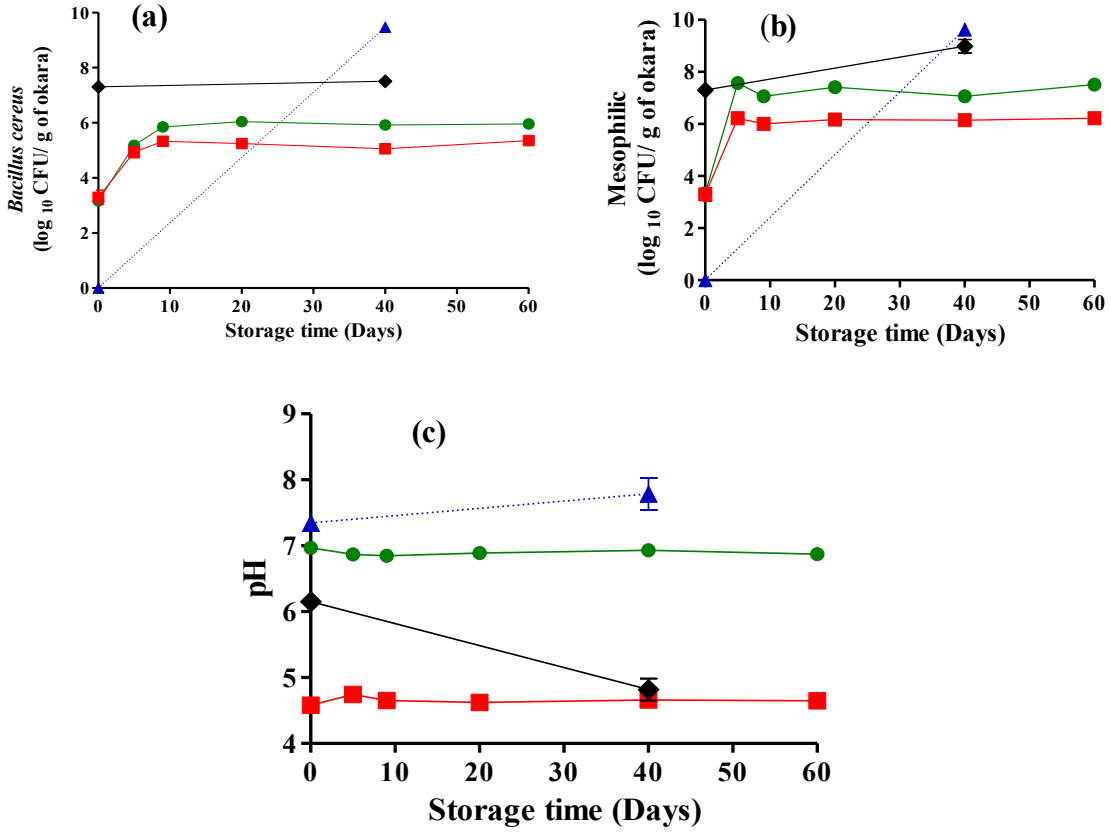
The lipid oxidation is generally associated with thermal treatment, when the decomposition of hydroperoxides may occur, however, the autoxidation continues to occur after that process due to free radicals (peroxides, hydroperoxides) formed.

3.4.5. Microbiological stability

The temporal evolution of the microbiological parameters and pH of okara during storage is reported in Fig.3.2. The pH value in initial stages were 7.37 ± 0.09, 6.15 ± 0.09, 6,97 ± 0.03, 4.58 ± 0.02, for FAOK, FNAOK, AOK and NAOK, respectively. The pH value ca. 7 is the most suitable for bacteria growth. The thermal process previously applied in fresh okara induced a significant ($P < 0.05$) reduction in the pH value. AOK, NAOK and FAOK showed a non-significant ($P > 0.05$) change in pH after 60 days of storage. Nevertheless, the pH in FNAOK decreased significantly ($P < 005$) from 6.15 ± 0.09 to 4.82 ± 0.17 after 40 days. This reduction may be associated with fermentation of the present carbohydrates (Luyun Cai et al., 2014).

The evolution of bacterial counts during the 40 and 60 days of storage for fresh and dry okara are reported in Fig. 3.2 and Table 3.4. FAOK showed a decrease of *Bacillus cereus* and mesophilic bacteria compared with FNAOK. Furthermore, the drying process also decreased the mesophilic bacteria and *Bacillus cereus*. At initial time, no *Enterobactereacea,* yeasts and molds were detected in any okara, but after 40 days of storage the fresh okara showed counts of 2.87 and 3.04 ($\log_{10}$ CFU/ g of okara) in FAOK and FNAOK, respectively, and dried okara ranged from 2.5 to 2.7 $\log_{10}$ CFU g^{-1} of okara after 60 days of storage, in NAOK and AOK, respectively. The yeasts and molds were not detected in dried okara, although the fresh okara had higher count 7.10 and 8.31- $\log_{10}$ CFU g^{-1} (FNAOK and FAOK)

indicating higher contamination after 40 days of storage (table 3.4), this also was observed for mesophilic and *Bacillus cereus* (Fig. 3.2). After 40 days the fresh okara storage in refrigerated conditions showed visible microbiological increase, for this reason this was the end time of storage for FAOK and FNAOK.



Days	0	5	9	20	40	60
AOK	Aa	Ab	Ab	Aab	Aab	Aab
NAOK	Ba	Bb	Bac	Bac	BC	Bac
FAOK	Ca				Ca	
FNAOK	Da				Db	

Figure 3.2. Enumeration of (a) *Bacillus cereus,* (b) total mesophilic bacteria and (c) pH of dried and fresh okara (autoclaved and not autoclaved) throughout the storage (-●- AOK; -■- NAOK, -▲-FAOK, -♦-FNAOK). Different lowercase letters in the same line and different uppercase letters in the same column are statistically different ($P < 0.05$).

Concerning for *Bacillus cereus* counts and mesophilic bacteria (Fig. 3.2) it was observed that both dried okara decreased the initial contamination and no viable cells were detected at initial stages (assuming the method limits), thus the thermal sterilization could inactivate or damage the spores (Warda et al., 2015), however, after 5 days the numbers increased, even for the previously autoclaved okara. Thus, both treatment showed an increase of *Bacillus cereus* and mesophilic bacteria after 5 days, but these values were maintained then after along storage time. Furthermore, the higher levels of *Bacillus cereus* in food can indicate risk for consumers because this bacterium may produce enterotoxins that cause food poisoning with low infectious dose. *Bacillus cereus* counts were previously detected in a fermented food soybean product by Han et al. (2001). On the other hand, the mesophilic bacteria presented similar numbers than *Bacillus cereus*. Although the autoclave inhibited the initial microbial contamination, throughout storage, the final counts were still relevant, probably due the drying process or initial contamination during the milling of sample.

Table 3.4. Enumeration of *Enterobacteriaceae*, yeasts and molds of okara throughout storage time.

Storage time (days)	*Enterobacteriaceae*				Yeasts and molds	
	AOK	**NAOK**	**FAOK**	**FNAOK**	**FAOK**	**FNAOK**
	log CFU g^{-1} of okara					
0	ND	ND	ND	ND	ND	ND
5	ND	ND	-	-	-	-
9	ND	ND	-	-	-	-
20	ND	ND	-	-	-	-
40	2.43 ± 0.10	2.30 ± 0.16	2.87 ± 0.03	3.04 ± 0.00	8.31 ± 0.07	7.10 ± 0.06
60	2.71 ± 0.11	2.48 ± 0.23	-	-	-	-

[a] ND: not detected and (-) not analyzed.

3.4.6. Isoflavones content in okara

During the soy beverage production, a fair amount of isoflavones (12-30 %) was retained in the okara (Jackson et al., 2002). These compounds are important secondary

metabolites present in soybeans and previous studies demonstrate that soy isoflavones have antioxidant properties and permit the reduction of oxidized low density lipoprotein (LDL) (Fritz et al., 2003). The main isoflavones quantified were glycosides (28.9%) and aglycones (15.4%) (Jackson et al., 2002). In this study four main isoflavones in okara samples were identified, including two aglycones (daidzein and genistein) and their glycosides (daidzin and genistin). The content of isoflavones were analyzed during the storage for the different okara samples as are shown in Figure 3.3. The analysis by HPLC confirm that the main isoflavones in okara were glycosides (daidzin and genistin); in Figure 3.3b is possible to observe that genistin was the most abundant glycoside and the autoclaving process increased its content 2.4-fold in fresh okara and 4-fold in dried okara. However, aglycones was found in lower concentration, the daidzein value was between 1.34 and 4.41 mg per 100 g of dry okara and genistein ranged from 2.13 to 3.75 mg per 100 g.

Isoflavones analyzed in this study increased with thermal treatment and the total isoflavones found in initial time of storage was 74.40, 20.06, 83.21 and 35.77 (mg isoflavones/100 g okara) for AOK, NAOK, FAOK and FNAOK, respectively. The value found for fresh and dried okara previously autoclaved was similar to previous studies of Genovese et al. (2006) and Jankowiak et al. (2014) with values ranging between 52.8 and 84.9 mg/100g of okara. However, the okara non-autoclaved showed the lower content, with a significant ($P < 0.05$) decreased in content of isoflavones of 3.7- fold and 2.3-fold for dried and fresh okara respectively. Previous studies reported that the total isoflavones can be influenced by different processes applied (Voss et al., 2018). Furthermore, the biological effect of each isoflavone was different and the distribution of isoflavones in the product was important for the functional value (Devi et al., 2009).

Since several studies reported the beneficial effect of isoflavones, according Izumi et al., (2000) the aglycones (genistein and daidzein) are absorbed faster in humans and in greater amounts than their glycosides, thus these can be more effective than their glycoside for preventing chronic diseases and suggested that products rich in genistein can be used to preventing the menopause symptoms, cancer, osteoporosis. Furthermore, in a study of He and Chen (2013) the genistein was considered a potent chemopreventive agent for prevention of breast cancer.

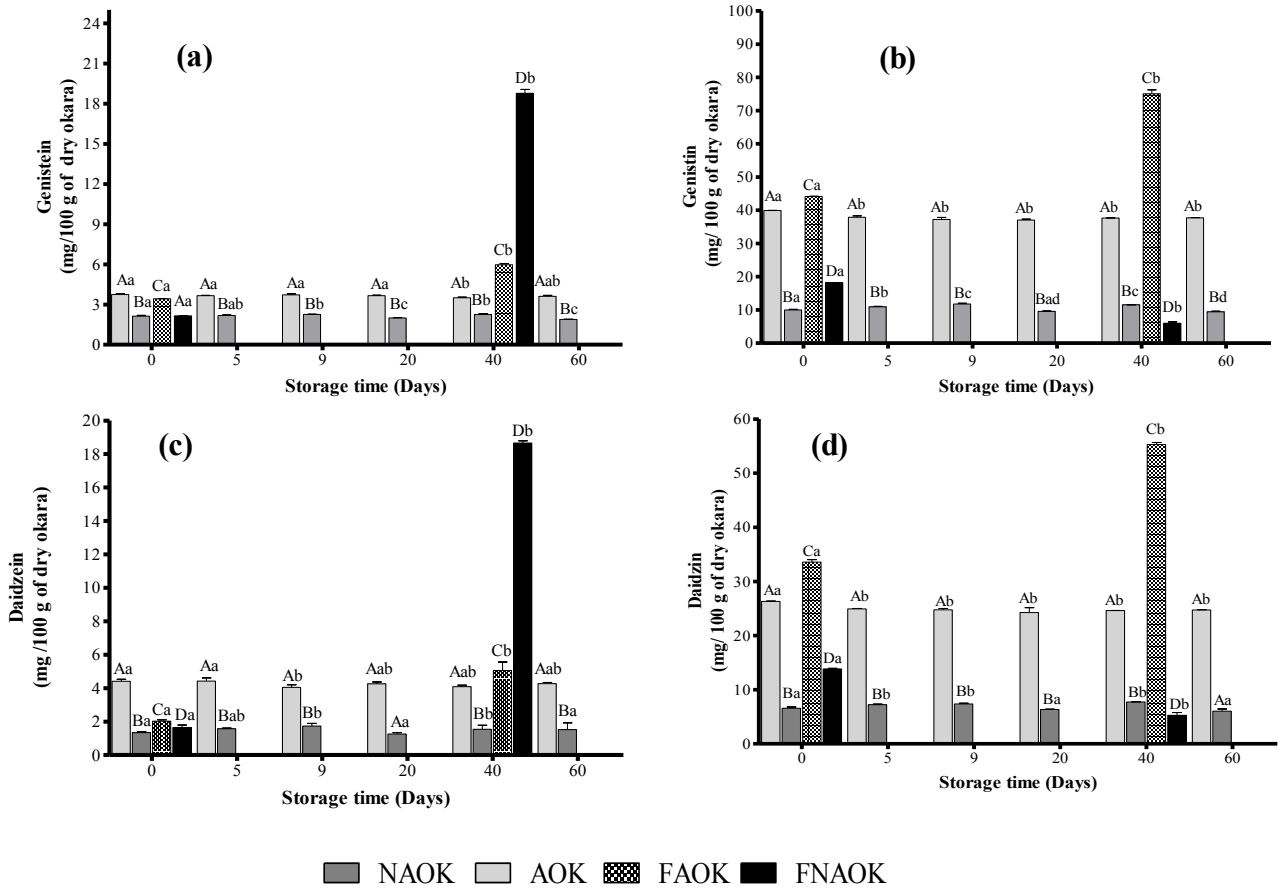


Figure 3.3. Isoflavones concentration genistein (a), genistin (b), daidzein (c) and daidzin (d) of dried and fresh okara (autoclaved and not autoclaved) throughout the storage. Different lowercase letters for the same sample are statistically different ($P < 0.05$). Different uppercase letters in the same storage time are statistically different ($P < 0.05$).

3.4.7. Antioxidant activity and total phenolics in okara

Dietary antioxidants can bring beneficial health effects eliminating free radical, hence it is important to study the antioxidant activity of okara with different thermal treatments. The antioxidant activity (ABTS and DPPH) and total phenolics of okara samples during storage are shown in Figure 3.4. FAOK presented the highest antioxidant activity in initial time with values of 77.16 ± 6.74 mg Trolox/ 100 g (ABTS) and 60.21 ± 2.14 mg Trolox/ 100 g (DPPH). However, the content of total phenolics (Fig. 3.4c) was significantly ($P < 0.05$)

lower than those found in dry okara. The values of antioxidant activity performed by DPPH was lower than ABTS, may be due to steric accessibility of DPPH radical, considering that their active sites radicals are in the middle of the structure, so the antioxidants can access the sites of radical DPPH to a lesser extent (Suvarnakuta et al., 2011b).

The fresh okara after autoclaving showed 1.8-fold more total phenolics and both dry okara also showed significant differences and increased 4.9-fold compared with fresh okara. However, after 40 days, as shown the Fig. 3.4c FAOK and FNAOK increased significantly ($P < 0.05$) by 23.4 and 13.9-fold, respectively. The content of total phenolics in dry okara was similar to previous studies in different soy cultivars while the fresh okara was lower (Devi et al., 2009).

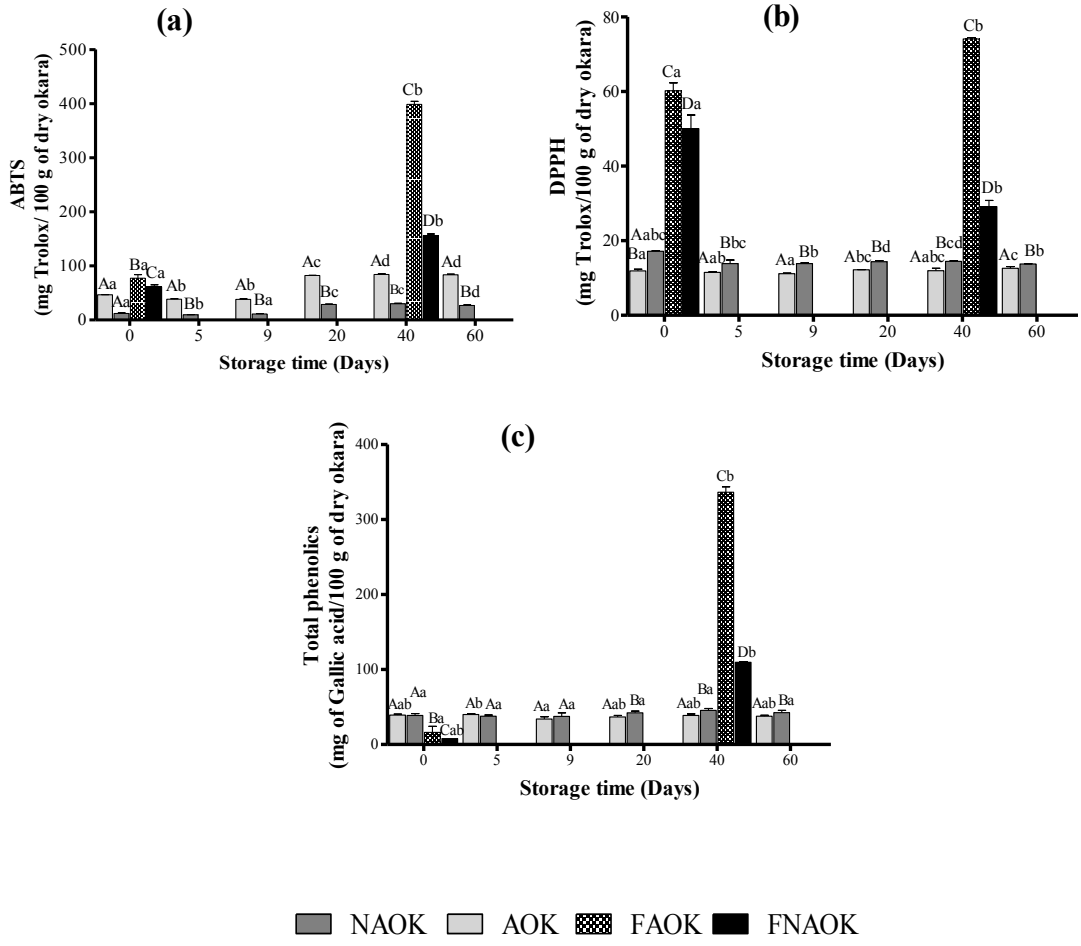


Figure 3.4. Antioxidant activity ABTS (a), DPPH (b) and total phenolics (c) over storage time in fresh and dried okara. Different lowercase letters in the same sample are statistically different ($P < 0.05$). Different lowercase

letters for the same sample are statistically different ($P < 0.05$). Different uppercase letters in the same storage time are statistically different ($P < 0.05$).

Through the correlation of Pearson, it was possible to evaluate the correlations between the bioactivities in okara. The antioxidant activity (DPPH) of AOK presented a strong and negative correlation with total phenolic compounds ($r = 0.95$). However, NAOK correlated well the ABTS and DPPH with total phenolic compounds ($r = 0.87$ and $r = 0.93$, respectively). In a previous study, Devi et al. (2009) also found a lower but positive correlation between antioxidant activity and phenols ($r = 0.60$) in soybeans and soy products. However, the correlation of isoflavones (genistin, daidzin, genistein and daidzein) with antioxidant activity was negative or non-significant for both dry okara samples. On the other hand, all isoflavones showed good correlation with antioxidant activity ($r > 0.97$) in FAOK. Nevertheless, FNAOK presented the highest and positive correlation with aglycones (genistein and daidzein). In a previous study, Ruiz-Larrea et al. (1997) also related the higher antioxidant activity with aglycones and considered that the number of free hydroxyls of the aromatic ring determines the antioxidant capacity and follows this order: genistein> daidzein> genistin> daidzin.

3.4.8 Trypsin inhibitor activity (TIA)

The trypsin inhibitors is the major antinutritional factors in soybeans and this can cause serious adverse effects in humans and animals (Hossain and Becker, 2002). The trypsin inhibitor activity (TIA) found in fresh and dried okara are shown in the Table 3.5. The fresh okara in this study showed lower trypsin inhibitor activity (10.45 ± 0.14) compared with values reported by Olguin et al. (2003) in raw soybeans (27.2 TUI/ mg dry grain raw soybeans). This reduced TIA observed in fresh okara can be due the thermal process applied for aqueous extraction of soy beverage that may reduce the trypsin inhibitors of the by-product (okara) obtained after this process. A previous study Stanojevic et al. (2013) showed that okara obtained from soymilk manufacture using Japanese method can have low trypsin inhibitor.

The thermal treatment (autoclave) applied in fresh okara or previously dried was significant ($P < 0.05$) for TIA, AOK and FAOK that showed about 10 times less TIA in relation to okara not autoclaved 1.02 ± 0.16 (FAOK) and 0.67 ± 0.23 (AOK), while the FNAOK and NAOK presented values between 10.45 ± 0.14 to 6.57 ± 0.24, respectively. These results for okara autoclaved were similar to those found in a previous study of the Siddhuraju, Vijayakumari and Janardhanan (1996) that used different heat treatments to inactive the TIA in seeds of Mucuna pruriensa. The major reduction was obtained using autoclave (ca. 96 %) but did not achieve the total inactivation of TIA. According to De Lumen and Salamat (1980) the heat resistance of these inhibitors is due to the proteins mainly located in the seed coat and associated with tannins.

Table 3.5. Trypsin inhibitor activity (TIA) obtained in fresh and dried okara at initial time of storage.

Sample	TUI/ mg dried okara
FAOK	1.02 ± 0.16^{a}
FNAOK	10.45 ± 0.14^{b}
AOK	0.67 ± 0.23^{a}
NAOK	6.57 ± 0.24^{c}

The results were expressed in average ± SD (n=3). Different lowercase letters are statistically different ($P < 0.05$).
TUI: trypsin inhibitor activity units.
FAOK: Fresh okara autoclaved.
FNAOK: Fresh okara not autoclaved.
AOK: Dry okara autoclaved.
NAOK: Dry okara not autoclaved.

3.5. Conclusion

In this study, the chemical composition, the amino acid and the fatty acid profile, the antioxidant properties and the isoflavones composition of fresh and dried okara (autoclaved and non-autoclaved) were evaluated along the storage. Differences were observed between the thermal treatments and during storage, especially for okara autoclaved. The mains fatty acid identified were linoleic acid for autoclaved okara and oleic acid for non-autoclaved okara. The dried okara showed a low microbiological charge and reduced growth during

storage compared with fresh autoclaved okara that showed total inhibition of contamination. Okara previously autoclaved presented higher total amino acid than non-autoclaved okara.

There was variation in the concentrations of isoflavones according to the different treatments applied; total isoflavones were higher in dry and fresh autoclaved okara. The results also suggest that okara is rich in genistin and daidzin. On the other hand, the amount of total phenolics in both dried okara was similar in initial stages and higher than in fresh samples. Antioxidant activity showed significant differences along storage and the fresh autoclaved okara had the highest activity. Besides, the non-autoclaved okara showed a higher lipid oxidation and a decrease in the total lipids. This study evidenced the high nutritional and functional value of okara flours and proved that heat process via autoclaving is a good processing to further improve flour nutritional and functional quality whilst reduce lipid oxidation and microbial growth, permitting better preservation for longer storage periods.

Part III- Enzymatic hydrolysis of soybean by-product (okara)

CHAPTER 4- Impact of thermal treatment and hydrolysis by Alcalase and Cynara cardunculus enzymes on the functional and nutritional value of Okara

4.1. Abstract

Enzymatic hydrolysis of dry okara (autoclaved and non-autoclaved) with Alcalase (AL) and proteases from *Cynara cardunculus* (CY) were studied, assessing the impact of heat treatment and hydrolysis on potential antioxidant and antihypertensive activities of final hydrolysates. This study showed that the thermal treatment (sterilization at 121 ºC for 15 min) in okara facilitated the enzymatic attack and the degree of hydrolysis (DH), especially for AL (37.9%), which presented a greater hydrolysis compared to CY. The antioxidant activity of dried Okara (either autoclaved or not) hydrolyzed with AL was higher (4.2 mg Trolox/mL) than that observed for CY. Additionally, the potential ACE-inhibitory activity was high for samples hydrolyzed with both enzymes, however the highest ACE inhibition was also found for AL (IC_{50}=9.97 μg/mL). This study allowed the identification of new peptide sequences in okara hydrolyzed with both enzymes, and some sequences could explain their bioactivities. The results indicate that okara hydrolysates can either be used as functional ingredient or as food supplement for blood pressure lowering or antioxidant applications in future.

4.2. Introduction

The soybean is an important grain originating in Asia and has been consumed worldwide (Moraes Filho et al., 2016). Many by-products are obtained from soybean processing, namely okara that constitutes a major insoluble by-product obtained from soy beverage, tofu and their derivatives (O´Toole, 1999).

Considering that about 250 kg of okara can result from the production of 1000 L of soy beverage, and based on soy beverage consumption, approximately 14 million tons of okara are estimated to be produced worldwide annually, what can be associated to serious environmental problems (Nguyen et al., 2013) and massive loss of valuable nutrients. Environmental problems caused by okara are mainly related to its high instability, since this

by-product is naturally decomposed when not refrigerated due to its high water content (~80%) and a rich composition in proteins, dietary fiber and lipids (MUFA and PUFA) (Ma et al., 1997; O´Toole, 1999). Thus, okara can be considered valuable ingredient for animal and human nutrition (Harthan and Cherney, 2017; Moraes Filho et al., 2016). Many studies based on okara have also reported fermentation applications (Moraes Filho et al., 2016), extraction of protein (Vishwanathan et al., 2011), extraction of polysaccharides (Villanueva-Suárez et al., 2013) and isoflavones (Jankowiak et al., 2014).

Studies performed with okara have shown some bioactivities, especially in fermented products (Moraes Filho et al., 2016). Other relevant compounds present in okara are the isoflavones, since although most soybean fraction is extracted during soymilk production ca. 12-30% of the isoflavones can be retained in okara (Jankowiak et al., 2014). Okara possesses ca. 30% (dry basis) of two main proteins - β-conglycinin (7S) and glycinin (11S) (O´Toole, 1999; Singh et al., 2014). These proteins resist to complete digestion by gastrointestinal enzymes and not all essential amino acids are bioavailable due to their poor solubility (Chan and Ma, 1999).

Through enzymatic hydrolysis of soy proteins, we can generate bioactive peptides and free amino acids. These peptides are specific protein fragments that can possess antioxidant activity, i.e. can control oxidative processes in food and in human, and also may have antihypertensive and antidiabetic activities (Singh et al., 2014) among other potential physiological activities.

Alcalase is an enzyme extract from *Bacillus licheniformis*, which present a several proteinases with different specificities (Osman et al., 2016). Alcalase has been used extensively to prepare hydrolysates of soy, whey, sweet potato and fish protein with bioactive peptides (Doucet et al., 2003; Rebeca et al., 1991; Zhang and Mu, 2017; Zhong et al., 2007). Aqueous extracts from cardoon (*Cynara cardunculus*) flowers can be considered the best-known vegetable coagulant used for production of some traditional ewe´s milk cheeses in Portugal and Spain (Liburdi et al., 2018). The enzymes Cardosin A and Cardosin B, present in the thistle, shown different amino acid sequences but both have proteolytic activity and promote a milk clotting (Barros and Malcata, 2002; Liburdi et al., 2018). Cardosin B has broader specificity than Cardosin A, in relation of activity and specificity, the Cardosin A is comparable with chymosin, while Cardosin B (~25%) is comparable with pepsin, however

this enzyme is less specific than pepsin and hydrolyses bonds of phenylalanine, tyrosine, leucine or valine residues (Barros and Malcata, 2002).

Despites some studies have been performed with okara, most of them explored its nutritional composition, and only few have tried to valorize the complete by-product but with scarce applications implemented in the market. Therefore, okara needs to be further valorized in order to transform this by-product into a food product with added value. Previous studies have shown that the pretreatment influence the molecular composition of protein, in addition the heat treatment provides the denaturation of protein and facilitates the enzymatic attack, because the denatured proteins are more easily hydrolyzed than native form and the peptide bonds are more accessible in unfolded protein molecules (Achouri et al., 1999; Márquez et al., 1998).

Moreover, the heat treatment influence the nutritional quality of food protein and several studies have shown that the wet heat treatment improve the quality compared with dry heat treatments (Márquez et al., 1998).So, in this study the enzymatic hydrolysis of dried okara (previously autoclaved and not autoclaved) with Alcalase and *C. cardunculus* extract was investigated and the antioxidant capacity and antihypertensive activity of the final hydrolysates were characterized. Furthermore, the peptides profile and respective sequence, as well as free amino acids composition of each hydrolysate was also evaluated.

4.3. Material and methods

4.3.1. Okara samples and drying process

The okara used in this study was kindly provided by a soy beverage producer (NUTRE Industry, Aveiro, Portugal). The collection of okara samples occurred immediately after the beverage production. Samples were readily transported under refrigerated conditions (not more than 2 h) and divided into 2 lots: one was immediately dried at 65 ºC for 68 h, to constant weight, whereas the other was firstly autoclaved (1 atm, 121 ºC and 15 min) and after was dried under the same conditions used for the non-autoclaved lot. Both samples were then grounded in a mill (Retsch, Haan, Germany) with 1 mm sieve.

4.3.2. Proximate composition analysis of okara

Moisture of okara was determined at 105 ºC for 24 h, according to the Association of Official Analytical Chemists (1995). The crude protein was determined using a nitrogen analyser (N × 6.25, Leco N analyzer, Model FP-528, Leco Corporation, St. Joseph, USA).

4.3.3. Enzymatic hydrolysis of okara

The hydrolysis of dried okara (autoclaved and not autoclaved) were performed in substrate solutions containing 27 mg protein/mL and using two enzymes - Alcalase 2.4 L FG (Novozymes, Bagsvaerd, Denmark) and proteases from *Cynara cardunculus* (Formulab, Maia, Portugal). For both enzymes were tested four different enzyme: substrate (E/S) ratios (0.4, 0.6, 0.7 and 1.1 v/w) and three reaction times (0, 2.5 and 5 hours). The summary of experiment conditions is shown in Table 4.1.

The enzymatic hydrolysis with Alcalase (AL) and proteases from *Cynara cardunculus* (CY) was performed in an orbital shaker (SI-100C, Wiggenhauser, Germany), according optimal conditions: AL (phosphate buffer (0.025 M), pH 8.3 at 50 ºC and 120 rpm) CY (citric acid – sodium citrate buffer (0.1 M), pH 5.2 at 55 ºC and 120 rpm). Afterwards, the samples were centrifuged at 2665 x g for 15 min at 4 °C (Universal 32R; Hettich, Tuttlingen, Germany) and the supernatant was analyzed.

Table 4.1. Summary of experiment conditions tested: okara sample, type and concentration of enzyme and time of hydrolysis.

Sample	Enzyme	E/S	Time (h)	Sample	Enzyme	E/S	Time (h)
NAOK	Control	0	0	AOK	Control	0	0
			2.5				2.5
			5.0				5.0
	AL	0.4	2.5		AL	0.4	2.5
			5.0				5.0
		0.6	2.5			0.6	2.5
			5.0				5.0
		0.7	2.5			0.7	2.5
			5.0				5.0
		1.1	2.5			1.1	2.5
			5.0				5.0
	CY	0.4	2.5		CY	0.4	2.5
			5.0				5.0
		0.6	2.5			0.6	2.5
			5.0				5.0
		0.7	2.5			0.7	2.5
			5.0				5.0
		1.1	2.5			1.1	2.5
			5.0				5.0

E/S – ratio of enzyme: substrate (v/w); NAOK- dry okara not autoclaved; AOK- dry okara previously autoclaved; AL – Alcalase; CY - proteases from *Cynara cardunculus.*

4.3.4. Determination degree of hydrolysis

The degree of hydrolysis (DH) of okara hydrolyzed with CY and AL was determined using the modified OPA method described by Nielsen, Petersen and Dambumann (2001). The method was modified concerning the volumes used, so the volume of 200 µl standard, blank (distilled water) or sample were added to each respective tube and 1.5 ml OPA reagents were added to all test tubes and let to react for 2 min. The absorbance was read at 340 nm. The % DH was calculated following–the equations reported by Adler-Nissen (1984) using as reference the control without enzyme.

$$\text{Serine } NH_2 = \frac{\text{OD sample} - \text{OD blank}}{\text{OD standart} - \text{OD blank}} * 0.9516 * 0.03 * \frac{100}{X + P}$$

Equation (4.1)

$$H = \frac{\text{Serine } NH_2 - \beta}{\alpha}$$

Equation (4.2)

$$DH = \frac{h}{h_{tot}} * 100$$

Equation (4.3)

Where: SerineNH_2 = meqv serine NH_2/ g protein; X = sample (g); P = protein (%); 0.03 is the volume in liter (L); h = number of hydrolyzed bonds (meqv/g protein); β = 0.342 (specific for soy); α = 0.970 (specific for soy); h_{tot} = total number of peptide bonds per protein equivalent (7.8 specific to soy).

4.3.5. Molecular weight pattern of okara hydrolysates

The molecular weight of okara hydrolysates was determined by gel filtration chromatography using the FPLC (fast protein liquid chromatography-gel filtration) AKTA Pure 25 system coupled with two gel filtration columns: Superdex 200 increase10/300 GL and Superdex peptide, 10/300 GL. The mobile phase was 25 mM phosphate buffer (pH 7.0), 150 mM sodium chloride and 0.2 g L^{-1} sodium azide. The flow of eluent was 0.5 mL min^{-1} and was monitored by absorbance at 280 nm.

4.3.6. Profile of peptides of okara hydrolysates by HPLC

Profile of peptide hydrolysates were analyzed by a modified method described by Garcia et al.(1997). Fifty µL of hydrolysate extract (obtained as explained in 4.3.3) were analyzed by HPLC (Waters Series 2695. Mildford, MA. USA) coupled to a diode array detector, using a Kromasil C-18 column (250 x 4.6 mm) to separate the peptides at 50 ºC. The mobile phase consisted of 0.1% trifluoracetic acid in water (v/v) (solvent A) and 0.1% trifluoracetic acid in acetonitrile (v/v) (solvent B). A linear gradient of 5 steps was used: 5-20% B; 20-25% B and 25-25% B for 10 minutes each, followed by a rapid increase of 35-46% B in 30 seconds and a linear reversed gradient 46%-5% B in 30 seconds to re-equilibrate

the column to starting condition. The flow rate was 1 mL/min. The proteins were detected by UV absorption at 254 nm. The data were processed on Empower 3 software. The profile of peptides was compared with the different hydrolysates.

4.3.7. Profile of free amino acids in hydrolysates

Free amino acids content was performed by derivatization with orthophthalaldehyde (OPA) methodology. The amino acids were separated by HPLC (Beckman coulter. California USA) coupled to a fluorescence detector (Waters, Milford, MA, USA) according to the procedure of Proestos, Loukatos and Komaitis (2008), 100 µL of each sample, at concentration 10 mg mL^{-1}, was derivatized according described method and injection volume of derivatives was 20 µL. The analysis were made in duplicate and quantified using a calibration curve built with amino acids standards (Sigma – Aldrich. St. Louis MO. USA) and expressed as mg g^{-1} of protein content.

4.3.8 Determination of antioxidant activity

Antioxidant activity of okara hydrolysates was determined using ABTS radical according the method described by Gião et al. (2007). The quantification was performed at 734 nm (mini UV 1240, Shimadzu, Tokyo, Japan) using 10 µl of samples (obtained as explained in 4.3.3) in 1 ml ABTS with absorbance limit of 0.7 (± 0.02). Trolox was used as a standard to prepare a calibration curve in the range 0.025 - 0.5 mg/mL. The results were expressed in mg Trolox per mL of hydrolysate.

4.3.9. Determination of ACE-inhibitory activity

The ACE inhibitory activity was performed based on the method modified by Quirós, Contreras, Ramos, Amigo and Recio (2009) using the fluorimetric assay. ACE working solution (42 mU/mL) was diluted with 150 mM Tris-HCl (pH 8.3) containing 0.1 mM $ZnCl_2$ and the substrate used was o-Abz-Gly-p-Phe(NO2)-Pro-OH (0.45 mM) dissolved in 150 mM Tris-HCl (pH 8.3) and 1.125 M NaCl. For reaction development a microplate with ninety-

six-well (Porvair, Leatherhead, UK) was used. In each test sample, the assay mixture was composed of the following components: 40 µL of ACE (42 mU/mL), 40 µL of hydrolysate and 160 µL of substrate (o-Abz-Gly-p-Phe(NO2)-Pro-OH, 0.45 mM). Control contained 40 µL of ACE (42 mU/mL), 40 µL of water distilled and 160 µL of substrate. Blank contained 80 µL of distilled water and 160 µL of substrate. The mixture was incubated at 37 °C and the fluorescence generated was measured at 30 min using a FLUOstar OPTIMA plate reader (BMG Labtech, Offenburg, Germany) and the wavelengths used were 350 and 420 nm for excitation and emission, respectively. The software used to process the data was FLUOstar control (version 1.32 R2, BMG Labtech). The activity of each sample was tested in duplicate and the inhibitory activity was expressed as the protein concentration required to inhibit the original ACE activity by 50% (IC50). The percentage of ACE-inhibitory activity was calculated using the formula 100 x (C - S)/(C - B) and IC_{50} values was calculated using the nonlinear fitting to the data. The protein content in hydrolysate extracts was analyzed by bicinchoninic acid, using albumin as standard.

4.3.10. MALDI-TOF/TOF

For mass spectrometry analysis, the okara hydrolysates were detected and acquired by a MALDI-TOF/TOF mass spectrometer (4800 Plus MALDI TOF/TOF Analyzer, SCIEX, Framingham, MA), equipped with a 200-Hz frequency Nd:YAG laser, operating at a wavelength of 355 nm. Peptides were purified by reversed-phase C18 chromatography (ZipTips, Millipore) following the manufacturer's instructions and eluted in the MALDI sample plate with the MALDI matrix alpha-Cyano-4-hydroxycinnamic acid (CHCA) elution solution at 8 mg/mL in 50% ACN, 0.1% TFA, 6 mM ammonium phosphate. Peptide mass spectra were acquired in reflector positive mode in the mass range of *m/z* 700–5000. Some peptide ions were selected for MS/MS peptide sequencing. Proteins were identified by the combined Peptide Mass Fingerprint (PMF) + MS/MS approach with the Mascot software (Matrix Science) using the UniProt protein sequence database for the taxonomic selection *Glycine max* (2015_12 release). The protein search settings were: methionine oxidation (variable modification), no enzyme specificity, peptide mass tolerance of 20 ppm and

fragment mass tolerance of 0.5 Da. Protein scores greater than 61 were considered significant ($P < 0.05$).

4.3.11. Statistical analysis

The results are expressed as the mean values ± standard deviations. Statistical analysis was performed using SPSS version 23.0 for Windows. Statistical significance of differences among means and group means was analyzed by One-Way analysis of variance (ANOVA) and student's T-tests. Interaction between factors (reaction time, thermal treatment and E/S ratio) for hydrolysis degree and antioxidant activity were analyzed by Two-Way analysis of variance (ANOVA). Correlations among hydrolysis degree and antioxidant activity were calculated using Pearson's correlation coefficient (r). In all cases a significance level of $P < 0.05$ was considered.

4.4. Results and discussion

The total protein of dry okara autoclaved (AOK) and dry okara not autoclaved (NAOK) was similar, with values of 27.03 % ± 0.24 and 26.84% ± 0.05, respectively. This protein content is similar to values reported in previous studies and protein from okara have shown high nutritive quality (Ma et al., 1997; O´Toole, 1999). The dried okara products had a moisture content of 3.39% ± 0.14 and 3.80% ± 0.11, for NAOK and AOK, respectively. The drying treatment led to stable powders that maintained stability under controlled storage conditions, overcoming the instability of okara due to the high initial moisture content (80%) (O'Toole. 1999).

4.4.1 Enzymatic Hydrolysis

As shown in Table 4.2, for DH of okara hydrolysates obtained from NAOK and AOK produced under different enzymes and conditions, it was observed that the degree of hydrolysis increased from 12.4% ± 3.1 to 33.8% ± 0.2 and from 21.7% ± 0.8 to 37.9% ± 1.4

% for NAOK and AOK, respectively in 5 hours of reaction. DH showed significant differences ($P < 0.05$) with increased E/S ratio for both reaction times evaluated. Hydrolysis of NAOK and AOK during 2.5 hours also showed significant differences ($P < 0.05$) for the different levels of enzyme. The values obtained for NAOK were similar to those observed by Sbroggio et al. (2016) for AL, however AOK hydrolysate presented higher DH values. Thus, this suggests that okara sterilization heat treatment improves the DH as observed in Table 4.2, for both enzymes. Previous studies (Achouri et al., 1999; Márquez et al., 1998) have also showed that the heat treatment can increase the hydrolysis degree. According to Achouri et al. (1999) the thermal denaturation facilitates the enzymatic attack making peptide bonds in the unfolded protein molecules more accessible. Nevertheless, according to previous studies in soy flour and meal, the nutritional value can be increased with the heat treatment, but may also reduce the functionality and bioavailability of amino acids (Radha and Prakash, 2009).

In relation to hydrolysis performed with CY for 5 hours of reaction a lower DH was observed comparing to AL, presenting values of 2.26% ± ≤ 0.01 and 3.57% ± 0.18 % for NAOK and AOK, respectively. The CY showed significant differences ($P < 0.05$) for DH of individual factors evaluated (E/S ratio, reaction time and thermal treatment of okara) and for the interactions between these factors. Moreover, for NAOK or AOK hydrolyzed for 2.5 hours of reaction no significant differences ($P > 0.05$) were found, whereas for 5 hours, the DH showed significant differences ($P < 0.05$). AL seemed to hydrolyze okara proteins to a greater extent than the CY. Alcalase is an endopeptidase and has a range of specificity of peptide bonds for hydrolysis, especially for aliphatic (Leu and Ala), aromatic (Phe, Trp and Tyr), sulphur-containing (Met), acidic (Glu), hydroxyl (Ser) and basic (Lys) residues (Doucet et al., 2003).

Table 4.2. Degree of hydrolysis (%) of hydrolysates obtained from dry okara autoclaved (AOK) and dry okara not autoclaved (NAOK) using Alcalase (AL) and proteases from Cynara Cardunculus (CY).

Enzyme	Time (h)	Sample	E/S[a]			
			0.4	0.6	0.7	1.1
AL	**2.5**	**NAOK**	13.14 ± 1.05[a]	15.38 ± 0.19[a]	15.65 ± 0.26[a]	28.56 ± 1.28[b]
		AOK	22.03 ± 0.27[a]	21.08 ± 0.12[a]	21.54 ± 0.54[a]	33.69 ± 2.33[b]
	5.0	**NAOK**	12.42 ± 3.11[a]	24.98 ± 2.23[b]	28.11± 0.10[bc]	33.80 ± 0.18[c]
		AOK	21.72 ± 0.81[a]	31.25 ± 1.64[b]	31.99 ± 0.49 [bc]	37.87 ± 1.43[c]
CY	**2.5**	**NAOK**	1.87 ± 0.05[a]	2.02 ± 0.09[a]	1.80 ± 0.11[a]	2.00 ± 0.10[a]
		AOK	1.93 ± 0.47[a]	1.52 ± 0.05[a]	1.94 ± 0.11[a]	2.49 ± 0.11[a]
	5.0	**NAOK**	2.16 ± 0.02[a]	1.42 ± 0.06[b]	1.85 ± 0.24[b]	2.26 ± ≤ 0.01[a]
		AOK	3.36 ± 0.04[a]	1.72 ± 0.04[b]	2.28 ± 0.23[c]	3.57 ± 0.18[a]

Values are expressed as average ± standard error (n=2). E/S – ratio of enzyme: substrate (v/w). Means in the same line with common lower letters are not significantly different ($P < 0.05$).

The CY is constituted by two types of Cardosins A and B, with similar specificity to chymosin and pepsin, respectively (Barros and Malcata, 2002). Both Cardosin have a preference for cleaving peptide bonds just past hydrophobic side chains (Barros and Malcata, 2002). In conclusion, the broader specificity of AL may explain a higher DH obtained by AL. Furthermore, in Table 4.5 it is possible to confirm the specificity of the cleaving though the terminal chain of the peptides obtained in both enzymatic hydrolysis: the CY mainly showed hydrophobic amino acid while AL showed many peptides with glutamic acid in terminal chain.

4.4.2. Molecular weight profiles of okara hydrolysates

The molecular weight distribution of the hydrolysates AOK and NAOK obtained by AL and CY, and the respective non-hydrolyzed samples, were monitored by FPLC as shown in Figure 4.1. Unhydrolyzed NAOK and AOK profile under similar conditions used for hydrolysis by AL (Fig. 4.1 A and B) exhibited both high and low molecular weight fractions. Unhydrolyzed AOK (Fig.4.1 A) showed a relevant peak with molecular weight (MW) range between 160-22 kDa, (peak 1), a peak 2 with MW lower than 8.5 kDa (average MW of 1.3 kDa) and from the peak 3 representing peptides of low MW (< 1 kDa). The unhydrolyzed NAOK showed differences compared to AOK, especially for peak 1 in Fig. 4.1 (A and C), for which is possible to observe a decrease of 1.5 times after thermal treatment. Thus, it was demonstrated that the applied thermal treatment (121 ºC, 1 atm and 15 minutes) broke part of the high weight proteins present in the okara. When AOK and NAOK were subjected to AL hydrolysis, at high DH, the high molecular weight polypeptides were broken into smaller fragments releasing smaller peptides (< 2 kDa) as shown in the Fig. 4.1 (B and D). Both samples (AOK and NAOK) showed a similar profile after hydrolysis. Likewise, in a previous study, Chiang, Shih and Chu (1999) obtained small peptides with molecular weight < 4 kDa from a soy protein isolate (SPI) hydrolyzed with a mixture of Flavourzyme: Alcalase enzyme. Furthermore, the concentration of peptides after hydrolysis increased as shown in Fig. 4.1 (B and D) since the area of peak increased 78% and 144% for AOK (37.9 % DH) and NAOK (33.8%), respectively, compared with unhydrolyzed samples.

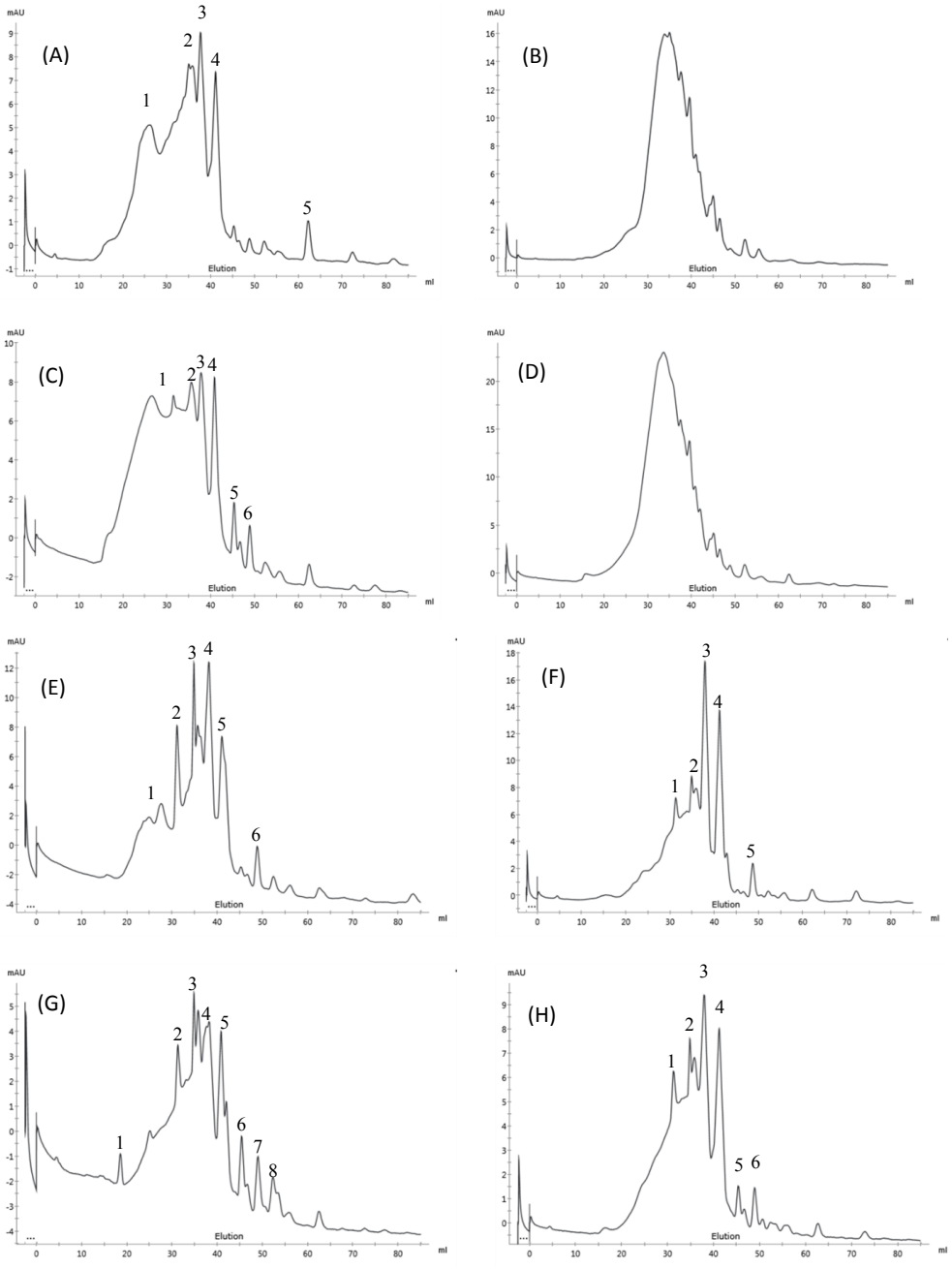


Figure 4.1. Typical size exclusion chromatograms of hydrolysate produced by Alcalase (AL) (A) control AOK, (B) AOK (37.9 % DH), (C) control NAOK and (D) NAOK (33.8 % DH) and by proteases from *Cynara cardunculus* (CY) (E) control AOK, (F) AOK (3.6 % DH), (G) control NAOK and (H) NAOK (2.26 % DH).

The enzymatic hydrolysis by CY induced a lower DH, however based on the observed MW profile, it is evident that the proteins with high MW were broken generating fragments with lower weight. Hydrolyzes of AOK by this enzyme generated two main peaks, as shown

in Fig. 4.1 (F), peak 3 and 4 (< 1 kDa). In addition, the signal of both peaks increased ca. 1.5 times compared to unhydrolyzed. The same was observed for NAOK hydrolyzed with CY, the peak 1 (30 KDa) and peak 8 (< 1 KDa) of unhydrolyzed sample Fig 4.1 (G) disappeared and the intensity of peak 3 (< 1 kDa) in Fig. 4.1 (H) increased 2.2 times. Studies evaluating okara hydrolysis are scarce, so these results showed for the first time that okara hydrolyzed by CY and AL generates smaller fragments mainly < 1 kDa, confirming previous studies with SPI obtained from soy meal hydrolyzed by Corolase PP (Coscueta et al, 2016) and with okara protein hydrolyzed by Trypsin (Chan and Ma, 1999). Additionally, results with CY are presented for the first time for vegetable protein, considering the fact that CY is an enzyme associated with milk coagulation (Liburdi et al, 2018), and applications were only tested on whey protein (Tavares et al., 2011) and on yeast hydrolysis (Amorim et al., 2016).

4.4.3 Free amino acids of okara hydrolysates

Previous studies showed the ratio of essential amino acids in okara was similar to tofu and soy milk, and this is explained by the fact that some soluble protein may remain in the interstices of the particles (O'Toole 1999). Wang and Cavins (1989) analyzed the profile of total amino acids in soybeans, soy beverage and okara, wherein the by-product presented a similar content for total amino acid analyzed.

The free amino acids (FAA) analyzed in NAOK and AOK, unhydrolyzed and hydrolyzed with AL and CY (E/S ratio of 1.1 and reaction time 2.5 h and 5 h) is shown in Table 4.3. The thermal treatment in okara affected the composition of free amino acid and the unhydrolyzed NAOK for both pH condition used in enzymatic hydrolysis contained three essential amino acids (valine, phenylalanine and leucine) while AOK showed only two for each pH condition: AL (phenylalanine and leucine) and CY (phenylalanine and isoleucine). Besides, the unhydrolyzed NAOK showed about two times more FAA than AOK. In previous studies, Fontaine et al. (2007) observed that autoclave treatment in soy meal may reduce the total lysine and reactive lysine content. On the other hand, Dajanta et al. (2011) compared the FAA in autoclaved and boiled soybeans, and observed that autoclaved soy present an increase around two times the content of FAA in relation to boiled soy.

The main free amino acids identified in both samples of okara were aspartic acid, glutamic acid, arginine and phenylalanine (Table 4.3). Furthermore, analysis of amino acid profiles presented in this study showed that FAA changed with the thermal treatment applied, enzyme and hydrolysis time. For example, hydrolysis with CY for 2.5 h presented the higher FAA for both samples, and the glutamic acid increased with enzymatic hydrolysis for AOK, while the hydrolysis using AL showed decrease in glutamic acid. This may be related to the fact that asparagine and glutamine can be converted to aspartic acid and glutamic acid respectively, while other amino acids are preserved, but this is more evident in strong acidic conditions (Ojha et al., 2016).

The different composition for FAA can be related with the specificity of these enzymes. Alcalase showed a large specificity, for example, due presence of a hydrophobic binding area on the enzyme molecule, and for that has preference for aromatic and hydrophobic amino acids, but Alcalase also has specificity for glutamic acid (Doucet et al., 2003). On the other hand, the *Cynara cardunculus* has aspartic proteases and cleave bonds flanked by hydrophobic amino acid residues with large side chains, such phenylalanine and leucine (Barros and Malcata, 2002).

Table 4.3. Free amino acids in unhydrolyzed and hydrolyzed NAOK and AOK with Alcalase and proteases from *Cynara Cardunculus* for 2.5 and 5 hours.

		NAOK[a]						AOK[b]					
		Unhydrolyzed		2.5 h[c]		5.0 h[d]		Unhydrolyzed		2.5 h[c]		5.0 h[cd]	
Enzyme	**Amino acid**	**mg of amino acid g^{-1} protein**	**SD**	**mg of amino acid g^{-1} protein**	**SD**	**mg of amino acid g^{-1} protein**	**SD**	**mg of amino acid g^{-1} protein**	**SD**	**mg of amino acid g^{-1} protein**	**SD**	**mg of amino acid g^{-1} protein**	**SD**
Alcalase (AL)	Aspartic acid	1.96	0.05	1.60	0.28	1.51	0.10	1.28	0.00	1.66	0.08	1.49	0.08
	Glutamic acid	3.30	0.67	1.84	0.05	0.68	0.04	2.02	0.02	2.03	0.01	1.25	0.41
	Arginine	1.09	0.03	0.88	0.20	0.71	0.01	0.64	0.04	1.17	0.11	0.75	0.01
	Tyrosine	-	-	0.29	0.09	0.50	0.01	-	-	1.00	0.06	0.54	0.03
	Valine	0.19	0.03	0.25	0.09	1.37	<0.01	-	-	0.80	0.02	0.25	0.05
	Methionine	-	-	-	-	1.01	<0.01	-	-	0.52	0.03	0.09	0.01
	Phenylalanine	1.48	0.08	1.07	0.39	1.83	0.65	0.64	<0.01	6.53	0.14	1.77	<0.01
	Leucine	0.09	0.01	0.09	0.02	0.30	0.01	0.06	<0.01	0.28	<0.01	0.18	0.01
	Total	8.11		6.02		7.90		4.64		13.98		6.32	
Proteases from *Cynara cardunculus* **(CY)**	Aspartic acid	2.10	0.05	2.34	0.07	2.08	0.03	1.40	<0.01	1.55	0.08	1.10	0.03
	Glutamic acid	3.53	0.71	3.25	0.23	3.44	0.04	0.56	<0.01	1.81	0.07	1.42	0.09
	Arginine	1.17	0.03	1.42	<0.01	1.24	0.04	0.78	0.05	0.72	<0.01	0.53	0.01
	Tyrosine	-	-	0.18	0.01	0.17	0.02	-	-	0.08	0.03	0.06	0.02
	Valine	0.21	0.03	-	-	0.17	0.07	-	-	-	-	-	-
	Phenylalanine	1.58	0.08	1.85	0.28	1.25	0.08	0.77	<0.01	1.00	0.02	0.77	0.05
	Isoleucine	-	-	-	-	0.31	0.02	0.20	<0.01	-	-	-	-
	Leucine	0.10	0.01	0.13	<0.01	0.11	0.01	-	-	0.09	<0.01	0.06	<0.01
	Total	8.69		9.24		8.77		3.72		5.18		3.95	

(-) Not detected. [a]NAOK (dry okara not autoclaved) and [b]AOK (dry okara autoclaved). [c]Enzymatic hydrolysis with E/S ratio of 1.1 (v/w) and 2.5 hours. [d]Enzymatic hydrolysis with E/S ratio of 1.1 v/w and 5 hours.

4.4.4. RP-HPLC peptide profile

Several studies have focused on bioactive peptides derived from food proteins and physiologically effects are related in peptide with low MW (Ambigaipalan and Shahidi 2017; Singh et al., 2014). Thus, the hydrolysates obtained in this work were studied by RP- HPLC that allows us a preliminary study of the peptide composition from okara (Garcia et al., 1997). The chromatograms showed differences between the profile of hydrolyzed and unhydrolyzed for peak 1, before 5 minutes and it is more evidenced in hydrolysates of AOK by CY (Fig 4.2 D). Besides, can be observed a major elution of molecules between 0 and 16 minutes (Fig.4.2) stage where the mobile phase is 75% water, i.e. more hydrophilic.

Thus, it is possible that okara present peptides on its composition with hydrophilic amino acids such as aspartic acid, glutamic acid, threonine, histidine, serine, lysine, arginine and glutamine (Amorim, Pereira, Gomes, and Dias, 2016). However, AOK hydrolyzed by AL (Fig. 4.2 H), showed peptides with more hydrophobicity (peak 5 and 6); according previous studies, these peptides are generally associated with biological activities such antioxidant (Ambigaipalan and Shahidi, 2017) and antihypertensive (Wang, Zhang, Sun, and Dai, 2013) activities. This can also be observed for this study, where AOK hydrolyzed by AL showed a higher antihypertensive and antioxidant activity (Fig.4.3 and Table 4.4) than for CY extracts.

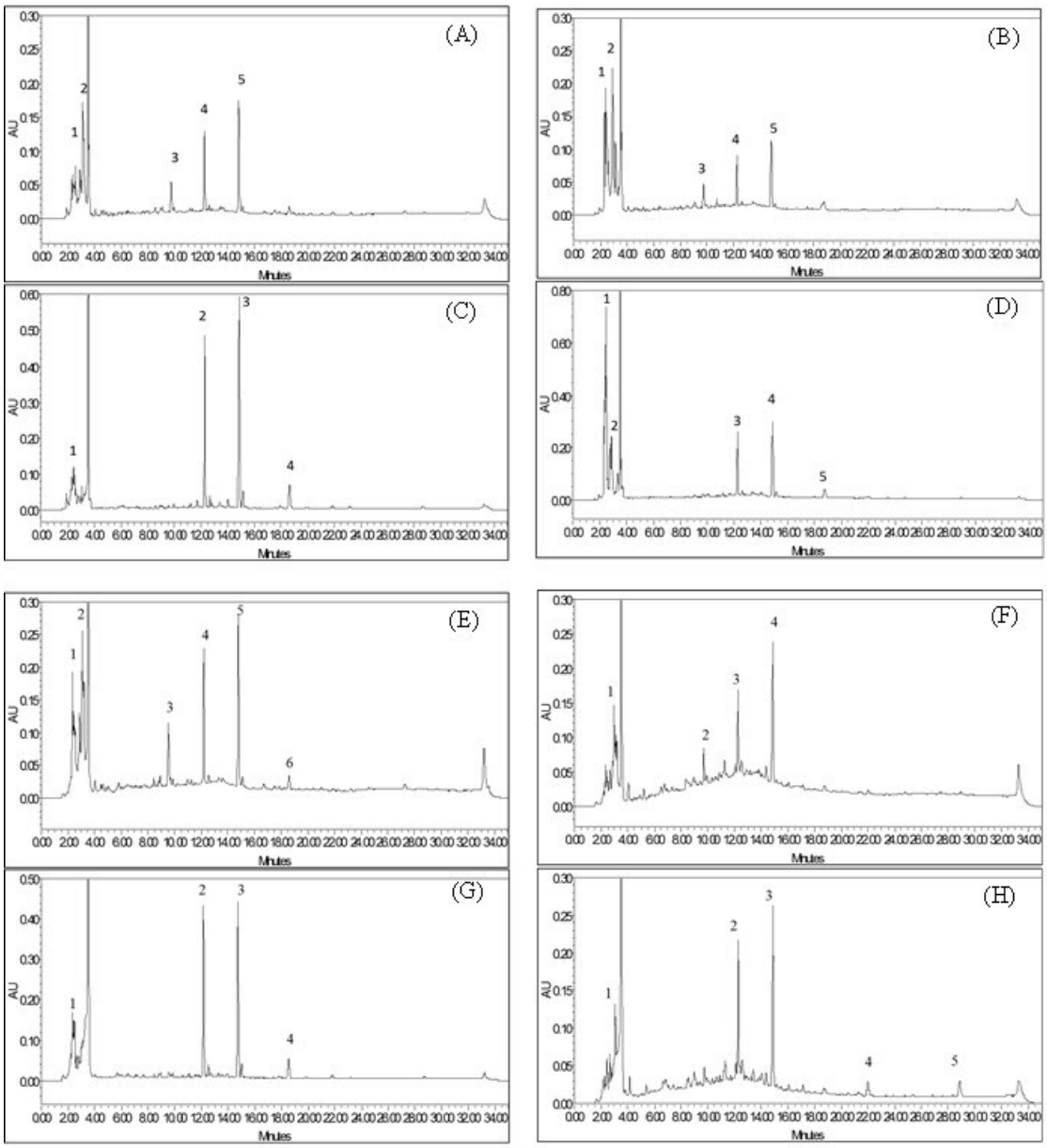


Figure 4.2. Peptide profile of hydrolysate produced by proteases from *Cynara cardunculus* (CY) (A) control NAOK, (B) NAOK (5 h and 1.1 E/S ratio), (C) control AOK and (D) AOK (5 h and 1.1 E/S ratio) and Alcalase (AL) (E) control NAOK, (F) NAOK (5 h and 1.1 E/S ratio), (G) control AOK and (H) AOK (5 h and 1.1 E/S ratio).

4.4.5. Antioxidant activity (ABTS radical)

Some reactive oxygen species (ROS) are responsible for oxidative reactions in the human body, which are generally converted into harmless species by enzymatic antioxidants of living cells. In addition, the intake of antioxidants through food can also lead also help

eliminating ROS producing beneficial health effects (Ambigaipalan and Shahidi, 2017). The antioxidant activity of the hydrolysate NAOK and AOK was measured *in vitro* using the ABTS scavenging assay, a colorimetric assay. Figure 4.3 shows the antioxidant activity obtained for hydrolysates where is evident the higher antioxidant activity generated by AL compared to CY (0.33 - 4.27 vs 0.03 - 0.20 mg Trolox mL^{-1}, respectively). Statistical analysis indicated significant differences between the factors analyzed (ratio of E/S, thermal treatment, reaction time) and comparing E/S ratio in same group (reaction time and thermal treatment).

CY extracts showed smaller antioxidant activity (Fig.4.3 B) than AL extracts (ca. 20 times less in AOK and ca. 50 times less in NAOK). In addition, the interaction between reaction time and the E/S ratio showed significant differences ($P < 0.05$) for hydrolysis performed under the same thermal treatment applied in okara (AOK or NAOK). Furthermore, comparing the thermal treatment in okara (NAOK and AOK) for different reaction time and E/S ratio, the antioxidant activity in AOK showed an increase ($P > 0.05$) compared with NAOK.

The high antioxidant activity observed for okara hydrolysates was attributed to peptides generated during the hydrolysis reaction and are related with its structure, composition and hydrophobicity. Peptides could react with free radicals stopping their chain reaction and converting them into more stable products. Furthermore, in AL hydrolysates the resulting antioxidant activity is highly correlated with DH in both treatment, AOK (r = 0.91) and NAOK (r = 0.88). The extensive hydrolysis induced formation of shorter peptides and free amino acids, that generated smaller and more hydrophilic groups and thus became more accessible to water-soluble radical-ABTS as previously suggested by Sbroggio et al. (2016). On the other hand, as expected, the hydrolysates of CY showed a low correlation between antioxidant activity and DH, AOK (r = 0.64) and NAOK (r = 0.04).

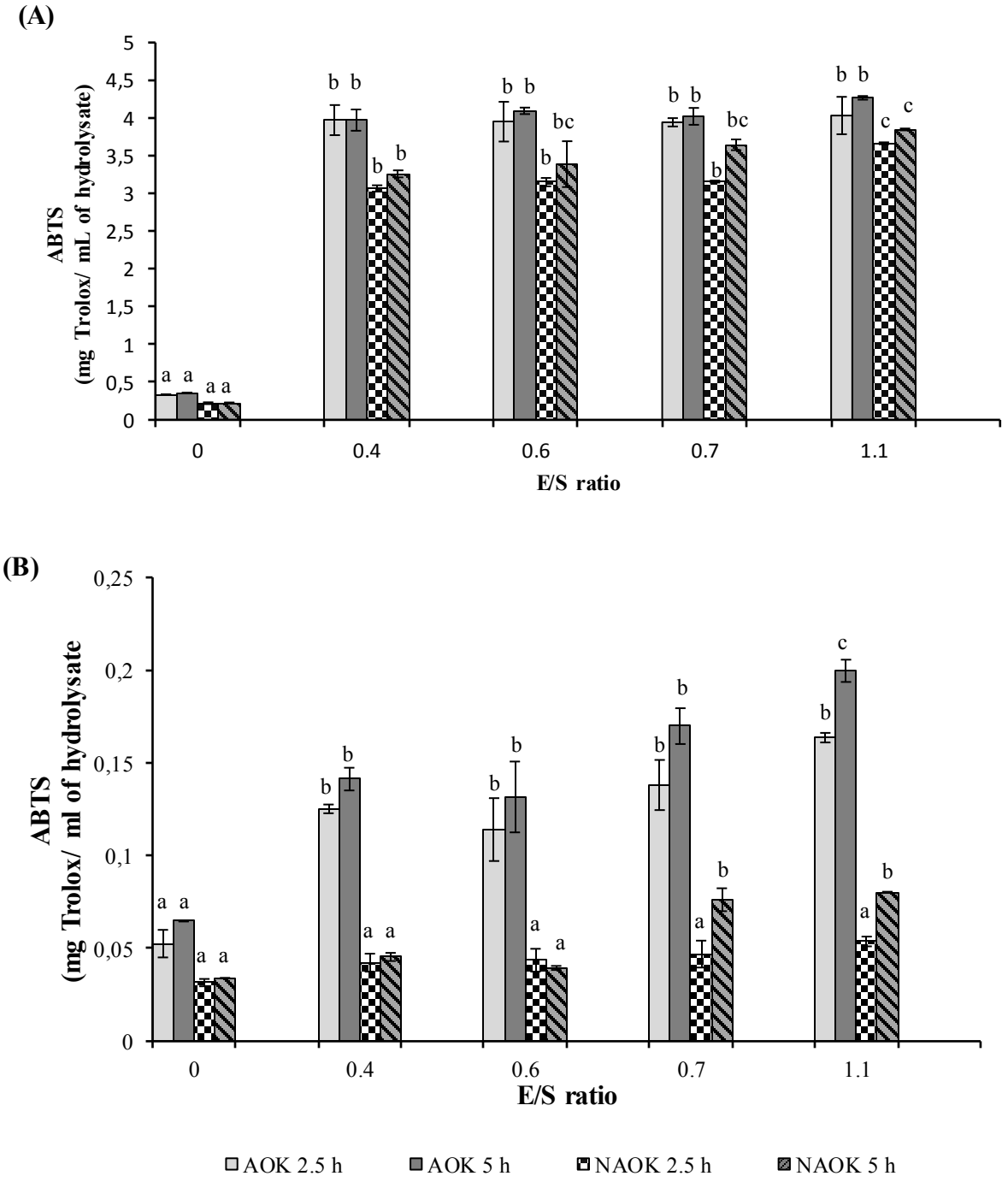


Figure 4.3. Antioxidant activity of hydrolysates from okara using (A) Alcalase (AL) and (B) proteases from *Cynara cardunculus* (CY). Different lower letters between different E/ S ratio in the same group (reaction time and thermal treatment) are significantly different ($P < 0.05$).

All 20 amino acids found in proteins have the potential to interact with free radicals, but some amino acids have been described as more prone to react, namely aromatic amino acids

(tyrosine, phenylalanine and tryptophan) and amino acids with nucleophilic sulphur in side chains (cysteine and methionine). However, the antioxidant activity of the amino acid residues is limited by their tertiary structure, but trough the enzymatic hydrolysis it is possible to increase this capacity since the protein cleavage leads to the exposure of antioxidant amino acids (Elias et al., 2008). Thus, considering the profile of FAA, AOK hydrolysates produced by AL showed high concentration of tyrosine and phenylalanine. Besides, the main protein in okara is glycinin (11S) that contains cysteine and methionine residues in their structure, and cysteine is considered one of the most active amino acids contributing towards the antioxidant activity assessed by the ABTS (Stanojevic et al., 2012).

4.4.6. ACE inhibitory activity

Several studies have evidenced the potential of antihypertensive peptides from foods or by-products, relating their activity with inhibition of angiotensin-converting enzyme (ACE). This is crucial for regulation of blood pressure by modulating the renin-angiotensin system. The angiotensin converting enzyme converts the decapeptide angiotensin I into octapeptide angiotensin II, that is a potent vasoconstrictor, which leads to an increase in blood pressure; thus the ACE inhibition has an antihypertensive effect (Wang and Mejia, 2005). Previous studies associated the positive health effects of soy with its antihypertensive activity, either fermented and hydrolyzed soy protein (Coscueta et al., 2016; Wang et al., 2013). For characterization of ACE-inhibition activity by NAOK and AOK hydrolyzed (with AL and CY) and non-hydrolyzed, IC_{50} was determined and results are presented in Table 4.4.

The unhydrolyzed samples showed low or no significant inhibition ($IC_{50} \geq 530$ µg protein/mL), but a significant increase in ACE-inhibition (lowering IC_{50} values) was observed for all samples hydrolyzed with AL and CY, indicating a potential for ACE inhibition. Considering that low values of IC_{50} represent high ACE-inhibitory activity, a small amount of protein was required to produce 50% ACE inhibition. Although all hydrolysates showed very good ACE inhibitory activity ($IC_{50} < 100$ µg protein/mL), those from AL showed higher ACE inhibitory activity (22.11 to 9.97 µg protein/mL) than CY (48.59-62.55 µg protein/ mL) (see Table 4.4). In addition, in a previous study, Nishibori, Kishibuchi, and Morita (2016) related

antihypertensive activity using an aqueous extract from okara sterilized and their oligopeptides fractions.

Table 4.4. ACE-inhibitory activity IC_{50} (µg protein/mL) for NAOK and AOK hydrolyzed with Alcalase and proteases from Cynara cardunculus and unhydrolyzed (control).

ACE-inhibitory activity IC_{50} (µg protein/mL)				
Sample	**AOK**		**NAOK**	
	AL	**CY**	**AL**	**CY**
Control	536.90 ± 0.91		>800	
E/S 1.1 and 2.5 h	21.87 ± 0.51	48.59 ± 7.22	22.11 ± 5.31	59.09 ± 6.70
E/S 1.1 and 5 h	9.97 ± 0.63	54.30 ± 0.70	12.33 ± 3.97	62.55 ± 3.74

Values are expressed as average ± standard error (n= 2). AOK: dry okara previously autoclaved, NAOK: dry okara not autoclaved, AL: Alcalase and CY: proteases from *Cynara cardunculus*.

Potential antihypertensive activity has been reported for previous studies for soy protein hydrolysates, with values for ACE inhibitory activity between 78 to 177 µg protein/ mL (Chiang et al., 2006; Coscueta et al., 2016). These values are lower than those observed in the present study which showed a higher potential of ACE inhibition especially when AL enzyme was used. But Chiang et al. (2006) reported for SPI hydrolysates obtained with AL an increase of ACE inhibitory activity from 668 to78 µg protein/mL in 6 h of hydrolysis. Additionally, the okara hydrolysates, obtained for the first time with CY have shown higher inhibition activity compared with hydrolysates obtained from whey protein concentrate using the same enzyme (48.6 vs 72 µg protein/ mL, respectively) (Tavares et al., 2011). However, it is important to highlight that the antihypertensive activity reported in most previous studies using AL corresponds to protein concentrates or isolates, while the present activity is associated with total hydrolyzed okara. In fact, this is a relevant result for a complete food ingredient that combines other health benefits such us functional lipids and fiber. It can hence be concluded that okara hydrolysates produced with AL or CY have a potential antihypertensive activity that has never been reported before.

4.4.7. Identification of peptide in hydrolyzed from okara by mass spectrometry

Samples with the highest DH and bioactivity values from each analyzed condition were selected for peptide sequencing by MALDI stabilities via resonance structures. This property improves the radical scavenging capacity of the amino acid residues. Thus, as depicted in Table 4.5, aromatic amino acids-TOF/TOF: NAOK and AOK either hydrolyzed by AL and CY (5 h of reaction time and an E/S ratio of 1.1). The main peptides were identified in each sample and the majority has never been previously reported in the literature (Table 4.5).

In general, peptides from AL and CY hydrolysates were rich in hydrophobic amino acids, such as proline (P), leucine (L) and isoleucine (I). According to Sarmadi and Ismail (2010) the presence of certain amino acids, their hydrophobicity and positioning in the peptide sequence determine the antioxidant activity. Some examples of amino acids that can have antioxidant activity are tyrosine (Y), tryptophan (W), methionine (M), lysine (K) and cysteine (C). The aromatic residues in amino acids can donate protons to electron deficient radicals maintaining their stabilities via resonance structures. This property improves the radical scavenging capacity of the amino acid residues. Thus, as depicted in Table 4.5, aromatic amino acids were present in both enzymatic hydrolysates and some peptides presented the aromatic residues located in the carboxyl terminus. Furthermore, the higher antioxidant activity found in hydrolysates obtained of AL compared with CY may be related with proline, which is present in greater quantity in the peptides generated by AL as shown in a previous study by Kitts and Weiler (2003) for antioxidant peptides isolated from soybean.

Table 4.5. Proteins and major peptide sequences identified by MALDI-TOF/TOF using PMF or PMF+MS/MS approaches in AOK and NAOK hydrolyzed with Alcalase (AL) and proteases from *Cynara cardunculus* (CY).

	AOK [a]				**NAOK** [b]			
	MW (DA)	PEPTIDES	PROTEIN	MS/MS SCORE	MW (DA)	PEPTIDES	PROTEIN	MS/MS SCORE
Proteases from *Cynara cardunculus* (CY)	862.44	V.SIIDTNSL.E	Glycinin G1- P04776		862.44	V.SIIDTNSL.E	Glycinin G2- P04405 and Proglycinin-Q549Z4	
	949.47	L.DQMPRRF.Y	Glycinin G2- P04405		906.49	H.RVEFEGGL.I and S.GFSKHFLA.Q	Glycinin-Q43452	
	1209.57	F.LVPPQESQRR.A						
	1331.67	Y.NFREGDLIAVPT.G			949,47	L.DQMPRRF.Y		
	1387,63	P.TDEQQQRPQEE.E			1149.52	S.EGGFIETWNP.N		
	1402.73	N.NPFKFLVPPQES.Q			1190,62	N.QLDQMPRRF.Y		
	1433.71	R.GSQSKSRRNGIDE.T			1331.68	F.NNQLDQTPRVF.Y and E.DEQIPSHPPRR.P		55
	1486.71	G.KGIFGMIYPGCPST.F	Glycinin G1- P04776		1515,82	R.SQRPQDRHQKVH.R	Glycinin G2- P04405 and Proglycinin-Q549Z4	
	1532,79	Y.TNGPQEIYIQQGKG.I			1523.76	L.IAVPTGVAWWMYN.N		
	1541,82	Y.ALNGRALIQVVNCNG.E			1607.81	M.PRRFYLAGNQEQE.F		
	1633.82	P.QDRHQKIYNFRE.G			1628,82	L.NGRALVQVVNCNGER.V		
	1699.91	V.FDGELQEGRVLIVPQ.N			1699.92	Q.EGGVLIVPQNFAVAAKS.Q		
	1741.91	L.NGRALIQVVNCNGERV.F			1741.92	A.LNGRALVQVVNCNGER.V		
	1892.97	L.KSQQARQVKNNNPFSF.L	Glycinin G2- P04405	77	1892.99	L.KSQQARQVKNNNPFSF.L		58
	1948.05	L.KSQQARQIKNNNPFKF.L	Glycinin G1- P04776	83	1892.99	S.KRSRNGIDETICTMRL.R		
	2075.08	A.NSIIYALNGRALIQVVNCN.G			1948.96	I.TTATSLDFPALWLLKLSA.Q		
Alcalase (AL)	703,36	P.HFNSKA.I	β-conglycinin:Q948X9, O22120, P13916 and Q94LX2	63	718.39	V.NKPGRF.E	β-conglycinin: P13916, Q94LX2.	
	774.386	P.SQVQELA.F			750.38	S.RDPIYS.N		
	795.38	P.QHPERE.P			755.44	E.KNPQLR.D		

718.4	V.NKPGRF.E			985,52	R.QFPFPRPP.H	F7J075 and	
750.38	S.RDPIYS.N			1068.56	E.REPQQPGEK.E	Q4LER5	
879.43	H.ADADYLIV.I			1098.64	L.AIPVNKPGRF.E		
1048,55	V.LQRFNQRS.P			1181.64	L.FKNQYGRIR.V		20
1068.57	E.REPQQPGEK.E			1361.71	F.PFPRPPHQKEE.R		
1302.74	E.GEIPRPRPRPQ.H		17	1378,7356	R.QFPFPRPPHQK.E		
1361.72	S.QVQELAFLGSAQA.V	β-conglycinin: Q948X9		1411.76	N.PDNNENLRLITL.A and S.EDSELRRHKNK.N		36
1378,74	R.QFPFPRPPHQK.E	β-conglycinin:Q948X9, O22120, P13916 and Q94LX2	34				
1665.9	E.GEIPRPRPRPQHPE.R		27	1665.9	E.GEIPRPRPRPQHPE.R		
1829.98	E.DEQPRPIPFPRPRQP.R	β-conglycinin: Q948X9	45	1829.98	E.DEQPRPIPFPRPQPR.Q		
1829.98	E.DEQPRPIPFPRPQPR.Q	β-conglycinin:Q948X9, O22120, P13916 and Q94LX2	45	2074.05	E.DEDEQPRPIPFPRPQPR.Q		38
2074.05	E.DEDEQPRPIPFPRPQPR.Q		63	2200.12	F.SREEGQQQGEQRLQESVIV.E		

[a] AOK: dry okara previously autoclaved. [b] NAOK: dry okara not autoclaved.

On other hand the antihypertensive activity is the most studied bioactivity in peptides generated from food. The ACE-inhibitory peptides are usually rich in hydrophobic amino acid residues and have a proline (P), lysine (L), arginine (R) or aromatic amino acids (F, Y, W) as a carboxyl terminal. In addition amino acids as R, H, W, Y and F, have important characteristics for the ACE inhibitory properties, especially for peptides with more than four amino acids residues (Kitts and Weiler, 2003). Thus, most of the peptides identified (Table 4.5) shared part of specific structural characteristics described above and their composition was rich in proline, arginine, leucine or phenylalanine. Furthermore, the positive charge on the C-terminal, contribute substantially to inhibitory potency, according Quirós et al. (2009) the substitution of proline for arginine in a model peptide increased twice the activity. Thus, this can explain the ACE inhibitory found for hydrolysates obtained by AL and CY.

4.5. Conclusions

The selected thermal treatment (sterilization at 121 ºC) facilitated the enzymatic attack and the DH (degree of hydrolysis) particularly when AL was used: a 1.7 time increase in DH was observed in autoclaved okara. The antioxidant activity of dried Okara (either autoclaved or not) hydrolyzed with AL was higher (4.2 mg Trolox mL^{-1}) than that observed when CY was used. On the other hand, the potential antihypertensive activity was high for samples hydrolyzed with both enzymes, AL and CY, however higher ACE inhibitory activity was also found for AL (22.11-9.97 µg /mL) than for CY (62.55 - 48.59 µg/mL).

The current study also enabled the identification of several new peptide sequences in dry okara (autoclaved and not autoclaved) hydrolyzed with AL and CY, and some sequences could explain the bioactivities found for these hydrolysates.

These results indicate that is possible to obtain valuable extracts from okara with different biological properties depending on the enzyme used to promote the hydrolysis. This suggests okara can either be used as functional ingredient or as possible food supplement for blood pressure lowering or antioxidant applications in future.

Part IV- Development of fermented okara beverage

CHAPTER 5- Impact of fructose and FOS supplementation upon the fermentation of hydrolyzed okara and its impact upon bioactive components

5.1. Abstract

Okara provide compounds of biological interest such as isoflavones and an increase of aglycone content can be attained through fermentation with food-grade bacteria. This study aimed to evaluate the effect of fructose and fructooligosaccharides (FOS) on the growth of probiotic bacteria in hydrolyzed (by *Cynara cardunculus* enzymes) okara beverage, as well as on the antioxidant activity, isoflavones and total phenolic content of fermented okara beverage. Okara beverages were fermented using *Bifidobacterium animalis* ssp. *lactis* Bb12 and *Lactobacillus rhamnosus* R11 alone or as mixture of both, at 37 ºC, until it reached a pH value of 4.5. The growth of lactobacilli and bifidobacteria was evaluated throughout the fermentation, with increases of viable cells ranging from 1.5 to 3 log CFU/mL. Moreover, okara samples supplemented with a carbon source presented a higher growth rate than their non-supplemented counterpart, as expected. In addition, significant differences in aglycone content and antioxidant activity were observed for the okara samples fermented by different species, with the overall content of isoflavone aglycones increasing, the genistein presented the highest increase (ca. 16-fold) in the fermented okara by *B. animalis* ssp. *lactis* Bb12. However, the antioxidant capacity was maintained in the okara without carbon source after fermentation by *L. rhamnosus* R11, while all fermented okara presented a decrease after fermentation process. Considering the hereby reported results, probiotic hydrolyzed fermented okara can be considered a good carrier for probiotic bacteria and a good source of aglycone isoflavones, combining all the other benefits resulting from probiotic metabolism and the hydrolyzed okara itself.

5.2. Introduction

The impact of probiotics (i.e. microorganisms capable of exerting a positive effect upon human or animal health when ingested in adequate amounts) consumption upon human health has been increasingly described in literature, with *Bifidobacterium* and *Lactobacillus* strains being the most used probiotic microorganisms, due to their perceived beneficial effects (Bedani

et al., 2014a; Gibson et al., 2017; Kerry et al., 2018; Liu et al., 2017; Panghal et al., 2018). On the other hand, prebiotics are nutrients that, while not easily digested by humans, modulate the gut microbiota in a beneficial manner for the host. They differ from most dietary fibers which stimulate the growth of a wide variety of intestinal microorganisms and not necessarily its beneficial constituents (Gibson et al., 2017; Kerry et al., 2018). Among several compounds fructooligosaccharides (FOS) and inulin have been used preferentially in the food market. According Gibson et al. (2017), prebiotics provoke a biased metabolism towards host health promotion rather than being extensively metabolized.

Previously, the benefits of probiotics consumption were only associated with milk-based fermented foods. However, factors such as lactose intolerance and milk's cholesterol content present limitations when considering some consumers' (e.g. lactose intolerant and hypercholesterolemic individuals) dairy probiotic intake (Panghal et al., 2018). Thus, previous studies have focused on the development of non-dairy probiotic or synbiotic foodstuffs using several plant raw materials, among which soy beverages are a prominent example due to their nutritional composition (Delgado et al., 2018). Okara, a by-product of the soy beverage, has also gained prominence in recent years due to its composition and bioactive compounds, with some studies focusing on the incorporation of okara into fermented products (Albuquerque et al., 2017; Bedani et al., 2014b). Nevertheless, the integral use of okara for the development of a fermented product has not been described, to the best of our knowledge, until now. Moreover, previous studies have shown that submitting soy to different process can affect their isoflavone profile and according to Chun et al. (2007) and Wei et al. (2007) the fermentation with lactic acid bacteria may contribute to increase the isoflavone aglycones. In parallel, fructose has been used as a sweetener in several food products, due to some features, such as the solubility in aqueous solutions and sweetness (ca. 1.7 times sweeter than sucrose) (Bray et al., 2004). In addition, fructose has been considered a therapeutic adjunct in the diet of diabetic patient, because its initial metabolic step do not require insulin and produces little rise in glycemia (Dirlewanger et al., 2000). It also elicits a more important increase in energy expenditure that has been suggested to be beneficial for obese diabetic and nondiabetic patients (Petersen et al., 2001). This makes fermented soy-based products more interesting as studies have reported that humans can absorb isoflavone aglycones faster than the glycosides and that high isoflavone

intakes have been previously associated with lower rates of cardiovascular and cancer diseases, in particular in Asian populations (He and Chen, 2013; Izumi et al., 2000; Jenkins et al., 2000).

Considering the above made arguments, the aim of this study was to investigate the influence of two concentrations of fructose and fructooligosaccharides (FOS) supplementation upon the growth of *Lactobacillus rhamnosus* R11, *Bifidobacterium animalis* ssp. *lactis* Bb12 and a mixture of both strains and upon specific bioactivities content (isoflavones and phenolic compounds) during the fermentation of hydrolyzed okara.

5.3. Material and methods

5.3.1. Microorganisms activation and inoculum preparation

The strains used as probiotics were *Bifidobacterium animalis* ssp. *lactis* Bb12 (Christian Hansen, Hørsholm, Denmark) and *Lactobacillus rhamnosus* R11 (Lallemand, Montreal, Canada). The aforementioned microorganisms were reactivated and pre-cultures were made in de Man–Rogosa and Sharpe (MRS; Biokar Diagnostics, Beauvais, France) broth, incubated overnight at 37 ºC. For *B. animalis* ssp. *lactis* Bb12 the MRS was supplemented with filter-sterilized L-cysteine·HC1 (0.5 g/L; Fluka, Switzerland), and incubated using an anaerobic chamber. The resulting cultures were then used as inoculum for the fermentation.

5.3.2. Enzymatic hydrolysis and fermentation of okara

Okara beverage was prepared as follows: 60 g of fresh okara were homogenized with 180 mL of citric acid – sodium citrate buffer (0.1 M, pH 5.2) using a blender under hygienic conditions. Afterwards, enzymatic hydrolysis was performed using *Cynara cardunculus* extract (Formulab, Maia, Portugal). The hydrolysis was performed in an orbital shaker (SI-100C, Wiggenhauser, Germany) at 55 ºC for 2.5 h using an enzyme: substrate (E/S) ratio of 1.1 (v/w) and under an orbital stirring of 120 rpm. The resulting solutions were autoclaved (1 atm, 121 ºC and 15 minutes) immediately after hydrolysis. Afterwards, fructose and FOS (Orafti® P95) concentrated solutions, both sterile, were added to the hydrolyzed okara, under aseptic conditions to reach final concentration of 3 or 6% (w/v) for fructose and 2% (w/v) for FOS.

Each mixture of hydrolyzed okara was inoculated with 1% of bacterial inoculum (as described in 5.3.1) and incubated in an orbital shaker at 37 ºC and 120 rpm to produce the fermented okara (OkaLR, OkaBb and OkaMix). Sampling was performed throughout the fermentation (0, 2, 4, 6. 8.10, 12 and 24 h) that was considered complete when the pH values reached 4.5.

A factorial design methodology was performed, encompassing two independent variables: fructose concentration (3 and 6%) and type of inoculum (*B. animalis* ssp. *lactis* Bb12, *L. rhamnosus* R11 and combination of both). In addition, a control (without fructose and FOS addition) was considered. Furthermore, the growth of bacteria in okara beverages was compared to their behavior when grown in MRS broth. Each experimental condition was performed in duplicate, so in total 24 fermented okara beverages were produced.

5.3.3. pH measurement

Potentiometric determination of pH values throughout the fermentation was performed at 20 ºC ± 2 ºC using a pH meter (Micro pH 2002, Crison, Barcelona, Spain).

5.3.4. Microbiological analysis

To determine the growth of microorganisms in fermented okara, 1 mL of each sample was blended with 9 mL sterile peptone water 0.1% (Peptone - Sigma Aldrich) and subjected to serial decimal dilutions with the same diluent, plated on using the Miles and Misra method on MRS agar (plain or supplemented with 0.5 gL^{-1} of cysteine-HC1 when *B. animalis* ssp. *lactis* Bb-12 was used) and incubated at 37 °C for 24-48 h under anaerobic conditions, using anaerobic chamber (Whitley DG250) (Lee and Lee, 2008; Miles and Misra, 1938). On each sampling time, throughout the fermentation, a new tube containing okara fermented from the same batch was used for the analysis.

Total mesophilic bacteria were determined by plating in Plate Count Agar (PCA; Biokar Diagnostics, Beauvais, France) and incubated aerobically at 30 ºC for 48 h. Yeasts and molds were enumerated in Potato Dextrose Agar (PDA; Biokar Diagnostics, Beauvais, France), incubated aerobically at 30 ºC for 5 d. Inoculation was performed also using the Miles and Misra method (Miles et al. 1938). *Enterobacteriaceae* viable count were determined through plating

on Violet Red Bile Glucose Agar (VRBGA, Oxoid, Basingstoke, United Kingdom) using the pour plate method and incubated aerobically at 37 ºC for 24 h.

5.3.5. Isoflavone analysis

The extracts were prepared according to Fernandes et al. (2017), using freeze dried fermented okara, previously degreased with hexane (1:10 w/v) and stirred for 1 h at room temperature. The extraction was performed using 200 mg of sample and 8.0 mL of extraction solvent (water, ethanol and acetone 1:1:1 (v/v/v)). The suspension was left to extract for 1 h at room temperature, with vortexing every 15 min. Afterwards, samples were placed in an ultrassound bath at room temperature for 15 min, centrifuged at 4105 xg for 15 min at 4 ºC and the supernatant was filtered through a 0.45 µm filter (Orange Scientific, Braine-l'Alleud, Belgium) before chromatographic analysis.

Isoflavones were analyzed by HPLC using an adaptation of the method described by Wang and Murphy (1996). Twenty microliters of each extract were analyzed by HPLC (Waters Series 2695, Milda ford, MA, USA) coupled to a diode array detector, using a Kromasil C-18 column (250 x 4,6 mm) to separate the isoflavones at 25 ºC. The mobile phase consisted of 0.1% acetic acid in water (v/v) (solvent A) and 0.1% acetic acid in acetonitrile (v/v) (solvent B). Elution started with 20% B, increased to 100% B in 20 min and decreased to 20% B in the next 12 min, with a flow rate was 0.8 ml/min. Spectral data from was accumulated in the 200–400 nm range and processed using Empower 3 software. Isoflavone quantification was achieved by comparing the absorbance spectrum at 255 nm with that of pure external standards. The standards were dissolved in analytical grade DMSO (Sigma-Aldrich, St Quentin Fallavier, France) and diluted in methanol. The calibration curve was performed with Genistin, Genistein, Daidzin and Daidzein (Sigma-Aldrich, St. Louis, USA). This procedure was done in duplicate for each sample, at the initial and final fermentation time (pH=4.5) and the results were expressed in mg of isoflavone per g of dried fermented okara.

5.3.6. Organic acids and fructose analysis

The organic acids and sugar contents were analyzed by HPLC according to the method described by Zeppa et al. (2001), with slight modifications. Briefly, 2 g of each sample were suspended in 10 mL of sulfuric acid 13 mM and homogenized with an Ultra-Turrax (IKA Ultra-turrax T18, Wilmington, USA) at 18.000 rpm for 3 min. Afterwards, the samples were centrifuged at 2665 x g for 10 min at 4 °C (Universal 32R; Hettich, Tuttlingen, Germany) and the supernatant was filtered with a 0.45 µm filter (Orange Scientific, Braine-l'Alleud, Belgium) before the chromatographic analysis.

The HPLC system used was comprised of a LaChrom L-7100 pump (Merck-Hitachi, Düsseldorf, Germany), an ion exchange Aminex HPX-87H column (300 × 7.8 mm) (Bio-Rad, Richmond CA, USA) maintained at 60 °C (L-7350 Column Oven; LaChrom, Merck-Hitachi) and two detectors assembled in a series: a refractive index detector (L-7490 RI Detector; LaChrom, Merck-Hitachi) to determine sugars and a UV-Vis detector (L-7400 UV Detector; LaChrom, Merck-Hitachi), acquiring chromatographic data at 220 nm, to analyze organic acids . The mobile phase used was 13 mM sulphuric acid at a flow rate of 0.8 mL/min. The running time was 35 min, and the injection volume was 50 µL. The data was collected and analyzed using D-7000 Interface (LaChrom, Merck-Hitachi) and HPLC System Manager 3.1.1 software (Merck-Hitachi). For each sample of fermented okara, two samples were analyzed at the beginning and at the end of the fermentation (pH = 4.5).

5.3.7. Radical scavenging activity determination (ABTS)

The scavenging activity of fermented okara (centrifuged at 20000 x g for 15 minutes) was measured using the ABTS radical cation method as described by Gião et al. (2007). Briefly, 20 µl of samples were mixed with 1 ml of ABTS (with an initial absorbance, at 734 nm, of 0.700 ± 0.020). After 6 min of reaction the absorbance (734 nm) was measured using a mini UV 1240 spectrophotometer (Shimadzu, Tokyo, Japan). Each sample was analyzed in triplicate at the beginning and at the end of the fermentation (pH=4.5). Trolox was used, as a standard, to prepare a calibration curve (0.05 - 0.2 mg/ mL) with the results being expressed in milligrams of Trolox per mL of fermented okara.

5.3.8. Total phenolic content determination

The total phenolic content of fermented okara (centrifuged at 20000 x g for 15 minutes) was evaluated using the Folin-Ciocalteu method (Singleton and Rossi, 1965). The samples were previously centrifuged at 20000 x g for 15 minutes and the quantification was carried out at 750 nm (UV mini 1240, Shimadzu, Tokyo, Japan). Total phenolic content of each sample was determined using a gallic acid standard curve (ranging from 0.025 to 0.5 mg/ mL). Each sample (at the beginning and at the end of the fermentation (pH=4.5) was analyzed in triplicate and the results expressed in milligrams of gallic acid equivalents per mL of fermented okara.

5.3.9. Statistical analysis

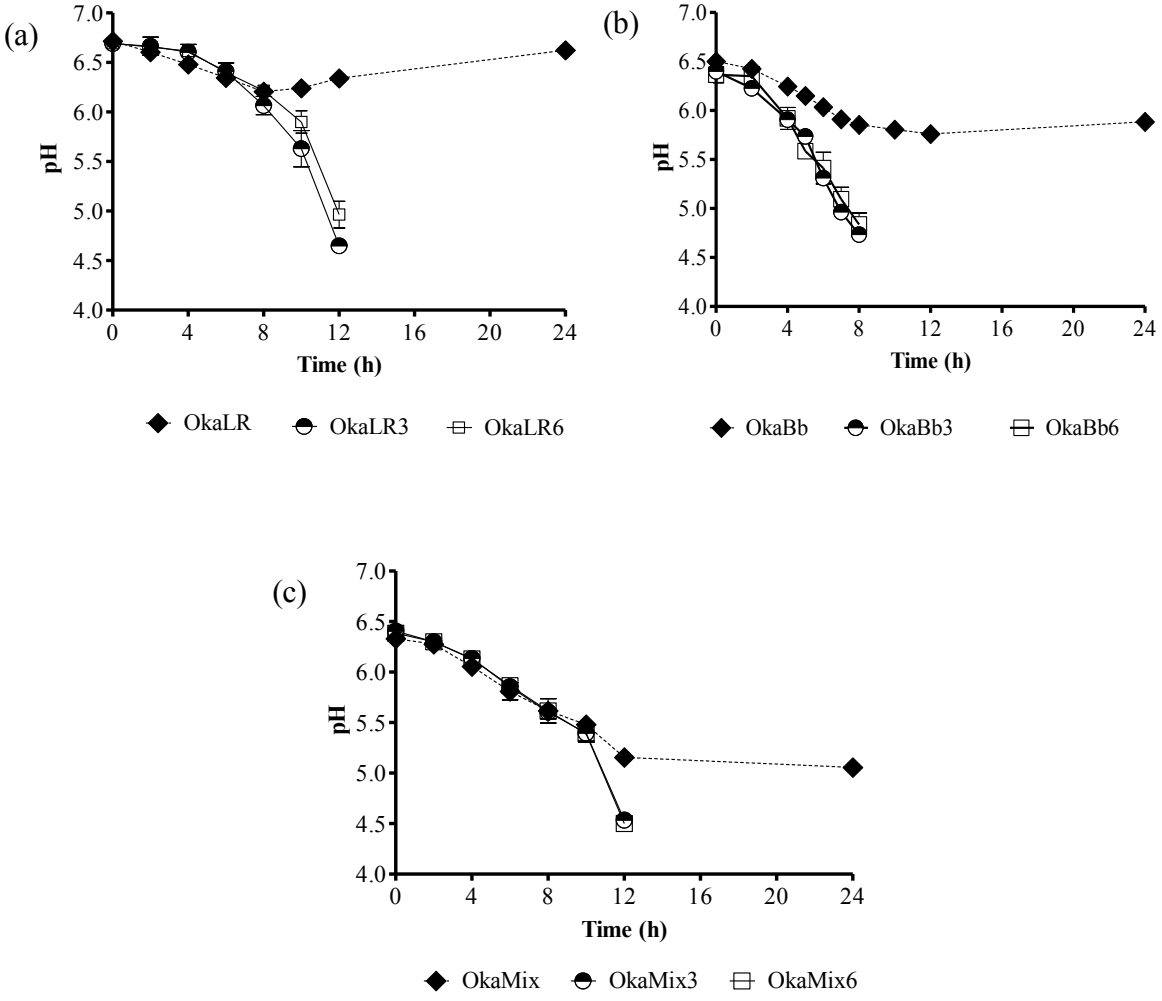
Statistical analysis of data on the microbial, physical and chemical parameters of okara beverages was performed using IBM SPSS STATISTICS version 23 (SPSS Inc., Chicago, IL, USA). Normality of the data was evaluated using Shapiro-Wilk's test. Data following a normal distribution was analyzed using T-Student or One-Way ANOVA coupled with Tukey's post-hoc test, with the differences between means being considered significant for p-values below 0.05.

5.4. Results and discussion

5.4.1. Microbial growth and acidification profile of okara beverage during fermentation

When the fermentation was carried out using B. animalis ssp. lactis Bb12 a stronger decrease of pH values (Figure 5.1) and higher viable cells counts (Figure 5.2) were observed than when the fermentation was carried out using L. rhamnosus R11 or a combination of both microorganisms. In this study, a pH value of 4.5 was defined as the end of the fermentation process in order to avoid an over-acidification of the fermented okara beverages. However, while all okara samples supplemented with fructose and FOS exhibited a continuous decrease in pH values until reaching the target pH of 4.5, when no carbon source was added, the pH values plateaued at 6 for *B. animalis* ssp. *lactis* Bb12 and *Lactobacillus rhamnosus* R11 and

(Fig. 5.1 a and b) at ca. 5 when the fermentation was carried out by both strains combined (Fig. 5.1 c).

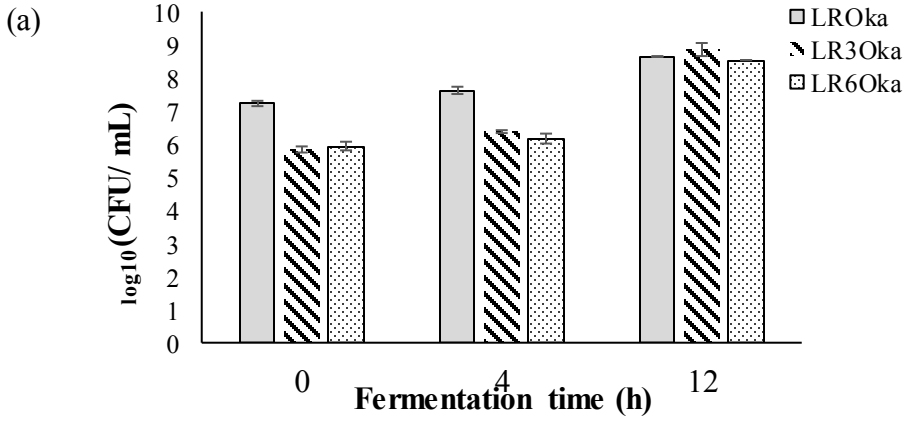


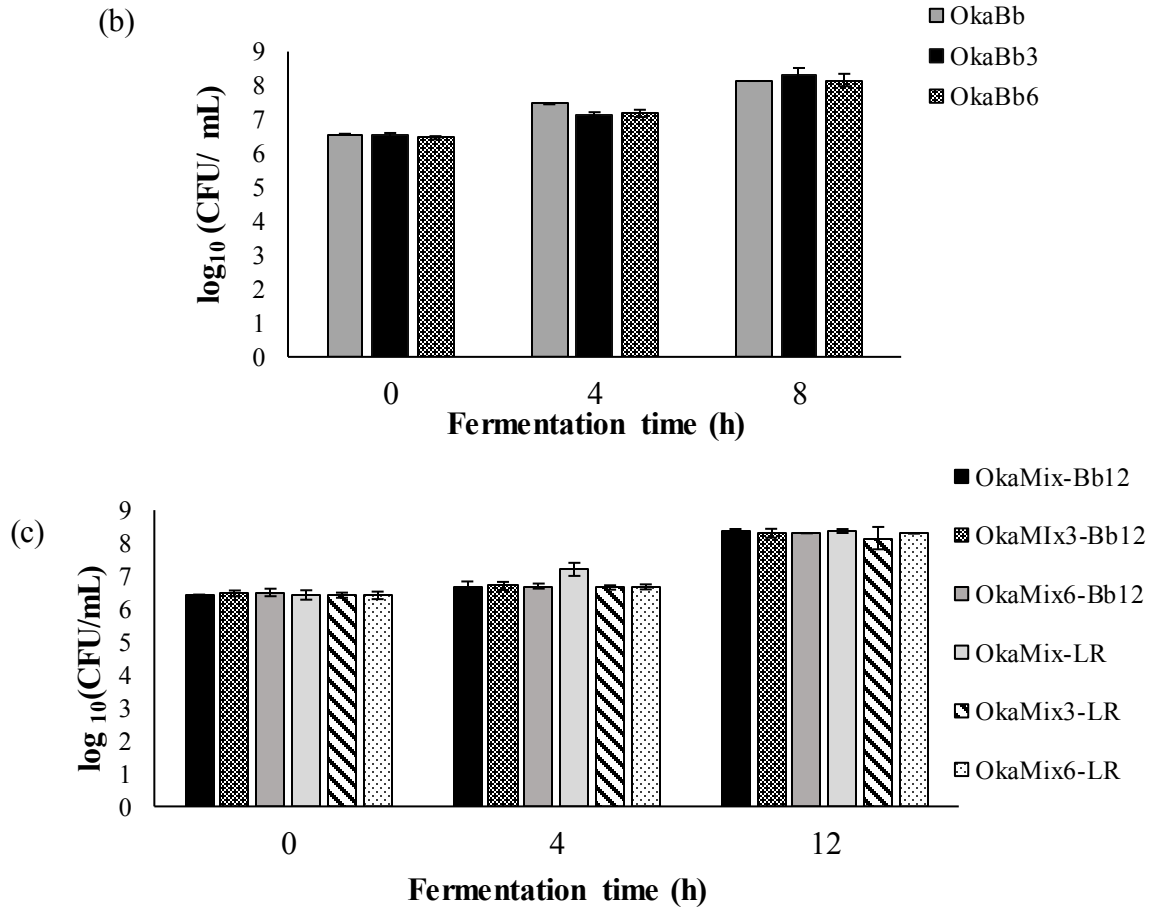
OkaLR: fermented by *L. rhamnosus* R11 and no carbohydrates added; **OkaLR3:** fermented by *L. rhamnosus* R11 with fructose (3%) and FOS (2%); **OkaLR6:** fermented by *L. rhamnosus* R11 with fructose (6%) and FOS (2%); **OkaBb:** fermented by *B. animalis* ssp. *lactis* Bb12 and no carbohydrates added; **OkaBb3**: fermented by *B. animalis* ssp. *lactis* Bb12 with fructose (3%) and FOS (2%); **OkaBb6:** fermented by *B. animalis* ssp. *lactis* Bb12 with fructose (6%) and FOS (2%); **OkaMix**: fermented by *B. animalis* ssp. *lactis* Bb12 and *L. rhamnosus* R11 combined and no carbohydrates added; **OkaMix3**: fermented by *B. animalis* ssp. *lactis* Bb12 and *L. rhamnosus* R11 combined with fructose (3%) and FOS (2%); **OkaMix6**: fermented by *B. animalis* ssp. *lactis* Bb12 and *L. rhamnosus* R11 combined with fructose (6%) and FOS (2%).

Figure 5.1. Variation of pH values of okara fermented by *Lactobacillus rhamnosus* R11 (OkaLR) (a), *Bifidobacteriun animalis* Bb-12 (OkaBb) (b) or a combination of both bacteria (OkaMix) (c) at two concentrations of fructose and FOS or no carbohydrates added

Data presented in Figure 5.2 (a-c) show the viable cell counts variation throughout the fermentation. It is possible to observe that the microorganism counts increased by ca. 1.5 to 3 log CFU/g from 0h to 8 or 12h. In turn, *B. animalis* Bb12 and *L. rhamnosus* R11's viable cells counts (after 8 or 12 h of incubation; Fig. 5.2 a-c) and specific growth rates (Table 5.1) revealed that, while there were no statistically significant differences between okara supplemented with fructose and FOS, okara supplemented with 6% fructose and 2% FOS appeared to be the best growth media among the three conditions tested.

The results of total viable cells observed for both probiotic bacteria (from 8.2 to 8.9 log CFU/mL) are comparable to those reported by other authors for soya beverages fermented by species and strains of the same bacterial groups (Delgado et al., 2018; Moraes Filho et al. 2016). On the other hand, to the best of our knowledge, to date there are no data reported in the literature focusing on the use of okara to grow and fermented by probiotic bacteria. In addition, as predicted, bacteria exhibited lower ($P < 0.05$) specific growth rates in okara fermented than in MRS medium (Table 5.1): 0.84 and 0.85 h^{-1} for *L. rhamnosus* R11 and *B. animalis* ssp. *lactis* Bb12, respectively, in monoculture fermentations while in the co-culture assay the specific growth changed between 0.58 to 0.68 h^{-1} for *L. rhamnosus* R11 and *B. animalis* ssp. *lactis* Bb12, respectively. Regardless, fructose and FOS's supplemented hydrolyzed okara showed to be a promising matrix for the growth of probiotic bacteria, exhibiting a μ_{max} of 0.5 h^{-1} (for each strain alone) and 0.30 and 0.39 h^{-1}, in the mixed culture, for *L. rhamnosus* R11 and *B. animalis* ssp. *lactis s* Bb12, respectively. Moreover, in the present study, no viable counts of *Bacillus cereus,* yeasts and molds and *Enterobacteriaceae* were detected in the fermented okara, indicating that these preparations should be safe for human consumption, microbiologically wise.





L. rhamnosus* R11 count: OkaLR:** fermented by *L. rhamnosus* R11 and no carbohydrates added; **OkaLR3:** fermented by *L. rhamnosus* R11 with fructose (3%) and FOS (2%); **OkaLR6:** fermented by *L. rhamnosus* R11 with fructose (6%) and FOS (2%); ***B. animalis* ssp. *lactis* Bb12 count**: **OkaBb:** fermented by *B. animalis* ssp. *lactis* Bb12 and no carbohydrates added; **OkaBb3**: fermented by *B. animalis* ssp. *lactis* Bb12 with fructose (3%) and FOS (2%); **OkaBb6:** fermented by *B. animalis* ssp. *lactis* Bb12 with fructose (6%) and FOS (2%); ***L. rhamnosus* R11 count in fermented okara by co-culture: OkaMix-LR**: fermented by *B. animalis* ssp. *lactis* Bb12 and *L. rhamnosus* R11 combined and no carbohydrates added; **OkaMix3-LR**: fermented by *B. animalis* ssp. *lactis* Bb12 and *L. rhamnosus* R11 combined with fructose (3%) and FOS (2%); **OkaMix6-LR**: fermented by *B. animalis* ssp. *lactis* Bb12 and *L. rhamnosus* R11 combined with fructose (6%) and FOS (2%).B. animalis* ssp. *lactis* Bb12 count in fermented okara by co-culture: OkaMix-Bb12**: fermented by *B. animalis* ssp. *lactis* Bb12 and *L. rhamnosus* R11 combined and no carbohydrates added; **OkaMix3-Bb12**: fermented by *B. animalis* ssp. *lactis* Bb12 and *L. rhamnosus* R11 combined with fructose (3%) and FOS (2%); **OkaMix6-Bb12**: fermented by *B. animalis* ssp. *lactis* Bb12 and *L. rhamnosus* R11 combined with fructose (6%) and FOS (2%).

Figure 5.2. Total viable cells (log10 CFU/ mL) in fermented okara by *Lactobacillus rhamnosus* R11 (OkaLR) (a), *Bifidobacteriun animalis* Bb-12 (OkaBb) (b) and combination of both bacteria (OkaMix), the cells growth of *Lactobacillus rhamnosus* R11 (OkaMix-LR) and *Bifidobacteriun animalis* Bb-12 (OkaMix-Bb12) (c) through the fermentation time (h).

Table 5.1. Growth parameters of *Lactobacillus rhamnosus* R11, *Bifidobacteriun animalis* spp *lactis* Bb-12 and for each of the bacteria in a mixture of both.

Bacteria	Carbon source	Specific growth rate $\mu_{max}(h^{-1})$
L. rhamnosus R11 (OkaLR)	MRS medium	0.85 ± 0.02^{a}
	No carbohydrates added (OkaLR)	0.33 ± 0.06^{b}
	Fructose (3%) and FOS (2%) (OkaLR3)	0.49 ± 0.02^{c}
	Fructose (6%) and FOS (2%) (OkaLR6)	0.53 ± 0.02^{c}
B. animalis ssp. *lactis* Bb12 (OkaBb)	MRS medium	0.84 ± 0.01^{a}
	No carbohydrates added (OkaBb)	0.39 ± 0.04^{b}
	Fructose (3%) and FOS (2%) (OkaBb3)	0.43 ± 0.04^{bc}
	Fructose (6%) and FOS (2%) (OkaBb6)	0.52 ± 0.01^{c}
B. animalis ssp. *lactis* Bb12 in a mixture of both (OkaMix)	MRS medium	0.68 ± 0.02^{a}
	No carbohydrates added (OkaMix)	0.26 ± 0.03^{b}
	Fructose (3%) and FOS (2%) (OkaMix3)	0.27 ± 0.01^{b}
	Fructose (6%) and FOS (2%) (OkaMix6)	0.30 ± 0.04^{b}
L. rhamnosus R11 in a mixture of both (OkaMix)	MRS medium	0.58 ± 0.01^{a}
	None carbohydrates added (OkaMix)	0.26 ± 0.03^{b}
	Fructose (3%) and FOS (2%) (OkaMix3)	0.31 ± 0.01^{b}
	Fructose (6%) and FOS (2%) (OkaMix6)	0.39 ± 0.01^{c}

Data indicate the mean ± standard deviation of two independent experiments. Different superscript letters for the same culture are significantly different ($P < 0.05$).

5.4.2. Organic acids and fructose in fermented okara

The decrease in pH values of the different fermented okara samples is related with the continuous production of organic acids that results from fermentation of carbohydrates by

probiotic bacteria. The citric acid levels (Table 5.2) remained stable before and after okara's fermentation regardless of the bacteria considered. For lactic and acetic acid, as could be expected, these acids were not detected in the unfermented beverage. The addition of carbon source (fructose and FOS), resulted in higher concentrations ($P < 0.05$) of lactic acid for all bacteria used. In addition, okara fermented by *B. animalis* ssp. *lactis* Bb 12 exhibited the highest lactic acid concentrations (8.95 ± 0.03 and 8.71 ± 0.24 mg/ mL of fermented, in OkaBb3 and OkaBb6, respectively) while, the okara fermented without carbon source exhibited values between 0.83 and 2.00 mg lactic acid/ mL of fermented okara.On the other hand, in Table 5.2 is possible to observe that all strains used in this study produced more acetic acid in fermented okara without carbon source and the addition of FOS and fructose results in a statistically significant decrease in the levels of acetic acid in all pure and co-culture. Moreover, the fermented okara by *B. animalis* ssp. *lactis* Bb 12 presented higher acetic acid production comparing to other fermented. In general, the metabolism of sugars by lactobacilli and bifidobacteria produces lactic and acetic acids as the major end-products, highly dependent on the strain. Consequently, the pH of the medium drops, leading to a decreased glycolytic flux and growth rate, compromising ultimately cell viability (Delgado et al., 2018). In addition, the lactic acid concentrations of fermented okara reported in the present work were higher than those reported by Wang et al. (2003) for a soymilk fermented by *Bifidobacterium infantis*, *L. acidophilus* and *S. thermophilus* (0.9 to 5.3 g of lactic acid/ mL of fermented soymilk). These differences in lactic acid production may be likely due differences in metabolism of the probiotic strains used in these studies (Delgado et al., 2018). Wang et al. (2003) also reported that the lactic acid bacteria and bifidobacteria were capable of metabolizing stachyose and raffinose present in soymilk.

Table 5.2. Concentrations of the fructose, citric, lactic and acetic acids measured in the fermented okara beverage for the two probiotic strains tested alone or combined with different carbon source

Sample	Citric acid (mg/ mL)		Lactic acid (mg/mL)		Acetic acid(mg/mL)		Fructose (mg/ mL)	
	T0	TF	T0	TF	T0	TF	T0	TF
OkaLR	6.03 ± 0.40^{A}	5.03 ± 0.75	ND	1.04 ± 0.16^{A}	ND	1.16 ± 0.21^{A}	ND	ND
OkaLR3	4.92 ± 0.25^{AB}	5.45 ± 0.36	ND	6.97 ± 0.47^{B}	ND	0.42 ± 0.05^{B}	21.07 ± 1.02	19.14 ± 2.29
OkaLR6	4.67 ± 0.10^{B}	$4.4\ 0 \pm 0.17$	ND	7.54 ± 0.14^{B}	ND	0.28 ± 0.03^{B}	50.85 ± 0.79	44.79 ± 2.54
OkaBb	4.31 ± 0.73	4.16 ± 0.90	ND	0.83 ± 0.03^{A}	ND	1.17 ± 0.21^{A}	ND	ND
OkaBb3	4.16 ± 0.35	4.33 ± 0.94	ND	8.95 ± 0.03^{B}	ND	1.09 ± 0.07^{A}	27.66 ± 3.05	24.51 ± 2.97
OkaBb6	4.16 ± 0.68	4.95 ± 0.05	ND	8.71 ± 0.24^{B}	ND	0.66 ± 0.07^{B}	50.35 ± 0.50^{a}	43.19 ± 2.07^{b}
OkaMix	4.82 ± 0.12^{A}	$4.57 \pm .07^{A}$	ND	1.85 ± 0.33^{A}	ND	0.87 ± 0.07^{A}	ND	ND
OkaMix3	3.91 ± 0.09^{B}	6.12 ± 1.16^{AB}	ND	5.99 ± 0.49^{B}	ND	0.44 ± 0.03^{B}	20.96 ± 0.28	18.96 ± 2.25
OkaMix6	5.00 ± 0.57^{A}	4.93 ± 0.14^{B}	ND	7.52 ± 0.42^{C}	ND	0.37 ± 0.05^{B}	51.48 ± 1.06^{a}	43.84 ± 2.05^{b}

Data indicate the mean ± standard deviation of two independent experiments. **OkaLR:** fermented by *L. rhamnosus* R11 and no carbohydrates added; **OkaLR3:** fermented by *L. rhamnosus* R11 with fructose (3%) and FOS (2%); **OkaLR6:** fermented by *L. rhamnosus* R11 with fructose (6%) and FOS (2%); **OkaBb:** fermented by *B. animalis* ssp. *lactis* Bb12 and no carbohydrates added; **OkaBb3**: fermented by *B. animalis* ssp. *lactis* Bb12 with fructose (3%) and FOS (2%); **OkaBb6:** fermented by *B. animalis* ssp. *lactis* Bb12 with fructose (6%) and FOS (2%); **OkaMix**: fermented by *B. animalis* ssp. *lactis* Bb12 and *L. rhamnosus* R11 combined and no carbohydrates added; **OkaMix3**: fermented by *B. animalis* ssp. *lactis* Bb12 and *L. rhamnosus* R11 combined with fructose (3%) and FOS (2%); **OkaMix6**: fermented by *B. animalis* ssp. *lactis* Bb12 and *L. rhamnosus* R11 combined with fructose (6%) and FOS (2%). Different superscript lowercase letters in the same row are statistically different ($P < 0.05$), as indicated by T-test. Different superscript uppercase letters in the same column for the same culture are significantly different ($P < 0.05$), as indicated by Tukey's test. Absence of superscript indicates no significant difference between samples.

In parallel, the ability of probiotic bacteria to ferment oligosaccharides may be an important characteristic, since the availability of carbohydrates that escape metabolism and adsorption in the small intestine have a major influence on the microflora that become established in the colon (Kaplan and Hutkins, 2000). Additionally, the fructooligosaccharides (FOS) are one specific group of oligosaccharides that has attracted much commercial interest and these have been self-affirmed by the manufacturers as GRAS (generally recognized as safe) and have been added to food products (such as yogurts) and food supplements (Speigel, et al., 1994; Kaplan and Hutkins, 2000). Moreover, in a previous study Kaplan and Hutkins et al. (2000) observed that FOS utilization did not require an induction period and that FOS was equally as good substrate as glucose in supporting growth for probiotic. Furthermore, in the present study, the growth of both probiotic may also be related to the addition of FOS, as can be seen on the table 5.1, the okara added showed a similar growth using different fructose concentration. In addition, all fermented okara supplemented with fructose (Table 5.2) showed a similar behavior, with lower fructose levels in the final of fermentation (pH 4.5) than the one observed in unfermented okara (T0). However, the fructose concentration in the fermented okara with 3% of fructose was similar ($P > 0.05$) to the one observed in unfermented okara.

5.4.3. Total phenolics, antioxidant activity and isoflavone content of fermented okara

The antioxidant activity, total phenolics and isoflavone contents of soy products can be affected by processes, such as microbial fermentation (Delgado et al., 2018; Donkor and Shah, 2008; Marazza et al., 2012). While previous studies have shown that this process typically contributes to an increase of phenolic compounds (Fernandes et al., 2017; Marazza et al., 2012), in this study a significant decrease in the total phenolic content and antioxidant activity was observed for most of the fermented okara (Table 5.3). In fact, while the total phenolic content, before fermentation, ranged from 0.18-0.25 mg of gallic acid/ mL, afterwards it ranged from 0.13 to 0.20 mg of gallic acid/ mL with the highest reduction in total phenolic content being observed for OkaLR6 (a 0.1 mg of gallic acid/mL reduction).

In parallel, Moraes Filho et al.(2016) also found a similar behavior, with a significative reduction of total phenolics during the fermentation of soymilk with okara flour by *Lactobacillus acidophilus* LA3 and *Lactobacillus plantarum* BG 112. However, in our study

the total phenolics were maintained after fermentation in the fermented OkaLR3 (0.25 mg of gallic acid/ mL). The antioxidant capacity variation was similar to the one observed for the total phenolic content, i.e. the ABTS radical cation scavenging capacity of the fermented okara (0.25 - 0.04 mg Trolox /mL) was lower than that of the unfermented sample (0.49 to 0.40 mg Trolox /mL). This might be a possible consequence of the degradation of phenolic compounds that results from the metabolism of the probiotic bacteria and according to Mujić et al. (2011) the content of phenolic compounds may promote antioxidant activity. Moreover, this also can be related with the conversion and final concentration of isoflavones. In a previous study, Ruiz-Larrea et al. (1997) reported that genistein presented a highest antioxidant activity comparing to other isoflavones (daidzein, daidzin and genistin) utilizing ABTS method. Furthermore, several attempts have been made to improve bioavailability of isoflavone aglycones from isoflavone glycosides in soya beverage milk by fermentation with lactobacilli and bifidobacteria species and large differences in the deglycosylation ability of these bacteria have been reported by several author (Chun et al., 2007; Delgado et al., 2018; Donkor and Shah, 2008; Gaya, et al., 2016; Raimondi et al., 2009).

The microorganisms used in the current work can synthesize β-glycosidase which is responsible for removing the sugar moiety from glycosylated isoflavones (Delgado et al., 2018; Fernandes et al., 2017; Wei et al., 2007). This transformation of glycosylated isoflavones, into their respective aglycones (mainly daidzin and genistin being converted into daidzein and genistein, respectively) has been reported in soya beverages fermented using different bacterial strains and mixed cultures (Delgado et al., 2018; Raimondi et al., 2009; Wei et al., 2007). However, although to the best of our knowledge, this was the first time that a study was performed using only okara.

Table 5.3. Total phenolic compounds and antioxidant activity (ABTS) in okara fermented in the initial time and end of each fermentation.

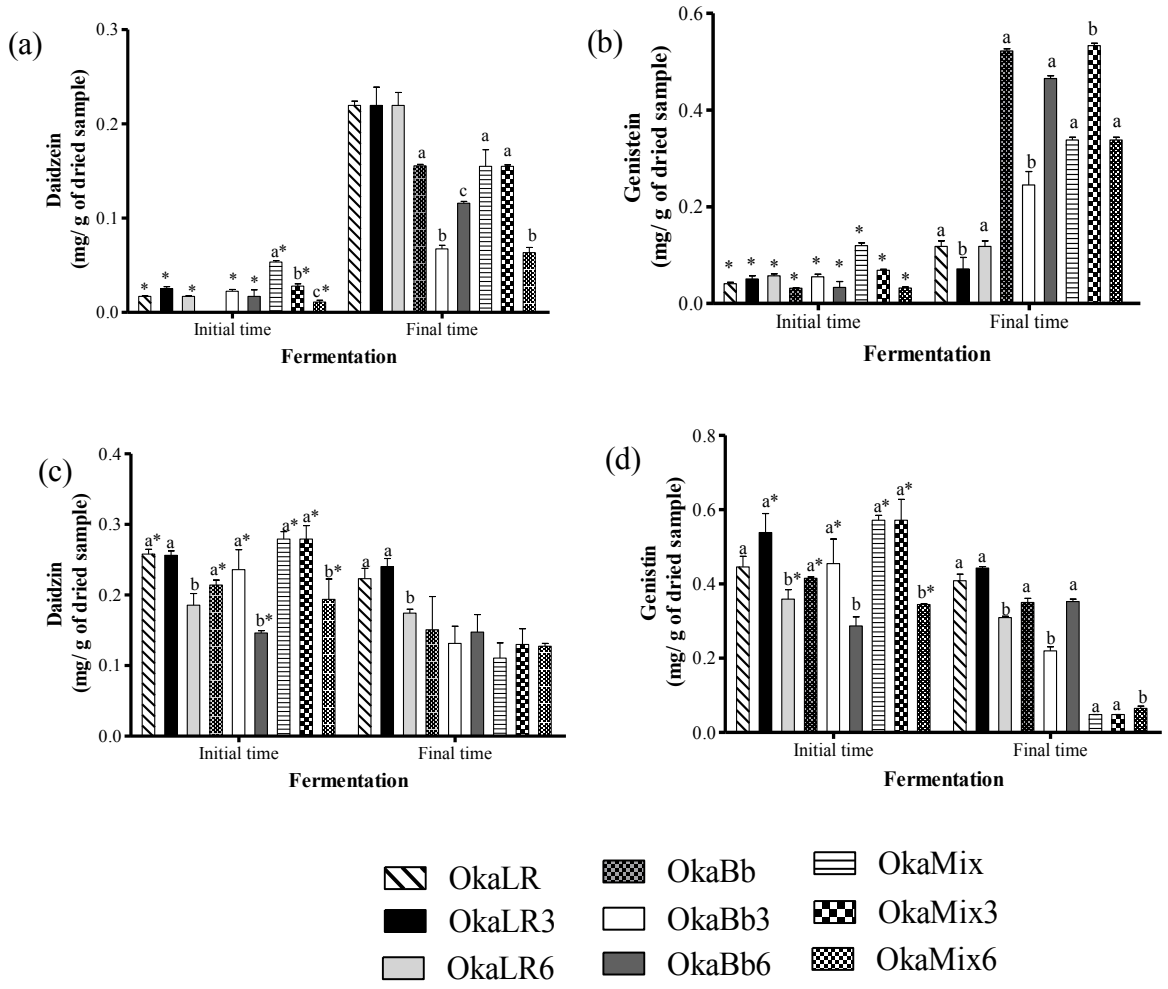
Bacteria	Sample	**Total phenolic compounds (mg gallic acid/ mL of fermented okara)**		**ABTS (mg of Troloxd/ mL of fermented okara)**	
		Before fermentation	After fermentation*	Before fermentation	After fermentation*
L. rhamnosus R11	OkaLR	0.18 ± 0.01 ^{Aa}	0.17 ± 0.01 ^{Aa}	0.44 ± 0.07 ^{Aa}	0.46 ± 0.04 ^{Aa}
	OkaLR3	0,25 ± 0.02 ^{Ba}	0.25 ± 0.01 ^{Ba}	0.46 ± 0.03 ^{Aa}	0.16 ± 0.01 ^{Bb}
	OkaLR6	0.25 ± 0.02 ^{Ba}	0.15 ± 0.01 ^{Cb}	0.40 ± 0.06 ^{Aa}	0.07 ± 0.01 ^{Cb}
B. animalis ssp. *lactis* Bb12	OkaBb	0.19 ± 0.01 ^{Aa}	0.16 ± 0.01 ^{Ab}	0.41 ± 0.01 ^{Aa}	0.25 ± 0.01 ^{Ab}
	OkaBb3	0.18 ± 0.02 ^{Aa}	0.13 ± 0.02 ^{Bb}	0.41 ± 0.10 ^{Aa}	0.04 ± 0.01 ^{Bb}
	OkaBb6	0.22 ± 0.01 ^{Ba}	0.15 ± 0.02 ^{ABb}	0.49 ± 0.02 ^{Aa}	0.05 ± 0.00 ^{Bb}
Combination of both (*B. animalis* ssp. *lactis* Bb12 and *L. rhamnosus* R11)	OkaMix	0.22 ± 0.01 ^{Aa}	0.16 ± 0.01 ^{Ab}	0.48 ± 0.01 ^{Aa}	0.18 ± 0.00 ^{Ab}
	OkaMix3	0.23 ± 0.02 ^{ABa}	0.18 ± 0.01 ^{ABb}	0.41 ± 0.01 ^{Ba}	0.11 ± 0.01 ^{Bb}
	OkaMix6	0.25 ± 0.01 ^{Ba}	0.20 ± 0.04 ^{Bb}	0.41 ± 0.02 ^{Ba}	0.07 ± 0.00 ^{Cb}

[a] Data indicate the mean ± standard deviation of two independent experiments. [b] **OkaLR:** fermented by *L. rhamnosus* R11 and no carbohydrates added; **OkaLR3:** fermented by *L. rhamnosus* R11 with fructose (3%) and FOS (2%); **OkaLR6:** fermented by *L. rhamnosus* R11 with fructose (6%) and FOS (2%); **OkaBb:** fermented by *B. animalis* ssp. *lactis* Bb12 and no carbohydrates added; **OkaBb3**: fermented by *B. animalis* ssp. *lactis* Bb12 with fructose (3%) and FOS (2%); **OkaBb6:** fermented by *B. animalis* ssp. *lactis* Bb12 with fructose (6%) and FOS (2%); **OkaMix**: fermented by *B. animalis* ssp. *lactis* Bb12 and *L. rhamnosus* R11 combined and no carbohydrates added; **OkaMix3**: fermented by *B. animalis* ssp. *lactis* Bb12 and *L. rhamnosus* R11 combined with fructose (3%) and FOS (2%); **OkaMix6**: fermented by *B. animalis* ssp. *lactis* Bb12 and *L. rhamnosus* R11 combined with fructose (6%) and FOS (2%).[c] Different lowercase letters in the same row are statistically different ($P < 0.05$), as indicated by t-test. [d] Different uppercase letters in the same column and for the culture are significantly different ($P < 0.05$), as indicated by Tukey's test.

* fermented okara when reached pH 4.5.

The results followed a similar trend, i.e. *B. animalis* ssp. *lactis* Bb12 and *L. rhamnosus* R11 were capable to metabolize the glycosylated isoflavones daidzin and genistin into their aglycones. Figure 5.3 shows the concentration of isoflavones of all the fermented okara samples. Overall, the isoflavone aglycones' content was higher in fermented okara samples than in the unfermented okara (initial time) (Fig. 5.3 a and b). Both *Lactobacillus* and *Bifidobacterium* resulted in a significant increase of the isoflavone aglycones in fermented okara. Daidzein (Fig. 5.3 a) showed the highest increase (from 9 to 13-fold) when the okara was fermented by *L. rhamnosus* R11, while for genistein *B. animalis* Bb12 resulted in the highest increase of aglycone form (when compared to the other fermented okara samples), with OkaBb presenting an increase of ca. 16-fold of genistein when comparing to the unfermented okara. At the same time, isoflavone glycosides' levels were either reduced or remained constant in the fermented okara samples (Fig.5. 3 c and d).

Overall these results show that both *B. animalis* ssp. *lactis* Bb12 and *L. rhamnosus* R11 are capable of exerting an important role in the hydrolysis of isoflavones, which stands in line with previous works that described a similar isoflavones profile behavior in probiotic fermented soymilk (Chun et al., 2007; Delgado et al., 2018; Marazza et al., 2012), and are therefore capable of exerting an important role in the improvement of the biological activity of soymilk (Raimondi et al., 2009; Wei et al., 2007). Moreover, according to Gaya et al.(2016), in soy beverages, these bacteria showed a higher affinity for the deglycosylation of genistin than for the other glycosylated isoflavones. This behavior differs to the one observed in okara fermented by *L. rhamnosus* R11wich exhibited a higher conversion of daidzin than genistin.



OkaLR: fermented by *L. rhamnosus* R11 and no carbohydrates added; **OkaLR3:** fermented by *L. rhamnosus* R11 with fructose (3%) and FOS (2%); **OkaLR6:** fermented by *L. rhamnosus* R11 with fructose (6%) and FOS (2%); **OkaBb:** fermented by *B. animalis* ssp. *lactis* Bb12 and no carbohydrates added; **OkaBb3**: fermented by *B. animalis* ssp. *lactis* Bb12 with fructose (3%) and FOS (2%); **OkaBb6:** fermented by *B. animalis* ssp. *lactis* Bb12 with fructose (6%) and FOS (2%); **OkaMix**: fermented by *B. animalis* ssp. *lactis* Bb12 and *L. rhamnosus* R11 combined and no carbohydrates added; **OkaMix3**: fermented by *B. animalis* ssp. *lactis* Bb12 and *L. rhamnosus* R11 combined with fructose (3%) and FOS (2%); **OkaMix6**: fermented by *B. animalis* ssp. *lactis* Bb12 and *L. rhamnosus* R11 combined with fructose (6%) and FOS (2%). Different letters are significantly different ($P < 0.05$) between fermented okara suing same bacteria and in the same time, as indicated by Tukey's test. Asterisks indicate significantly different ($P < 0.05$), comparing initial and final time of fermentation for the same sample, as indicated by T-test.

Figure 5.3. Isoflavone compounds daidzein (a), genistein (b), daidzin (c) and genistin (d) in okara unfermented and after fermentation by *B. animalis* ssp. *lactis* Bb12, *L. rhamnosus* R11 and mixture of both.

5.5. Conclusion

This work demonstrated that okara could be effectively fermented using *Bifdobacterum animalis* Bb12, *Lactobacillus rhamnosus* R11 or a combination of both. However, the time required to reach a pH value of 4.5 (value at which the fermentation was interrupted) varied according to the cultures used, and the shortest acidification was observed for fermented okara by *Lactobacillus rhamnosus* R11without carbon source. Additionally, both the specific growth rate and acid production during okara fermentation were primarily dependent on the carbon source present although the strain used to ferment showed also to influence. Fructose and FOS addition to okara is likely to be the main reason contributing to the enhanced growth and acid production observed in the supplemented samples although a synergistic effect between the carbohydrate components and okara is still possible. This work also showed that fermented okara presented higher concentrations of isoflavone aglycones than its unfermented counterpart, which is likely to result in an enhancement of isoflavones' bioavailability when comparing to their glycosylated forms. Therefore, the use of probiotic microorganisms to ferment okara appears to improve functional benefits for consumers through the modification of the bioavailability of the isoflavone aglycones. Furthermore, regardless of the cultures used, the higher levels of fructose (6%) combined with a fix prebiotic concentration of FOS (2%) supplementation resulted in higher specific growth rates for both bacteria than when okara was supplemented with 3% fructose and 2% FOS or not supplemented at all.

CHAPTER 6- Development of a novel synbiotic okara beverage: stability, functionality and impact of gastrointestinal tract

6.1. Abstract

This study aimed to produce four beverages from okara (a by-product from soybeans) previously hydrolyzed by *Cynara cardunculus* enzymes and fermented by probiotic bacteria: *Lactobacillus rhamnosus* R11, *Bifidobacterium animalis* ssp. *lactis* Bb12 or both (OkaLR6, OkaBb6 and OkaMix6), and unfermented beverage (OkaNF). The probiotic viable cells were determined in the fermented beverage and the isoflavones profile and organic acids were evaluated in all beverages. In addition, total phenolic content, antioxidant and antihypertensive activities were evaluated at storage time and during *in vitro* gastrointestinal digestion of all okara beverages. The probiotic bacteria were viable throughout storage (ca. 9 log CFU/mL) in all fermented beverages. The significant bioconversion of the isoflavone glycosides (daidzin and genistin) into their responding bioactive aglycones (daidzein and genistein) was observed in fermented beverage compared to the OkaNF. Furthermore, the beverages showed a good antihypertensive activity, especially the OkaNF (442.50 ± 21.02 μg of protein mL-1). In addition, after gastrointestinal tract, all beverages showed an increase in the antioxidant (between 27 and 41 times) and antihypertensive activities (IC_{50}) (ca. 172 and 252 μg of protein mL-1 in fermented beverages and OkaNF, respectively). However, for a better overall liking by consumers, the fermented beverages need to be reformulated, especially in relation to acidity and sweetness. In conclusion, this study shows that the application of okara for a multifunctional beverage could be a promising strategy in the disease prevention and contribution to a zero waste approach in Soya milk industry.

6.2. Introduction

The development of probiotic products, which are food containing live microorganisms, has shown a good progress in last years, despite the regulatory restrictions on health claims imposed by the European Food Safety Authority (EFSA) (Regulation 1924/2006) (European Parliament and The Council of the European Union, 2006). The probiotic foods can provide

health benefits to the consumers when administered in adequate amounts (FAO/WHO, 2001) and previous studies suggested levels of probiotics must be between 10^6 and 10^8 of viable probiotic cells per milliliter or milligram of product (Donkor et al., 2007; Martinez et al., 2015; Mondragón-bernal et al., 2017). In addition, the food products with incorporation of the prebiotic ingredients may also confer relevant health benefits, the prebiotic are non-viable substrates, that are selectively utilized by host microorganisms and can include non-carbohydrate substances and other categories than food. Besides that, the definition includes the application of this substances to body sites other than the gastrointestinal tract (Gibson et al., 2017).

The combination of probiotic species and prebiotic substances, so-called synbiotic foods, might promote a positive interaction *in vivo*, besides that the synbiotic may confer the stability of the final product (Martinez et al., 2015; Mondragón-bernal et al., 2017).

Additionally, most of probiotic or synbiotic food products use milk as base matrix, but factors such as allergenicity or intolerance to dairy-food and the preference for vegetarian food have stimulated the use of plant raw materials for the development of these products, among which is the soya beverage (Bedani et al., 2014b). Moreover, the consumption of this oilseed and their products are increasing due to its valuable nutritional characteristics, such as high quality proteins, polyunsaturated fatty acids, dietary fibers and isoflavones, as well the health benefits, such as reduction of the risk of cardiovascular diseases, diabetes (Bedani et al., 2014a; Chen et al., 2010; Fritz et al., 2003; Izumi et al., 2000; Villares et al., 2010).

On the other hand, some studies have reported the potential use of okara, a by-product from soya beverage and tofu, in food industry, due their nutritional and functional properties (Bedani et al., 2014a; Jankowiak et al., 2015; O´Toole, 1999; Redondo-Cuenca et al., 2008), however limited food applications have been studied. Although, okara is usually treated as industrial waste, because of its short shelf life and their high humidity (80%) (O´Toole, 1999), previous studies have suggested that okara present a rich nutritional composition (O´Toole, 1999; Redondo-Cuenca et al., 2008) and might be useful as a dietary supplement for a weight-loss (Préstamo et al., 2007), decrease of risk factors in cardiovascular diseases (Bedani et al., 2014a), possesses hypocholesterolemic and hypolipidemic effects (Villanueva et al., 2011) and protected the gut environment in terms of antioxidant status and prebiotic effect (Jiménez-Escrig et al., 2008).

In the last 10-15 years, much research has focused on food protein derived peptides, as alternative to angiotensin converting enzyme (ACE) inhibitory drugs that are used for the regulation of blood pressure. However, many adverse effects are associated with the use of synthetic ACE inhibitory drugs (Majumder and Wu, 2009). In addition, previous studies, have suggested that the enzymatic hydrolysis and fermentation increase the antihypertensive activity in soy protein or beverage (Coscueta et al., 2016; Margatan et al., 2013; Martinez-Villaluenga et al., 2012).

In addition to protein, soy contains a diverse group of biologically active compounds, such as phytoestrogens, called isoflavones (Delgado et al., 2018), which have been reported to have antioxidant properties and they can contribute to positive health effects, including cancer prevention (He and Chen, 2013; Izumi et al., 2000; Sarkar and Li, 2003). Nevertheless, the conditions used to obtain soymilk affect the content of the isoflavones in okara, but about 12 - 40% of the raw soybean isoflavones still remains in this by-product (Muliterno et al., 2017). Moreover, application of some processes can affect the content and profile of the isoflavones in food and according to previous studies, the fermentation by lactic bacteria can increase the isoflavone aglycones (Chun et al., 2007; Wei et al., 2007). Additionally, these isoflavones are faster absorbed by the organism compared to their glycosides isoflavones (Izumi et al., 2000). Although the process of fermentation in soymilk can be a promising alternative to increase the economic value and provide health benefit to consumers (Albuquerque et al., 2017) only few studies have investigated the impact of the incorporation of okara combined with soymilk (Bedani et al., 2014a, 2014b), but no study has been performed using only okara as base for a fermented probiotic beverage.

Therefore, the objective of this work was to obtain four different beverages using okara previously hydrolyzed by proteases from *Cynara cardunculus*: (1) beverage fermented by *Lactobacillus rhamnosus* R11, (2) beverage fermented by *Bifidobacterium animalis* ssp. *lactis* Bb12, (3) beverage fermented by a mix of both bacteria and (4) beverage without fermentation. In addition, the antioxidant activity, isoflavones profile, viable cells of probiotics of the beverages, microbiological stability throughout 28 days of storage at 4 ºC were evaluated, as well as, the sensory acceptability of these products. Furthermore, the total phenolics, antioxidant and antihypertensive activities were also evaluated throughout simulated gastrointestinal conditions.

6.3. Material and methods

6.3.1. Microorganisms activation and inoculum preparation

The strains used as probiotics were *Bifidobacterium animalis* ssp. *lactis* Bb12 (Christian Hansen, Hørsholm, Denmark) and *Lactobacillus rhamnosus* R11 (Lallemand, Montreal, Canada). The aforementioned microorganisms were reactivated, and pre-cultures were made in de Man–Rogosa and Sharpe (MRS; Biokar Diagnostics, Beauvais, France) broth, incubated overnight at 37 ºC. For *B. animalis* ssp. *lactis* Bb12 the MRS was supplemented with filter-sterilized L-cysteine·HC1 (0.5 g/L; Fluka, Switzerland), and incubated using an anaerobic chamber. The resulting cultures were then used as inoculum for the fermentation.

6.3.2. Preparation of okara beverages

The okara used in this study was kindly provided by a soy beverage producer (NUTRE Industry, Aveiro, Portugal). The okara was transported immediately after the beverage production and refrigerated at 4 ºC. Preparation of okara beverage not fermented (Figure 6.1 a) and okara beverage fermented (Figure 6.1 b) was as following: 200 g of fresh okara was homogenized with 600 mL of citric acid – sodium citrate buffer (0.1 M, pH 5.2) using a blender under hygienic conditions. Afterwards, enzymatic hydrolysis of fresh okara was performed using proteases from *Cynara cardunculus* (Formulab, Maia, Portugal). The hydrolysis was performed in an orbital shaker (SI-100C, Wiggenhauser, Germany) at 55 ºC for 2.5 h using an enzyme: substrate (E/S) ratio of 1.1 (v/w) and under an orbital stirring of 120 rpm. The resulting solutions were autoclaved (1 atm, 121 ºC and 15 minutes) immediately after hydrolysis.

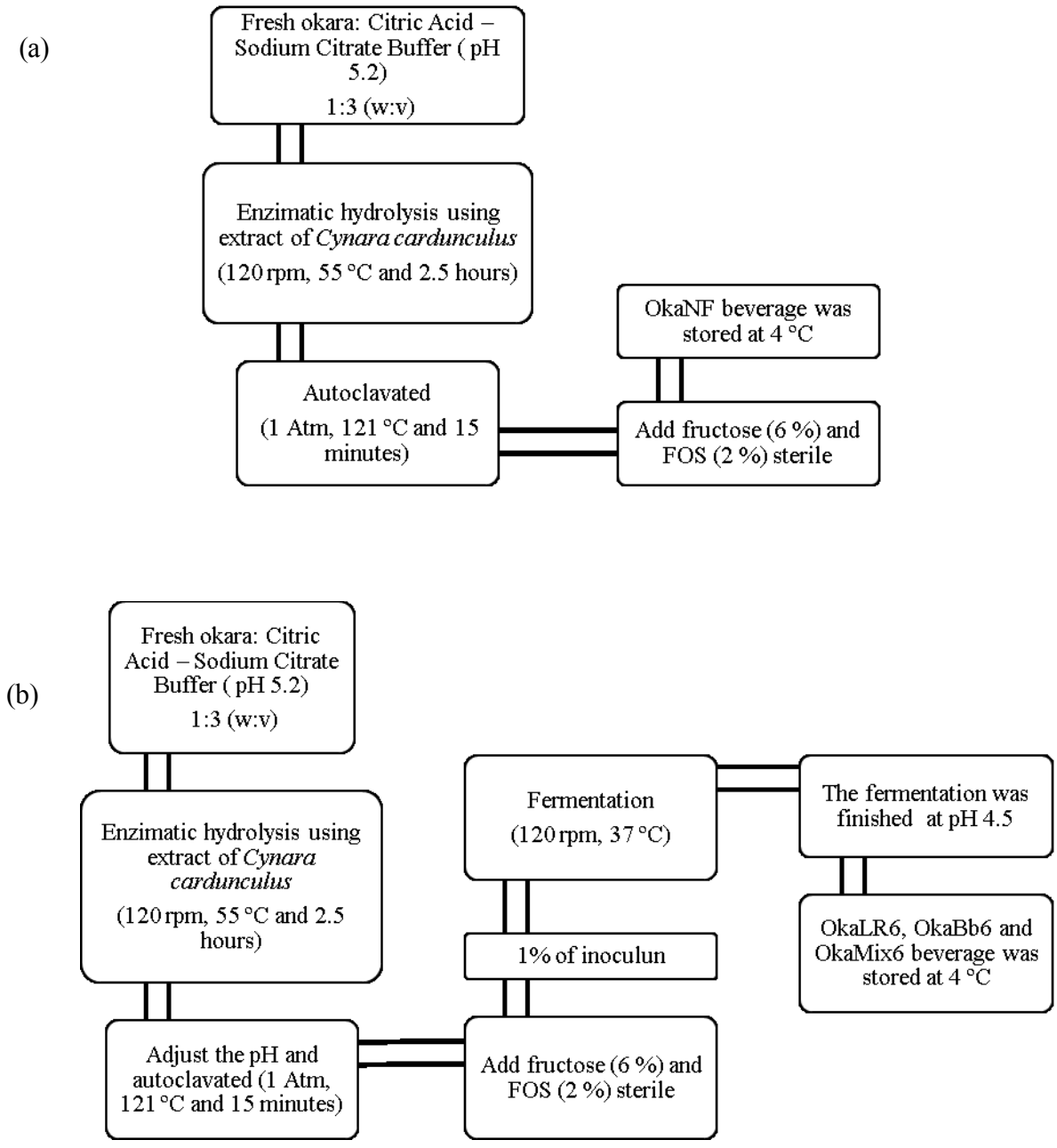


Figure 6.1. Flowchart of okara beverage production: (a) beverage from okara hydrolyzed and (b) beverage from okara previously hydrolyzed and fermented by *Lactobacillus rhamnosus R11, B. animalis* Bb-12 or a mixture of both.

Then, for all okara beverages, 100 mL of fructose solution (60 g) and 100 mL of fructooligosaccharides (FOS) Orafti® P95 solution (20 g), both sterile (Figure 6.1 a) were added to 800 mL of hydrolyzed okara, under aseptic conditions. Each okara fermented beverage

(OkaLR6, OkaBb6 and OkaMix6) was inoculated with 1% of bacterial inoculum (as described in 6.3.1) and incubated in an orbital shaker at 37 ºC and 120 rpm. The fermentation was finished at 4.5 pH, with an average duration of 8 h for *B. animalis* Bb12 and 12 h for *L. rhamnosus* R11 and combination of both bacteria (*B. animalis*. Bb12 and *L. rhamnosus* R11). Each experimental condition was performed in duplicate, so in total 8 okara beverages were produced.

6.3.3. Stability test of fermented beverages

After processing (fermentation or autoclaving), the beverages were bottled in sterile 100 mL and stored under refrigeration (4 °C) for 28 days. Every 7 days, a bottle of each treatment (OkaLR6, OkaBb6, OkaMix6 and OkaNF) was used to analyze pH, Brix, titratable acidity, isoflavones, antioxidant activity, total phenolics, sugar, organic acids and enumerate probiotics and microbial contamination.

6.3.3.1. Physical-chemical analyses

Brix degree was measured at 20.0 ºC ± 0.5 ºC using digital refractometer (model PR-32, Atago, Co. Ltd, Tokyo, Japan). Potentiometric determination of pH was performed at 20 ºC ± 2 ºC using a pH meter (Micro pH 2002, Crison, Barcelona, Spain). Titratable acidity (g of acid/100 mL) was determined by titration of a 5 mL aliquot with NaOH 0.01 M solution using 1% phenolphthalein as an indicator.

6.3.3.2. Microbiological analysis

To determine the growth of microorganisms in fermented okara beverage during the storage period (0, 7, 14, 21 and 28 days), 1 mL of each sample was blended with 9 mL sterile peptone water 0.1% (Peptone - Sigma Aldrich) and subjected to serial decimal dilutions with the same diluent, plated on using the drop method on MRS agar (plain or supplemented with 0.5 g L^{-1} of cysteine-HCl when *B. animalis* ssp. *lactis* Bb-12 was used) and incubated at 37 °C for 24-48 h under anaerobic conditions, using anaerobic chamber (Whitley DG250) (Lee and

Lee, 2008; Miles and Misra, 1938). On each sampling day, a new bottle containing okara beverage from the same batch was used for the analysis.

Total mesophilic bacteria were determined by plating in Plate Count Agar (PCA; Biokar Diagnostics, Beauvais, France) and incubated aerobically at 30 ºC for 48 h. Yeasts and molds were counted in Potato Dextrose Agar (PDA; Biokar Diagnostics, Beauvais, France), incubated aerobically at 30 ºC for 5 d. Inoculation was performed using the drop method as described by Miles et al. (1938). *Enterobacteriaceae* viable count were determined through plating on Violet Red Bile Glucose Agar (VRBGA, Oxoid, Basingstoke, United Kingdom) using the pour plate method and incubated aerobically at 37 ºC for 24 h.

6.3.3.3. Isoflavones analysis

The extracts were prepared according to Fernandes et al. (2017), using freeze dried okara beverage, previously degreased with hexane (1:10 w/v) and stirred for 1 h at room temperature. The extraction was performed using 200 mg of sample and 8.0 mL of extraction solvent (water, ethanol and acetone 1:1:1 (v/v/v)). The suspension was left to extract for 1 h at room temperature, with vortexing every 15 min. Afterwards, samples were placed in an ultrassound bath at room temperature for 15 min, centrifuged at 4105 x g for 15 min at 4 ºC and the supernatant was filtered through a 0.45 µm filter (Orange Scientific, Braine-l'Alleud, Belgium) before chromatographic analysis.

Isoflavones were analyzed by HPLC using an adaptation of the method described by Wang and Murphy (1996). Twenty microliters of each extract were analyzed by HPLC (Waters Series 2695, Milda ford, MA, USA) coupled to a diode array detector, using a Kromasil C-18 column (250 x 4,6 mm) to separate the isoflavones at 25 ºC. The mobile phase consisted of 0.1% acetic acid in water (v/v) (solvent A) and 0.1% acetic acid in acetonitrile (v/v) (solvent B). Elution started with 20% B, increased to 100% B in 20 min and decreased to 20% B in the next 12 min, with a flow rate was 0.8 ml/min. Spectral data from was accumulated in the 200–400 nm range and processed using Empower 3 software. Isoflavone quantification was achieved by comparing the absorbance spectrum at 255 nm with that of pure external standards. The standards were dissolved in analytical grade DMSO (Sigma-Aldrich, St Quentin Fallavier, France) and diluted in methanol. The calibration curve was performed with Genistin, Genistein, Daidzin and

Daidzein (Sigma-Aldrich, St. Louis, USA). This procedure was done in duplicate for each sample, and the results were expressed in mg of isoflavone per g of dried beverage.

6.3.3.4. Organic acids and fructose analysis

The organic acids and sugar contents were analyzed by HPLC according to the method described by Zeppa et al. (2001), with slight modifications. Briefly, 2 g of each sample were suspended in 10 mL of sulfuric acid 13 mM and homogenized with an Ultra-Turrax (IKA Ultra-turrax T18, Wilmington, USA) at 18.000 rpm for 3 min. Afterwards, the samples were centrifuged at 2665 x g for 10 min at 4 °C (Universal 32R; Hettich, Tuttlingen, Germany) and the supernatant was filtered with a 0.45 µm filter (Orange Scientific, Braine-l'Alleud, Belgium) before the chromatographic analysis.

The HPLC system used was comprised of a LaChrom L-7100 pump (Merck-Hitachi, Düsseldorf, Germany), an ion exchange Aminex HPX-87H column (300 × 7.8 mm) (Bio-Rad, Richmond CA, USA) maintained at 60 °C (L-7350 Column Oven; LaChrom, Merck-Hitachi) and two detectors assembled in a series: a refractive index detector (L-7490 RI Detector; LaChrom, Merck-Hitachi) to determine sugars and a UV-Vis detector (L-7400 UV Detector; LaChrom, Merck-Hitachi), acquiring chromatographic data at 220 nm, to analyze organic acids . The mobile phase used was 13 mM sulphuric acid at a flow rate of 0.8 mL/min. The running time was 35 min, and the injection volume was 50 µL. The data was collected and analyzed using D-7000 Interface (LaChrom, Merck-Hitachi) and HPLC System Manager 3.1.1 software (Merck-Hitachi). For each sample of different beverage two samples were analyzed.

6.3.4. In vitro gastrointestinal digestion

An *in vitro* gastrointestinal digestion of okara beverages was performed according Maccaferri et al. (2012) and Mills et al. (2008) with some modification. The lyophilized beverages (6 g) were mixed with 15 mL of distilled water, homogenized and incubated at 37 ºC, α-amylase (2 mg) was mixed with 1 mM $CaCl_2$ (0.6 mL, pH 7.0) and added to the beverage solution, then incubated at 37 ºC for 30 min, under shaking. Afterwards, the pH was adjusted to 2.0 and pepsin (0.27 g) in 0.1 M HCl (0.25 mL) was added, prior to a further incubation cycle, under shaking conditions, at 37 ºC for 2 h. Finally, bile (0.35 g) and pancreatin (0.56 mg) were

mixed with 0.5 M $NaHCO_3$ (12,5 mL) at pH 7.0 and then with beverage sample solution. Samples of each step were stored immediately at −20 °C and then lyophilized for analysis.

6.3.4.1. Soluble protein content and degree of hydrolysis

The total soluble protein content during gastrointestinal digestion was determined by bicinchoninic acid, using albumin as standard. The degree of hydrolysis was determined using the OPA method (Nielsen et al., 2001). Serine was used as a standard.

6.3.4.2. Molecular weight of okara beverage peptides

The molecular weight of protein and peptides present on okara beverage before and during the gastrointestinal system (mouth, stomach and intestine) was determined by gel filtration chromatography using the FPLC (fast protein liquid chromatography-gel filtration) AKTA Pure 25 system coupled with two gel filtration columns: Superdex 200 increase10/300 GL and Superdex peptide, 10/300 GL. The mobile phase was 25 mM phosphate buffer (pH 7.0), 150 mM sodium chloride and 0.2 g L-1 sodium azide. The flow of eluent was 0.5 mL min^{-1} and was monitored by absorbance at 280 nm.

6.3.4.3. Profile of peptides of okara beverage and after gastrointestinal digestion by HPLC

Profile of peptides in okara beverage before and during the gastrointestinal system (mouth, stomach and intestine) was analyzed by a modified method described by Garcia, Torre, Laborba and Marina (1997). Fifty µL of samples were analyzed by HPLC (Waters Series 2695. Mildford, MA. USA) coupled to a diode array detector, using a Kromasil C-18 column (250 x 4.6 mm) to separate the peptides at 50 ºC. The mobile phase consisted of 0.1% trifluoracetic acid in water (v/v) (solvent A) and 0.1% trifluoracetic acid in acetonitrile (v/v) (solvent B). A linear gradient of 5 steps was used: 5-20% B; 20-25% B and 25-25% B for 10 minutes each, followed by a rapid increase of 35-46% B in 30 seconds and a linear reversed gradient 46%-5% B in 30 seconds to re-equilibrate the column to starting condition. The flow rate was 1 mL/min. The proteins were detected by UV absorption at 254 nm. The data were processed on Empower

3 software. The profile of peptides was compared with the different okara beverages throughout the gastrointestinal digestion.

6.3.4.4. Radical scavenging activity determination (ABTS)

The scavenging activity of okara beverages (before and during gastrointestinal digestion and after being centrifuged at 20000 x g for 15 minutes) was measured using the ABTS radical cation method as described by Gião et al. (2007). Briefly, 20 µl of samples were mixed with 1 ml of ABTS (with an initial absorbance, at 734 nm, of 0.700 ± 0.020). After 6 min of reaction the absorbance (734 nm) was measured using a mini UV 1240 spectrophotometer (Shimadzu, Tokyo, Japan). Each sample was analyzed in triplicate and Trolox was used, as a standard, to prepare a calibration curve (0.05 - 0.2 mg/ mL) with the results being expressed in milligrams of Trolox per mL of okara beverage or digestion solution.

6.3.4.5. Total phenolic content determination

The concentration of total phenolic compounds was determined in okara beverage and in samples during gastrointestinal digestion (previously centrifuged at 20000 x g for 15 minutes) by the Folin-Ciocalteu method (Singleton and Rossi, 1965) and the quantification was done at 750 nm (UV mini 1240, Shimadzu, Tokyo, Japan). Total phenolic content in each sample was determined using a standard curve prepared by gallic acid in the range 0.025 - 0.5 mg/ mL. The results were expressed in as milligrams of gallic acid per mL of okara beverage or digestion solution.

6.3.4.6. Determination of ACE-inhibitory activity

The ACE inhibitory activity of okara beverage, before and after gastrointestinal digestion was performed based on the method modified by Quirós, Contreras, Ramos, Amigo and Recio (2009) using the fluorimetric assay. ACE working solution (42 mU/mL) was diluted with 150 mM Tris-HCl (pH 8.3) containing 0.1 mM $ZnCl_2$ and the substrate used was o-Abz-Gly-p-Phe(NO2)-Pro-OH (0.45 mM) dissolved in 150 mM Tris-HCl (pH 8.3) and 1.125 M

NaCl. For reaction development a microplate with ninety-six-well (Porvair, Leatherhead, UK) was used. In each test sample, the assay mixture was composed of the following components: 40 µL of ACE (42 mU/mL), 40 µL of hydrolysate and 160 µL of substrate (o-Abz-Gly-p-Phe(NO2)-Pro-OH, 0.45 mM). Control contained 40 µL of ACE (42 mU/mL), 40 µL of water distilled and 160 µL of substrate. Blank contained 80 µL of water distilled and 160 µL of substrate. The mixture was incubated at 37 °C and the fluorescence generated was measured at 1 h using a FLUOstar OPTIMA plate reader (BMG Labtech, Offenburg, Germany) and the wavelengths used were 350 and 420 nm for excitation and emission, respectively. The software used to process the data was FLUOstar control (version 1.32 R2, BMG Labtech). The activity of each sample was tested in duplicate and the inhibitory activity was expressed as the protein concentration required to inhibit the original ACE activity by 50% (IC50). The percentage of ACE-inhibitory activity was calculated using the formula 100 x $(C - S)/(C - B)$ where C is control, S is sample and B is the blank of sample. IC_{50} values was calculated using the nonlinear fitting to the data. The protein content in hydrolysate extracts was analyzed by bicinchoninic acid, using albumin as standard.

6.3.5. Sensory evaluation

Sensory evaluation of four okara beverages was performed by 60 participants, including students and staff of the university (11 male and 49 female). Samples were served in white cups containing 20 mL of product. No information about the samples was provided to the participants and each sample was identified by a random 3-digit code. Overall liking was evaluated using a nine point hedonic scale (Jones et al., 1955; Peryam and Pilgrim, 1957). 1- "dislike extremely" and 9- "like extremely". The appropriateness of intensity of sensory attributes sweetness and acidity were evaluated using 5-point Just-About-Right (JAR) scales ranging from much too low (1) a much too high (5) sweetness/acidity (Popper, 2014).

6.3.6. Statistical analysis

Statistical analysis of data on the microbial, physical and chemical parameters of the okara beverage analyzed in this work was performed using IBM SPSS STATISTICS version 23 (SPSS

Inc., Chicago, IL, USA). Normality of the data was checked by the Shapiro-Wilk test. Data following a normal distribution were subjected to the Student's t test (antihypertensive activity different stage of digestion) and ANOVA-one way followed by Tukey test (antihypertensive activity in the same stage of digestion, genistein, daidzein, daidzin and total phenolics, protein, hydrolysis degree, Brix) to establish the significance of differences between means, while the non-parametric Kruskal-Wallis and Mann-Whitney's U test (genistin, acidity, lactic acid, citric acid, fructose, pH, acidity, ABTS, sensory analysis) were used otherwise. Statistical significance was set at $P < 0.05$.

6.4. Results and discussion

6.4.1. Chemical and physical evaluation of okara beverage stored

The acidity, total solids soluble and pH values of the different okara beverages during refrigerated storage are shown in Table 6.1. All fermented beverages presented a significant reduction of the pH ($P < 0.05$) throughout the storage time, while the unfermented beverage (OkaNF) maintained its pH over the 28 days of storage. Additionally, the decrease in pH of the different okara beverage can be related with the continuous production of organic acids resulting from fermentation of carbohydrates by probiotic bacteria and can be defined as "post-acidification" (Bedani et al., 2014b). In parallel, an opposite behavior to the pH was observed for the acidity profile (Table 6.1). The fermented beverage presented a significant increase in the acidity and at 28 days of storage the acidity was twice the initial acidity. In addition, the acidity showed significant differences ($P < 0.05$) between the different bacteria used in fermentation. In Table 6.1 is possible to observe that the OkaBb6 showed the highest acidity. However, the acidity was negatively correlated to pH evolution during storage time for all fermented beverages (r= -0.97 (OkaLR6) and r= -0.98 (OkaBb6 and OkaMix6), while for OkaNF beverage the Pearson correlation was not significative.

The total solids soluble (ºBx) in fermented beverage (OkaBb6 and OkaMix6) showed slight difference ($P < 0.05$) over storage. In general, the TSS was maintained during the 28 days of storage, with values between 9.4 to 9.8 ºBx. The sugar metabolism of the lactic acid bacteria and bifidobacteria produce lactic and acetic acids as the major end-products (Delgado et al.,

2018). The amounts of acetic, lactic and citric acid produced by different culture in fermented okara beverage are shown in Table 6.1. All cultures (mixed or alone) produced, as expected, more lactic than acetic acid. However, *B. animalis* Bb12 had the highest acetic acid production (0.65 mg/ mL) comparing to *L. rhamnosus R11* (0.24 mg/mL) and co-culture (0.52 mg/mL). In parallel, the lactic acid concentration also showed significative differences ($P < 0.05$) between the fermented beverages. Corroborating the higher acidity observed (Table 6.1), the OkaBb6 produced higher amount of lactic acid as compared to OkaLR6 (8.59 and 7.58 mg/ mL respectively). Furthermore, the lactic acid concentration also presented a significant increased ($P < 0.05$) during storage time in all fermented beverage and after 28 days of storage, the lactic acid was twice than initial storage time; these values confirm the acidity found using the titratable method (Table 6.1). According to Delgado et al. (2018) these differences among the culture used might come from of metabolic activities such as stress response and proteolytic activities of the strains used in this study. In addition, these results can be related with the fact that the bifidobacteria are nutritionally less fastidious than lactobacilli and lactic and acetic acid are major metabolites (Van Der Meulen et al., 2006). On the other hand, the results obtained in our study can also be explained by the ability of *B. animalis* Bb-12 to ferment FOS. According to previous studies the degradation of inulin or FOS by bifidobacterial increased the acetic acid production at the cost of lactic acid (Van Der Meulen et al., 2006, Van Der Meulen et al. 2004). Similarly, Østlie et al.(2003) also found higher acetic acid concentration in the fermented milk by bifidobacteria comparing to lactobacilli strains. In parallel, as expected in unfermented beverage (OkaNF), the lactic acid and acetic acid was not detected. Moreover, the fructose and citric acid (Table 6.1) showed a similar behavior over 28 days of storage at 4 ºC for all beverages.

Table 6.1. Viable counts of *L. rhamnosus* R11 and *B. animalis* spp *lactisBb-12* (log_{10} CFU. mL^{-1}), pH, total soluble solids (TSS) (º Bx), acidity (% lactic acid), citric acid, lactic acid, acetic acid and fructose in four okara beverage (OkaLR6, Okab6, OkaMix6, OkaNF) during storage period for 28 days at 4 °C.

Time (days)	**OkaLR6**	**OkaBb6**	**OkaMix6**		**OkaNF**
	Viable counts of bacteria log_{10} (CFU/mL)				
	L. rhamnosus R11	*B. animalis* Bb-12	*L. rhamnosus* R11	*B. animalis* Bb-12	NF
0	9.31 ± 0.027 ^{aA}	9.17 ± 0.117 ^{abA}	8.92 ± 0.097 ^{cA}	9.05 ± 0.067 ^{bcA}	ND
7	9.21 ± 0.04 ^{aAB}	9.03 ± 0.07 ^{abAB}	8.80 ± 0.14 ^{cA}	8.86 ± 0.07 ^{bcB}	ND
14	9.06 ± 0.047 ^{aAC}	9.23 ± 0.127 ^{bA}	8.98 ± 0.047 ^{acA}	8.86 ± 0.077 ^{cB}	ND
21	9.16 ± 0.07 ^{aBC}	8.99 ± 0.10 ^{abAB}	8.79 ± 0.15 ^{bcA}	8.73 ± 0.09 ^{cB}	ND
28	8.80 ± 0.02 ^{aD}	8.90 ± 0.15 ^{aB}	8.95 ± 0.17 ^{aA}	8.90 ± 0.10 ^{aAB}	ND
pH					
0	4.55 ± 0.05 ^{aA}	4.48 ± 0.00 ^{aA}	4.53 ± 0.00 ^{aA}		5.27 ± 0.01 ^{aA}
7	4.11 ± 0.02 ^{aB}	4.03 ± 0.00 ^{aB}	4.04 ± 0.00 ^{aB}		5.20 ± 0.00 ^{aA}
14	4.02 ± 0.08 ^{aBC}	3.90 ± 0.02 ^{aC}	3.92 ± 0.00 ^{aC}		5.26 ± 0.06 ^{aA}
21	3.91 ± 0.03 ^{aCD}	3.84 ± 0.02 ^{aD}	3.84 ± 0.01 ^{aD}		5.15 ± 0.01 ^{aA}
28	3.77 ± 0.01 ^{aD}	3.72 ± 0.02 ^{aE}	3.72 ± 0.01 ^{aD}		5.18 ± 0.02 ^{aA}
TSS (º Bx)					
0	9.65 ± 0.06 ^{aA}	9.70 ± 0.23 ^{aA}	9.63 ± 0.05 ^{aA}		9.78 ± 0.10 ^{aA}
7	9.50 ± 0.08 ^{aA}	9.38 ± 0.17 ^{aA}	9.45 ± 0.06 ^{aBC}		9.75 ± 0.17 ^{aA}
14	9.60 ± 0.00 ^{aA}	9.48 ± 0.10 ^{bA}	9.58 ± 0.05 ^{aAB}		9.80 ± 0.08 ^{cA}
21	9.60 ± 0.12 ^{abA}	9.40 ± 0.08 ^{abA}	9.40 ± 0.14 ^{aC}		9.80 ± 0.12 ^{bA}
28	9.58 ± 0.10 ^{aA}	9.63 ± 0.15 ^{aA}	9.55 ± 0.06 ^{aABC}		9.80 ± 0.08 ^{aA}

Acidity (% Lactic acid)				
0	0.62 ± 0.01 ^{aA}	0.72 ± 0.01 ^{bA}	0.68 ± 0.04 ^{cA}	0.32 ± 0.00 ^{dA}
7	0.88 ± 0.03 ^{aB}	0.99 ± 0.02 ^{bB}	0.97 ± 0.01 ^{cB}	0.31 ± 0.01 ^{dA}
14	1.07 ± 0.02 ^{aC}	1.22 ± 0.00 ^{aC}	1.18 ± 0.02 ^{cC}	0.32 ± 0.01 ^{Da}
21	1.13 ± 0.03 ^{aD}	1.26 ± 0.01 ^{aD}	1.24 ± 0.02 ^{cD}	0.31 ± 0.00 ^{dA}
28	1.23 ± 0.01 ^{aE}	1.39 ± 0.02 ^{aE}	1.37 ± 0.02 ^{cE}	0.32 ± 0.00 ^{dA}
Citric acid (mg/ mL)				
0	5.15 ± 0.32 ^{aA}	4.93 ± 0.03 ^{aA}	5.02 ± 0.06 ^{aAB}	4.99 ± 0.07 ^{aA}
7	4.90 ± 0.08 ^{abAB}	5.38 ± 0.38 ^{bcAB}	4.71 ± 0.03 ^{aA}	5.63 ± 0.23 ^{cB}
14	4.54 ± 0.27 ^{aB}	5.07 ± 0.46 ^{abAB}	5.23 ± 0.25 ^{bAB}	4.97 ± 0.24 ^{abA}
21	5.11 ± 0.10 ^{aA}	5.70 ± 0.46 ^{aB}	5.49 ± 0.61 ^{aB}	5.17 ± 0.24 ^{aA}
28	5.13 ± 0.17 ^{abA}	5.08 ± 0.06 ^{aAB}	5.11 ± 0.08 ^{abAB}	5.31 ± 0.09 ^{bAB}
Lactic acid (mg/mL)				
0	7.58 ± 0.64 ^{aA}	8.59 ± 0.25 ^{bA}	8.42 ± 0.17 ^{bA}	ND
7	7.64 ± 0.48 ^{aA}	9.91 ± 2.87 ^{aAB}	8.17 ± 0.36 ^{aA}	ND
14	10.89 ± 0.64 ^{aB}	12.65 ± 1.23 ^{bBC}	13.78 ± 0.15 ^{bB}	ND
21	13.08 ± 0.42 ^{aC}	15.60 ± 1.28 ^{aC}	15.85 ± 0.61 ^{aC}	ND
28	15.48 ± 0.47 ^{aD}	16.14 ± 0.49 ^{aD}	16.18 ± 0.24 ^{aC}	ND
Acetic acid (mg/ mL)				
0	0.24 ± 0.05 ^{aAB}	0.65 ± 0.09 ^{bA}	0.52 ± 0.07 ^{bA}	ND
7	0.21 ± 0.03 ^{aA}	0.67 ± 0.07 ^{bA}	0.54 ± 0.12 ^{cA}	ND
14	0.22 ± 0.02 ^{aAB}	0.63 ± 0.10 ^{bA}	0.50 ± 0.04 ^{cA}	ND
21	0.26 ± 0.04 ^{aAB}	0.72 ± 0.11 ^{bA}	0.48 ± 0.05 ^{cA}	ND
28	0.29 ± 0.03 ^{aB}	0.75 ± 0.08 ^{bA}	0.53 ± 0.04 ^{cA}	ND

Fructose (mg/ mL)				
0	40.91 ± 1.67 [aA]	43.84 ± 0.95 [aA]	40.99 ± 0.84 [aAB]	46.03 ± 0.75 [bA]
7	45.88 ± 2.67 [abB]	45.51 ± 4.24 [abA]	40.61 ± 0.84 [aA]	51.01 ± 1.29 [bB]
14	38.93 ± 3.10 [aA]	40.00 ± 3.85 [abA]	39.35 ± 0.56 [abA]	44.64 ± 1.89 [bA]
21	42.43 ± 1.04 [aAB]	45.17 ± 4.19 [Aa]	43.67 ± 2.34 [aB]	45.31 ± 2.02 [aA]
28	41.66 ± 0.21 [aAB]	40.66 ± 1.79 [Aa]	39.79 ± 0.83 [aA]	46.76 ± 0.97 [bA]

[a] ND- not detected. [b]NF-not fermented. [c] Data indicate the mean ± standard deviation of two independent experiments. [d] Means with different uppercase superscript letters in the same column for each analyze are significantly different ($P < 0.05$) and different lowercase superscript letters in in the same row are significantly different ($P < 0.05$). OkaLR6- Okara beverage fermented by *L. rhamnosus R11;* OkaBb6- Okara beverage fermented by *B. animalis* Bb-12; OkaMix6- Okara beverage fermented by *B. animalis* Bb-12 and *L. rhamnosus R11;* OkaNF- Okara beverage not fermented.

6.4.2. Probiotic counts in okara beverage during storage

Several studies reported that the probiotic food can provide beneficial effects for individuals, such as maintaining composition and good balance of intestinal flora, increase the resistance against invasion of pathogens, when administered in appropriate amounts (Tripathi and Giri, 2014). However, the ideal level of probiotic microorganisms to be administered to lead to beneficial health effects in individual is not easy to be determined, considering that the sufficient dose of probiotic may vary depending on the strain and the product used (Martinez et al., 2015). However, no general agreement has been reached on the recommended levels, and suggested levels of probiotics in a functional product must be between 10^6 and 10^8 cells per milliliter or gram of product of end product of viable cells to get most advantageous therapeutic effects (Donkor et al., 2007; Martinez et al., 2015; Mondragón-bernal et al., 2017). Based on the previous statements all okara fermented beverages in the present study would be considered probiotic during over 28 days of storage. The behavior of *L. rhamnosus* R11 and *B. animalis* Bb-12 viability in the fermented beverage throughout storage is shown in Table 6.1, both populations remained around 9 log CFU/mL between the first and the day 28 of storage in the okara beverage. A small and significative decrease ($P < 0.05$) in *L. rhamnosus* R11 and *B. animalis* Bb-12 populations throughout 28 days of storage was observed in OkaLR6 and OkaBb6 beverages. In addition, for beverage with bacteria (OkaMix6) the *B. animalis* Bb-12 presented a slight decrease ($P < 0.05$) during storage, while *L. rhamnosus* R11 maintained the viable counts during the storage in the OkaMix6, however, the viable count of this bacteria into OkaMix was lower ($P < 0.05$) than other beverages. Even though certain variations in *L. rhamnosus* R11 and *B. animalis* Bb-12 populations were observed between the fermented beverages in each storage period evaluated, these changes are negligible microbiologically, since they are always below 0.5 $\log_{10}$ CFU/mL.

Bacillus cereus, yeasts and molds and *Enterobacteriaceae* were not detected in the beverages analyzed in the present study, indicating that these preparations were microbiologically safe for human consumption.

6.4.3. Antioxidant activity, total phenolics and isoflavones in okara beverages stored

The antioxidant activity and total phenolic compounds present in the okara beverages over 28 days of storage are shown in the Figure 6.2 (a and b). The bacteria *L. rhamnosus* R11 and *B. animalis* Bb-12 showed a similar behavior during okara fermentation with a slight reduction ($P < 0.05$) in total phenolics (ca. 0.020 mg of gallic acid/ mL of beverage) and antioxidant activity (0.007 mg of Trolox/ mL of beverage) compared with the OkaNF (0.024 and 0.009, total phenolics and antioxidant activity, respectively). The reduction of phenolic compounds levels after the fermentation process may contribute to the reduction of antioxidant activity (Moraes Filho et al., 2016). Additionally, the high levels of isoflavone aglycones (daidzein and genistein) caused a high antioxidant activity due to the availability of a greater amount of hydroxyl groups that stabilize the free radicals through of resonance (Lee, 2005; Moraes Filho et al., 2016). However, this correlation was not observed in fermented okara, and this may be related with the concentration of this compounds, that in general was lower compared with the total of glycosides in the OkaNF. In addition, during the storage, the okara beverages maintained the total phenolics with slight changes (Fig. 6.2 a). Moreover, in relation to antioxidant activity, the fermented beverages presented a slight decrease (from 0.07 to 0.05 mg of Trolox/ mL of beverage) over 28 days ($P < 0.05$) (Fig. 6.2 b).

The isoflavone compounds analyzed by HPLC in this work include the isoflavone glycosides daidzin and genistin and their derived aglycones daidzein and genistein (Fig. 2 c, d, e and f). The largest amount of both glycosides, daidzin (0.16 ± 0.01 mg/ g of dried sample) and genistin (0.23 ± 0.01 mg/ g of dried sample) were identified in the OkaNF. Small quantities of both genistein (0.015 ± 0.001) and daidzein (0.0084 ± 0.0005) were also identified in OkaNF.

In recent years, isoflavones have gained interest and the effects of these compounds have been studied in humans. According to Izumi et al. (2000) the isoflavone aglycones are absorbed faster and in greater amounts than their glycosides (daidzin and genistin). This is due to the fact that the bioavailability of glycosides *in vivo* is possible only after hydrolysis of the sugar by intestinal *β-glycosidases* (Chun et al., 2007). In addition, in previous studies He and Chen (2013) reported a lower incidence of breast and prostate cancer in Asian countries, where the traditional diet is rich in soy products.

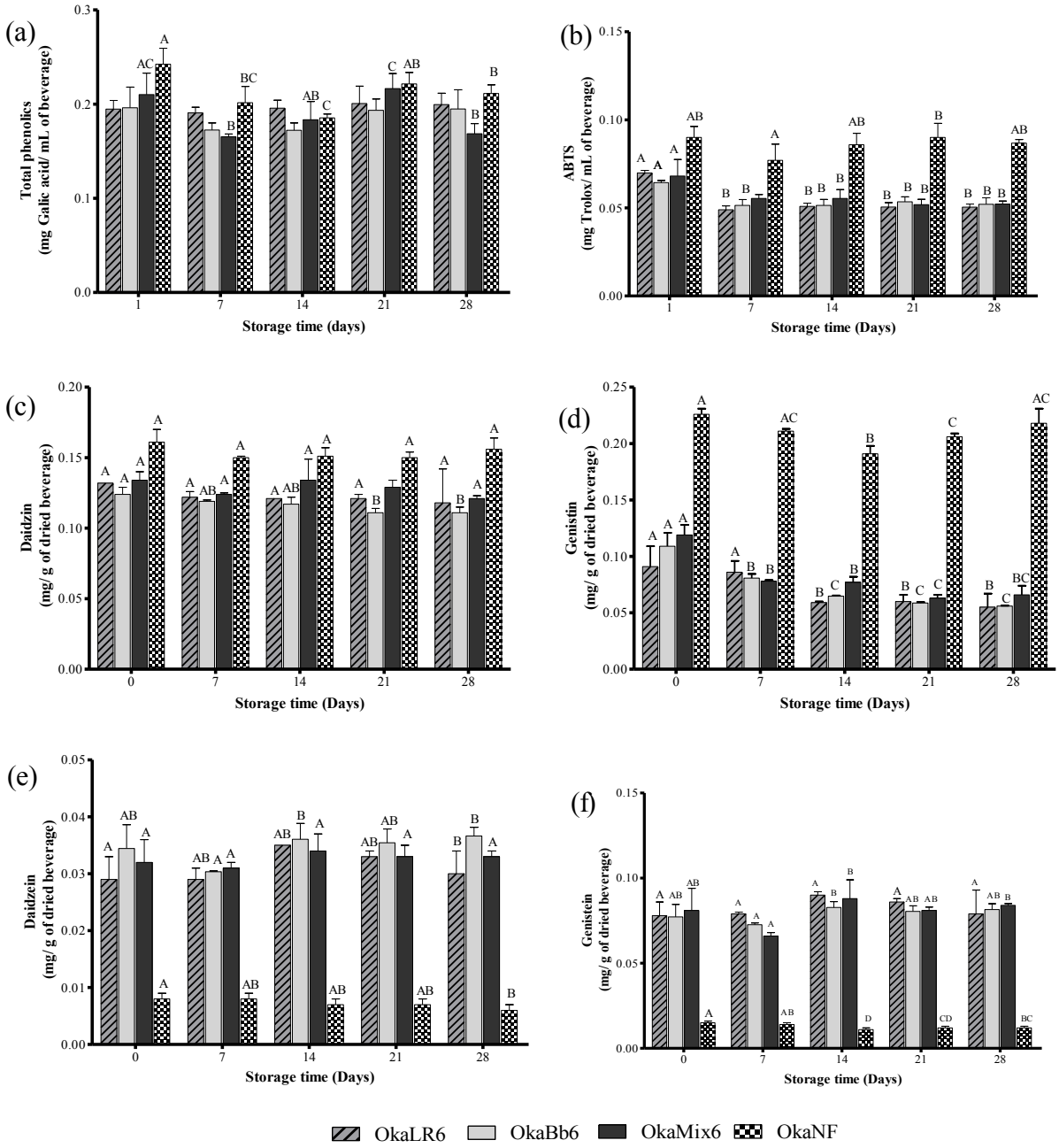


Figure 6.2. Antioxidant activity (ABTS) (a), total phenolics (b) and individual isoflavones: daidzin (c), genistin (d), daidzein (e), genistein (f) in okara beverage. Different letters denote significant differences between storage time in the same beverage ($P < 0.05$). Absence of letters indicates no significant difference.

Thus, many attempts have been performed to improve bioavailability of isoflavone aglycones from isoflavone glycosides in soya beverage fermented by different species of *bifidobacterial* and *lactobacilli* (Chun et al., 2007; Delgado et al., 2018; Wei et al., 2007).

Several studies, have shown an increase of isoflavones aglycones and the growth of dairy bacteria, such as *lactobacilli* and *bifidobacteria* in soya beverage (Chun et al., 2007; Moraes Filho et al., 2016; Wei et al., 2007). However, there isn´t data available using okara without soya beverage addition, and in this work some differences compared to the soya beverage were observed. It is important to highlight that the isoflavones compounds in okara are the isoflavones not extracted during soya beverage production. This fact influences the different composition and modification of isoflavones during the fermentation process in okara. In addition, the soya beverage also can show differences in the isoflavone profile, such as related by Delgado et al. (2018) that found a different profile of isoflavones in two different commercial soya beverages. In our study, the isoflavones glycosides decreased in all fermented beverages compared to OkaNF. On the other hand, the isoflavones aglycones increased in all fermented beverages (Figure 6.2 e and f), such as related in previous studies about fermentation in soya beverage (Delgado et al., 2018; Wei et al., 2007).

In general, aglycones and glycosides isoflavone analyzed in beverages showed significant differences ($P < 0.05$) between OkaNF and fermented beverages. The use of probiotic microorganisms in okara beverage was beneficial to alter the biological isoflavone aglycone, that consequently can improve the functional benefits for the consumers. Thus, the fermented beverage showed a higher amount of aglycones, the daidzein and genistein increased ca. 3 and 8 times in all fermented beverages compared to the OkaNF (Fig. 6.2 e and f), while the glycosides were lower than that the OkaNF (Fig. 6.2 c and d). The values observed for the content of isoflavones glycosides and aglycones were maintained with slight differences ($P <$ 005) during the 28 days of storage.

6.4.4. In vitro gastrointestinal digestion of okara beverages

6.4.4.1. Soluble protein and degree of hydrolysis in okara beverages during gastrointestinal tract

The *in vitro* gastrointestinal digestion was divided into three phases, namely: mouth, gastric digestion, intestine digestion. During gastric phase, all beverages had higher soluble protein content than other stages of digestion (Fig. 6.3a). After gastric phase, the amount of soluble protein increased to 11.45-13.68 (mg of the soluble protein/mL of beverage) at the end

of the gastric digestion phase. The OkaLR6 and OkaBb6 had similar soluble protein content (11.98 and 11.45 mg of soluble protein/mL of beverage, respectively) and were significantly lower than that of OkaMix6 (13.68 mg of soluble protein/ mL of beverage). In addition, the *in vitro* gastrointestinal digestion has not yet been studied in okara. However, these results showed a similar behavior to that obtained in soymilk curd in a previous study of (Rui et al., 2016). On the other hand, these authors found a decrease in the soluble protein of the soymilk digested. In addition, in the intestine digestion, the beverages presented a significant decrease in the soluble protein (ca. 6 mg of soluble protein/mL of beverage). These values were similar to those found in initial time of the beverages digestion. On the other hand, Rinaldi et al. (2015) found an increase in the soluble protein in yogurts during the gastrointestinal tract.

Moreover, the degree of hydrolysis (DH) obtained during gastrointestinal digestion is presented in Figure 6.3b. All beverages showed a steadily increase in DH through the digestion process, from 4-8 % (T0) to 66-76 % (intestine digestion), indicating effective action of digestive enzymes in the okara proteins. In addition, during the intestine digestion, the DH has increased sharply, showing slight differences ($P < 0.05$) between beverages analyzed. Nevertheless, the values of DH found in the intestine were greater to previous studies in soymilk or soymilk curd (Rui et al., 2016), in addition, the higher values of DH at each phase of digestion, found in beverages demonstrated that okara has a greater susceptibility to proteolytic enzymes than soymilk and soymilk. However, the DH of okara beverages was similar to found by Rinaldi et al. (2015) in different yogurt formulation digested. Still according Rinaldi et al. (2015) those results suggest, that during a intestine stage, the fast hydrolysis, promoted a high increase in the peptides with low molecular weight. This behavior can also be observed in the Fig. 6.4.

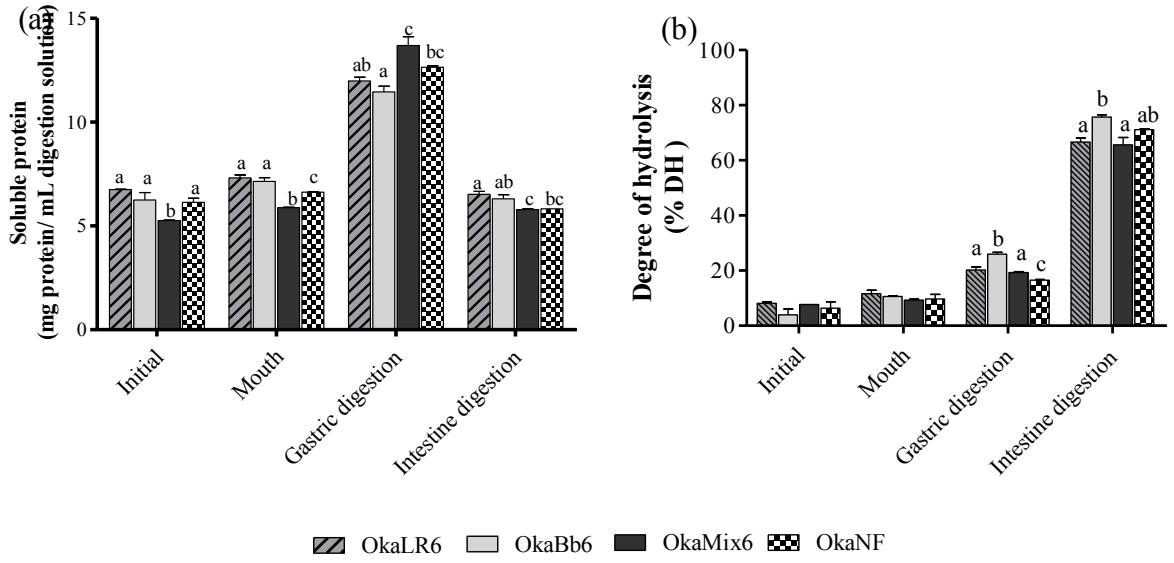


Figure 6.3. Soluble protein (a) and degree of hydrolysis (b) during the gastrointestinal system (beverage initial, mouth, gastric digestion and intestinal digestion) for okara beverage. Values are the average of two independent experiments. Error bars indicate the standard deviation. Means with different letters indicate significant difference between beverages for the same stage of the gastrointestinal tract (initial time, mouth, gastric digestion and intestine digestion) ($P < 0.05$). Absence of letters indicates no significant difference

6.4.4.2. Molecular weight profiles of okara beverages in FPLC

The molecular weight distribution of the okara beverages (OkaLR6, OkaBb6, OkaMix6 and OkaNF) were monitored by FPLC as shown in Figure 6.4 (a, b, c and d). In general, all beverages exhibited similar profile with low molecular weight (MW) fractions (< 3 kDa). One relevant peak with MW lower than 1 kDa, was observed for all beverages at 47 mL of elution.

The OkaLR6 and OkaNF showed one peak at 49 mL of elution, while in the OkaBb6 and OkaMix6, this peak disappeared, probably due the different metabolism of bifidobacteria. However, after the gastrointestinal tract (Fig. 6.4 e, f, g and h) the mean peak for all beverages (48 mL of elution) disappeared while the peak between 50-55 mL of elution increased. These changes in the profile, can be related whit the antihypertensive activity that increased after gastrointestinal tract (Table 6.2).

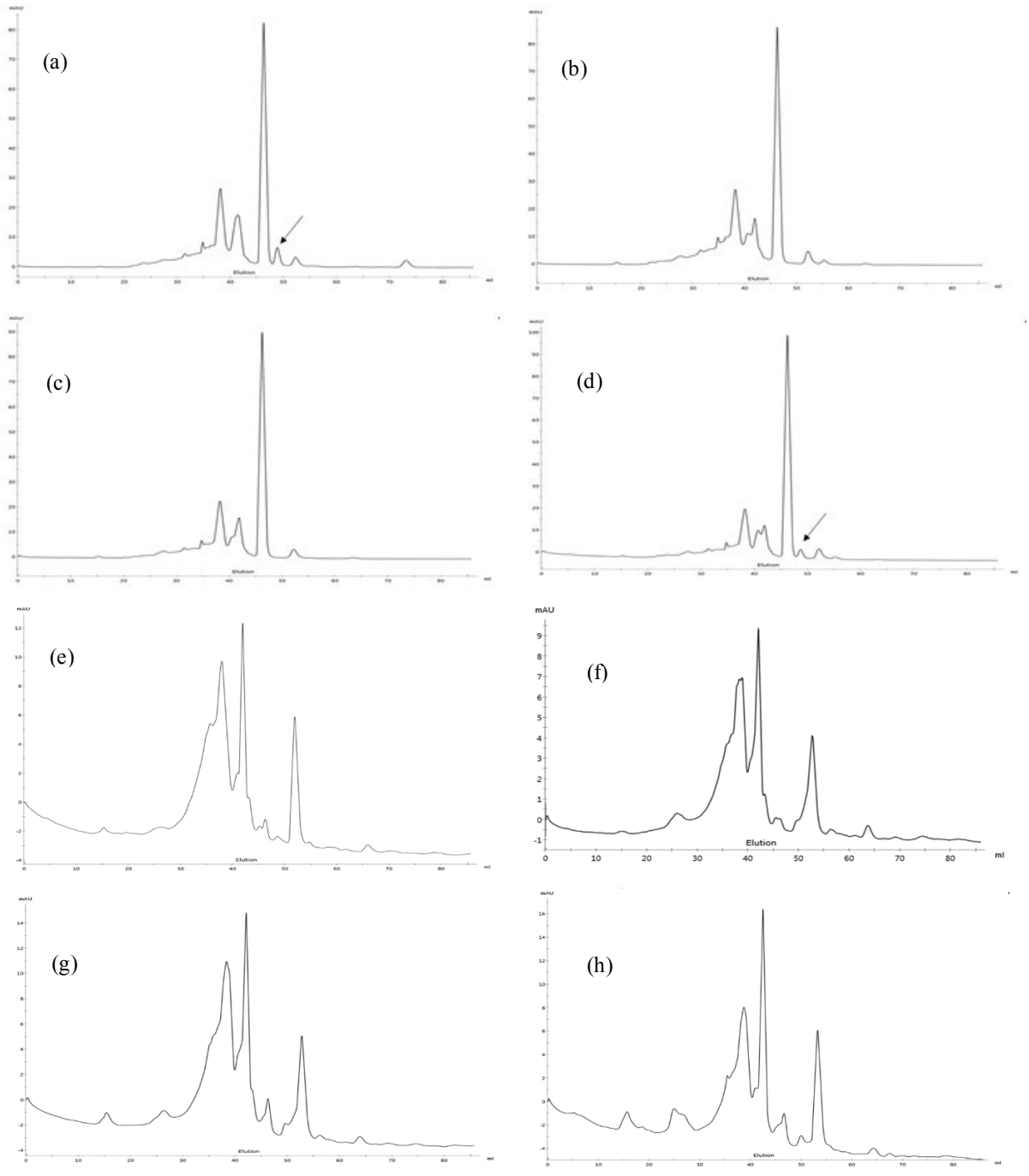


Figure 6.4. Typical chromatogram of protein profile in FPLC of beverage: (a) OkaNF, (b) OkaMix6, (c) OkaBb6, (d) OkaLR6 and beverage after intestine digestion: (e) OkaNF, (f) OkaMix6, (g) OkaBb6 and (h) OkaLR6.

6.4.4.3. Peptide profile in okara beverages in HPLC

The okara beverages, before and during the gastrointestinal digestion were analyzed by RP- HPLC that allows us a preliminary study of the peptide composition. Considering, that previous studies have evidenced many physiologically effects in bioactive peptides from food protein (Saleh et al., 2016). The chromatograms obtained from beverages (Figure 6.5) showed differences between the profile of initial beverage and during the tract gastrointestinal. Besides, it can be observed a major elution of molecules between 0 and 16 minutes (Fig. 6.5 b) during the mouth step, indicating a strong presence of the more polar peptides.

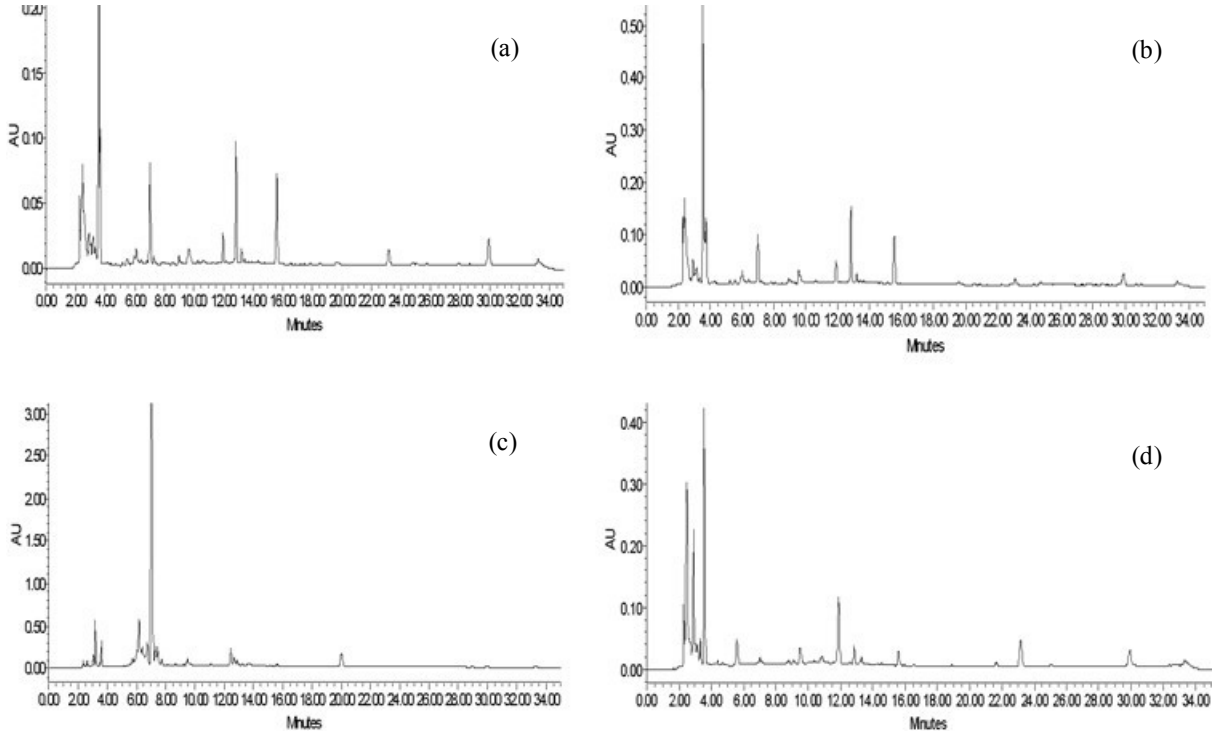


Figure 6.5. Typical Chromatogram in HPLC of the peptides in beverage gastrointestinal digestion of beverage OkaLR6 (a) Initial, (b) Mouth, (c) Gastric digestion and (d) Intestine digestion.

In addition, for gastric digestion, it's possible to observe the same, however the peaks between 6 and 8 minutes showed a concentration ca. 15-times higher than in the beverage sample at the start of digestion or mouth phase. Thus, this suggests that okara has a major hydrophilic peptide. However, for intestine digestion as shown Figure 6.5d is possible to observe a peak at 7 minutes, generated in gastric digestion disappear, on the other hand, two

peaks appear after 16 minutes, thus hydrophobic peptides can be generated during the tract gastrointestinal. The hydrophobic characteristics in peptides are generally associated with potential biological activities, namely antihypertensive (Escudero et al., 2012; Wang et al., 2013) and antioxidant (Ambigaipalan and Shahidi, 2017; Escudero et al., 2012). This can also be observed in this study, where intestine digestion led to a higher antihypertensive and antioxidant activity (Table 6.2 and Figure 6.6, respectively) than okara beverage before digestion.

6.4.4.4. Antihypertensive activity in okara beverages

The potential use of peptides from food to inhibit angiotensin-converting enzyme (ACE) has been suggested in several studies. Moreover, previous studies also associated the positive health effects with the ACE-inhibitory present in soya fermented and hydrolyzed (Coscueta et al., 2016; Wang et al., 2013). However, the antihypertensive activity from okara, previously hydrolyzed and fermented has not studied previously. In Table 2 the IC_{50} for all beverages analyzed in this study before and after the gastrointestinal tract are shown; the lower the IC_{50} the higher the potency of the ACE inhibitory activity. The OkaNF presented a higher ($P < 0.05$) inhibition activity than that the fermented beverages. In addition, is possible to observe in Table 6.2, a significant decrease ($P < 0.05$) in ACE activity potency in the fermented beverages (IC_{50} ca. 750 μg mL^{-1}) compared to the nonfermented beverage OkaNF (442.50 ± 21.02 μg mL^{-1}). Recently, some studies have used lactic bacteria to produce fermented milk or soymilk with antihypertensive activity. Moreover, IC_{50} values found for fermented okara beverages shown in this study were lower than those reported by Martinez-Villaluenga et al. (2012) in soy milk fermented with different Lactobacilli strains (24 to 79 mg mL^{-1}). Nevertheless, Muguerza et al. (2006) found lower IC_{50} in milk fermented using different *L. rhamnosus* (175 to 609 μg mL^{-1}). Thus, our results are promising and present for the first time the application of *L. rhamnosus* R11, *B. animalis* Bb-12 and combination of both cultures to produce fermented beverage from okara with antihypertensive activity.

Table 6.2. Antihypertensive activity (IC_{50}) in okara beverage non-digested and after intestinal digest.

Okara beverage	Non-digested	Intestine digestion
	IC_{50} (μg protein/mL)	
OkaLR6	750.83 ± 27.37^{Aa}	183.04 ± 6.31^{Ba}
OkaBb6	752.21 ± 6.10^{Aa}	149.83 ± 22.36^{Ba}
OkaMix6	752.37 ± 7.82^{Aa}	136.75 ± 12.85^{Ba}
OkaNF	442.50 ± 21.02^{Ab}	273.54 ± 13.17^{Bb}

[a]Data indicate the mean ± standard deviation (n=3). [b]OkaLR6- Okara beverage fermented by *L. rhamnosus R11;* Okab62- Okara beverage fermented by *B. animalis* Bb-12; OkaMix6- Okara beverage fermented by *B. animalis* Bb-12 and *L. rhamnosus R11;* OkaNF- Okara beverage not fermented. [c]Different upper letters in same line are significantly different and different small letters in same column are significantly different ($P < 0.05$).

Furthermore, after gastrointestinal digestion all beverages showed a significant increase in the antihypertensive activity (lower IC_{50}). In Table 6.2 is possible to observe that in all fermented beverages (136.75 to 183.04 μg mL^{-1}) the antihypertensive activity was higher than to OkaNF (273.54 μg mL^{-1}). Thus, the peptides generated after fermentation were more instable and easily hydrolyzed. These results suggest that physiological digestion may promote the formation of active peptides from the proteins as during the digestion the high hydrolysis degree and peptides with lower molecular weight are released, and these are responsible for the antihypertensive activity. Furthermore, the results obtained after gastrointestinal digestion indicate that the antihypertensive activity found in our okara beverages, could be efficiently absorbed through the intestine (Hernandez-Ledesma et al., 2004).

6.4.4.5. Antioxidant activity and total phenolics of okara beverage during gastrointestinal tract

The effect of *in vitro* gastrointestinal digestion in the total phenolics and antioxidant activity from okara beverage is presented in Figure 6.6. For all stage of gastrointestinal digestion was performed one control (using all reagents of digestion without sample) and this control was discount in each digestion stage. As can be seen in Figure 6.6a, the gastric digestion increased the total phenolics compared to non-digested beverages. These results suggest that the gastric

digestion improves the release of the phenolic compounds. On the other hand, during the intestine digestion the total phenolics showed a slight decrease compared to the gastric digestion. The intestine digestion step also showed higher amount of total phenolics than the non-digested beverage, allowing state that the phenolic compounds that make up these beverages are resistant to gastrointestinal digestion conditions. In addition, the behavior of all beverages analyzed during gastrointestinal tract was contrary to previous studies of digestion in soya beverage (Rodríguez-Roque et al., 2013).

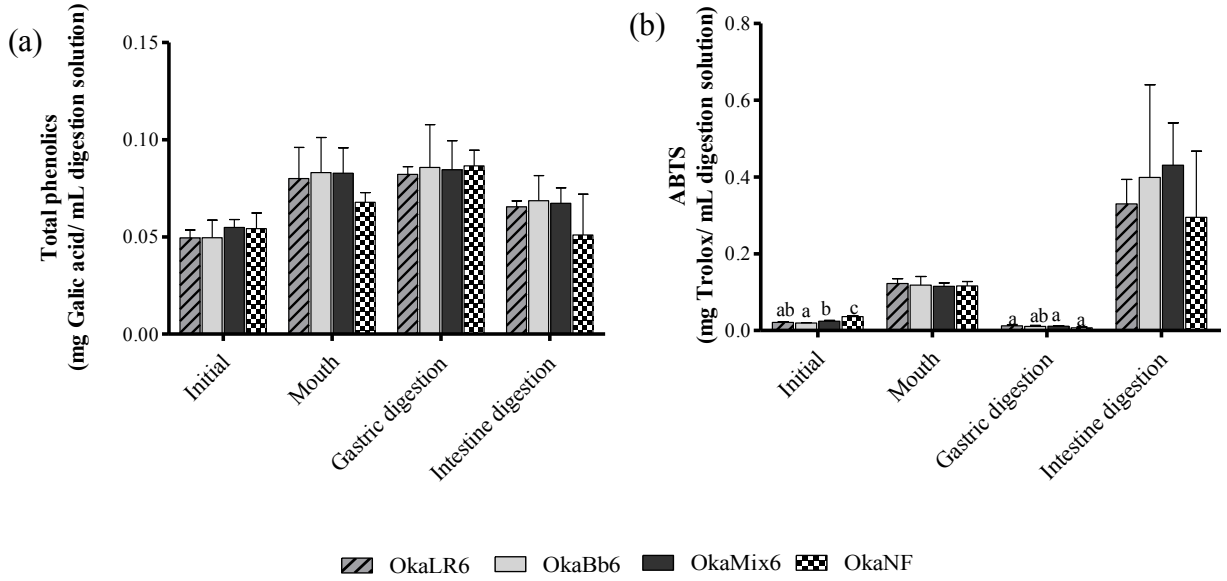


Figure 6.6. Antioxidant activity (a) and total phenolics (b) during the gastrointestinal system (beverage initial, mouth, gastric digestion and intestinal digestion) for okara beverage. Values are the average of two independent experiments. Error bars indicate the standard deviation. Means with different letters indicate significant difference between beverages for the same stage of the gastrointestinal tract (initial time, mouth, gastric digestion and intestine digestion) ($P < 0.05$). Absence of letters indicates no significant difference.

The antioxidant activity of non-digested beverages showed ca. 0.03 mg Trolox. mL^{-1} of digestion solution (Fig. 6.6b) and during the gastric digestion was observed a decrease of the antioxidant activity for all beverages. Nevertheless, in the intestine digestion stage, the antioxidant activity increased from 27 to 40-times compared to the gastric digestion. The results obtained are also contradictory with reported by Rodríguez-Roque et al. (2013) in soya beverage, that found a highest antioxidant activity during gastric digestion, while for intestine digestion the antioxidant activity was ca. 2.3-times lower than to gastric digestion and 1.3 times

lower than non-digested soymilk. However, in a previous study, Wootton-Beard et al. (2011) found an increase in antioxidant activity (ABTS) after gastric and intestinal digestion using 23 commercially vegetable juices. Furthermore, according to Rodríguez-Roque et al. (2013) the decrease of the antioxidant activity during intestine step for some food, may to be due the fact that some compounds, such as phenolic are transformed into different structural forms with different chemical properties.

6.4.5. Sensory evaluation

The mean overall liking ratings obtained for all okara beverages evaluated are shown in Figure 6.7 a. Mean rates ranged between 2.8 (Dislike moderately) and 4.6 (Dislike slightly/ Neither like nor dislike). The unfermented okara beverage (OkaNF) presented a significantly higher mean rate than the fermented ones (OkaLR6, OkaBb6 and OkaMix6) ($P < 0.05$), which did not differ in liking among each other.

The percentage of JAR ratings corresponding to much too low and too low intensities were compiled in a too low category whereas too high and much too high ratings were compiled in the too high category for each attribute. The percentage of too low, just-about-right and too high ratings for sweetness and acidity are shown in Fig. 6.7 b. OkaNF was judged to be too low in sweetness and too high in acidity by a much lower percentage of participants ($P < 0.05$) than the fermented beverages. In fact, 58-77% of the participants considered the acidity of fermented okara beverages (OkaLR6, OkaBb6 and OkaMix6) too high and 63-78% considered their sweetness too low. These evaluations can contribute to explain the lower overall liking ratings observed for the fermented beverages when compared to the unfermented okara.

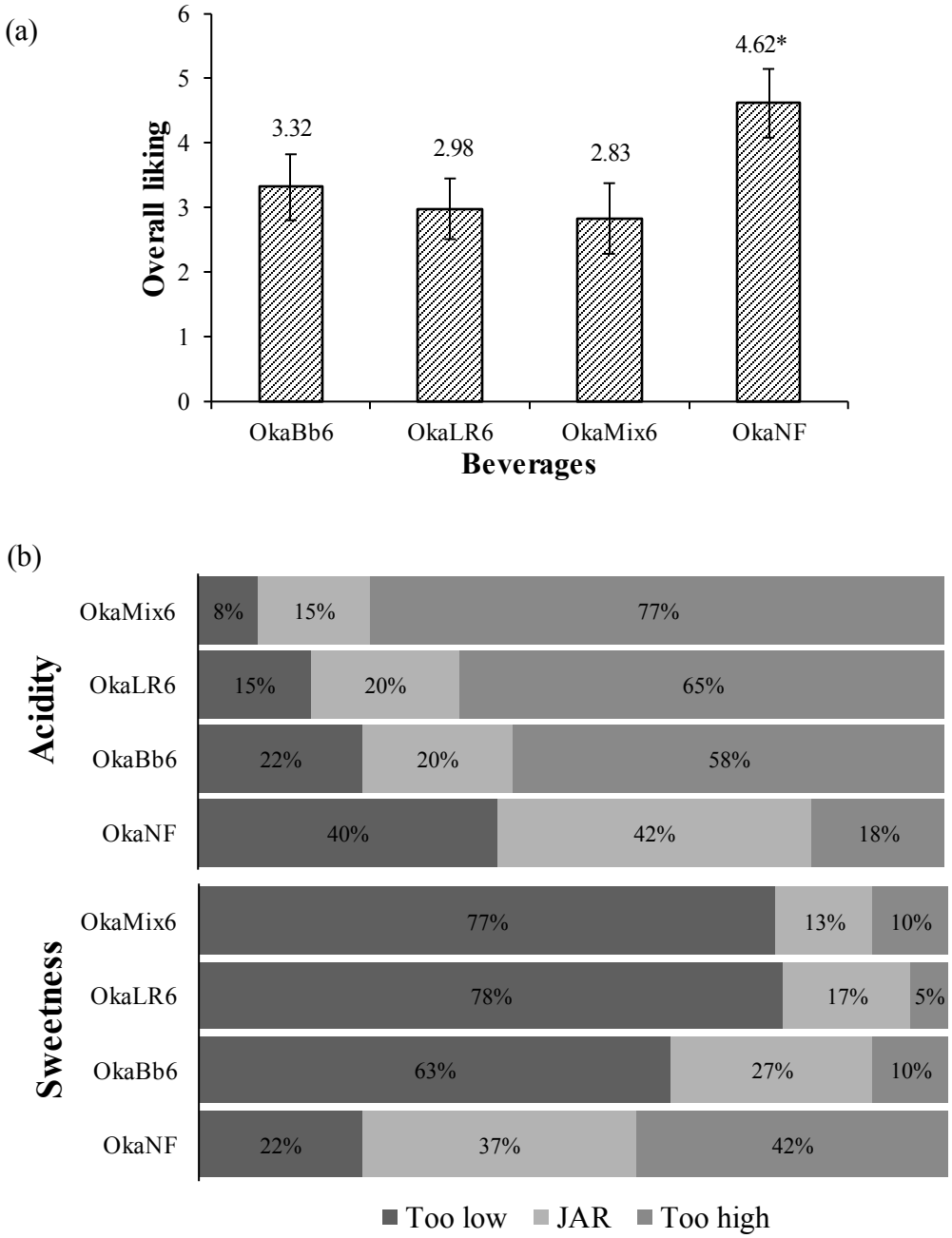


Figure 6.7. Sensory analysis of overall liking (a) and Just-About-Right (JAR) ratings for sweetness and acidity (b) of okara beverages (OkaLR6, OkaBb6 and OkaMix6). Values of acceptability was presented as mean ± confidence interval (n=60). Asterisks indicate significant differences between beverages ($P < 0.05$).

The generally low overall liking ratings obtained for okara beverages can also be due to the participants lack of consumption habits of soy products, and in particular fermented soy products, as previously found by Bedani et al. (2014b). According to Drake and Gerard (2003), the information about the health benefits of soy protein has led, nevertheless to an increase in

the acceptability of yogurts fortified with soy protein, in recent years. In another study Bedani et al. (2014b) evaluated the overall liking of synbiotic soy yoghurts with 5% of okara during 21 days of storage and found mean rates (from 5.3 to 4.4) similar to those obtained for the OkaNF beverage (4.6). Additionally, these authors did not observe a significant increase on the overall liking with the addition of 12.5% of mango or guava pulp to synbiotic soya yoghurts. On the other hand, Kumar and Mishra (2003) found a correlation between the increase in the overall linking of mango soy fortified yoghurt and the proportion of mango pulp incorporated. According to the authors, the fruit pulp can mask the beany flavor of soya beverages.

In future work, the incorporation of fruit pulp to okara beverages could be studied to decrease the beany taste, to increase the ratio of sweetness to acidity of the products and to expectedly increase the okara beverages acceptability.

6.5. Conclusion

In conclusion, our results showed that okara beverages fermented maintained the viable cells through storage. Thus, the okara fermented can be a good alternative to milk derivatives which contain lactose and thus unsuitable for those with lactose intolerance. In this study was also possible to show that okara hydrolyzed beverages presented antihypertensive activity. In addition, during *in vitro* gastrointestinal digestion, the antioxidant and antihypertensive activities increased, while the total phenolic compounds were maintained for all okara beverages. Moreover, extensive research has centered on the process for increase the antihypertensive activity in soy or milk, but few have been done so on soy waste. To our knowledge, this study was the first to report on the use of probiotic in okara fermentation and obtain the beverage with an *in vitro* antihypertensive effect. This suggest that okara beverages might be used as potential functional food, and simultaneously reducing accumulation of agricultural waste. However, based on the sensory results, some modifications are required to improve acceptability, whereas the rates of overall liking obtained for the okara beverages is also related to lack of habit in relation to the consume of soy products. For a future work, incorporating fruit pulp may be the alternative to a better overall liking of the beverages.

Part V- Valorisation of okara for Aquaculture: Incorporation in diets for Nile tilapia

CHAPTER 7- Apparent digestibility coefficients and growth of Nile tilapia (*O. niloticus*) fed differently processed by-product from soy beverage (okara)

7.1. Abstract

The apparent digestibility coefficients (ADCs) of differently processed okara meals were assessed in Nile tilapia (*Oreochromis niloticus*) to further evaluate the potential use of the most digestible(s) okara meal(s) as an alternative protein sources in juveniles. A commercial based diet was used as reference and each experimental diet was obtained by replacing 30% of the reference diet with one of the following test ingredients: dried okara not autoclaved (NAOK), dried okara autoclaved and (AOK), okara hydrolyzed with Alcalase (ALOK), okara hydrolyzed with *Cynara cardunculus* protease (CYOK), okara previously hydrolyzed and fermented with lactic bacteria: *Lactobacillus rhamnosus* R11(CYR11OK) or *Bifidobacterium animalis* ssp. lactis Bb12 (CYB12OK). The results showed that the different treatments applied to okara significantly affected nutrient digestibility. The dry matter ADC of the test ingredients was highest in CYR11OK (80%) and lowest in NAOK (40%). The highest protein digestibility values were obtained in AOK (97%), CYOK (91%) followed by CYB12OK, NAOK and ALOK, showed intermediate values for protein ADC (84, 82 and 81%, respectively); the lowest protein digestibility was observed in CYR11OK (72%). The ingredients with the best digestibility (AOK and CYOK) were used in the aquafeed of Nile tilapia, in a nutrition and growth trial. The inclusion of up to 20% okara meal did not significantly affect Nile Tilapia growth performance, nutrient utilisation or whole body composition in relation to a reference diet. Besides that, the energy and nutrient gain remained similar among dietary treatments. Altogether results suggest that okara meal (hydrolyzed or autoclaved) is a valid ingredient to be used in feed formulations for Nile tilapia. The highest protein digestibility was observed in AOK followed by CYOK, clearly demonstrating the effectiveness of the autoclave and the use of a protease form Cynara without fermentation to increase okara nutrient bioavailability. Okara is an ingredient obtained from local food industries that could help decrease the importation of feedstuffs and consequently decrease the carbon footprint in the aquafeed sector.

7.2. Introduction

The soybean is an important oilseed consumed worldwide, especially in Asian countries (Nishinari et al., 2014). The world harvest of this oilseed in 2016/2017 was of 351.25 million tons (USDA, 2017). In general, soybean has a great commercial interest especially due to its derived products namely soy oil, soy proteins, soy beverage and derivatives. In the last years, the aquaculture research community has been looking for suitable sustainable ingredients able to replace fish meal in aquafeeds assuring fish nutrition and growth (El-Sayed, 1999; Hardy, 2010; Patterson and Gatlin, 2013). Among plant protein ingredients, soybean meal has high nutritional value (Dajanta et al., 2011) and is the most widely feedstuff used by the aquaculture industry. But, soybean meal contains a range of antinutritional substances, such as phytic acid, protease inhibitors and saponins (Francis et al., 2001; Yuan et al., 2013) that often limits its use in large amount. The high inclusion of soybean in fish diets can result in the limitation of certain essential amino acids, reduce nutrient digestibility and diet palatability (Hassaan et al., 2015) and be associated with fish intestinal abnormalities (Yamamoto et al., 2010). On the other hand, the growing use of soy in aquafeeds not only directly competes with human nutrition, but also plays an increasing role in promoting agricultural expansion and LUC (land-use change) in the tropics (Henders et al., 2015).

The consumption of soy products increases worldwide either whole or as an ingredient in soy beverage and tofu (Belén et al., 2012). The food industry generates large amounts of by-products which are often discarded, but that can still be valuable sources of nutrients and natural antioxidants for animal nutrition (Harthan and Cherney, 2017; Jankowiak et al., 2014; O'Toole, 1999; Van der Riet et al., 1989). Okara is a soybean by-product that remains after filtering the water soluble fraction during the production of soya beverage and tofu (O'Toole, 1999). A large amount of okara is annually produced, considering that 1.1 kg of fresh okara is produced from every 1 kg of soybean processed into soymilk/tofu (Khare et al., 1995). In Asian countries, the amount of okara generated reaches 800,000 tons in Japan, 310,000 tons in Korea and 2,800,000 tons in China (Vong and Liu, 2016). This by-product has high moisture content (70–80%) that makes it susceptible to spoilage, being often discarded, but it still has high protein (~30%), lipid (10-20%) and fiber (20-50%) levels (O´Toole, 1999; Van der Riet et al., 1989). Consequently, okara was recently considered a nutritive ingredient for incorporation into diets for an herbivorous

fish like Nile tilapia, *Oreochromis niloticus* (El-Saidy, 2011). Moreover, and contrarily to soybean meal that has high level of antinutritional factors (Floreto et al., 2000; Francis et al., 2001), the production of soya beverage includes a thermal process that reduces most of the antinutritional factors present in soybeans (Stanojevic et al., 2013). Previous studies demonstrated that autoclaving process can inhibit the antinutritional factors in soybean (Hossain and Becker, 2001), whereas lactic fermentation not only reduced soybeans antinutrient factors and indigestible carbohydrates but also improved the digestibility of lipids in Atlantic salmon (Refstie et al., 2005). Moreover, the fermentation of soybean meal by *Lactobacillus* was shown to enhance fish performance and nutrient digestibility; in addition the fermented soybean also reduced the pathomorphological changes in the distal intestine in turbot (Wang et al., 2016). Likewise, some processing techniques, like hydrolysis, fermentation and thermal treatment, can further enhance the nutrient bioavailability of okara (Sbroggio et al., 2016) by improving digestibility and assimilation of nutrients by fish (Zheng et al., 2012). On the other hand, protein hydrolysates have gained prominence in recent years due to the antihypertensive, antioxidant and antidiabetic activity of bioactive peptides (Coscueta et al., 2016; Gibbs et al., 2004; Singh et al., 2014).

Nile tilapia is the most common finfish species farmed worldwide (FAO, 2017). Tilapia is an omnivorous fish with a dietary protein requirement estimated between 26 and 40% (as fed basis), depending on fish size (NCR, 2011). Although fishmeal is the best protein source for tilapia and other fish due to its equilibrated amino acid profile, alternative protein sources have been widely considered (El-Sayed 1999). Previous studies have evaluated the inclusion of 25 to 100 % of soybean meal as fish meal replacement in diets for Nile tilapia (Goda, 2007; Hassaan et al., 2015). According to Hassaan et al. (2015) the fish meal protein could be replaced (up to 37%) by SBM fermented in Nile tilapia diets without any adverse effect on growth performance, nutrient digestibility and physiological condition. On the other hand, previous studies reported a lower growth with increasing SBM levels to replace fish meal protein in Nile tilapia diets (Goda, 2007; Shiau et al., 1987). Moreover, the use of soybean by-products is gaining attention due to its large production worldwide and associated environmental problems (Nguyen et al., 2013). Okara could be a good nutrient source for Nile Tilapia diets, taking into account its protein and lipid composition, namely its high PUFA content (Voss et al., 2018). Nevertheless, this by-product is highly perishable, due to its high moisture content (70-80%) and rich nutritional composition that are determinant factors for rapid degradation by chemical reactions

and action of microorganisms. Previous studies focused on okara processing and stabilization to generate a stable high quality flour (Voss et al., 2018), but the nutritional value of such feedstuffs for aquafeeds remains to be evaluated.

The present study has a dual objective: 1) to assess the *in vivo* apparent digestibility coefficients (ADCs) of differently processed okara meals in Nile tilapia (*Oreochromis niloticus*); and 2) to further evaluate the potential use of the most digestible okara meals as an alternative protein sources in tilapia juveniles. The okara was used after appropriate technological processing or application of bioprocess, such enzymatic hydrolysis and fermentation with lactic bacteria. The effects of increasing levels (10 and 20%) of okara meals with best ADC values on growth performance and nutrient utilization was evaluated, after 10 weeks of feeding the experimental diets to Nile tilapia juveniles.

7.3. Materials and methods

The present study was performed by accredited scientists in laboratory animal science by the Portuguese Veterinary Authority (1005/92, DGV-Portugal, following FELASA category C recommendations) and conducted according to the Directive 2010/63/EU of the European Parliament and of the Council on the protection of animals for scientific purposes.

7.3.1. Okara processing and experimental diets

The okara generated by soy beverage producer (NUTRE Industry, Aveiro, Portugal) was used as test ingredient for Nile tilapia diets after appropriate technological processing or application of bioprocess, such enzymatic hydrolysis and acid lactic fermentation, as shown in Table 7.1.

The fresh okara was divided in two lots: one was immediately dried at 65 ºC until constant weight (OK), whereas the other was firstly autoclaved, then dried under the same condition (AOK). The fresh okara was further hydrolyzed using two enzymes - Alcalase 2.4 L FG (Novozymes, Bagsvaerd, Denmark)- ALOK or proteases from *Cynara cardunculus* (Formulab, Maia, Portugal)- CYOK. The enzymatic hydrolysis with Alcalase was performed in phosphate buffer (0.025 M), pH 8.3, E/S ratio of 0.7 (v/w) in an orbital shaker at 50 ºC and 5 h.

Table 7.1. Process applied in by-product from soy beverage production process (okara) for obtain of okara meal used as test ingredients.

Ingredient	Processing method
Dried okara (NAOK)	Fresh Okara dried until constant weight (65 ºC for 68 hours)
Autoclaved and dried okara (AOK)	Autoclaved okara (1 atm, 121 ºC for 20 min) followed by drying until constant weight (65 ºC for 68 hours)
Hydrolyzed okara using Alcalase (ALOK)	Okara hydrolyzed with Alcalase and autoclaved, followed by drying until constant weight (65 ºC for 68 hours)
Hydrolyzed okara using protease from *Cynara cardunculus* (CYOK)	Okara hydrolyzed with protease from *Cynara cardunculus* and autoclaved followed by drying until constant weight (65 ºC for 68 hours)
Hydrolyzed okara using protease from *Cynara cardunculus* and fermented with R11[a] (CYR11OK)	Okara hydrolyzed with protease from *Cynara cardunculus*, autoclaved, added fructose and fermented with *Lactobacillus rhamnosus* R11 followed by drying until constant weight (65 ºC for 68 hours)
Hydrolyzed okara using protease from *Cynara cardunculus* and fermented with Bb12 [b] (CYB12OK)	Okara hydrolyzed with protease from *Cynara cardunculus*, autoclaved, added fructose and fermented with *Bifidobacterium animalis* ssp. *lactis* Bb12 followed by drying until constant weight (65 ºC for 68 hours)

[a] *Lactobacillus rhamnosus* R11; [b] *Bifidobacterium animalis* ssp. *lactis* Bb12.

The enzymatic hydrolysis with proteases from *Cynara cardunculus* was performed in citric acid – sodium citrate buffer, 0.1 M and pH 5.2, E/S ratio of 1.1 (v/w at 55 ºC and 2.5 h.

All hydrolysates were autoclaved after finish of enzymatic reaction for stopping the hydrolysis and for elimination of antinutritional factors. The okara hydrolyzed with *C. cardunculus* was further added fructose (3 % w/v) and fermented. The fermentation was performed using two lactic bacteria: *Lactobacillus rhamnosus* R11 from Lallemand (Montreal, Canada) (CYR11OK) and *Bifidobacterium animalis* ssp. *lactis* Bb-12 from Christian Hansen (Hørsholm, Denmark) (CYB12OK), both fermentation was conducted at 37 ºC and 120 rpm, resulting in pH 4.5. After drying, all samples were ground in a mill (Retsch, Haan, Germany) with 1 mm sieve. The proximate composition of each test ingredient is presented in Table 7.2.

For the digestibility trial, a commercial-based diet for Nile tilapia was formulated and extruded by SPAROS, Portugal, and used as basal mixture. To this mixture, 1% chromic oxide (Cr_2O_3, Merck KGaA, Germany) was added as an inert marker for the evaluation of the apparent digestibility coefficient (ADC). The reference diet (REF, Table 7.3 and Table 7.4) consisted in 100% of the basal mixture. Six test diets were produced by mixing 70% of the basal mixture and 30% of each test ingredient (NAOKd – fresh okara meal diet; AOKd – autoclaved okara meal diet; ALOKd – enzymatic hydrolyzed (Alcalase) okara meal diet; CYOKd – enzymatic hydrolyzed (*C. cardunculus* protease) okara meal diet; CYR11OKd – enzymatically hydrolyzed by *C. cardunculus* protease and fermented with *L. rhamnosus* R11 okara meal diet and CYB12OKd – enzymatically hydrolyzed by *C. cardunculus* protease and fermented with *B. animalis* ssp. *lactis* Bb12 okara meal diet). The experimental diets were extruded by SPAROS, Lda. (Portugal), and pellet size was 3 mm. Upon extrusion, all batches were dried in a convection oven (OP 750-UF, LTE Scientifics, UK) for 3 h at 60 °C and allowed to cool at room temperature.

For the growth trial, four isolipidic and isonitrogenous diets for Nile tilapia juveniles were formulated with selected okara meals included at 10 and 20% at the expense of soybean meal, corn meal and wheat. Formulation and chemical composition of the experimental diets is shown in Table 7.4. The diets were supplemented with L-Lysine and DL-Methionine to meet the amino acid requirements for Nile Tilapia (Santiago and Lovell, 1988). Diets were extruded by SPAROS, Lda. (Portugal), and pellet size was 3 mm. Upon extrusion, all batches were dried in a convection oven (OP 750-UF, LTE Scientifics, UK) for 3 h at 60 °C and allowed to cool at room temperature.

Table 7.2. Proximate composition of the test ingredients used in digestibility trial.

	NAOK[a]	AOK[b]	ALOK[c]	CYOK[d]	CYR11OK[e]	CYB12OK[f]
Dry matter (DM, %)	97.2	97.4	92.0	94.0	88.7	88.7
Ash (% DM)	4.0	3.9	7.3	12.0	11.1	10.7
Crude protein (% DM)	31.6	31.0	26.9	25.2	20.1	19.4
Crude fat (% DM)	17.2	16.4	15.4	13.2	9.7	9.3
Gross energy ($kJ.g^{-1}$ DM)	22.6	22.5	21.5	20.0	19.7	20.0
Phosphorus (% DM)	0.4	0.4	1.1	0.3	0.3	0.3
TDF [g] (% DM)	27.0	21.8	27.3	26.2	28.6	20.3
IDF [h] (% DM)	14.7	13.6	15.2	15.0	9.6	10.6

[a]NAOK: Dried okara; [b]AOK: Autoclaved and dried okara; [c]ALOK: Hydrolyzed okara using Alcalase; [d]CYOK: Hydrolyzed okara using protease from *Cynara cardunculus;* [e]CYR11OK: Hydrolyzed okara using protease from *Cynara cardunculus* and fermented with R11; [f]CYB12O: Hydrolyzed okara using protease from *Cynara cardunculus* and fermented with Bb12.–. [g]TDF: Total dietary fiber; [f]IDF = Insoluble dietary fiber

Table 7.3. Formulation and proximate composition of the basal mixture and experimental diets of the digestibility trial.

	Experimental diets						
	REF	**NAOKd**	**AOKd**	**ALOKd**	**CYOKd**	**CYR11OKd**	**CYB12OKd**
Basal mix. %	100	70	70	70	70	70	70
NAOK. %		30					
AOK. %			30				
CYB12OK. %				30			
CYR11OK. %					30		
CYOK. %						30	
ALOK. %							30
Proximate composition							
Dry matter (%) (DM)	93.9	93.8	93.0	94.5	92.5	94.1	93.7
Ash (% DM)	9.0	7.2	7.2	8.5	9.3	9.2	9.0
Crude protein (% DM)	43.0	36.6	38.4	35.4	34 .9	35.4	37.1
Crude fat (% DM)	8.1	11.7	11.2	11.0	10.3	9.3	9.2
Gross energy ($kJ.g^{-1}$ DM)	20.5	21.0	21.1	20.4	20.2	19.9	20.1
Phosphorus (% DM)	1.2	0.9	0.9	1.2	0.9	0.9	0.9
TDF (% DM)	22.7	25.6	22.4	15.9	22.7	23.9	23.8
IDF (% DM)	8.2	10.2	10.1	8.4	11.0	12.7	7.3

The abbreviations for the experimental diets stand for: REF – reference diet; NAOKd – okara meal diet; AOKd – autoclaved okara meal diet; ALOKd – enzymatic hydrolyzed (Alcalase) okara meal diet; CYOKd – enzymatic hydrolyzed (*Cynara cardunculus* protease) okara meal diet; CYR11OKd – enzymatically hydrolyzed by *Cynara cardunculus* protease and fermented with R11 okara meal diet and CYB12OKd – enzymatically hydrolyzed by *Cynara cardunculus* proteases and fermented with Bb12 okara meal diet.

Table 7.4. Formulation and proximate composition of the experimental diets and ingredient used during the growth trial.

	REF	AOK10	AOK20	CYOK10	CYOK20
Ingredients %					
Fishmeal 60[a]	10.0	10.0	10.0	10.0	10.0
Corn gluten[b]	17.0	17.0	17.0	17.0	17.0
Soybean meal 48[c]	17.5	12.5	7.5	14.0	10.5
Rapeseed meal[d]	15.0	15.0	15.0	15.0	15.0
Wheat meal[e]	5.0	4.5	3.0	4.0	1.0
Wheat bran[f]	10.0	10.0	10.0	10.0	10.0
Rice bran full fat[g]	12.0	12.0	12.0	12.0	12.0
Corn meal[h]	8.0	4.3	1.5	3.3	0.5
Soybean oil[i]	2.7	2.0	1.2	2.0	1.2
Vit & Min Premix[j]	1.0	1.0	1.0	1.0	1.0
Betaine HCl[k]	0.2	0.2	0.2	0.2	0.2
Antioxidant[l]	0.2	0.2	0.2	0.2	0.2
Sodium propionate[m]	0.1	0.1	0.1	0.1	0.1
MCP[n]	0.2	0.2	0.2	0.2	0.2
L-Lysine[o]	0.7	0.7	0.7	0.7	0.7
DL-Methionine[p]	0.4	0.4	0.4	0.4	0.4
AOK[q]		10.0	20.0		
CYOK[r]				10.0	20.0
Proximate composition					
Dry matter (DM. %)	90.5	89.9	90.9	92.3	90.1
Ash (% DM)	7.2	7.1	7.1	7.8	8.4
Crude protein (% DM)	42.9	43.8	43.8	43.0	43.8
Crude fat (% DM)	7.2	8.3	8.8	8.0	8.3

Gross energy (kJ.g^{-1} DM)	21.3	21.7	21.9	21.5	21.5
TDF[s] (%DM)	17.3	16.9	16.8	15.6	17.1
IDF[t] (%)	9.6	9.1	10.4	9.6	10.8
Phosphorus (% DM)	2.6	2.7	2.0	2.4	2.5
Essential amino acids (mg/ g sample)					
Arginine	20.8	20.2	22.3	21.7	23.1
Histidine	7.9	6.0	7.3	9.8	8.4
Lysine	31.8	30.9	34.2	31.7	29.7
Threonine	15.7	14.7	16.0	16.5	17.4
Isoleucine	17.8	17.3	19.1	18.5	18.9
Leucine	37.1	36.0	40.1	38.5	38.1
Valine	21.5	20.9	23.2	22.0	22.3
Methionine	9.3	9.8	10.6	10.6	9.4
Phenylalanine	17.7	17.2	19.2	18.6	17.1
Cystine	5.7	5.0	5.8	5.5	5.4
Tyrosine	13.7	13.5	15.0	14.5	14.8
Aspartic acid	53.4	52.9	58.4	57.2	59.8
Glut acid + Glutamine	99.4	96.1	105.7	104.3	109.3
Alanine	24.5	23.8	26.1	25.6	25.4
Glycine	7.4	6.3	6.4	6.8	7.8
Serine	17.7	17.2	19.2	18.8	19.8

The abbreviations for the experimental diets stand for: REF-based diet; AOK10 and AOK20 – diets with 10 and 20% autoclaved okara meal, respectively; CYOK10 and CYOK20 - diets with 10 and 20 % of okara hydrolyzed by protease from extract *Cynara cardunculus* meal. respectively.

[a] CONRESA 60: 61.2% crude protein (CP), 8.4% crude fat (CF), Conserveros Reunidos S.A., Spain.

[b] Corn gluten meal: 61% CP, 6% CF, COPAM, Portugal.

[c] Dehulled solvent extracted soybean meal: 47% CP, 2.6% CF, CARGILL, Spain.

[d] Defatted rapeseed meal: 34% CP, 2% CF, Premix Lda, Portugal.

[e] Wheat meal: 10.2% CP; 1.2% CF, Casa Lanchinha, Portugal.

[f] Wheat brean, 15% CP, 4% CF, Ribeiro & Sousa Cereais, Portugal.

[g] Rice bran full-fat: 13.3% CP; 16.3% CF, Casa Lanchinha, Portugal.

[h] Corn meal: 8.1% CP; 3.7% CF, Ribeiro & Sousa Cereais, Portugal.
[i] Henry Lamotte Oils GmbH, Germany.
[j] PREMIX Lda, Portugal: Vitamins (IU or mg/kg diet): DL-alpha tocopherol acetate, 100 mg; sodium menadione bisulphate, 25mg; retinyl acetate, 20000 IU; DL-cholecalciferol, 2000 IU; thiamin, 30mg; riboflavin, 30mg; pyridoxine, 20mg; cyanocobalamin, 0.1mg; nicotinic acid, 200mg; folic acid, 15mg; ascorbic acid, 500mg; inositol, 500mg; biotin, 3mg; calcium panthotenate, 100mg; choline chloride, 1000mg, betaine, 500mg. Minerals (g or mg/kg diet): copper sulphate, 9mg; ferric sulphate, 6mg; potassium iodide, 0.5mg; manganese oxide, 9.6mg; sodium selenite, 0.01mg; zinc sulphate, 7.5mg; sodium chloride, 400mg; excipient wheat middlings.
[k] Premix Lda, Portugal.
[l] Paramega PX, Kemin Europe NV, Belgium.
[m] Sodium propionate, Premix Lda, Portugal.
[n] MCP: 22% P, 18% Ca, Fosfitalia, Italy.
[o] Biolys 54.6%, EVONIK Nutrition & Care GmbH, Germany.
[p] DL-METHIONINE FOR AQUACULTURE 99%, EVONIK Nutrition & Care GmbH, Germany.
[q] Autoclaved okara meal dry matter dry matter (95.3%), ash (4.8%) protein (38.4%), crude fat (18.2 %), gross energy (23.5 kJ.g-1), phosphorus (1.3%), TDF (20.6%), IDF (16.5%)).
[r] Okara hydrolyzed by protease from extract Cynara cardunculus meal (dry matter (91.5%), ash (10.6%) protein (33.7%), crude fat (15.5%), gross energy (17.2 kJ.g-1) , phosphorus (1.1%), TDF (13.0%), IDF (8.4%)).
[s] Total dietary fiber.
[t] Insoluble dietary fiber.

7.3.2. Digestibility trial

The digestibility trial was conducted in the experimental Unit of UTAD (Vila Real, Portugal), with juvenile Nile tilapia (*O. niloticus*). Three homogeneous groups of 18 fish (weight 154.2 ± 1.4 g) were randomly distributed by 3 cylinder-conical tanks of 75 L in a recirculation freshwater system at 25 ºC with automatic feces collection system (Choubert system) and fed the reference diet. After the conditioning period, the experimental diets were fed once a day until apparent satiation of the fish, during 4 days to adapt to each experimental diet before the feces collection began. Fish continued to be fed once a day, during the period in which feces were collected. About 30 minutes after feeding, each tank was carefully cleaned to assure that no remains of uneaten feed were left in the tanks. Feces were daily collected and stored at -18 ºC until obtaining an amount considered enough for chemical analysis (5-8 days). At the end of the trial, feces were freeze-dried prior to analysis. Since the recirculating water system used was only constituted by three tanks, this procedure had to be repeated over time with different diets. Fish were fasted for 24 h between diets, allowing the first 5 days of feeding for adaption to the new diet. The remaining procedure was performed as described above.

7.3.3. Growth trial

The growth trial was conducted in the experimental Unit of UTAD, Vila Real, Portugal, with juvenile Nile tilapia (*O. niloticus*). In order to adapt to the experimental conditions, fish were kept in quarantine for 4 weeks and fed with the reference diet (43% crude protein and 8% crude fat). After acclimatization, fish were individually weighed (g) and measured (total length, cm) and fifteen homogeneous groups of 20 fish (mean body weight 17 g and mean body length 10 cm) were randomly distributed by 300 L tanks within a water recirculation system. Fish were adapted to the new conditions for 2 days (water temperature of 25 ºC, flow rate at 5 L min^{-1} and 12 h light/12 h dark photoperiod regime) and then each diet was randomly assigned to triplicate tanks and fed until apparent satiation, twice a day. The trial lasted 10 weeks, and fish were bulk weighed once during this period (at 4 weeks) to monitor and register feed consumption and weight gain. Before the growth trial began, 10 fish from the initial fish stock were collected after a 48 h fasting period and euthanized by a sharp blow on the head, and then kept at −20 ºC, until

initial whole body composition was analyzed. At the end of the 10 weeks' period, fish were anesthetized with MS-222 (50 mg L^{-1}) and were individually weighed (g) and measured (total length, cm). Three fish per tank, sacrificed by anesthetic overdose (150 mg L^{-1} of MS222; Sigma-Aldrich Co. LLC, Bellefonte, USA), were collected for weight determination of liver and viscera and for sampling the dorsal muscle, which was immediately frozen in liquid nitrogen and kept at -20 ºC until analysis of pH and water activity. Five fish per tank, sacrificed by anesthetic overdose, were also collected for whole body composition analysis and frozen at −20 °C until analysis.

7.3.4. Chemical and physical analysis

Fish collected from each tank were ground, pooled and moisture was determined (105 °C for 24 h). Afterwards, fish were freeze-dried, ground and homogenized before proceeding to the analysis. The experimental diets, ingredients and feces collected were also ground (feces were sifted) and homogenized before analysis. Proximate composition analysis was performed in duplicate and according to the Association of Official Analytical Chemists methos (AOAC, 1995). All samples were analyzed for dry matter (105 ºC for 24 h); ash by combustion in a muffle furnace (Nabertherm L9/11/B170, Bremen, Germany; 500 ºC for 5 h); crude protein (N × 6.25, Leco N analyzer, Model FP-528, Leco Corporation, St. Joseph, USA); crude fat content by petroleum ether extraction using a Soxtherm Multistat/SX PC (Gerhardt, Germany); and gross energy was determined in an adiabatic bomb calorimeter (Werke C2000, IKA, Staufen, Germany). Phosphorus content was analyzed in ingredient, diets and feces by digestion at 230 ºC in a Kjeldatherm block digestion unit followed by digestion at 60 ºC in a water bath and absorbance determination at 820 nm (adapted from AFNOR V 04-406). Ingredients and diet were analyzed regarding their total dietary fiber (TDF) and insoluble dietary fiber (IDF) content according modified method of Goering and Van Soest (1970). Chromic oxide content in diets of digestibility test and feces was determined according to Bolin et al.(1952), both analysis were performed in triplicate.

7.3.5. Amino acid analysis

Samples from test ingredients and experimental diets were hydrolyzed with HCl (6M) at 105 ºC over 20 h in sealed glass vials with previous injection of nitrogen for remove the oxygen, then the pH were adjusted for 9.5 in all samples. Amino acids content for all sample were performed by derivatization with orthophthalaldehyde (OPA) methodology. The amino acids were separated by HPLC (Beckman coulter. California USA) coupled to a fluorescence detector (Waters, Milford, MA, USA) according to the procedure of Proestos, Loukatos, and Komaitis (2008), 100 µL of each sample, at concentration 10 mg mL^{-1}, was derivatized according described method and injection volume of derivatives was 20 µL. The analysis was made in duplicate and quantified using a calibration curve built with amino acids pure standards (Sigma – Aldrich. St. Louis MO. USA) and expressed as mg g^{-1} of protein content.

7.3.6. Calculations

The apparent digestibility coefficients (ADCs) of the experimental diets were calculated according to the NRC (2011): dry matter ADC (%) =100 × (1 – (dietary Cr_2O_3 level/feces Cr_2O_3 level)) and nutrients ADC (%)= 100 × (1 – (dietary Cr_2O_3 level/feces Cr_2O_3 level) × (feces nutrient or energy level/dietary nutrient or energy)). The ADCs of nutrients and energy of the test ingredients were estimated according to NRC (2011): ADC_{ing} (%) = ADC_{test} + [(ADC_{test} - ADC_{ref}) × (($0.7 \times D_{ref}$)/($0.3 \times D_{ing}$))]; where ADC_{test} = ADC (%) of the experimental diet, ADC_{ref} = ADC (%) of the reference diet, D_{ref} = % nutrient (or $kJ.g^{-1}$ gross energy) of the reference diet (DM basis); D_{ing} = % nutrient (or $kJ.g^{-1}$ gross energy) of the test ingredient (DM basis).

Average body weight (ABW) = (final body weight + initial body weight)/2; N, P, L or E gain= (final carcass N, P, L or E content – initial carcass N, P, L or E content)/ABW/days; Condition factor (K)= (final body weight/(final body length)^3) × 100; Daily growth index (DGI) = 100× ((Final body weight)$^{1/3}$ – (Initial body weight)$^{1/3}$)/days; Voluntary feed intake (VFI) =100 × crude feed intake/ ABW/day; Feed conversion ratio (FCR)= dry feed intake/weight gain; Protein efficiency ratio = weight gain/ crude protein intake; Hepatosomatic index (HSI) = 100 × liver weight/ body weight; Viscerosomatic index (VSI) = 100 × weight of viscera/ body

weight; Specific growth rate (SGR)=(Ln final body weight − Ln initial body weight) × 100 / days.

7.3.7. Statistical analysis

Data were tested for normality and homogeneity of variances by Shapiro-Wilk and Levene's tests, respectively, and transformed whenever required before being submitted to a one-way ANOVA with the statistical program IBM SPSS STATISTICS version 23.0 (SPSS Inc., Chicago, IL, USA). When this test showed significance, individual means were compared using HSD Tukey Test. In all cases significant differences were considered when $P < 0.05$.

7.4. Results

7.4.1. Digestibility trial

The proximate composition of the test ingredients is presented in Table 7.2. Crude protein content of ingredients from differently processed okara ranged from 19 to 32%, crude fat varied between 9 and 17% and gross energy from 20 to 23 $kJ.g^{-1}$. Phosphorus content of the test ingredients ranged from 0.3 to 1.1% and the total fiber varied from 22 to 29 % among test ingredients.

The apparent digestibility coefficients (ADCs) of the experimental diets and feed ingredients are reported in Table 7.5. In the experimental diets, ADC of the protein was high for all diets (85 to 90 %), but the CYR11OKd showed a significantly lower value compared to REFd, whereas AOKd had the highest ADC value. Contrarily, the CYB12OKd and CYR11OKd showed the highest value for dry matter ADC (75%), while NAOKd registered the lowest dry matter ADC (63%). Energy digestibility ranged between 74 to 81%; NAOKd showed the lowest energy ADC value ($P < 0.05$) and differed significantly from all other diets.

Concerning the ADC of the ingredients tested, the dry matter ADC values varied between 40 to 80%, and NAOK meal had the lowest value ($P < 0.05$). NAOK showed the lowest energy (63%) ADC value. Protein ADC values differed significantly among ingredients (72 –

97%), and the AOK showed the highest ADC value, followed by CYOK (91%). These two ingredients were hence selected for the growth trial.

7.4.2. Growth trial

After 10 weeks of feeding the experimental diets, all groups of Nile Tilapia increased 6-fold the initial weight (Table 7.6). Mortality was very low (1.7%) and only in control and CYOK20 diet. Final body weight (104 g) and length (17 cm) and the overall growth performance (SGR) of the fish were similar among the different experimental diets. All diets were well accepted by Nile tilapia, resulting in similar voluntary feed intake among dietary treatments. There were no significant differences among dietary treatments for the FCR (1.1) and PER (2.1-2.2).

The hepatosomatic and viscerosomatic indexes of fish remained similar among experimental conditions (Table 7.6). The dietary inclusion of different okara meal (autoclaved or hydrolyzed) did not significantly affect final whole-body composition of the fish. Although lipid retention was highest in fish fed the REF diet, nutrient gain remained similar among dietary treatments (Table 7. 6).

Table 7.5. Apparent digestibility coefficients (ADC) of diets and test ingredients.

.	**REFd**	**NAOKd**	**AOKd**	**ALOKd**	**CYOKd**	**CYR11OKd**	**CYB12OKd**
ADC Diets (%)							
Dry matter	73.4 ± 0.4^{ab}	63.2 ± 1.2^{c}	70.7 ± 0.7^{b}	72.9 ± 1.7^{ab}	73.4 ± 1.0^{ab}	75.1 ± 0.8^{a}	74.9 ± 0.7^{a}
Protein	87.7 ± 1.1^{bc}	86.3 ± 0.4^{bd}	89.8 ± 0.6^{a}	86.3 ± 0.4^{bcd}	88.3 ± 0.4^{ac}	85.1 ± 0.8^{d}	87.1 ± 1.0^{bcd}
Energy	79.8 ± 0.1^{a}	74.4 ± 1.1^{b}	78.4 ± 1.3^{a}	79.3 ± 0.9^{a}	80.4 ± 0.2^{a}	80.3 ± 1.1^{a}	80.8 ± 0.9^{a}
ADC Ingredients (%)		NAOK	AOK	ALOK	CYOK	CYR11OK	CYB12OK
Dry matter		40.4 ± 4.0^{c}	64.6 ± 2.2^{b}	72.0 ± 5.8^{ab}	73.6 ± 3.4^{ab}	79.6 ± 2.9^{a}	78.6 ± 2.4^{a}
Protein		81.9 ± 1.7^{bc}	96.6 ± 2.6^{a}	81.5 ± 1.9^{bc}	91.1 ± 1.8^{ab}	72.1 ± 4.9^{c}	84.1 ± 5.9^{b}
Energy		63.1 ± 2.4^{b}	75.5 ± 2.9^{a}	78.4 ± 8.5^{a}	81.9 ± 2.2^{a}	81.7 ± 3.6^{a}	83.5 ± 2.2^{a}

The abbreviations for the experimental diets stand for: REF – reference diet; NAOKd – okara meal diet; AOKd – autoclaved okara meal diet; ALOKd – enzymatic hydrolyzed (Alcalase) okara meal diet; CYOKd – enzymatic hydrolyzed (*Cynara cardunculus* protease) okara meal diet; CYR11OKd – enzymatically hydrolyzed by *Cynara cardunculus* protease and fermented with R11 okara meal diet and CYB12OKd – enzymatically hydrolyzed by *Cynara cardunculus* proteases and fermented with Bb12 okara meal diet. Values are presented as mean ± standard deviation (n = 3). Values in the same row without a common superscript letter differ significantly ($P < 0.05$).

Table 7.6. Final growth performance, somatic indexes and whole body composition (% or kJ g−1 of wet weight. WW) of Nile tilapia fed the experimental diets for 10 weeks.

	REF	AOK10	AOK20	CYOK10	CYOK20
Growth					
Initial body weight (g)	17.4 ± 3.6	17.2 ± 3.6	17.4 ± 3.8	17.3 ± 3.7	17.2 ± 3.6
Final body weight (g)	110.6 ± 35.7	103.5 ± 32.2	99.4 ± 33.6	105.6 ± 31.4	102.1 ± 26.7
Final body length (cm)	17.5 ± 2.0	17.2 ± 2.0	16.9 ± 2.1	17.2 ± 2.1	17.2 ± 1.5
K	2.2 ± 0.1	2.0 ± 0.1	2.2 ± 0.2	2.1 ± 0.0	2.1 ± 0.2
DGI	3.1 ± 0.1	2.9 ± 0.1	2.8 ± 0.1	3.0 ± 0.1	2.9 ± 0.1
SGR	2.6 ± 0.1	2.5 ± 0.0	2.5 ± 0.1	2.5 ± 0.1	2.1 ± 0.1
Dry matter intake (g DM/100g ABW/day)	2.1 ± 0.1	2.1 ± 0.0	2.1 ± 0.0	2.1 ± 0.1	2.2 ± 0.1
Protein intake (g protein/100g ABW/day	0.9 ± 0.0	0.9 ± 0.0	0.9 ± 0.0	0.9 ± 0.0	1.0 ± 0.0
Lipid intake (g lipid/ 100 g ABW/day)	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0
Energy intake (kJ/100 g energy/g ABW/day)	0.5 ± 0.0	0.5 ± 0.0	0.5 ± 0.0	0.5 ± 0.0	0.5 ± 0.0
FCR	1.1 ± 0.1	1.1 ± 0.0	1.1 ± 0.0	1.1 ± 0.1	1.1 ± 0.0
PER	2.2 ± 0.2	2.1 ± 0.1	2.1 ± 0.1	2.2 ± 0.1	2.1 ± 0.1
HIS	1.3 ± 0.2	1.4 ± 0.1	1.5 ± 0.1	1.2 ± 0.3	1.2 ± 0.2
VSI	6.7 ± 0.4	6.0 ± 0.3	6.4 ± 0.1	6.7 ± 0.5	6.8 ± 0.2
Final whole body composition (% WW)					
Dry matter	28.0 ± 0.2	28.0 ± 1.8	27.4 ± 0.4	26.9 ± 0.8	27.2 ± 0.7

Crude protein	16.3 ± 0.6	16.0 ± 0.9	15.6 ± 0.5	15.8 ± 0.4	15.7 ± 0.7
Crude fat	8.0 ± 0.7	8.3 ± 1.0	7.9 ± 0.5	7.3 ± 0.3	7.8 ± 0.4
Gross energy (kJ g^{-1})	6.8 ± 0.1	6.8 ± 0.6	6.7 ± 0.2	6.4 ± 0.1	6.6 ± 0.2
Ash	3.6 ± 0.3	3.7 ± 0.2	3.4 ± 0.2	3.6 ± 0.3	3.5 ± 0.2
Retention per consumption					
Dry matter	26.8 ± 1.7	26.9 ± 1.6	25.8 ± 0.6	26.1 ± 0.9	25.0 ± 0.3
Protein	36.5 ± 3.3	35.0 ± 1.7	33.3 ± 0.5	35.8 ± 1.0	33.0 ± 1.4
Lipid	107.6 ± 6.7^{a}	97.1 ± 13.3^{ab}	85.1 ± 7.5^{b}	88.0 ± 3.3^{ab}	86.3 ± 3.4^{b}
Energy	29.8 ± 1.2	29.7 ± 2.7	28.1 ± 1.6	28.3 ± 1.0	28.0 ± 0.1
Gain					
Dry matter	5.8 ± 0.1	5.7 ± 0.4	5.5 ± 0.1	5.5 ± 0.1	5.5 ± 0.1
Protein (ABW Kg/day)	3.4 ± 0.2	3.3 ± 0.2	3.1 ± 0.1	3.2 ± 0.1	3.2 ± 0.1
Lipid (ABW Kg/day)	1.7 ± 0.2	1.7 ± 0.2	1.6 ± 0.1	1.5 ± 0.0	1.6 ± 0.1
Energy (ABW Kg/day)	1.4 ± 0.0	1.4 ± 0.1	1.3 ± 0.1	1.3 ± 0.0	1.3 ± 0.0
Energy (Total)	6.4 ± 0.2	5.9 ± 0.6	5.5 ± 0.5	5.8 ± 0.2	5.7 ± 0.1

The abbreviations for the experimental diets stand for: REF-based diet; AOK10 and AOK20 – diets with 10 and 20 % autoclaved okara meal, respectively; CYOK10 and CYOK20 - diets with 10 and 20 % of okara hydrolyzed by protease from extract *Cynara cardunculus* meal. respectively. Values are presented as mean ± standard deviation (n = 3). Values in the same row without a common superscript letter differ significantly ($P < 0.05$). Absence of superscript indicates no significant difference between treatments.
K condition factor.
DGI, daily growth index.
SGR, specific growth ratio.
FCR, feed conversion ratio.
PER, protein efficiency ratio.
SGR, specific growth ratio.
HSI, hepatosomatic index.
VSI, viscerosomatic index.

7.5. Discussion

7.5.1. Digestibility trial

The food industry generates large amounts of okara which is often discarded, but that can still be a valuable nutrient source to include in fish diets. Previous studies showed that the required processing and stabilization of okara to generate a stable flour affects its nutritional value (Voss et al., 2018), but the feasibility of considering okara as nutrient source for fish diets had never been evaluated before. According to Mo et al., (2018) this is a low cost by-product that can be used to feed carp, tilapia and other fish in Asia. However, the extensive use of okara is problematic due to its high humidity (ca. 80 %) and rich composition; okara is difficult to conserve and can putrefy quickly (Vong and Liu, 2016). So, a drying process is essential for okara conservation and to increase the time for use of this by-product. Besides, the thermal treatment used for extraction of soya beverage eliminates most antinutrients, as protease inhibitors and phytates which is an advantage for fish feeding (Francis et al., 2001; Voss et al., 2018). In fact, the present study shows that the nutrients in okara meal are highly digestible, when okara is properly processed. The dry matter ADCs values observed for the hydrolyzed and fermented okara meals (ALOK, CYOK, CYR11OK, CYB12OK) were well within the range of values previously reported for soybean in Nile tilapia, whilst NAOK and AOK dry matter ADCs values correspond to those reported in pea seed meal and faba bean meal, respectively (Fontaínhas-Fernandes et al., 1999). In fermented soybean, Dong et al. (2010) found a lower ADC for dry matter (69.9%) than that observed in our study for both fermented okara meals (CYR11OK, CYB12OK). However, Köprücü & Özdemir, (2005) and Vidal et al. (2015) obtained higher values for dry matter ADC in soybean coproducts (full-fat soybean meal, expeller pressed soybean meal, low-protein soybean meal, high-protein soybean meal and soybean protein concentrate) fed to Nile tilapia than the results obtained for okara.

In general, the energy ADC values depend on the energy ADCs for carbohydrate, lipid and protein; as these constituents account for most of the dry matter (NRC, 2011). In this study it was possible to observe that the energy ADC varied significantly among okara meals, being lowest in fresh okara meal (NAOK). This meal had the highest protein and fat content, and also had one of the highest levels of fiber. However, the lower energy ADC found in NAOK may be

related to some antinutritional factors, which might have remained in NAOK compared to AOk. Furthermore, the application of thermal treatments were previously related to a higher digestibility of different plant ingredient, including soybeans (Francis et al., 2001). According to Ngo et al. (2015), the dry matter and energy ADC values are correlated: a low dry matter digestibility may be reflected in a poor energy ADC value. In fact, the NAOK showed the lowest dry matter and energy ADC. The energy ADC values in NAOK and AOK were lower than those previously reported for soybean coproducts fed to Nile tilapia (Vidal et al., 2015). However, energy ADC of fermented and hydrolyzed okara (ALOK, CYOK, CYR11OK and CYB12OK) were similar to those reported for deffated soybean meal and full-fat toasted soybean (Fontaínhas-Fernandes et al., 1999; Köprücü and Özdemir, 2005) and even higher than those observed by Dong et al. (2010) in fermented soybean and soybean meal.

The ADCs' of protein (72.1–96.6%) observed in the test ingredients are in general agreement with values reported for soybean and its coproducts used as fed ingredients in Nile tilapia. Moreover, the highest apparent digestibility coefficient (ADC) of protein was obtained in okara submitted to a thermal treatment (AOK) (96.6 %), whereas NAOK had a lower value (81.9 %) than AOK. This clearly demonstrates the effectiveness of the autoclave for eliminating most antinutrients (trypsin inhibitors) in okara. The values for ADCs of protein in AOK and CYOK were similar and correspond to values reported in previous studies in Nile tilapia using different vegetable protein sources (lupin seed, extruded pea seed and defatted soybean) (Fontaínhas-Fernandes et al., 1999). Likewise, Köprücü & Özdemir (2005) also reported a protein value ADC value for soybean meal similar to that found in the CYB12OK. However, the ADC of protein in CYR11OK (72%) was lower than that previously reported for either soybean meal or fermented soybean (Dong et al., 2010; Fontaínhas-Fernandes et al., 1999; Guimarães et al., 2008; Köprücü and Özdemir, 2005; Vidal et al., 2015). In general, is possible to observe that the different hydrolysis performed by the two different enzymes in okara resulted in different protein ADC values in Nile Tilapia. This can be a result of the protein profile obtained after enzymatic hydrolysis: the peptide fractions in okara hydrolyzed by Alcalase had a lower molecular weight than okara hydrolyzed by proteases from *Cynara cardunculu*s that resulted in peptides with high and low molecular weights. Previous studies have also reported that the enzymatic hydrolysis of soy products has improved its functional and nutritional properties compared to intact soy protein (Coscueta et al., 2016; Gibbs et al., 2004; Guan et al.,

2017). Thus, the inclusion of soy hydrolysates in aquafeeds was reported to have positive results on growth performance, blood biochemistry parameters, disease resistance, gastrointestinal digestion or muscle composition of starry flounder (*Platichthys stellatus*) (Song et al., 2014) and red sea bream (*Pagrus major*) (Khosravi et al., 2015). Moreover, peptides obtained from protein hydrolysis present an easier digestibility compared to native proteins (Carvalho et al., 2004; Zambonino Infante et al., 1997).

In conclusion, the present results show that the nutrients in okara are highly digestible when okara is properly processed, confirming previous studies with soybean coproducts (Refstie et al., 2005; Vidal et al., 2015). The highest protein digestibility was observed with AOK followed by CYOK, clearly demonstrating the effectiveness of the autoclave for eliminating most antinutrients (trypsin inhibitors) in okara, and the use of a protease form *C. cardunculus* without fermentation for *L. rhamnosus* R11 and *B. animalis* ssp. *lactis* Bb12.for promoting peptide absorption in the intestine. These two okara meals were hence selected for a growth trial in Nile tilapia.

7.5.2. Growth Trial

Soybean meal has high nutritional value and is the most widely feedstuff used by the aquaculture industry for replacing fish meal. Although several studies have focused on different products from soybean to partial or total replace fish meal in aquafeeds (Elangovan and Shim, 2000; Hassaan et al., 2015; Mamauag et al., 2011; Peres et al., 2003; Tibaldi et al., 2006), studies using okara are very scarce. In the present study, it was clearly demonstrated that okara meal can replace at least 20% of soybean meal, without any negative impact on growth performance, feed utilization or body composition of Nile tilapia juveniles.

It is generally known that the nutritional value and amino acid profile of soybean products depends on such products, but also on the industrial process used in grain processing (Vidal et al., 2015; Voss et al., 2018). In general terms, the AA profiles of the selected okara meals observed in this study are consistent with those reported in the literature. Lysine and methionine levels ranged between 26-37 and 4-5 mg AA/g of sample, in CYOK and AOK, respectively, whilst previous studies reported values of 32-53 and 5.4-8.7 mg AA/ g, respectively, in other soybean coproducts (Vidal et al., 2015).

In the present study, the replacement of soybean meal by up to 20% of okara meal in diets for juvenile Nile Tilapia resulted in similar fish growth to that observed with a control diet. Likewise, in a previous study El-Saidy (2011) reported that the use of okara up to 75% to replacing fish meal didn´t affect the growth performance of Nile tilapia. Moreover, according to Mamauag et al., (2011) a 20% inclusion of soy peptide in a diet for Japanese flounder, *Paralichthys olivaceus,* promoted growth performance and improved blood biochemical parameters. However, this result could not be confirmed in the present study. To our knowledge, there are no published reports of okara or soybean hydrolysate fed Nile Tilapia.

Additionally, according to Kim and Kaushik (1992) similar growth should be expected in fish fed diets containing the same level of digestible energy intake and digestibly protein intake. Although diets digestibility were not determined in the present study, energy and protein intake remained similar among okara diets. Moreover, the protein and energy ADC values of the selected okara meals (AOK and CYOK) did not differ significantly. Gomes et al. (1995) showed that a 100 % replacement of fish meal by vegetable protein led to a decrease of voluntary feed intake and growth performance in rainbow trout. Nonetheless, Mamauag et al.(2011) reported that the inclusion of 20% soy peptide in diets for Japanese flounder was an effective feed attractant and improved diets palatability, due the presence of small molecular weight compounds as free amino acid. This could not be confirmed in this study where a similar voluntary feed intake (DM, protein, energy and lipid) was observed for every diet.

The feed conversion efficient ratio (FCR) obtained in this study for all diets was generally low (1.1), and lower than that previously reported in studies using different levels of okara (1.7-1.8) (El-Saidy, 2011) or yeast fermented soybean meal (1.6) (Hassaan et al., 2015) in Nile tilapia. Besides that, our results show that weight gain (82-90 g/fish) and the PER (2.2) were higher than values reported by El-Saidy (2011) (ca. 0.6 and 10-11g/ fish, respectively). These results may be related to the antinutrients present in soybeans, such as protease inhibitors, which may reduce the bioavailability of protein and minerals, and thus have detrimental effects on fish growth (Spinelli et al., 1983; Storebakken et al., 1998). So, only one appropriate heat-treatment can inactivate these compounds. Thus, the okara showed lower antinutritional factors than soybean meal due the thermal process for obtaining of the soya beverage, though this fresh by-product still presents ca. 8.8 (TUI/ mg dry sample) (Voss et al., 2018). Moreover, the okara meal used in this work was previously autoclaved, which can eliminate almost completely the

antinutritional factors, mainly protease inhibitors and thus can improve the absorption of nutrients on fish. Similarly, previous studies employing a heat treatment in soybeans reported improved growth performance and feed utilization in channel catfish (Peres et al., 2003) and Coho Salmon (Arndt et al., 1999).

Moreover, in the present study, voluntary feed intakes were similar for every diet, indicating that different okara meal have a good palatability. So, this study showed that up to 20 % of okara meal could be included in Nile tilapia diets without any adverse effect on growth performance.

Whole body composition (dry matter, crude protein and crude lipid) of the fish did not vary significantly between AOK and CYOK dietary treatments. Similar results were also reported replacing fish meal with fermented soybean meal in Chinese sucker diets (Yuan et al., 2013). On the other hand, fermented soybean induced lower lipid content in rockfish using (Lee et al., 2016), and in Nile tilapia (El-Saidy, 2011), but similar whole body protein levels to those found in our study. In addition, the whole body composition in our study was higher than that reported using soy peptide in Japanese flounder (Mamauag et al., 2011). In contrast, Elangovan and Shim (2000) reported that the whole body moisture content increased and body fat decreased with replacing of fish meal for soybean meal in diet of juvenile tin foil barb. On the other hand, Yuan et al. (2013) used fermented soybean meal for replacement of fish meal in *Myxocyprinus asiaticu*s and found slightly lower final values of protein in the whole body than those obtained in this study. The hepatosomatic and viscerosomatic indexes of fish also were similar ($P > 0.05$) among experimental conditions. In accordance with the present study, similar values were recorded by Lee et al. (2016) in the VHI and VSI of grower rockfish using levels different of soybean fermented by *Bacillus subtilis*.

7.6. Conclusion

The results obtained in this study showed that all different okara meals were well digested by Nile Tilapia. The highest protein digestibility was observed in AOK followed by CYOK, clearly demonstrating the effectiveness of the autoclave and the use of a protease form *Cynara* without fermentation to increase okara nutrient bioavailability. Additionally, the inclusion of up to 20% of AOk or CYOK did not affect growth, nutrient utilization or whole

body composition of Tilapia Nile in relation to a reference diet. Since this ingredient can be obtained from local food industries, the use of okara could decrease the importation of feedstuffs and consequently decrease the carbon footprint in the aquafeed sector. More studies on the use of okara are required with different fish species and increasing incorporation levels to achieve an adequate destination for this by-product, which could also reduce the costs of aquafeed.

Part VI- Conclusions and futures perspectives

CHAPTER 8- Conclusions

The hereby proposed work aimed at characterizing effect of thermal processing and storage upon the nutritional and functional properties of okara and as well exploit the application of biotechnological processes to increase their intrinsic properties. From the experimental work undertaken it was possible to observe that okara is a by-product with a high nutritional value possessing significant amounts of fiber, protein and lipids, although a great variability was observed between each studied batch of this by-product. Moreover, it should also be emphasized that other factors, such as processing conditions and soybean variety, may also play an important role in the compositional variations observed between batches.

Freezing can result in the stabilization of fresh okara and therefore the maintenance of the original nutritional quality, although the issue of the initial microbial counts remained unaffected and, therefore a potential problem that may be potentiated after defrost. Overall, while the thermal treatment used to reduce the water content of okara resulted in a reduction of microbial contamination (ca. 45 %), it was not enough to allow for microbial stabilization. In addition, the higher temperatures appeared to promote secondary reactions that resulted in oxidations and acidifications of okara during storage. In fact, it was the lower temperature (80 ºC) that appeared to be the best thermal treatment at stabilizing okara. Furthermore, while okara's monounsaturated and polyunsaturated fatty acid remained unchanged (during storage) regardless of the thermal treatments applied, the high temperature treatment (D200) resulted in an increase of isoflavones (mainly daidzin and genistin, that are present in okara in high amounts) and in the strongest reduction in trypsin inhibitor activity (from 8.75 ±0.65 to 1.52 ± 0.66 TUI/ mg dry sample for fresh okara and D200, respectively). After autoclaving dried okara (AOK) a reduction in lipid oxidation and microbial growth was observed, which allowed for a better preservation and longer storage periods when comparing with NAOK. In addition, an increase in amino acids and total isoflavone contents was also observed for AOK along with a significantly decrease in trypsin inhibitor activity for both types of autoclaved okara (FAOK and AOK) (when compared NAOK and FNAOK). Overall, this study evidenced the high nutritional and functional potential value of okara flours and proved that heat treatment (namely autoclaving) could be an important processing step to further improve the flour's nutritional value. In fact, autoclaving proved to play an important role in the pre-treatment of hydrolyzed

samples with AOK showing a higher degree of hydrolysis (DH) than NAOK, with the heat appearing to aid the enzymatic attack of the protein.

When comparing the effect of both enzymes (AL and CY) it was observed that AL resulted in a fraction with higher antioxidant activity than the okara flour (autoclaved or not) hydrolyzed by CY. Similarly, while both enzymes (AL and CY) resulted in flours with a high antihypertensive activity, the highest ACE-inhibitory activity was also found for okara hydrolyzed by AL (at 9.97 μg /mL). Additionally, it was possible to identify peptide sequences (in dry okara, AOK and NAOK, after enzymatic hydrolysis) that, to the best of our knowledge, have not been published in scientific literature so far.

When considering the fermentation assays it can be seen that, overall, after hydrolysis with CY okara can be fermented by probiotic bacteria being considered a viable matrix for probiotic strains. When producing the fermented beverages, a pH value of 4.5 was established as point where the fermentation process was interrupted regardless of the probiotic bacteria (*B. animalis* Bb12, *L. rhamnosus* R11 or combination of both) used. In addition, the incorporation of an additional carbon source (fructose and FOS) to the hydrolyzed okara resulted in higher specific growth rates than the those observed for okara with a low concentration of added fructose (3%) and okara without an added carbon source. Moreover, all of fermented okara samples showed an increase in the levels of isoflavones' aglycones when comparing to unfermented okara. This can be translated into an enhanced bioavailability of isoflavone compounds as the aglycones are absorbed faster than their glycoside counterpart.

In the okara beverage's stability assessment (that encompassed the formulations with 6% (w/v) fructose and 2% (w/v) of FOS), all fermented okara beverages (OkaLR6, OkaBb6 and OkaMix6) maintained the total viable cells counts throughout storage (ca. 9 log CFU/mL) and registered an increase in acidity. However, the non-fermented okara beverage (OkaNF) maintained all its characteristics over the 28 days of storage and registered a stronger Ace-inhibitory activity than all the fermented beverages.

The *in vitro* simulation of the gastrointestinal tract's impact upon of okara beverages, showed an increase in the antioxidant and ACE-inhibitory activity after gastrointestinal simulation, while the total phenolic compounds remained stable throughout this process. In addition, the fermented beverages registered a higher increase in ACE-inhibitory activity, at intestinal level, than OkaNF. Overall, this work demonstrated the importance of how several

extrinsic factors, such as processing conditions of the product (e.g. fermentation and pH), could interfere with the release of bioactive compounds after *in vitro* gastrointestinal digestion.

With respect to the sensory analysis of the beverages, they were not so well accepted by participants with the mean values of overall liking lower than 5.0, ranging from 2.8 (Dislike moderately) to 4.6 (Dislike slightly/ Neither like nor dislike) for fermented okara (OkaLR6, OkaBb6 and OkaMix6) and OkaNF, respectively. Moreover, the JAR ratings showed that most of the participants considered that the fermented beverages were too acidic and lacked sweetness. Thus, some organoleptic modifications are required in order to improve the acceptability of these beverages.

When considering the work focused on the potential aquafeed applications of okara, it was observed that the apparent digestibility coefficients (ADCs) of the okara meals (NAOK, AOK, ALOK, CYOK, CYR11OK and CYB12OK), in Nile Tilapia (*Oreochromis niloticus*), largely depends on the processes used to produce them. The highest protein digestibility values were observed for AOK (97%) and CYOK (91%). Therefore, these ingredients were selected to be used in a growth trial using Nile Tilapia juveniles. During this assay it is observed that all diets were well accepted by Nile tilapia (exhibiting a similar voluntary feed intake) and that the different diets used had no significant impact on the overall growth performance of the fish. These results showed that okara flour can be included up to 20% in diets for tilapia, at the expense of other vegetable sources (soybean, wheat and corn meals), without significantly affecting the growth performance or nutrient utilization.

CHAPTER 9-Future prospects

The results described in the present PhD program provide some interesting insights into possible uses of okara (by-product of soya beverage), regardless further studies are still required. For instance, while the composition and stability results of okara flour demonstrated that its incorporation into baked food products (e.g. bread or cakes) could be an interesting solution to improve the functional and nutritional quality (e.g. fiber content) of these foodstuffs, a better understanding of it's impact upon the final products quality (sensorial, nutritional and functional) and safety is still required. Moreover, when considering the potential use of okara to produce a synbiotic beverage some issues must still be addressed, namely the improvement of some of its organoleptic characteristics, such as sweetness and acidity. In this aspect, the use of other fructose proportions could be considered as well as the use of alternative sugars. Another aspect that could contribute to the improvement of the consumer's acceptance of okara's fermented beverages is the incorporation of fruit pulps (such as banana or apple), which could contribute to improve not only the flavour but also texture of the beverage, particularly in hiding the beany flavour usually present soy-based products. From a different perspective, and considering the results obtained for the beverages' *in vitro* bioactivities (namely antihypertensive activity) it would be interesting to further study their potential, through *in vivo* assays, using mice or even humans. Additionally, it could also be interesting to consider, for the fermented beverages, the study of other biological properties as well as evaluate the effect of the gastrointestinal tract upon the survival of the probiotic bacteria.

Regarding the potential application of okara in aquafeeds, while it was showed to be a promising alternative for the substitution of other vegetable protein ingredients in Nile tilapia diets, the study of higher concentrations of okara flour could be of particular interest when seeking to further reduce feed costs. Additionally, it would also be important to evaluate other parameters that are associated with the muscle quality of Nile tilapia (such as fatty acid profile or antioxidant capacity) as well as consider some biochemical blood parameters that are marks of disease resistance in Nile Tilapia. Additionally, to expand the potential application of okara flours in aquafeed, extending these studies to other fish species would be of great interest.

Appendix

Appendix 1. Sensory analysis sheet

Idade _____

Sexo **Feminino** ❑ **Masculino** ❑

Nacionalidade: **Portuguesa** ❑ **Outra** ❑ **Especifique** _____

Pais de residência: **Portugal** ❑ **Outro** ❑ **Especifique** _____

Escolaridade:

- ❑ **1º Ciclo do Ensino Básico (4º ano)**
- ❑ **2º Ciclo do Ensino Básico (6º ano)**
- ❑ **3º Ciclo do Ensino Básico (9º ano)**
- ❑ **11º ou 12º ano**
- ❑ **Bacharelato**
- ❑ **Licenciatura ou Mestrado**
- ❑ **Doutoramento**

Costuma consumir produtos á base de soja? **Sim** ❑ **Não** ❑

Com que frequência consume bebidas de soja?

Todos os dias ❑ **1 ou mais vezes por semana** ❑ **1 ou mais vezes por mês** ❑

Por favor prove a amostra 706:

Globalmente, QUANTO GOSTOU da amostra 706?

(Por favor assinale com X a caixa que melhor representa a sua opinião)

- **9 - Gostei extremamente** ❑
- **8 - Gostei muito** ❑
- **7 - Gostei moderadamente** ❑
- **6 - Gostei ligeiramente** ❑
- **5 - Não gostei nem desgostei** ❑
- **4 - Desgostei ligeiramente** ❑
- **3 - Desgostei moderadamente** ❑
- **2 - Desgostei muito** ❑
- **1 - Desgostei extremamente** ❑

Avalie por favor a amostra 706 assinalando para cada um dos seguintes atributos sensoriais a caixa que melhor representa a sua opinião:

Doçura	**Insuficiente**		**Como gosto**		**Demasiado doce**
	❑	❑	❑	❑	❑
Acidez	**Insuficiente**		**Como gosto**		**Demasiado ácido**
	❑	❑	❑	❑	❑

Appendix 2. Fermented okara beverage

Appendix 3. Sampling of Nile Tilapia after 10 weeks of the growth and nutrition trial

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