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NUTRITION AND FOOD TECHNOLOGY

Development of gluten free biscuits to control glycaemia

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Table of contents

ملخص الدراسة

في الآونة الحديثة ازدادت الحاجة إلى نظام غذائي خال من الغلوتين وذلك لانتشار الاضطرابات الصحية المرتبطة به مثل (الداء الزلاقي (سيلياك)، حساسية القمح ، وحساسية الغلوتين غير السيلياك). ومن الجدير بالذكر ايضا ان هناك علاقة واضحة بين هذه الاضطرابات بالاخص السيلياك وتطور داء السكري. وفي هذا الصدد ، فان المتتبع لمشاكل المنتجات الخالية من الغلوتين المتواجدة حاليا في الاسواق يجد ان ارتفاع المؤشر الجلايسمي (مؤشر تحلون الدم) لها يعد معضلة لانه يزيد من احتمالية تطور السكري او يزيد من احتمالية فقدان السيطرة على سكر الدم عند مرضى السكري.

تهدف هذه الرسالة الى التحقيق في استجابة نسبة السكر في الدم المحتملة من البسكويت الخالي من الغلوتين المدعم بمستخلص بروتين الصويا والكازين.

للحصول على هذه النتيجة تم انتاج بسكويت خالي من الغلوتين بدون اضافة اي من البروتين له، بالاضافة الى اربع عينات تحتوي على مستخلص الصويا او الكازيين بنسبة 4.5% او 13% .ومن ثم تم تطويع الخمسة عينات لعملية الهضم الانزيمي خارج جسم الانسان في المختبر بطريقة تطابق الى حد كبير الهضم داخل جسم الانسان ، ومن ثم تم حساب تركيز سكر الجلوكوز والحموض الامينية في العصارة في اوقات مختلفة من الهضم (مرحلة المعدة ومرحلة الامعاء).

بناءً على التحليل الاحصائي للنتائج تبين ان اضافة مستخلص بروتين الصويا او الكازيين للبسكوت الخالي من الغلوتين قلل من سرعة هضم الكربوهيدرات في مرحلة هضم الامعاء مما قلل من الاستجابة الجلاسيمية طردياً مع تركيز البروتين المضاف. وذلك التأثير ربما يكون ناتج عن وجود بعض الببتيدات في الصويا والكازيين والتي لها قدرة على تثبيط عمل الانزيمات الهاضمة.

Abstract

A gluten-free diet is needed to the management of gluten-related disorders (celiac disease, wheat allergy, and non-celiac gluten sensitivity). There is a well-known relation between these gluten-related disorders especially celiac disease and diabetes. In this regard, the high glycemic index of the available gluten-free products is considered a dilemma.

The aim of this study is to investigate the potential glycemic response of gluten-free biscuits enriched with soy protein and casein hydrolysates. A control gluten free biscuit (without protein hydrolysates) as well as soy protein and casein hydrolysates-enriched biscuits at 4.5% and 13% w:w were developed. The five types of biscuit were subjected to an *in vitro* enzymatic digestion mimicking human digestion and the concentrations of glucose and free amino acids were measured in the digesta collected at different time points during both the gastric and the intestinal phases. The addition of soy and casein protein hydrolysate to the samples reduces the starch digestion in the intestinal phase which reduces the glycemic response depending on the hydrolysate dose. This may be due to an effect of digestive enzyme inhibition operated by some peptides forming from the digestion of soy and casein hydrolysates.

Chapter 1
Introduction

1.1 Gluten

Gluten is the main structural protein in wheat as well as rye, barley, and oats. (Biesiekierski, 2017). Gluten can be defined as "the rubbery mass that remains when wheat dough is washed by salty solutions to remove starch granules and water-soluble constituents" (Wieser, 2007). From biochemists overview it is a complex of heterogeneous proteins mainly soluble gliadins and the insoluble glutenins which have a high amount of prolines and glutamines. Glutenins and gliadins work together in order to give the dough sensory properties like air entrapping and leavening. In the past, gliadins were divided into four groups of monomers depending on their mobile ability in gel electrophoresis which were (α–,β–,γ–,o-gliadins) but recently gliadins have divided into more than hundreds of gliadin components depending on their molecular weight, and amino acid sequences and composition (Wieser, 2007).

1.2 Gluten Related Disorders

Gluten is not easily digested in human digestive system. This resistance results from its characteristics as it contains some resistant gliadin peptides and large size glutenins macro polymers. These tow fractions contain high amount of prolines and glutamines (Biesiekierski, 2017; Wieser, 2007). Regarding these properties it is important to mention that there are three well known health conditions related to gluten and wheat products consumption (Catassi et al., 2013). Which are Celiac disease (CD), wheat allergy (WA) and non-celiac gluten sensitivity (NCGS) or wheat intolerance syndrome (WIS).

Celiac disease is a condition developed when gluten-derived peptides trigger T-cells autoimmune reactions leading to inflammation in the small intestine, enteropathy of the wall of the intestine and nutrition malabsorption (Elli et al., 2015). The prevalence of the celiac disease is increasing as many chronic diseases (Lebwohl et al., 2014). In the current decade celiac prevalence is around 1-1.5% in American and European populations (Gujral et al., 2012).

Celiac disease risk increases among peoples who suffer from Down syndrome, Turner syndrome, Williams's syndrome, and other autoimmune diseases (Husby et al., 2012). The problem in celiac diagnosis is that it could be asymptomatic, or it can cause heterogeneous symptoms such as diarrhea, constipation, weight loss, alterations in liver function tests, anemia and others. Which differs from patient to another (Fasano and Catassi, 2012).

Wheat allergy is the allergy that caused by some wheat proteins which can result in IgE-mediated non-IgE (cell) mediated or mix (Sampson HA, 2016). WA is a third allergy after cow's milk and egg allergy with a prevalence rate of 3.6% (Nwaru BI, et al 2014). Many symptoms can result from WA such as itching, mouth, nose, eyes swelling, cramps, bloating, and anaphylaxis (Tovoli et al., 2015). The wheat allergy can be diagnosed by Skin prick tests (SPT) and wheat-specific IgE testing (Constantin et al., 2005).

Non-celiac gluten sensitivity is a clinical condition in which gluten ingestion causes intestinal and extra-intestinal symptoms. With unknown prevalence. In NCGS there is no mucosal inflammation result from gliadins as what happens in CD (Bucci et al., 2013).

1.3 Gluten-Free Diet

All the patients with gluten related disorders need a gluten free diet. Gluten related disorders makes a 5% of the digestion disorders worldwide (Elli et al., 2015). There is a well-known relation between these gluten related disorders especially celiac disease and diabetes. While the studies estimated that 16% of celiac disease patients are diabetic (El Khoury et al., 2018). In 2014 Antvorskov and colleagues showed that gluten may be a factor that enhances the development of diabetes. Diabetes is a heterogeneous group of metabolic diseases characterized by hyperglycemia, which may result in long-term complications leading to damages to many body's systems, especially kidneys, nerves, eyes, and blood vessels (Gülden et al 2015). According to WHO statistics there were 382 Million diabetic people around the world in 2013 and it is expected to be about 592 Million by 2035 (Upadhyaya & Banerjee, 2015). **In this regard**, it is worth to mention that high glycemic index is one of the most gabs in the gluten-free products the feature that causes loss of glycemic control among diabetic patients (Scaramuzza et al., 2013). Depending on these facts the scientists in the recent decades are searching on varied approaches to reduce the gluten free products glycemic responses. For instance, the addition of **inulin-type**

fructans prebiotics, other kinds of dietary fibers and resistant starch (Capriles & Arêas, 2013). Moreover, some researchers changed in the processing technologies like, germination of GF grains and sourdough fermentation (Capriles & Arêas, 2016).

1.4 Gluten free products: technological challenges

Gluten has many different roles in the bakery products, as it is responsible for dough toughness, elasticity, viscosity, and other sensory properties. So the absence of gluten affects the physical properties of gluten-free products badly. In order to imitate gluten functionality the food technologists used many approaches.

First of all, gluten-free starches are used to improve gelling ability, water holding capacity, and thickening and to get specific volume, texture and color. They are mainly starches from potato, quinoa, sorghum, tapioca, chickpea, navy bean, arracacha, arrowroot and others (Horstmann et al., 2017).

On the other hand, various gluten-free flours with different characteristics are used to get better gluten-free products like corn flour, rice flour, legumes flours, amaranth flour, quinoa flour, and other flours used individually or in mixes (Rai et al., 2018). In this regard, maize and rice flours are the best wheat flour replacers. However, the **corn protein** zein has primary structure different from gluten and this **affects negatively the elasticity of dough**. Therefore, to improve the elasticity in corn flour products it is important to add non-gluten high molecular weight glutenine co-protein such as casein (Fevzioglu, Hamaker, & Campanella, 2012). Moreover, using rice flour in gluten free products has two main challenges. Since rice flour bread does not have a preferred flavor, it can be improved by enriching the bread with rice bran which can slow the mineral bioavailability due to the presence of phytic acid (Kadan & Phillippy, 2007).

The gluten-free products lack some vitamins, minerals, and dietary fibers. In this regard highly nutritious pseudo cereals like quinoa, amaranth, and buckwheat are used to improve nutritional quality because they have high biological value proteins (albumins and globulin) (Bergamo et al., 2011). In 2002 Gambus and co-workers replaced corn starch with amaranths flour and they obtained an increase in protein content by 32% and fiber contents by 152% with the same sensory quality (Gambus et al., 2002). Furthermore, the 65% increase in the moisture of gluten free dough was obtained by using amaranth with

quinoa. This improved the dough volume, softness, and nutritional values (Schoenlechner and Berghofer, 2002).

1.5 Soy, casein and glycaemia

There are many studies that tested the effect of soy and soy product on glycaemia and glycemic response. For example, there was an improvement in the glycemic levels in diabetic rats when they had diet enriched with isolated soy protein (Mendes et al., 2014). Furthermore, in 2016 Lacroix and co-workers conducted a review on the foods that can have an effect on glycemia and they found that soy peptides can reduce the glycemic response (Lacroix et al., 2016). Other study demonstrated that the consumption of soy milk with bread reduces the glycemic response (Sun et al., 2015). On the other hand the role of casein in glucose control has been studied in many researches. In 2018 a research used cell screening method to test the effect of casein hydrolysate on the post-prandial glycemia and found a significant reduction concomitant with increased insulin levels. (Drummond et al 2018)

Chapter 2
Aim of the thesis

In the last years the demand of gluten-free products increased more and more, to manage gluten-related disorders and other health conditions. Since, diabetes is highly associated with celiac disease (El Khoury, et al 2018), glycemic control is an important issue for these patients. Since many of the gluten free products have a high glycaemic index (Scaramuzza, et al 2013), developing gluten free products that can control glycaemia is a urgent challenge for food technologists.

Recently, some scientific studies have been conducted focusing on the glycaemia control in people on gluten free diet. Some scientists focused on addition to gluten free products of the prebiotics inulin-type fructans, while others enriched gluten free flours with proteins or dietary fibers (Capriles & Arêas, 2013).

Casein and soy protein hydrolysates to enrich gluten-free products in order to control the glycemia are under investigated despite they have been reported to show a glycemic control effect.

The aim of this study will be to investigate the potential ability to control glycaemia of soy protein and casein hydrolysates added to gluten free biscuits into two different concentrations.

To this purpose the ingredients were characterized physic-chemically (by measurements of water holding capacity and oil holding capacity), the recipes of enriched biscuits were formulated and the prototypes were produced and analyzed through enzymatic digestion in order to follow the digestion of starch and proteins over gastric and intestinal simulated digestion *in vitro*.

Chapter 3

Material and Methods

3.1 Materials

Four ingredients were used in the biscuit recipe. They were: maize flour (Conad Farina di mais, Bergamo and Italy), rice flour (Le Farine Magiche, Frigento AV, Italy), white sugar, chemical dried yeast (PANEANGELI, Genova, Italy). All ingredients were purchased from local supermarket. OPA, pancreatin, pepsin, dithiothreitol, bovine bile extract were obtained from Sigma chemical Co.(Dorset Gillingham, United Kingdom).

3.2 Equipment

Flasks: 200 mL, Test tubes: 10 mL, A 4-decimal analytical balance. Pipettes: 5 mL, 1 mL, and 10 µl, Magnetic stirrer, Spectrophotometer at 340 nm and 540nm, Cuvettes, Water bath capable of maintaining temperature at 37 ± 1 °C and 60 °C, Eppendorf's (1.5 , 2 ml), Falcons (50 ml , 15 ml). Cylinder 100ml, 30ml. centrifuge. pH meter.

3.3 Biscuit Recipes and Preparation

Gluten-free biscuits were prepared using two kinds of gluten-free flours, i.e. rice and maize flours. The ingredients (g) used to prepare 154.32 g of biscuits were 146.25 g flour, 43.75 g white sugar, 18.75 margarine, and 1.25 g yeast. Then this mixture was hydrated by 45 ml of water. Biscuit dough was prepared by hand, cut in round shapes (3.5 cm diameter), transferred in the baking tray and baked for 15 minutes at 190 °C in pre-heated oven.

The same recipe was repeated to prepare soy, and casein containing samples in two different percentages. In the protein-enriched biscuits, 4.5% and 13% of flours were replaced by the soy proteins and casein hydrolysates.

The nutritional composition of the biscuits (except starch content that was chemically analyzed), was determined by considering the macronutrient composition of the recipes. The results are reported in Table 1.

Table 1: macronutrients composition of the different biscuits for 100 gm

Macronutrient	Control	4.5% protein hydrolysate	13% protein hydrolysate
Energy	325 kcal	327.5 kcal	331.7 kcal
Fat	7.6 g	7.6 g	7.5 g
Proteins	4.1g	8.2 g	16.1 g

3.4 Methods

3.4.1 Determination of Water Holding Capacity and Oil Holding Capacity

The determination of water holding capacity (WHC) and oil holding capacity (OHC) was conducted according to Fuentes-Alventosa et al., (2009) with some modifications. In summary, 200 mg of each sample and 12 ml of distilled water were mixed and stirred for 24 hours at room temperature. After that, the mixtures were centrifuged at 4000 rpm for 45 min. Finally, the wet precipitates were weighted after removing the supernatants. The weight difference between wet pellet and the initial sample represents the WHC that was expressed as mL of water per gram of sample.

For OHC measurement, sunflower seeds oil (12 ml) was mixed with 200 mg of sample, stirred and held for 24 hours at room temperature. Then sample was centrifuged for 45 minutes at 4000 rpm. The weight difference between oil contained in the final sample and the starting sample weight was calculated to determine the OHC that was expressed as mL of sunflower seeds oil per gram of sample.

3.4.2 In vitro Digestion

In vitro sequential enzymatic digestion of the biscuits was performed according to Minekus et al. (2014) with some adjustments. This method was done in three phases oral, gastric, and intestinal phases.

Oral phase:

2.5 grams of sample were weighted in 50 ml falcon tubes and mixed with 2.5 ml of distilled water. The mixture was shaken in a water bath at 37°C for 2 minutes (160 rpm).

Gastric phase:

At pH (3), 3.75 mL of Simulated Gastric Fluid (SGF) stock solution, 2.5 µl of 0.3 MCaCl2 solution, 347.5 µl of distilled water and 0.8 mL of pepsin (25000 U/mL) (dissolved in SGF) were added to the oral phase mixture, to a final volume of 10 mL. The sample was maintained in a shaking water bath at 37°C for 2 hours (130 rpm). After this period, the pH of the solution was adjusted to 7 by using 1 M NaOH solution.

Intestinal phase:

5.5 ml of Simulated Intestinal Fluid (SIF) stock solution, 20 µl of 0.3 M CaCl 2 solution, 0.655 ml of distilled water, 1.25 ml of 160 mM bile extract, 2.5 ml of pancreatin (800 U/ml) and 32,5 µL of amyloglucosidase (3300 U/mL), were added to the gastric chyme to a final volume 20 ml. The sample was maintained in a shaking water bath at 37°C (100 rpm). Aliquots (200 µL) were taken at 0, 15, 30, 60, 90, 120 and 180 min intervals and mixed with absolute ethanol (800 µL) to stop the digestion. These solutions were centrifuged at 800g for 10 min and were stored at -40°C before OPA and glucose analysis.

3.4.3 Determination of Total Starch

The methods described by Goni et al., (1997) and Holm et al. (1986) were used. Each sample (100 mg) was mixed with 6 ml of 2 M KOH and shaken at room temperature for 30 minutes. After that, according to Holm et al. (1986) 3 ml of 0.4 M sodium acetate buffer PH 4.75 was added with about 3,5 mL of HCl 2 M to obtain solution pH 4,75. Then, 85 µl of amyloglucosidase (EC 3.2.1.3., 3300 U/ mL, Megazyme) was added. The samples were maintained at 60 °C in a water bath with shaking at 100 rpm.

Aliquots (200 µL) were taken and added to 800 µL of absolute ethanol in order to be analyzed by glucose oxidase-peroxidase assay.

3.4.4 Glucose Analysis

Sample preparation: First, the collected aliquots were centrifuged at 14800 rpm per 10 min (4°C). Second, sodium acetate 0.4 M buffer solution was prepared and adjusted to reach

5.2 pH. After that, 100 µl of amyloglucosidase (3300 U/mL) was added to 10 ml of sodium acetate buffer prepared previously. Then 100 µl of each sample supernatant was placed in 0,5 mL of a solution that was made of sodium acetate buffer with the enzyme as described above.

The reaction continued for 30 minutes at room temperature before filtering the sample with cellulose acetate 0,22 µm filters.

Glucose Analysis:

Glucose oxidase-peroxidase assay was used in order to determine the hydrolyzed glucose (as following) and 0.9 conversion factor was used to measure starch content in the samples.

Glucose Determination

Glucose concentration in the digested samples was determined by a GLUCOSE ASSAY KIT (Sigma, Dorset Gillingham, United Kingdom). Glucose oxidase/peroxidase reagent was prepared by using dissolving the contents of the capsule in an amber bottle with 39.2 ml of deionized water and adding 0.8 ml of a solution composed of 5 mg of o-dianisidine dihydrochloride in 1 ml of distilled water to the solution.

The glucose oxidase/peroxidase reagent (400 µL) was added to 200 µL of diluted samples with 30 seconds intervals, then the reaction continued for 30 minutes at 37°C. After that, the reaction was stopped by adding 12 N H2SO4 in 30 seconds interval. Finally, the absorption was measured by spectrophotometer at 540 nm.

3.4.5 Determination of protein hydrolysis

Protein hydrolysys was assessed in the digesta collected from the gastric phase at 0, 15, 30, 60, 120 min and the intestinal phase at 0, 15, 30, 60, 90, 120, 180 min. Hydrolysed proteins were determined by using the OPA method described by Nielsen (2002).

Diluted samples (50 µL) were incubated for 2 minutes in 950 µL OPA reagent and absorbances were read on a spectrophotometer at 340 nm. OPA reagent was prepared by dissolving 3.81g Borax and 1g SDS in 80 ml of distilled water with the addition of 2ml of

ethanol contains 80 mg of OPA, 88mg of DTT, and 18 ml of distilled water to reach a final volume 100 ml. All these reagents were mixed and the final solution was filtered using a syringe with a 0.45µl filter and stored in a dark container. The standard curve was conducted with an Isoleucine stock solution of 10000 ppm and using solutions in the range 20-300 ppm.

3.4 Statistical Analysis

All the analyses were performed in triplicates and results were expressed as mean ± standard deviation. Statistical analysis was carried out using Excel statistical software and the analysis of variance ANOVA, Tukey test ($p<0.05$) was conducted to determine the differences between biscuits. In addition bivariate correlation analysis was performed between results from glucose and protein hydrolysis analysis and a level of $p<0.05$ was set as significant between variables.

Chapter 4

Results and Discussion

4.1 Water Holding Capacity and Oil Holding Capacity

Figure1 shows the results of WHC for the different samples. Casein 4.5% showed the highest WHC followed by soy 4.5%, and soy 13% which was similar to the control. Casein 13% exhibited the lowest value.

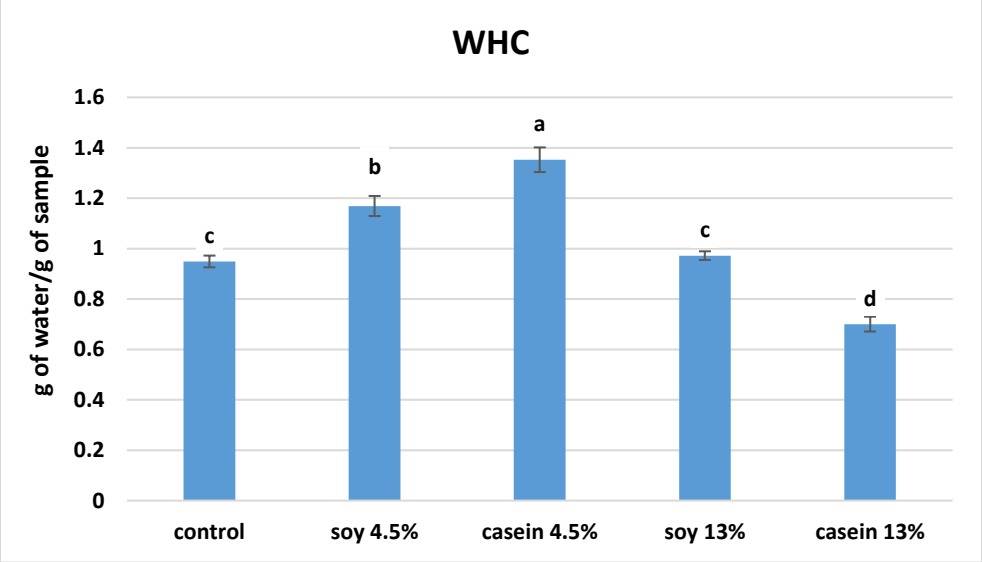


Figure 1: Water holding capacity of the different types of biscuit. Different letters on the bars indicate significant differences between samples with $p<0.05$ by ANOVA and Tukey post hoc test

OHC results are reported in figure 2. While 13% of soy and casein samples had the highest OHC with no significant difference between the two different protein hydrolysates added in the biscuits. Followed by 4.5% soy (P value <0.005) then 4.5% casein which is equal to the control sample. Which mean the higher protein amounts more oil holding capacity.

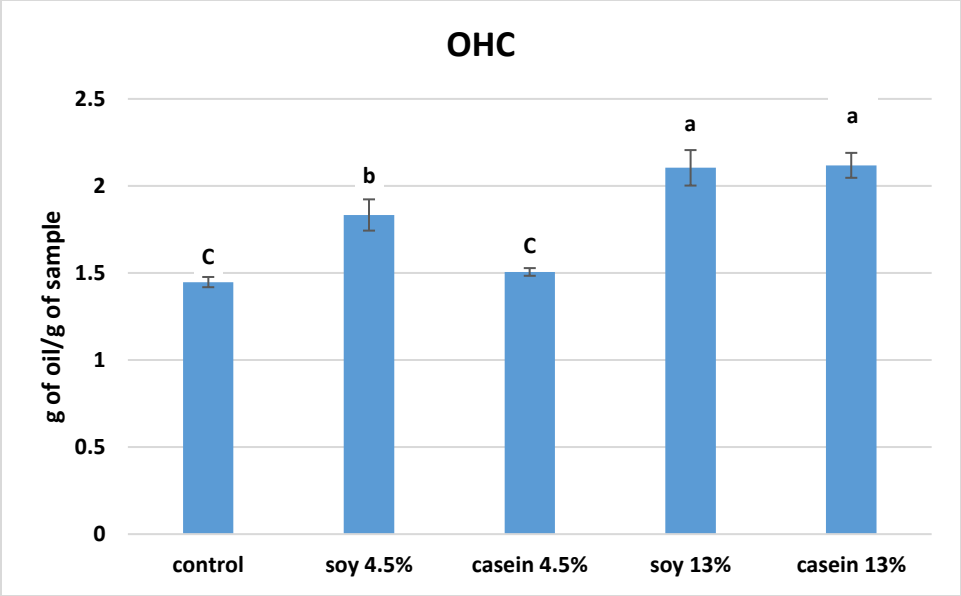


Figure 2: Oil holding capacity of different types of biscuit. Different letters on the bars indicate significant differences between samples with $p<0.05$ by ANOVA and Tukey post hoc test

The data suggested a negative correlation between the protein percentages in the samples and WHC. Yin et al. (2008) got the same results when they tested hemp proteins hydrolysate. They ascribed the reduction in WHC after adding the protein hydrolysate to the fact that protein hydrolysis process may solubilize the hydrophilic groups which are responsible of water attraction and WHC. In addition to that, Hera et al. (2013), Jamal et al. (2016) and Tang et al. (2012) report WHC reduction in peanut with no explanation for this effect. Furthermore, the reduction of the protein molecular mass can decrease the WHC by reducing the physical ability to trap water or, contrarily, it can increase the availability of hydrophobic groups negatively impacting the WHC of protein hydrolysates (Wouters et al. (2016).

Finally, increased OHC with higher amount of hydrolysates may is related to the increase of the availability of the hydrophobicity in the sample which helps in oil entrapping. (Wouters et al., 2016).

4.2 Total Starch

Total starch content of each sample was measured and reported in the Table 2. Total starch in the control sample was around 0.51 mg per mg of sample and was similar to that present in the biscuits added with 4.5% of both soy and casein hydrolysate. As expected, the 13%-enriched samples had lower starch because of the replacement of the starch sources (rice and maize flours) by the protein hydrolysates.

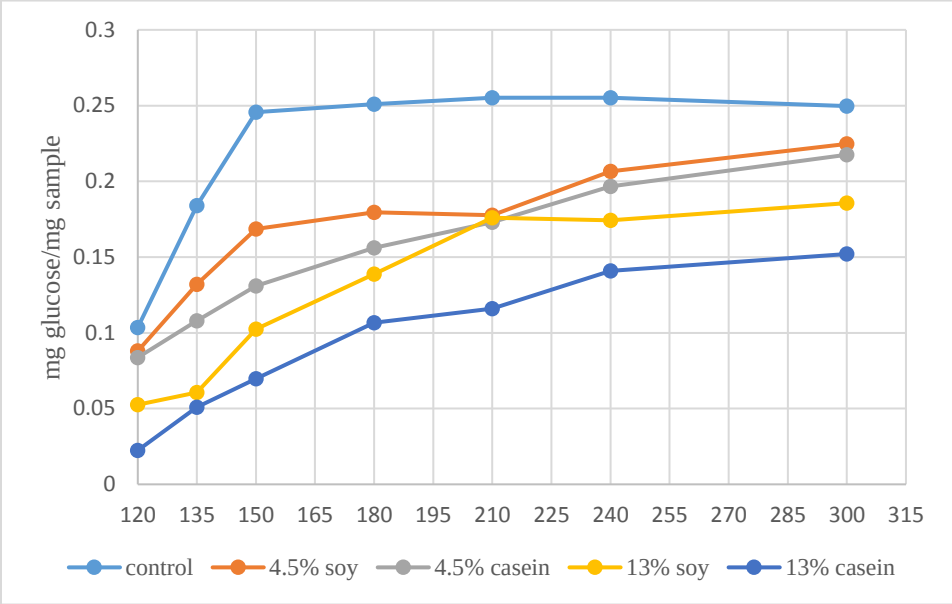
Table 2: Total starch mg/mg sample

Biscuit	**STARCH (mg/mg biscuit)**
control	0.51
soy 4.5%	0.50
soy 13%	0.44
casein 4.5%	0.50
casein 13%	0.48

Starch digestion in the intestine

The digestion of starch was monitored by the concentration of glucose in the digesta collected over time during the intestinal phase (from 120 min to 300 min). The data expressed per mg of sample at each time point and the AUC of glucose are reported in (Figure 3 A and B respectively). It shows that the hydrolysis of starch is higher for control biscuits followed by 4.5% soy, 4.5% casein, 13% soy and 13% casein. A significant difference between the samples was found in the AUCs of glucose released from each biscuit.

A)



B)

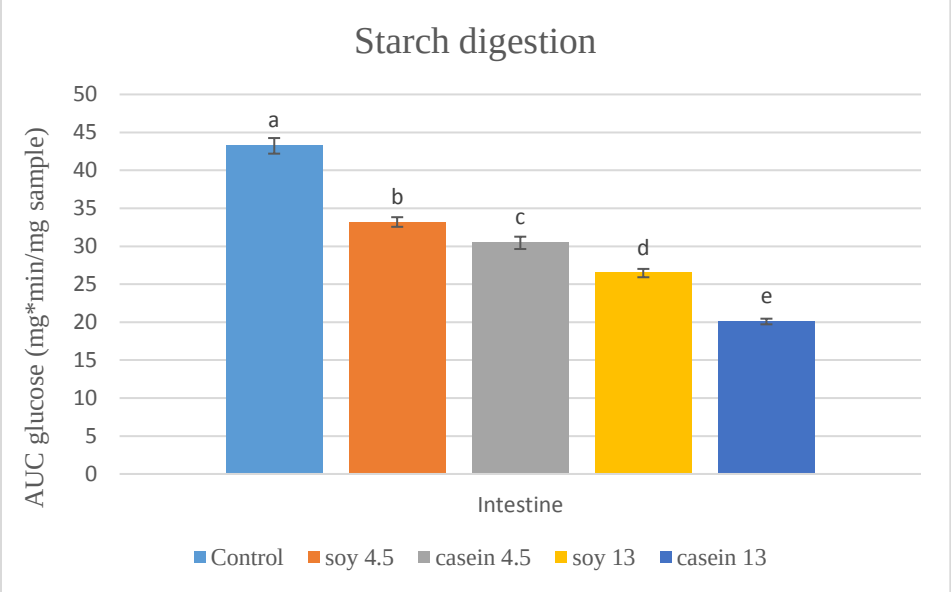
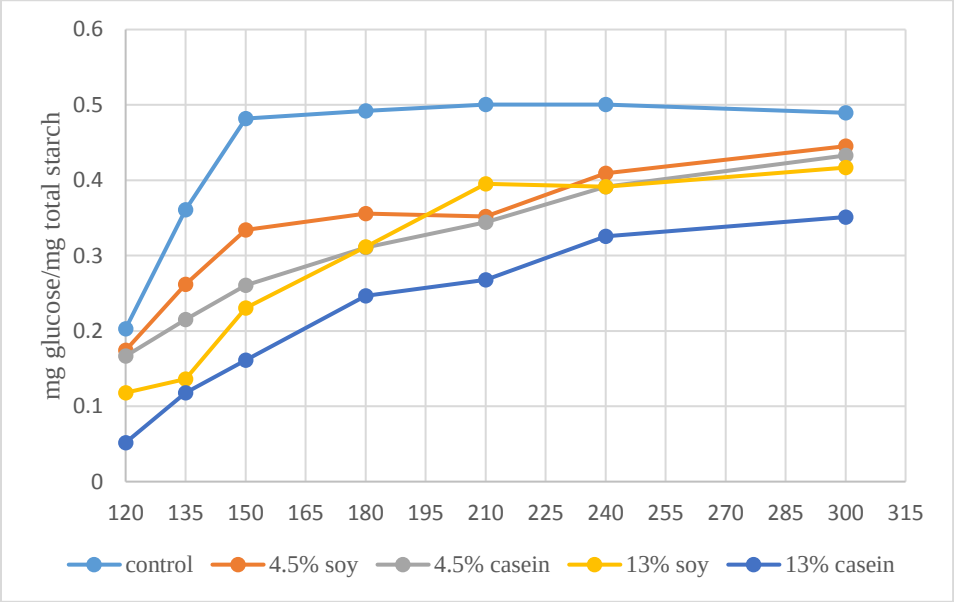


Figure 3: Starch digestion over time expressed as mg glucose /mg sample (A) and AUC of glucose (B). Different letters on the bars indicate significant differences between samples with $p<0.05$ by ANOVA and Tukey post hoc test

In an attempt to evaluate the effect of protein on starch hydrolysis, the amount of glucose released from the biscuits over digestion was normalized for both the starch and the amount of proteins into the samples and results are reported in Figure 4 and Figure 5, respectively. Figure 4A shows that the rate of starch hydrolysis in the 5 biscuits follows the order (control > (4.5% soy, 4.5% casein, and 13% soy)> 13% casein. The percentage of starch hydrolysis was compared to that of white bread (reference food to calculate glycaemic index of starchy foods, Wolever et al., 2001) and the glycaemic index was measured (Figure 4B). Data showed that all the biscuits here developed can be classified as products at medium glycaemic index because their GI are all within the range 55%-70%. However, the addition of 4.5% soy or casein hydrolysates significantly decreased the GI in comparison with the control sample. The same effect was observed with the addition of 13% soy hydrolysate. The lowest reduction of the glycemic index was shown by addition of casein at a concentration of 13%.

A)



B)

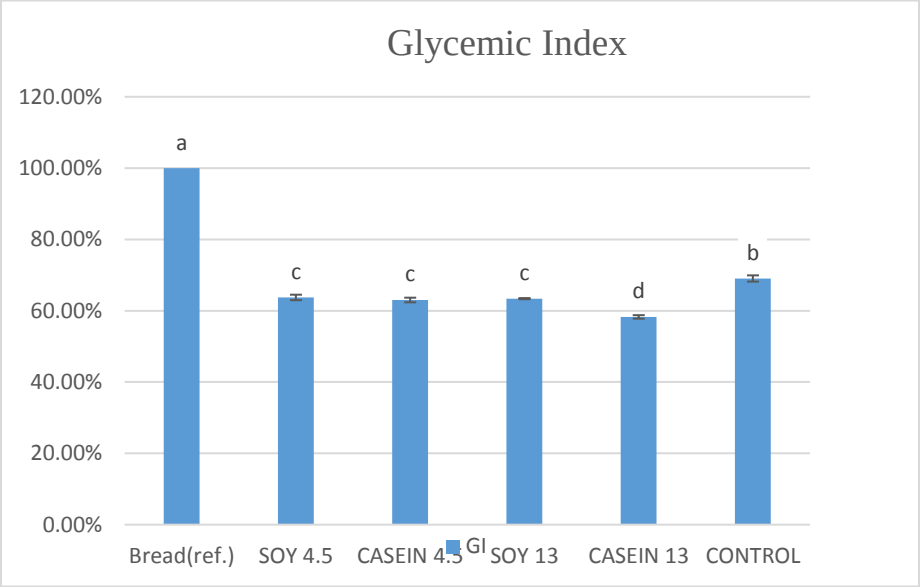


Figure 4 Starch digestion over time expressed as mg glucose /mg starch (A) and glycaemic index of the biscuits compared to white bread (B). Different letters on the bars indicate significant differences between samples with $p<0.05$ by ANOVA and Tukey post hoc test.

We hypothesized that the reduction of the GI by addition of soy protein and casein hydrolysates was due to bioactive peptides already present in the ingredient or forming over digestion which inhibited the activity of enzymes involved in starch digestion.

Indeed, soy protein possesses peptides (LLPLPVLK, SWLRL, and WLRL) which exhibit α-glucosidase activity inhibition (Wang, et al., 2019). Moreover, in another study low molecular weight (< 5 kDa) peptides in soy inhibited DPP-IV, α-amylase, maltase, α-glucosidases (González-Montoya et al., 2018). Similarly, milk-derived bioactive peptides suppress the functions of alpha-glucosidase and DPP-IV (Patil, Mandal, Tomar, & Anand, 2015).

This hypothesis was supported by data obtained normalizing the concentrations of glucose and the AUC of glucose from each biscuit by the amount of proteins in the relative biscuit. The results, reported in figure 5, show that there was a dose dependent effect of soy protein and casein hydrolysates in reducing starch hydrolysis indeed the glucose released by the

biscuit with soy 13% and casein 13% were both higher than that released from the relative biscuits having the hydrolysate at 4.5%.

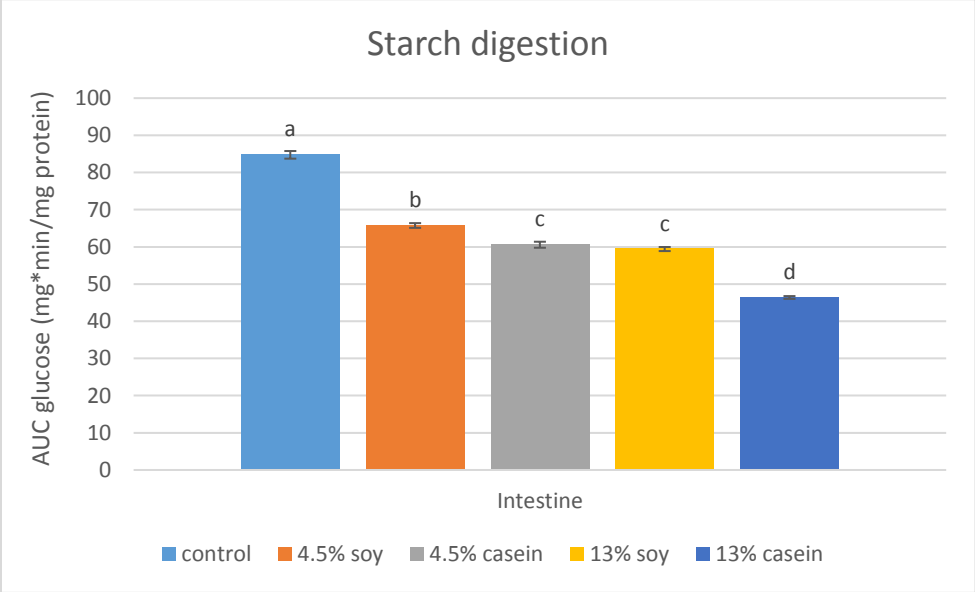


Figure 5: AUC of glucose normalized by the amount of protein in the sample (B). Different letters on the bars indicate significant differences between samples with $p<0.05$ by ANOVA and Tukey post hoc test

4.4 Protein Hydrolysis

Protein hydrolysis in biscuits was monitored over time along gastric and intestinal digestion by determining the concentration of free amino groups (FAG) in the digesta. Results are reported in Figure 6.

Figure 6A shows that the FAG released in the intestinal phase are always higher than in the gastric phase. That is because during gastric digestion pepsin enzyme was used and this enzyme is an endopeptidase which breaks the peptidic bonds inside the proteins and large peptides to produce more peptides, while in the intestinal phase pancreatin enzyme contain other enzymes which continue the peptide digestion and also produce FAG (Berg et al., 2002**).** Figure 6 also shows that, as expected, the curve of FAG and the AUC obtained from digestion of biscuits with higher content of proteins are higher than the others. In other words more protein hydrolysates was added more free amino acids were released.

Moreover**,** all the samples but control were digested slowly in the intestinal phase. This observation indicates that the addition of hydrolyzed proteins reduce protein digestion in the intestinal phase. This result can be related to the ability of protein hydrolysate in the intestinal phase to bind the bile acids which may lead to slow down the digestion as reported in Pak et al. (2005). They found that some peptides from soy protein such as 11S-Globulin have the ability to reduce blood glucose through its binding with the bile acids in the intestine. In addition, casein hydrolysate has binding affinity and binding capacity to bile acid (Lanzini, Fitzpatrick, Pigozzi, & Northfield, 1987).

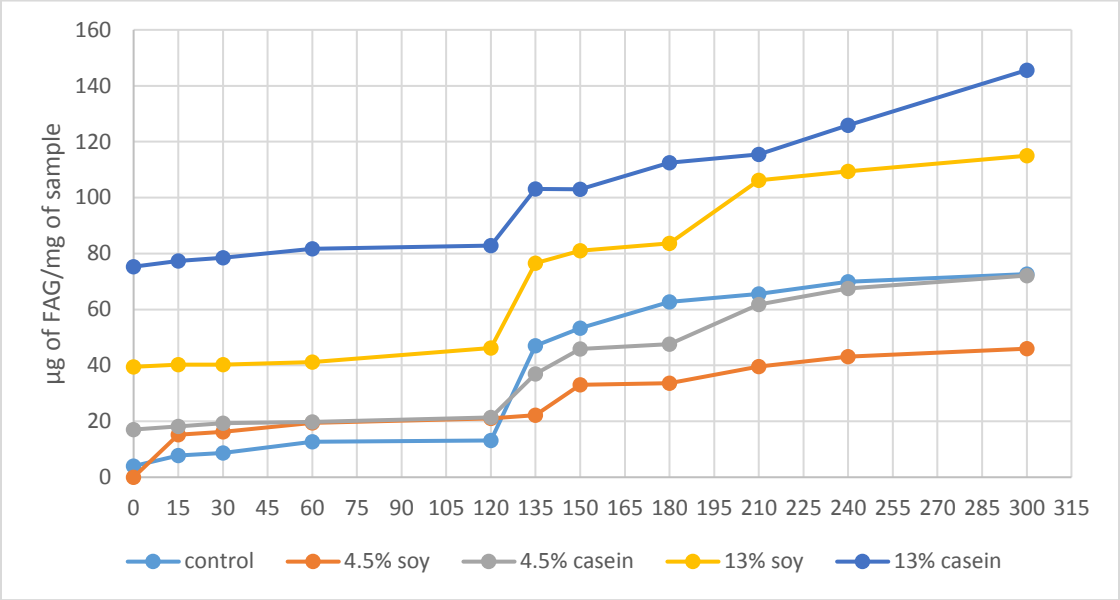
Finally**,** during gastric phase there is no variation or difference between 4.5% soy and 4.5% casein unlike the intestinal phase where biscuits with casein 4.5% released more FAG than the soy counterpart. Moreover, both 4.5% soy and casein showed a lower protein digestibility than the control biscuit in the intestine despite they contained a double amount of proteins. The lower digestibility of soy samples than casein samples may be related to the fact that soy protein has some proteases inhibitors like Lunasin and Bowman-Birk peptides (Hernández-Ledesma et al., 2009).

Normalizing the results by the amount of protein in the biscuits, data showed that all curves (Figure 7A) are different except some time points in the gastric phase mainly for the curve of casein 13% as well as for soy 4.5% and casein 13% during the intestinal phase.

From the beginning of the time course, casein sample released higher amounts of FAG than soy samples at the 13% concentrations. We hypothesized that this might be due to the presence of some protease inhibitor peptides in soybean as previously shown by Hernández-Ledesma et al., (2009). Despite this initial higher amount of casein, the digestion rate of soy and casein are similar over time.

On the other hand, the intestinal digestion of proteins in the control sample is the strongest because it released around double amount of FAG than other samples. Considering digestion within the same hydrolysate, as expected, there was a dose dependent effect of FAG releasing.

A)



B)

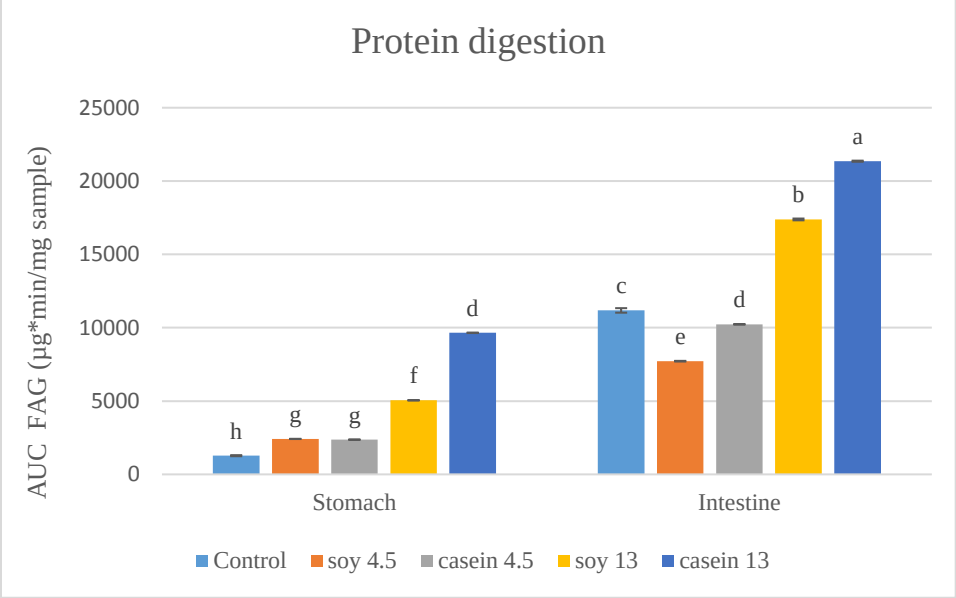
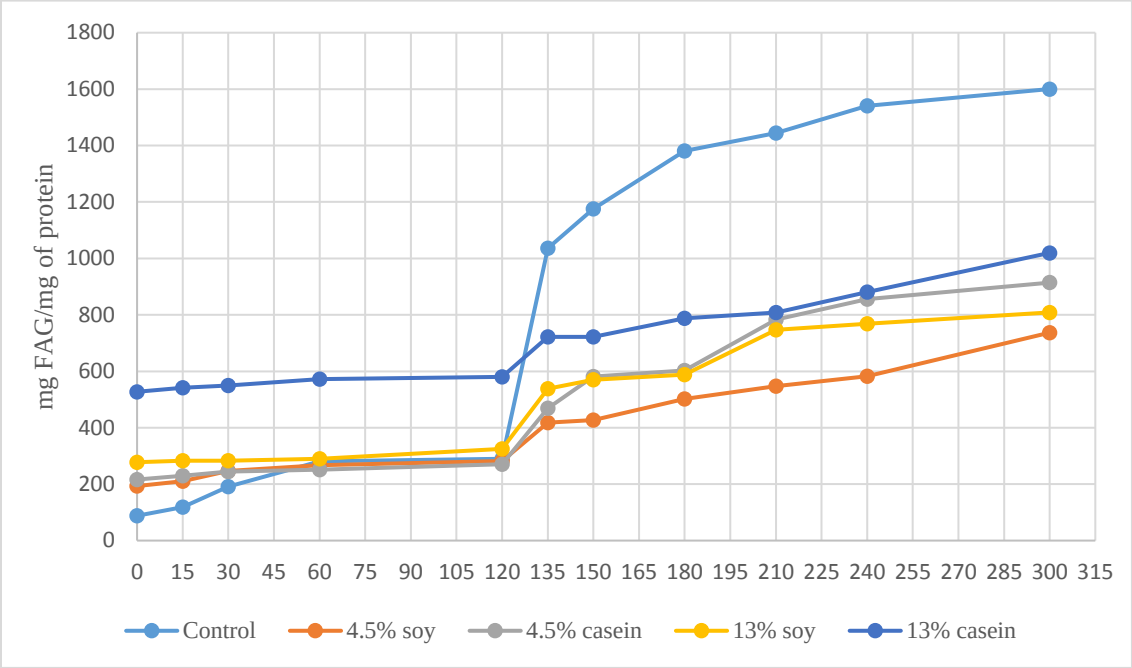


Figure 6: Amount of free amino groups (FAG) available during time (A) and AUC of free amino groups (FAG) available during digestion. Different letters on the bars indicate significant differences between samples with $p<0.05$ by ANOVA and Tukey post hoc test

A)



B)

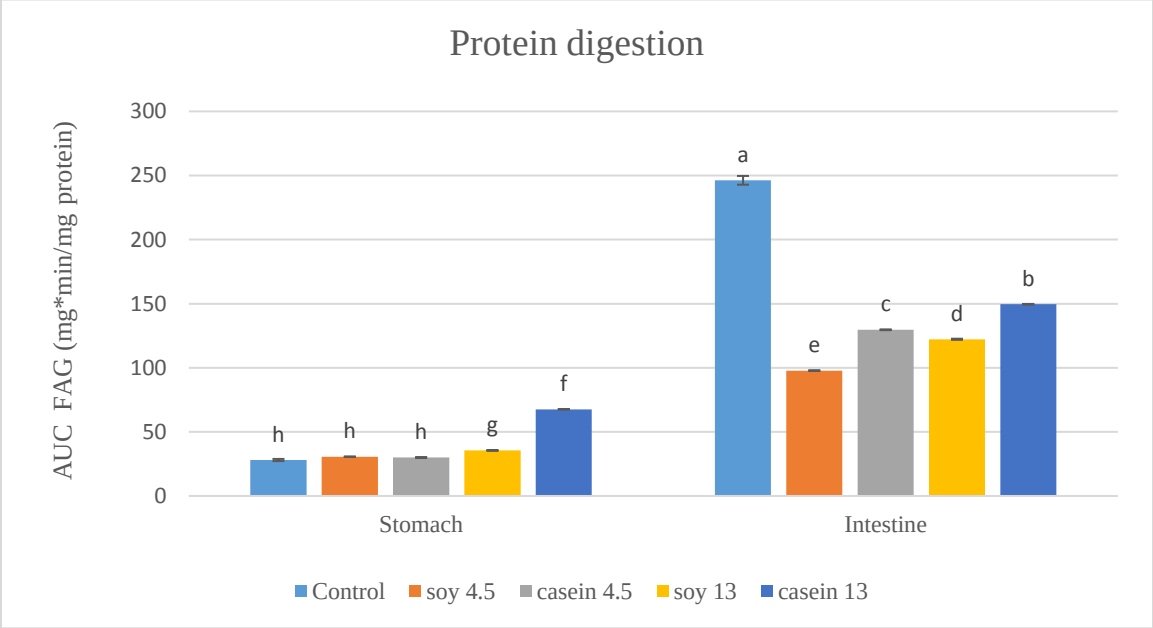


Figure 7: Protein digestion over time expressed for mg free amino groups (FAG) available per amount of proteins in the biscuits (A) and AUC of FAG. Different letters on the bars indicate significant differences between samples with $p<0.05$ by ANOVA and Tukey post hoc test.

Correlation between protein and starch digestion over time

To evaluate the relationship between starch and protein digestibility a correlation analysis of the data was performed and result is reported in Figure 7.

It shows a negative correlation between AUC of glucose and AUC of FAG released during digestion of samples per amount of sample. In other words, more FAG are delivered lower is the glucose available for the absorption. This result may be related to the presence in soy and casein hydrolysates of bioactive peptides, which inhibit the activity of enzymes responsible of starch hydrolysis (α-glucosidase and α-amylase) as discussed above.

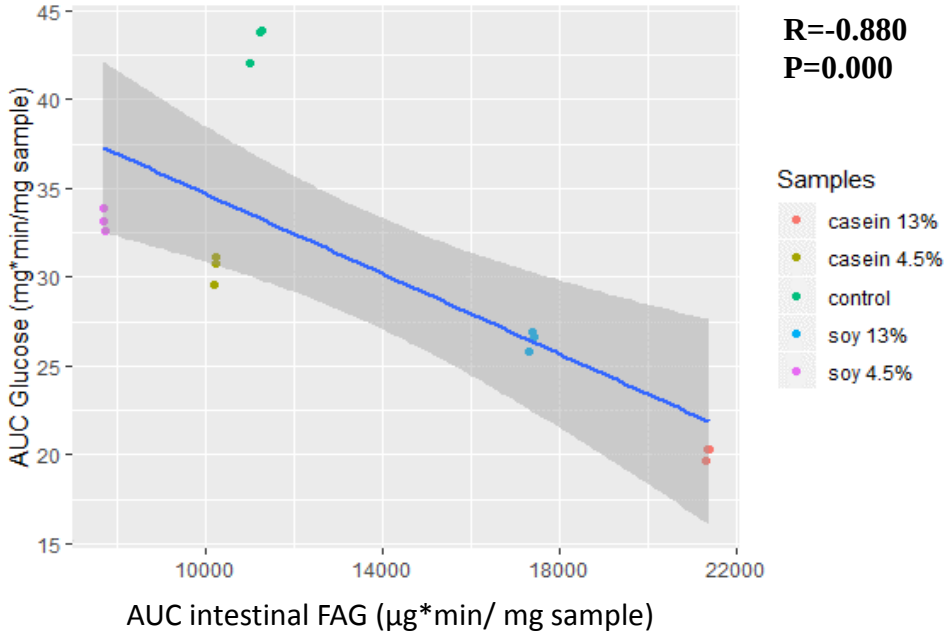


Figure 7: Correlation between AUC glucose and AUC FAG in the intestinal digestion

Chapter 5
Conclusions

The addition of protein hydrolysates to the recipe of gluten free biscuits based on a blend of rice and maize flours affects biscuits properties.

Compared to the control biscuit, the addition of the protein hydrolysates:

- Reduces the water holding capacity with an effect that is in the order casein 4.5% > soy 4.5%> soy 13% > casein 13%)
- Increases oil holding capacity with an effect in the order 13% soy and 13% casein > 4.5% soy > 4.5% casein and control.
- Reduces the glycemic index of the biscuits that was in the order control > 4.5% soy > 4.5% casein > 13% soy > 13% casein. We hypothesized it was due to the ability of some soy and casein peptides to inhibit the digestive enzymes α-glucosidase and α-amylase.
- Increases the concentration of free amino groups in the intestinal phase (mainly 13% casein and 13% soy) but halves the rate of protein digestion (mainly casein hydrolysate) that is in the control > 13% casein > 4.5% casein > 13% soy > 4.5% soy.

Interestingly, a negative correlation between protein digestibility and starch digestion was found in this study.

Altogether, the data indicated that the addition of soy protein and casein hydrolysates to gluten free biscuits based on rice and maize flours can further reduce the glycaemic index of the biscuits in a dose dependent manner. This is explained by the modification of the physical structure of the product that affects the digestion of proteins and starch as well as by the delivery, during the digestion, of bioactive peptides which can inhibit the activity of enzymes responsible of starch digestion.

Chapter 6

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كلية الدراسات العليا

درجة الماجستير في التغذية وتكنولوجيا الغذاء

تطوير بسكوت خال من الغلوتين له القدرة على تنظيم سكر الدم

الطالبة: ايمان شوخة ، رقم الجامعي 11750135

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