Effect of enzymatic and physical gelation methods and Xanthan gum in the gelation capacity of *Acheta domesticus* flour

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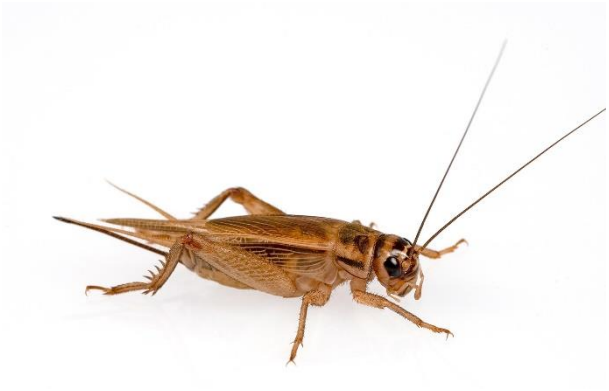

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Todas as correções determinadas pelo júri, e só essas, foram efetuadas.
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Resumo

A população mundial está em constante crescimento e, assim, é necessário fazer uma gestão sustentável dos recursos alimentares disponíveis. Para isso, alternativas à proteína animal convencional, mais sustentáveis e com caraterísticas nutricionais igualmente positivas, como os insetos, estão a ser amplamente estudadas. Uma possível aplicação dos insetos, ainda pouco explorada, é a sua incorporação em géis alimentares que podem, posteriormente, ser utilizados como componente em outros produtos alimentares. Dessa forma, o objetivo desta dissertação foi estudar a capacidade gelificante do pó de *Acheta domesticus* quando submetido a diferentes tratamentos físicos, enzimáticos e/ou combinados com goma xantana. Na primeira fase, tratamentos físicos, temperatura (85ºC) e altas pressões hidrostáticas (300 e 500 MPa) e tratamentos enzimáticos, protéases (Alcalase, Flavourzyme e iZyme BA) e polimerases (transglutaminase e glucose oxidase) foram aplicados individualmente e de forma combinada a uma solução de pó de *Acheta domesticus,* 2% w/v NaCl e pH 7. Os resultados foram expressos em quantidade de sólidos solúveis em água presentes no sobrenadante da solução centrifugada. Numa segunda fase, tendo em conta os resultados da primeira fase, onde foram eleitos os tratamentos mais vantajosos, foi combinada com o pó de *Acheta domesticus,* goma xantana em diferentes concentrações. Esta solução foi submetida a tratamentos de temperatura (85ºC) e de pressão (300MPa) com e sem enzimas (iZyme BA e glucose oxidase) com a adição da goma xantana em dois momentos diferentes. Foram realizadas análises de viscosidade, firmeza, concentração de sólidos solúveis em água e capacidade de retenção de água. Na primeira fase, a menor concentração de sólidos solúveis, foi encontrada nos tratamentos físicos de temperatura e pressão de 300 MPa com a combinação de enzimas iZyme BA e glucose oxidase. Na segunda fase, verificou-se um aumento da viscosidade da solução com o aumento da concentração de goma xantana, uma alteração da estrutura interna da solução com a adição das enzimas incubadas e um aumento da reatividade da xantana com o aumento da exposição ao calor. A aplicação de pressão levou a uma diminuição de textura e viscosidade. Após centrifugação verificou-se a formação de um gel a 3% w/w de goma xantana em ambos os tratamentos físicos. Em conclusão, verificou-se que dependendo da técnica de manipulação aplicada ao pó de *Acheta domesticus* diferentes perfis são revelados o que reflete a sua versatilidade para aplicação em diferentes produtos alimentares com diferentes propósitos.

Palavras-chave: *Acheta domesticus,* goma xantana, gel alimentar, entomofagia

Abstract

The world population is constantly growing and, therefore, it is necessary to make a sustainable management of the available food resources. For this, alternatives to conventional animal protein, more sustainable and with equally positive nutritional characteristics, such as insects, are being widely studied. A possible application of insects, still little explored, is their incorporation in food gels that can later be used as a component in food products. Thus, the objective of this dissertation was to study the gelling capacity of *Acheta domesticus* flour when subjected to different physical, enzymatic, and/or combined treatments with xanthan gum. In the first phase, physical treatments, temperature (85ºC) and pressure (300 and 500 MPa) and enzymatic treatments, proteases (Alcalase, Flavourzyme and iZyme BA) and polymerases (transglutaminase and glucose oxidase) were applied individually and in combination with a solution of *Acheta domesticus* flour 2% w/v NaCl and pH 7. The results were expressed as the quantity of water-soluble solids present in the supernatant of the centrifuged solution. In a second phase, considering the results of the first phase, the most advantageous treatments were chosen and then *Acheta domesticus* powder was combined with xanthan gum at different concentrations. This solution was subjected to temperature (85ºC) and pressure (300MPa) treatments with and without enzymes (iZyme BA and glucose oxidase) with the addition of xanthan gum at two different times. Analysis of viscosity, firmness, concentration of water-soluble solids and water binding capacity were performed. In the first phase, the lowest concentration of soluble solids was found in the physical treatments of temperature and pressure of 300 MPa with the combination of enzymes iZyme BA and glucose oxidase. In the second phase, there was an increase in the viscosity of the solution with the increase of the concentration of xanthan gum, a change in the internal structure of the solution with the addition of the incubated enzymes and an increase in the reactivity of xanthan with an increase of the exposure to heat. The application of pressure led to a decrease in texture and viscosity. After centrifugation, a 3% w/w xanthan gum gel was formed in both physical treatments. In conclusion, it was found that depending on the processing technique applied to *Acheta domesticus* powder different profiles are revealed which reflects its versatility for application in different food products with different purposes.

Ketwords: *Acheta domesticus*, xanthan gum, food gel, entomophagy

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List of abbreviations

A: Alcalase

AD: *Acheta domesticus*

BA: iZyme BA

C: Control Sample

$CaCl_2$: Calcium Chloride

CFU: Colony Forming Unit

CP: Cricket Powder

cP: Centipoise

EFSA: European Food Safety Authority

EU: European Union

FAO: Food and Agriculture Organization of the United Nations

Fla: Flavourzyme

g: Grams

GHGs: Green-House Gases

GO: Glucose Oxidase

H: Heat treatment

HHP: High Hydrostatic Pressure

HPP: High Pressure Procedure

NaCl: Sodium Chloride

P: Proteases

Poly: Polymerases

RVA: Rapid Viscoanalyzer

SDGs: Sustainable Development Goals

SDS-PAGE: Sodium Dodecyl Sulphate- Polyacrylamide Gel Electrophoresis

SS: Saline Solution

TGase: Microbial Transglutaminase

WBC: Water Binding Capacity

WRC: Water Retention Capacity

WSS: Water Soluble Solids

WTT: Willingness to try

XG: Xanthan Gum

ºC: Degree Celsius

1.Introduction

1.1 Entomophagy

1.1.1. The need of new protein alternatives

The world is facing an environmental and humanitarian crisis. Knowing that, in 2015 the United Nations Member States adopted the 17 Sustainable Development Goals (SDGs) to ensure the goals of no poverty, peace and a healthy planet by 2030. One of the SDGs is zero hunger where the objective is to “end hunger, achieve food security and promote sustainable agriculture”. Food security must be approached by the quantitative and the qualitative direction that are extremely dependent of each other. The first one focuses on the access of the proper amount of food to meet the physiological need of a population or age group and the second one is related with the nutritional quality of the food consumed in a way that the health of the population is not affected (FAO, 2001). According to FAOs state of food security and nutrition in the world (2020) the people facing food insecurity is increasing gradually since 2014 with about 8.9% of the world population hungry and roughly 2 billion people with no access to nutritious and quality food (Figure 1). According to FAO report (2019) the main drivers for the hunger problems, mainly in the developing countries, are the conflicts, the natural disasters caused by the increasing anthropogenic pollution and the economic shocks, distributed in different proportions around the world (figure 2). Besides that, it is predicted that the world population will increase at least 34% than today especially in developing countries. To feed all this future population, the food production would have to increase more than

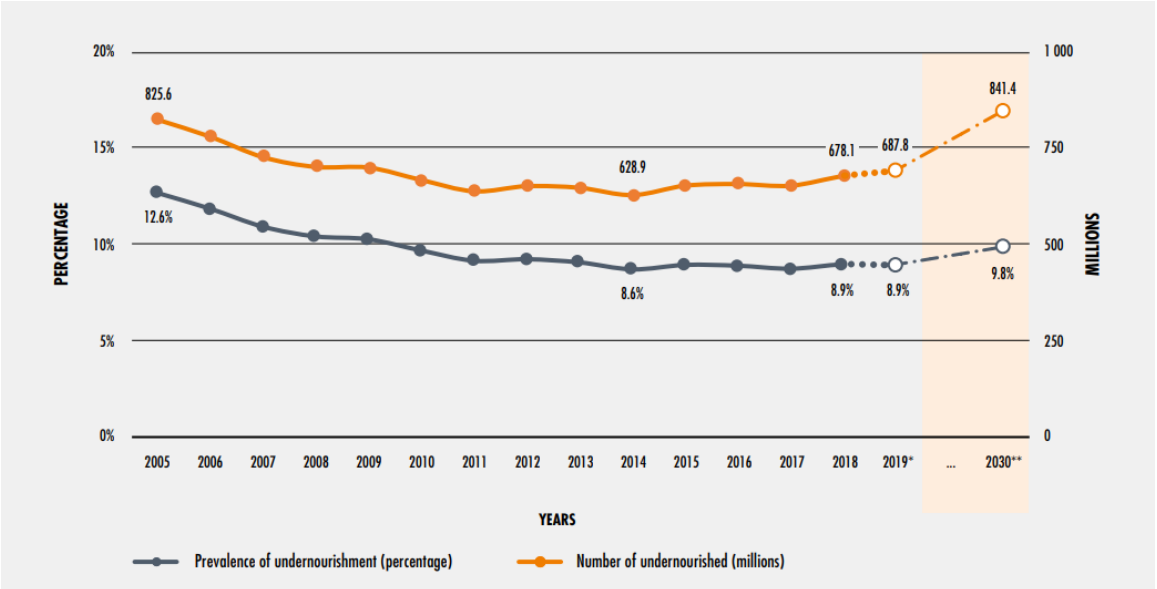

Figure 1. Number of undernourished people in the world since 2015 to 2019 with 2030 prospects. (source: FAO, 2020)

70%. This means that, if the food habits continue like they are now, the production of meat, the ultimate responsible for the emission of green-house gases (GHGs) is projected to increase by 80% (Tilman & Clark, 2014). Apart from that, the natural resources, as water and land, will be scare faster than expected and will comprise the needs of the population and the world (Tao & Li, 2018) . So, any alternative to food that is nutritionally interesting and have a sustainable production system should be explored (Simion *et al.*, 2019)

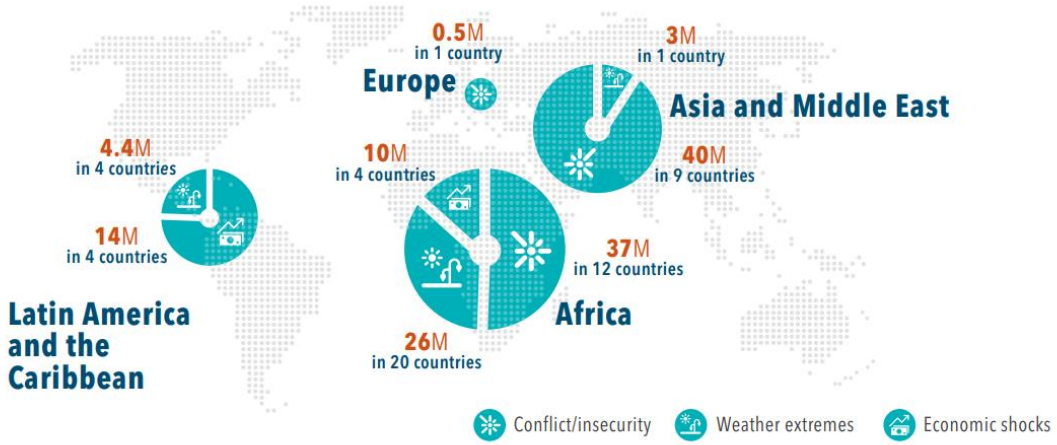

Figure 2. Main drivers for world's undernutrition. (source: FSIN, GRFC 2020)

1.1.2 Insects as food alternative

According with the information in chapter 1.1 a sustainable way of eating, with protein sources that have lower impact on the environment need to be explored, so insects appear to be a good alternative (Miglietta *et al.*, 2015). Insects are good nutritional alternatives to the conventional meat, they are not healthier but they are equally good for health (Payne *et al.*, 2016). They are good sources of protein, high in monounsaturated and polyunsaturated fatty acids, show an amino-acid profile appropriated for humans and a complete micronutrient content with high values of zinc, iron, among others (Zielinska *et al.*, 2015). Even though entomophagy (the practice of eating insects by humans and no humans) appears to have a lot of advantages, some disadvantages are strong, and both will be discussed in the next two chapters.

1.1.3 Advantages of entomophagy

These animals show a huge economic and sustainable advantage that makes them a really good alternative to the production of the main animal proteins (beef, pork, and chicken). This last demonstrates a large impact in the environment because of the high quantities of food, land and water needed to produce that kind of protein and, as important, because of the GHGs emitted (carbon dioxide, methane and nitrogen oxide) (Wegier *et al.*, 2018). The animal protein sector is responsible for most of the GHGs emissions inside the food sector which is the least sustainable (figure 3). Insects, economically speaking, display a very gainful rate of food conversion, a high quantity of off springs that acquire maturity in very few days (Bessa *et al.*, 2017). Also, their food resources are cheapest than other animals. In terms of environment friendly production, the main focus is the low emission of GHGs that show a huge difference when compared with other meats (Oonincx, 2015). Likewise, they use quite small areas of land to produce big quantities of insects and their needs in water are almost insignificant because they can utilize the humidity that they gain from food (Halloran, 2017; Tao & Li, 2018) (figure 4).

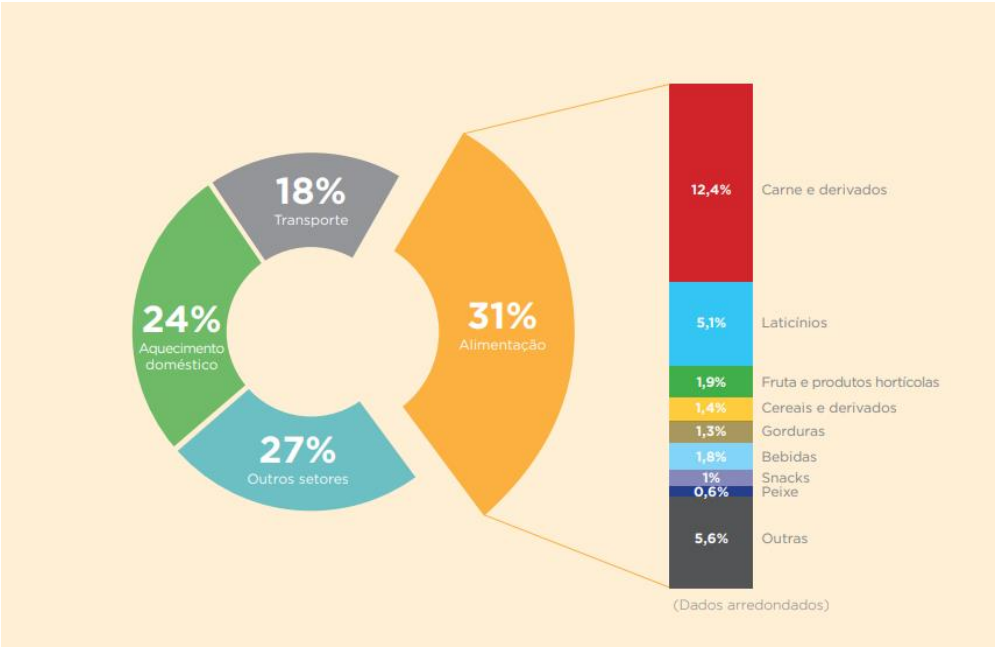

Figure 3. European emissions of green-house gases in function of the various sectors. Source APN (2017) adapted from Tukker and Jansen (2006)

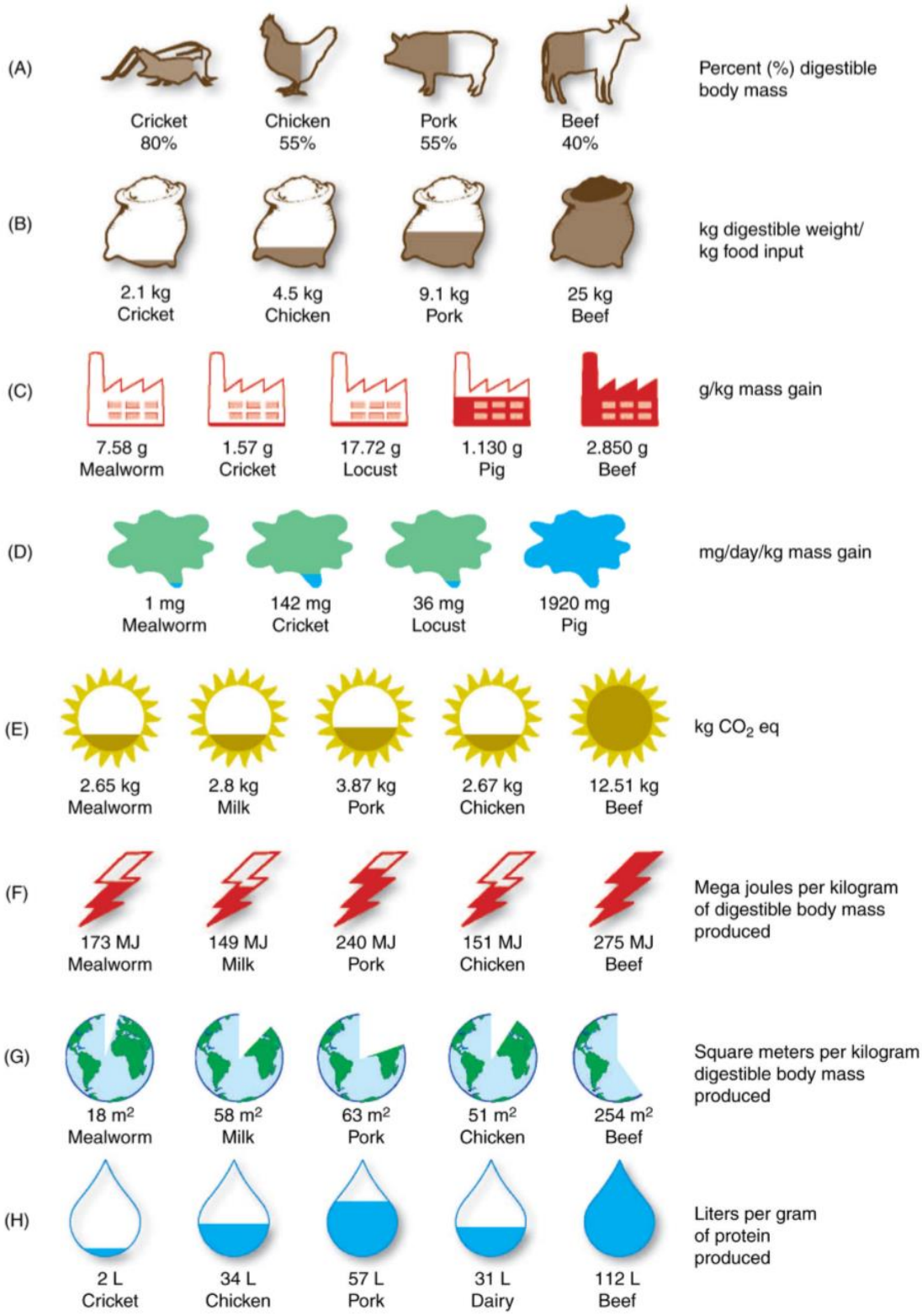

Figure 4. Environmental impact and resource use of insect farming versus other livestock. A: Source: Gahukar (2016)

1.1.4 Disadvantages of Entomophagy

1.1.4.1 Food safety problems

Insects are a potential new source of protein that can be used as human and animal feed. However, as any other novel food, the risks for health associated with the consumption of that kind of food must be analysed. EFSA (2015) indicated that possible risks may be chemical, microbiological and allergenic. Since 2015, profound studies had been conducted and other safety problems had been researched, as the presence of prions (H. J. van der Fels-Klerx *et al.*, 2018). Chemical hazards are mainly related with their presence in the rear substrate. They include heavy metals and arsenic accumulation where essential metals do not seem to accumulate and are efficiently expelled by insects, and non-essential metals such cadmium and arsenic (semimetal) that are often present in insects from different life stages and species (H. Van der Fels-Klerx *et al.*, 2016). Toxins, may be also produced by the insects which, according with the actual studies, do not appear in the list of proposed insects for food and feed, or can be present in the substrate, where studies shown that the values of mycotoxins present in their bodies is much lower when compared with the environment where they are reared. This reflects none or very low concentrations of toxins that might be explained by the presence of metabolic enzymes (Charlton *et al.*, 2015), chemical residues (veterinarian drugs, hormones, etc.) which their impact is not yet profoundly studied (Lalander *et al.*, 2016). Relatively with microbiological hazards it is important to take in account two possible sources, the microorganisms inherent to insects, that is, the insect microflora, and the ones that may be present in their farming conditions and processing processes (EFSA, 2015). There are various organisms related with this hazard., Viruses that often use insect as a vector for disease spread, so, when they are present in the substrate a rigorous post harvesting treatment may be performed. Bacteria can also contaminate insects, as they represent a friendly environment for the developing of a whole diversity of microorganisms (mainly Enterobacteriaceae and spore-forming bacteria), which can be highly harmful for human health, so heat treatment as shown to be effective to reduce the CFU of most of them (Amadi & Kiin-Kabari, 2016). At the same time, allergens related with the consumption of insects have been reported over the years. Therefore, it is important to analyse the causes which may be from occupational exposure, direct contact, or inhalation. A food allergy is composed by a 2-step procedure. There is a primary exposure to the specific allergen which causes sensibilization to that molecule but not a reaction, and then a second exposure that can trigger an allergic reaction. This occurs because the food proteins have a specific antigen-IgE which will bind to the specific antibody IgE leading to the release of histamine or derivates (H. J.

van der Fels-Klerx et al., 2018). Most studies report a cross-reactivity/co-sensation between edible insects and crustaceans, even without the first contact with their ingestion. This is related with the presence of tropomyosin and arginine kinase (Known arthropod pan-allergens) (Ribeiro *et al.*, 2018). Chitin was largely studied as an allergen in insects. This polysaccharide is present in the cell walls of fungi and in arthropod exoskeletons. It can appear in various forms, mainly in alfa-chitin form for insects. The percentage and organization of this molecules will depend on its life stage, body part or specie. Literature demonstrate that this molecule can be either beneficial, by reducing inflammatory effects when present in small particles or damaging for health leading to allergic inflammation when present in medium particles (Chandran *et al.*, 2016; Mack *et al.*, 2015).

1.1.4.2 Legal framework of insects

Insects are often consumed by a lot of people around the world essentially in Asia, Africa, Central America, and South America. However, in western societies, their consumption is considered exotic and less accessible (Costa-Neto & Dunkel, 2016). Consequently, the legislation in the European Union (EU) was only applied recently and is not very extensive. First, there was a need to identify insects as a food which is accepted by the article 2 of the regulation (EU) 178/2002. Then, it was important to take in account the food safety risks related with the consumption of that kind of food that have been reported by EFSA (2015) established based on article 22 of regulation (EU) 178/2002. Like that, the population have valuable information for production and consume of insects. Thereby, edible whole insects, insect parts and ingredients derived from insects were only recently considered a novel food to the UE by the regulation 2015/2283 that entered in vigour at 11 de December of 2015 and turned applicable at 1 of January of 2018. However, no insect species were present in the EU list that comprise all the novel foods until 4 of October of 2019 (because of regulation (EU) 2017/2283). So, for an insect species to be on that list the applicant must complete an application according with article 10 et seq. of regulation (EU) 2015/2283. Nevertheless, more legislation needs to be done for edible insects mostly about the quality requirements for selling edible insects (A Van Huis & Dunkel, 2017)

1.1.4.3 Consumer's acceptance of insects as food

As said before, edible insects are considered a peculiar food for occidental countries. Previous studies showed that people of these countries demonstrate very low level of acceptance for that kind of food (Verbeke, 2015). This aversion is mainly connected with food neophobia, which is the fear and disgust for unfamiliar food products (Sogari *et al.*,

2019). In this part of the world, insects are generally associated with food contamination, health risks and primitive diets and so, are misquoted by the population as a food source. It is possible to perceive that the willingness to try (WTT) insects is highly dependent of cultural and social parameters but can be related with other parameters like, taste expectation, visual appearance, consumer sex or previous tasting experience (Hartmann *et al.*, 2015; Hartmann & Siegrist, 2016). Many studies show that consumers that already experienced insects or food products with insects were more willing to eat them more often in the future (Caparros Megido *et al.*, 2014). It was demonstrated in various studies that a more culturally closed country is further reluctant to food products that are not implicated in their costumes and, consecutively simpler and more open-minded countries, with few dietary traditions have higher WTT insects and other different foods (Menozzi *et al.*, 2017). Regarding the consumer gender, men reveal a slightly superior WTT this food. That fact can be related with less sensitive to disgust and lower animal reminder disgust than women (Hamerman, 2016). Different ways to present the insect to the participant may trigger different WTT. It is proved that whole and alone insects boiled or fried have lower values of that factor than processed insects mixed in familiar food products like cookies, pasta, energy bars, etc. (Tan *et al.*, 2016). In all this studies the participants had to choose from products that contained insects. Though, when they have the power to choose between foods with or without insects, almost all of them select the samples with no insects (de Boer *et al.*, 2013). Taking it in account, the main problem for the companies that enter in the market with insects' products is to understand how to increase the WTT and more important, to buy it over the traditional ones. Conducted studies regarding with this topic demonstrated the importance of information about the advantages of entomophagy in order to sensitized the population and ways to make insects' products more familiar with known flavours, textures or appearance (Sogari et al., 2019). More studies must be done in that area to better understand the opinion of consumers relatively to edible insects in order to develop strategies to enhance their opinion and change the eating patterns.

1.1.5 *Acheta domesticus* as an insect for human consumption

EFSA (2015) considered *Acheta domesticus* (AD) one of the species with the best potential to be used in food and feed in the EU. Therefore, this will be the specie that will be used in this work. That specie takes part of the order *Orthoptera* and the family *Gryllidae* and its life cycle comprises three life stages: egg, nymph, and adult. Adult form shows a more beneficial nutritional profile and so, it is the most utilized for entomophagy. Nutritionally, it can show values of protein about 53,90g in 100g of fresh weight basis,

contain a good content in essential and non-essential amino-acids and can be a good source of iron, zinc and magnesium (Köhler *et al.*, 2019). This specie is also very advantageous in the farming and rearing process, showing advantages in many levels. Because of that and the good characteristic taste, this specie has been produced and domesticated for animal feed over the years and could not survive in their natural habitat. Nowadays, it is mainly produced in Thailand. They have short life circles of 30 to 45 days where each female can produce around 1500 eggs being a rentable production process. As any other insect they do not need much space, low maintenance, can consume a whole variety of foods and have a low diseases incidence, showing adaptability of farming (Gahukar, 2016; Gere *et al.*, 2019; Arnold Van Huis, 2020) .

The nutritional and sustainable characteristics of insects may have various applications on the food industry. The development of new products with isolated insect protein, like food gels, is one of them.

1.2 Gels in the food industry

1.2.1 Food gel characteristics

Gels are dispersed systems composed by a disperse phase and a dispersant phase forming a cohesive network. They are characterized by its lack of fluidity and deformability caused by the polymer crosslinking in covalent and non-covalent links (Belitz, 2009). In a simpler way its “an intermediate between a solid and liquid, possessing both elastic (solid) and viscous (liquid) characteristics” (Banerjee & Bhattacharya, 2012).

The formation of a gel is a very simple process that can be extremely controlled by external conditions that will determine how the formation of the gel occurs or if it occurs (Clark, 1992). It is a spontaneous process that implicates simple polymer dispersion or particles suspension. They can be physical, chemically or enzymatically induced to make the gelation process happen (Totosaus et al., 2002). So, there is not only one factor that determines the gel formation but a set of factors. Gels, in general have multiple ways to be classified according with its formulation (simple, binary/mixed or composite), type of gelation mechanism (temperature, pH, salts concentration, pressure or enzymes), morphology, type of interaction of the molecules and physical structure of the network.

There are numerous types of polymers that can be used as a gelling agent. These can be either polysaccharides or proteins and can be obtained from animals, microorganisms or plants (Einhorn-Stoll & Drusch, 2015). Among polysaccharides, we can list for instance agar and starches, and for proteins, gelatines, and myosin, among others.

Depending on the source, gels have different ways to be formed. The first ones need lower critical concentration of the gelling agent to start the gelling process, comparatively with proteins, and after the hydration of the polymer, the dispersion starts to become a gel with a firm structure. The second ones, are very different, the gelation process is established by a partial denaturation unleashed by diverse means and then a reconnection of the molecules which will increase the intermolecular interactions and form a gel (Nishinari *et al.*, 2000). These interactions (covalent or non-covalent) will vary among different types of proteins and way of gelation (Wijayanti *et al.*, 2014). So, for protein gels, it is important to search ways that will allow to increase the solubility of the solution to produce molecules with the capacity to rebound and form a gel.

Insects contain the same myofibrilar proteins composition that appear in muscle tissue, mainly composed by myosin (higher percentage), actin, tropomyosin, troponin and regulatory proteins (Urbina, 2018). Montowska *et al.* (2019) performed an SDS-PAGE of the protein content of 3 different *Acheta domesticus* powders proving the presence of the main proteins described above (table 1).

Table 1. Three principal protein present in 3 different house cricket powders. Adapted from Montowska et al. (2019)

Order of apperarence	Identified protein	Reference specie
1º	Myosine heavy chain, muscle	DROME (*Drosophila melanogaster*, fruit fly)
2º	Actin, muscle-type or 87E	BOMMO (*Bombyx mori*, domestic silkworm)
3º	Calcium-transporting ATPase sarcoplasmic/endoplasmic reticulum type	DROME (*Drosophila melanogaster*, fruit fly)

1.2.2 Myofibrillar proteins and its gelation capacity

Myosin has been studied broadly showing to be a promising gelling agent to improve the quality of some food products. Temperature, pH, salts, pressure and additives (protein or non-protein) are the most important factors on myosin gelation (Sun & Holley, 2011) (figure 5).

This protein can form a gel alone under certain conditions or under a combination of factors described above. Many studies where made to understand the gelation behaviour of the myofibrilar and myosin alone from various animals.

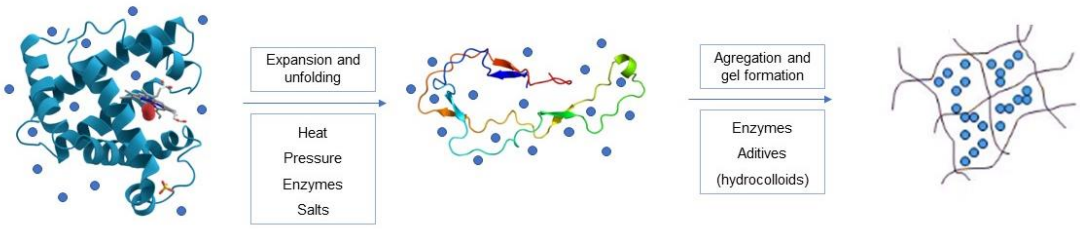

Figure 5. Protein-gel formation and factors influencing this process. Adapted from Totosaus *et al.* (2002)

1.2.3 Food engineering techniques for myosin and myofibrilar proteins

1.2.3.1 Heat

Heat is the most used technique in the gelation process. However, every article uses it associated with other parameters like pH and salts. Núñez-Flores *et al.* (2018) studied the heat induced gel formation in Alaska pollack surimi with and without NaCl (0% and 3%) and assessed that without the presence of the ionic forces of salt the quality of the gel is worst with negative rheological characteristics. The same was perceived in shrimp gel formation at shrimp surimi at 2,5% NaCl (Y. Yang *et al.*, 2020). The temperature plays a denaturation role that will increase the solubility turning the protein available for restructuration. So, relatively high temperatures (75 to 90ºC) are used normally for 30 min (L. Li *et al.*, 2018; Mishyna *et al.*, 2019; Ndiritu *et al.*, 2019; Núñez-Flores et al., 2018; Y. Yang et al., 2020; Yi *et al.*, 2013). Shifts in the pH of the solution can lead to changes in the gel conformation, rheological characteristics and thermal denaturation when combined with temperature treatments (Lee *et al.*, 2017). The pH alkali treatment was performed in Alaska pollock surimi fortified with fish oil by Gao *et al.* (2018). The surimi gels were subjected to pH values from 5.5 to 9.0 increasing the alkalinity of the solution and then exposed one-step (90ºC/30 min) or two-step (30ºC/ 30 min- 90ºC/ 30 min) heating treatments. It was assessed that higher solubility that consequently lead to stronger gels were obtained under higher pH's (pH 8) in the one-step heating procedure. The gelation of insect protein, although little is known, was studied regarding the effects of temperature, pH and salts. Mishyna et al. (2019) showed an increase of protein surface hydrophobicity, making them available for new connections, net charge by changing the attraction and repulsion forces in the solution and, availability of buried sulphydryl groups under neutral pH and a heating treatment of 85ºC for 30 minutes. These lead to a better covalent and non-covalent aggregation leading to the formation of gels. Yi et al. (2013) extracted and characterized proteins by five different species of insects including the one in study, *Acheta domesticus.* Chemical characteristics, foaming capacity and gelation capacity where assessed at 4 different pH (3, 5, 7 and 10), 2

concentrations (3% and 30% w/v) at 86ºC for 10, 20 and 30 minutes. Gel forming capacity was only obtained for the house cricket supernatant at 30% w/v for every pH's, however, the best quality gels appear at pH 7. Even though gel was formed, only visual analysis was performed and so, the gel formation assessment will depend on the analysis technique performed. Finally, Ndiritu et al. (2019) analysed the effect of salts (NaCl) and pH on different functional properties like, protein solubility, water holding capacity and least gelation concentration of *Acheta domesticus* extracted protein when submitted to a water bath of 80ºC for 30 minutes. The least gelation concentration was 30% w/v which means that this is the minimum concentration possible of protein for gelation to occur. In this concentration, gels were formed only for the pellet derived from the aqueous extraction in all salt percentages and for all pH's but pH 2. These articles related with edible insects were the starting point for the experimental design of this thesis.

1.2.3.2 High Pressure Processing (HPP)

HPP is studied in literature as a chemically and environmentally friendly procedure that can modify the structure of proteins leading to enhancement or deterioration of protein gels (X. Chen *et al.*, 2016). This treatment is studied for different applications like, protein gelation, modification of the rheological properties of gels, reduction of the microorganisms content, among others (Vanacolcha, 2003). The modification of the protein structure by HPP is dependent on the pressure applied. Lower pressure between 100 and 150 MPa affect its quaternary structure, above 200 MPa the hydrophobic and di-sulphide bonds of the tertiary structure are altered and only in very high pressures, above 700MPa, the secondary structure is affected leading to irreversible denaturation which makes rebounding impossible (Cao *et al.*, 2012). Sometimes the pressure applied can disrupt or collapse the gel form instead of enhance its functional characteristics, so, additives like polysaccharides, ions or aminoacids are often applied in order to reduce this pressure consequence (Z. Li *et al.*, 2019). Xue *et al.* (2018) analysed the microstructural composition of gels formed by myosin extracted from meat proteins exposed to a high-pressure treatment (100-300 MPa for 9 minutes at 25°C), myosin solution without treatment displayed a control role. They observed that at pressures of 200 and 300 MPa the thermal stability and the thickness increased. Enhancement of rheological and chemical properties at this pressure scales, were evidenced by other investigators like Y. Wang *et al.* (2018) that submitted chicken breast myofibrilar proteins to $CaCl_2$ (20-100 mM) and HPP (200 MPa for 10 minutes at 20°C) under low Na conditions and obtained an increases on the solubility of proteins. Z. Guo *et al.* (2019)

treated golden threadfin bream myosin with HPP (300MPa/ 3 minutes), $CaCl_2$ and heat where stronger gels and higher water holding capacity were achieved. In the same way, Z. Li et al. (2019) submitted threadfin beam myosin to HPP (0,1 to 300MPa) and 2% w/v deacetylated konjac glucomannan treatments and obtained stronger gels with higher values of water trapped in the gel network. It was also seen a higher increase in myosin unfolding and transformation of α-helix motifs in other structures. HPP treatment will be considered as a physical treatment in this thesis.

1.2.3.3 Enzymatic treatments: proteases (P) and polymerases (Poly)

Enzymes are often used in food industry as processing aids to improve the functional and stability properties of colloidal foods. These enzymes can enhance the covalent cross-linking of the biopolymers (cross-linking enzymes) like, GO and TGase or hydrolyse side groups of the biopolymers very specifically to turn them more receptive for new ligations (degrading enzymes) as proteases (Zeeb *et al.*, 2017).

Glucose oxidase (GO, EC 1.1.3.4) is an oxidoreductase that is responsible for degrading glucose in gluconic acid with H_2O_2 formation. This is transformed in $OH^{\bullet}$ that can lead to crosslinking reactions on myofibrilar proteins and consequently gel formation (Wang *et al.*, 2016) (figure 6). Literature demonstrates that the addiction of GO on gelation of myofibrillar proteins at different concentrations (0,5%; 0,8% and 1.0%) leads to a reduction of the sulphydryl and amines molecules which reflects an increase in the disulphide and non-disulphide bonds. Increases are also remarkable in surface hydrophobicity and gel strength that reflects in higher elastic modulus (G'), breaking force and breaking deformation (A. Q. Guo & Xiong, 2019; Omura *et al.*, 2020; L. Wang *et al.*, 2018) Microbial transglutaminase (TGase; EC2.3.2.13) is an acyltransferase that is accountable to transfer acyl groups between γ- carboxyamine group from glutamyl residues to aminoacidic group and consequently leads to new covalent links between proteins (Buchert *et al.*, 2010) (Figure 7). The use of TGase at its optimal temperature and time is highly reported in literature as an important factor in the enhancement of the functional properties of gels. N. Yang *et al.* (2020) observed that frozen stored long tail southern cod mince when treated with TGase and optimal temperature of 35ºC increased the myosin crosslinking leading to stronger gels. Regarding to edible insects, Kim *et al.* (2020) analysed the effect of TGase on the functional properties of protein solutions from *Protaetia brevitarsis* and observed an increasing on the crosslinking which allowed the solution to increase its thermal stability, a very important characteristic for gelling capacity.

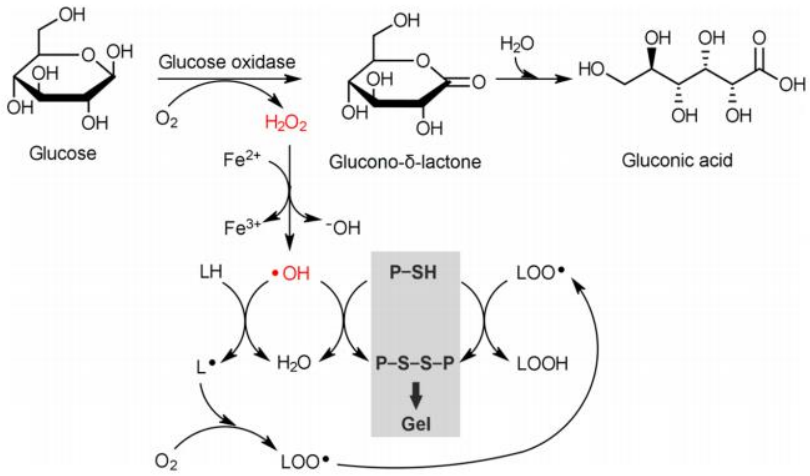

Figure 6. Glucose oxidase-induced cross-linking and gelation of myofibrillar proteins. **LH:** lipid molecule; **P−SH**: protein sulfhydryl; **P−S−S−P**: cross-linked proteins with disulphide bond.

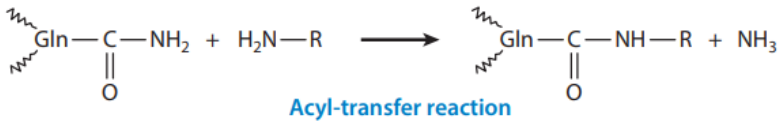

Figure 7. Transglutaminase-induced cross-linking and gelation of myofibrillar proteins.

Proteases hydrolyse peptide bonds in smaller polypeptides or proteins. The formed small-chain peptides provide new functional properties to the solutions as, solubility and gelation (Ju & Kilara, 1998). Among the huge variety of proteases this work is going to use Alcalase (A), Flavourzyme (Fla) and iZyme BA (BA). Alcalase is an endopeptidase (serine type) obtained by the fermentation of *Bacillus licheniformis* with a broad specificity. This means that they can hydrolyse most peptide bonds within a protein molecule. They present their optimal action at pH from 6.5 and 8.5 and temperatures from 45 to 65 ºC. Flavourzyme is a blend of endo and exopeptidases extracted from the fermentation of *Aspergillus oryzae.* It is widely used because of its capacity to produce flavour active compounds in consequence of the hydrolyse process. Its maximum activity is perceived at pH from 4 to 8 and temperatures from 30 to 65ºC. iZyme BA is an aspartate protease and its origin is not defined. This enzyme shows a very specific action hydrolysing dipeptides with hydrophobic residues. Its optimal range of action for pH is 2 to 4 and for temperature 50ºC (Baron *et al.*, 2017). Those proteases were selected because they are food grade and show different hydrolytic activity.

1.2.3.4 Additives (Xanthan Gum)

Hydrocolloids are largely used in food industry for enhancement of the functional properties and restructuring of food matrices because of its emulsifying, stabilizing, thickening and gelling properties. Xanthan gum (XG) is an anionic polysaccharide used as hydrocolloid obtained by *Xanthomonas campestris* (figure 8) (Majzoobi *et al.*, 2017). J. X. Chen *et al.* (2020) proved that in low concentrations XG could improve the gel strength and the water holding capacity of silver carp surimi gels. Binsi *et al.* (2017) tested the effect of XG on microwave extracted fish gelatine and verified an increase in hardness and bloom strength of the obtained gels. In this thesis, XG will be tested as an adjuvant to improved gels formed.

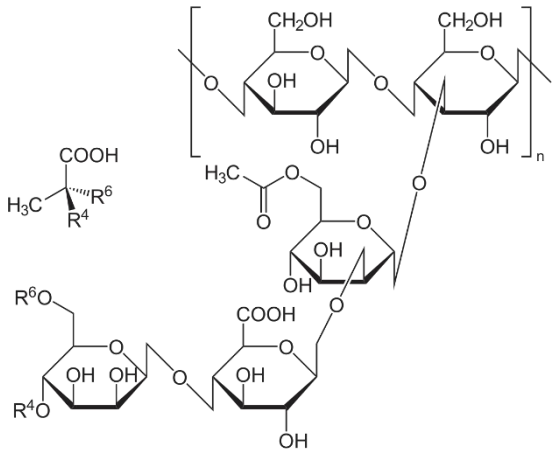

Figure 8. Xanthan gum structure.

2. Objectives

2.1 Main objective

- The main objective of the work is to study the gelling capacity of insect flour (*Acheta domesticus)* subjected to different physical and biochemical processes with the ultimate objective of advancing towards its incorporation as an ingredient in real foods.

2.2. Specific objectives

- Assessment of physical and chemical treatments in the gelation capacity of *Acheta domesticus* powder under neutral conditions and 2% NaCl by measuring the water-soluble solids (WSS).
- Assessment of the effect of different Xanthan Gum concentration in the gelation capacity of *Acheta domesticus* powder under neutral conditions and 2% NaCl treated with enzymes, pressure and/or heat measured by viscosity, firmness, WSS, and water binding capacity (WBC).

3. Line of work

The performed work was divided in 2 different parts. In the first part, the gelation capacity of *Acheta domesticus* powder alone submitted to physical (heat, pressure) and biochemical (polymerases and proteases) treatments, was indirectly measured as the total water-soluble solids present in the solution. In the second part, viscosity, texture, water-soluble solids, and water binding capacity were measured in *Acheta domesticus* powder submitted to physical and biochemical treatments with the addiction of Xanthan gum. In figure 9, the line of the work is better explained. The main steps of the laboratory research are described next.

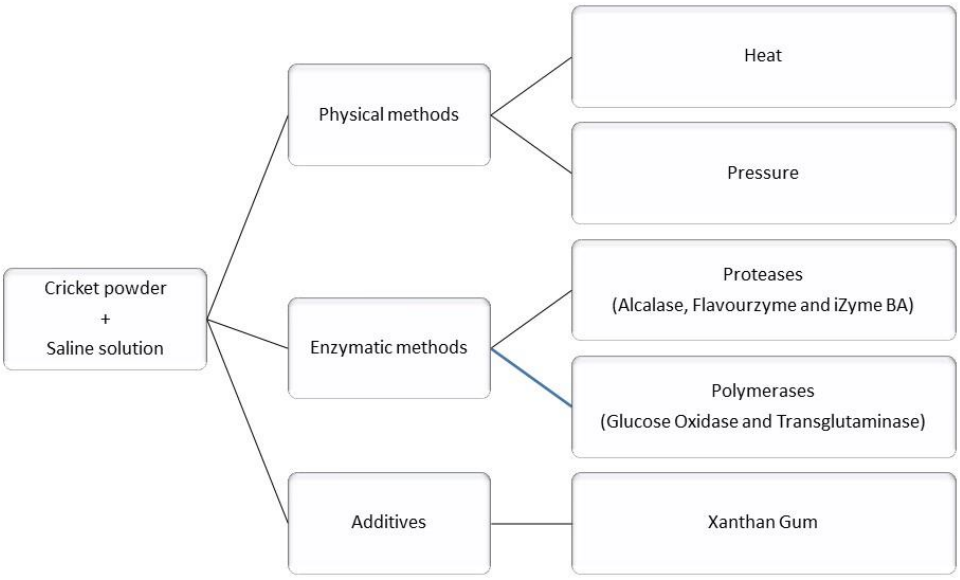

Figure 9. Line of work: Gelation capacity of *Acheta domesticus* flour with physical and enzymatic methods or additive addiction.

1. The cricket powder (CP) and the saline solution (SS) were submitted to the heat treatment (H).
2. The CP and the SS were submitted to high hydrostatic pressure treatments (HHP).
3. The CP and the SS were submitted to proteases (P) and H.
4. The CP and the SS were submitted to P and HPP.
5. The CP and the SS were submitted to P, polymerase (Poly), and H.
6. The CP and the SS were submitted to P, Poly, and HHP.

7. The CP and the SS were mixed with Xanthan gum (XG) and submitted to H.
8. The CP and the SS were mixed with XG and submitted to HPP and H.
9. The CP and the SS were mixed with XG and submitted to P, Poly and H.
10. The CP and the SS were mixed with XG and submitted to P, Poly, HPP and H.

4.Materials and methods

4.1 Material acquisition

The *Acheta domesticus* flour was acquired from the brand Thailand Unique and made from small adult crickets and 100% defatted. The proteases (Alcalase (A), Flavourzyme (Fla) and iZyme BA (BA)) were from the brand Novozymes and the polymerases (Glucose oxidase (GO) and transglutaminase (TGase)) from the brand Novonordisk.

4.2 Saline solution preparation

Distilled water was combined with solid NaCl to a concentration of 2% w/v. Then the pH was adjusted with NaOH at 0,01; 0,1 and 1% w/v until neutral pH of 7. All the measurements were made with the Crison pH meter GLP 21 (Barcelona, Spain).

4.3 Evaluation of the gelation capacity of *Acheta domesticus* powder treated with enzymes, heat, and high hydrostatic pressure

4.3.1 Sample preparation

The sample preparation and the treatments are described in figure 10. Eppendorf tubes (1,5 ml) were prepared with 30% w/v of *Acheta domesticus* flour (0,3 mg) and 1 ml of at 2% NaCl (1 ml) as used in Ndiritu et al. (2019) at pH 7.

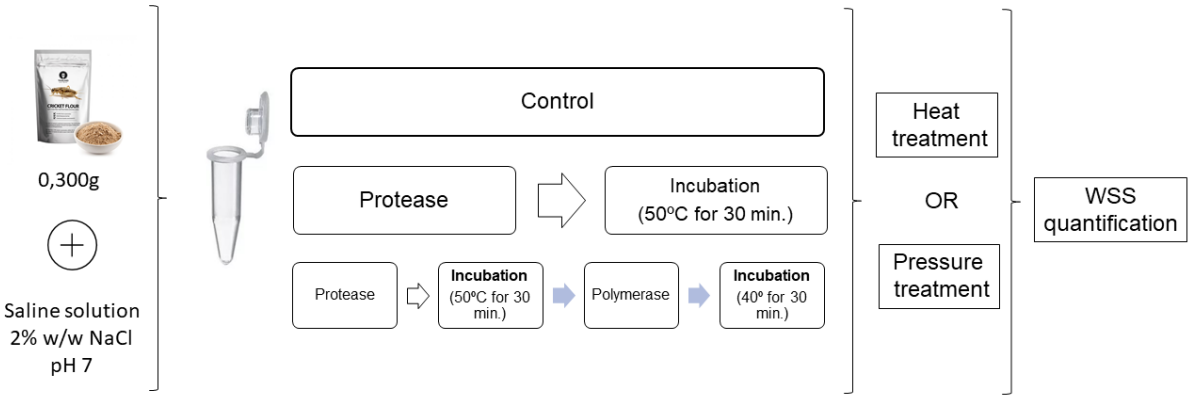

Figure 10. Sample preparation scheme.

The enzymes used were from two different types, proteases, and polymerases. The proteases (A, Fla and BA) were used as a pre-treatment (increase the solubility of the myofibrilar proteins) that will lead to a major molecules availability to reconnect and form a gel. The polymerases (TGase and GO) are proteins that can catalyse the polymerization of the aminoacids that can lead to gel formation. The tubes with enzymes

were submitted to an incubation time of 30 minutes at its optimal temperature with a SKI4 shaker incubator (40 ºC for polymerases and 50 ºC for proteases). All the thirteen enzymatic treatments are described in table 2. After the enzyme treatment, a physical processing technology (heat or high hydrostatic pressure) was applied. The standard processing technology applied was heat. The treatments, 2, 4, 6, 7, 9, 11 and 13 were chosen between all the treatments as the ones with the best apparent gelation capacity, to be submitted to pressure treatments.

Table 2. Enzymatic and non-enzymatic treatments from 1 to 13.

Treatment			No polymerase	Polymerase	
				GO 1,0%	TG 2,0%
Proteases	Alcalase	1%	1.	———	———
		5%	2.	7.	8.
	Flavourzyme	1%	3.	———	———
		5%	4.	9.	10.
	iZyme BA	1%	5.	———	———
		5%	6.	11.	12.
No pre-treatment (no protease)			13.	———	———

4.3.2 Heat treatment

The heat treatment is widely used in gelation process so it will be used as a standard process in this work. That means that all the treatments are associated with a heat process except for the ones where pressure is applied. The samples were submitted to 85ºC for 30 minutes in a PST-100HL (BioSan) thermo-shaker. Then were removed, cooled down at room temperature and stored at 4ºC until analysis.

4.3.3 Pressure treatment

The samples 2, 4, 6, 7, 9, 11 and 13 from table 2 were submitted to a HHP treatments of 300 MPa or 500 MPa for 10 min in order to assess its effect in the gelation capacity of cricket flour and to compare with the standard (heat treatment). These sample were chosen by the analyse of the WSS explained in results and discussion section. The eppendorf tubes were sealed in vacuum on ROVAC bags and placed in the high hydrostatic pressure equipment of the brand Engineered Pressure Systems International (EPSI) Belgium, with 2,35L of capacity (figure 11).

Figure 11. Pressurizer EPSI utilized in the pressure treatments.

4.3.4 Water-soluble solids (WSS) quantification

The water-soluble solids quantification indicates the weight of the molecules that are present in the supernatant after centrifugation. In the context of this work they will be read in two different ways. In one hand, in solubility tests (proteases alone) it is imperative to have high values of WSS which means that the protein molecules became more hydrophilic and are soluble in water which will make the re-ligation to form a network more favourable. On the other hand, low values of WSS mean that the treatment have good gelation capacity because the molecules are trapped in the network.

For the quantification of WSS the eppendorf tubes with the respective treatment were centrifuged with an eppendorf 5702 R centrifuge at 13.2 RFC, at 4 ºC for 5 min, then the supernatant (SB) was separated from the pellet. The SB was submitted to the oven at 120 ºC for 2 h30 min to dry all the water present in the sample and then the tubes were weighted to quantify the quantity of residue that remained in the tube. This measure gives the WSS in grams. All the steps are summarized in figure 12.

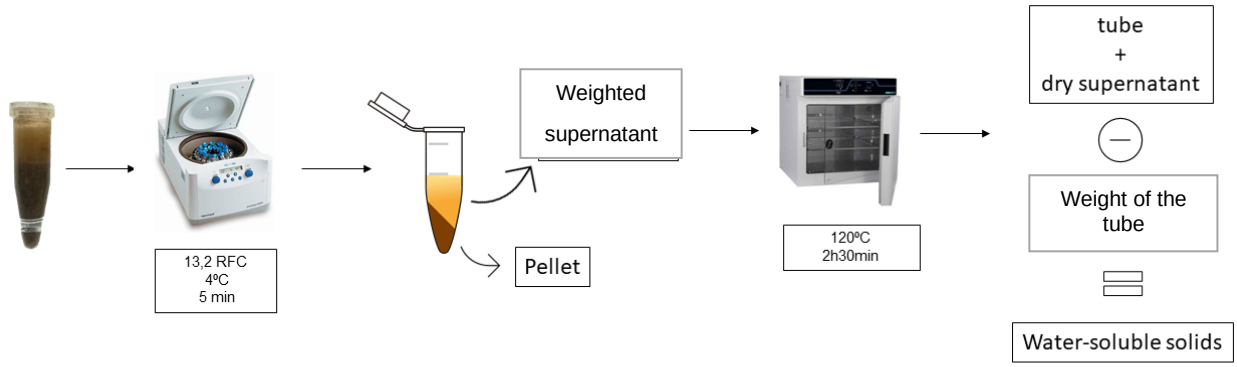

Figure 12. Water-soluble solids quantification scheme.

4.4 Evaluation of the combined effect of enzymes, heat, HHP and xanthan gum on the gelation capacity of *Acheta domesticus* powder

4.4.1 Sample preparation

The samples prepared in this phase are explained in figure 13. They were prepared with the same base of 7,5 g of *Acheta domesticus* and 25 ml of the saline solution of 2% NaCl pH 7. To determine the effect of the XG, different systems were prepared:

i. Combination of enzymatic treatment and XG. This combination was carried out in two different ways. First, enzymatically treated *Acheta domesticus* powder (as previously described) applying BA with GO addition were blended with XG to reach different concentrations (0,5%, 1% and 3%). Those blends were subjected to the heating-cooling cycle using the Rapid Viscoanalyzer (RVA 4500 viscosity meter, Perten laboratories, Sweden). RVA settings were, initially sample suspension was hold at 50 °C for 1 min, then heated up to 85 °C at 12.2 °C min-1, hold at 85 °C for 2.5 min and cooled down to 50 °C at 11.8 °C min-1, with a total assay duration of 13 min (Figure 14). The rotational speed of the paddle was 960

rpm during 10 s and kept at 160 rpm during the rest of the assay. Apparent viscosity was recorded during the assay. Second method consisted in mixing insect powder with enzymes and xanthan gum and this suspension was subjected to two different incubation temperatures of 50 ºC for 30 min and 40ºC for 30min and then to the RVA cycle previously described.

ii. Combination of heat treatment and XG. Insect powder was blended with XG (different concentrations) and then subjected to thermal incubation using the RVA as previously described but reaching 85 ºC instead of 95 ºC to resemble the heat treatment of the first part of the study.

iii. Combination of HHP and XG. Insect powder suspension was blended with different amounts of XG (to reach 0,5%, 1% and 3%) and subjected to HHP (300 MPa for 10 min). Then the sample was subjected to RVA heating-cooling cycle recording the apparent viscosity.

A control or reference was also prepared in which the insect powder was directly blended with XG at different concentrations and submitted to RVA heating-cooling cycle. The gels obtained from the different treatments were cooled down till room temperature prior to measure the solid soluble contents, water binding capacity and the texture.

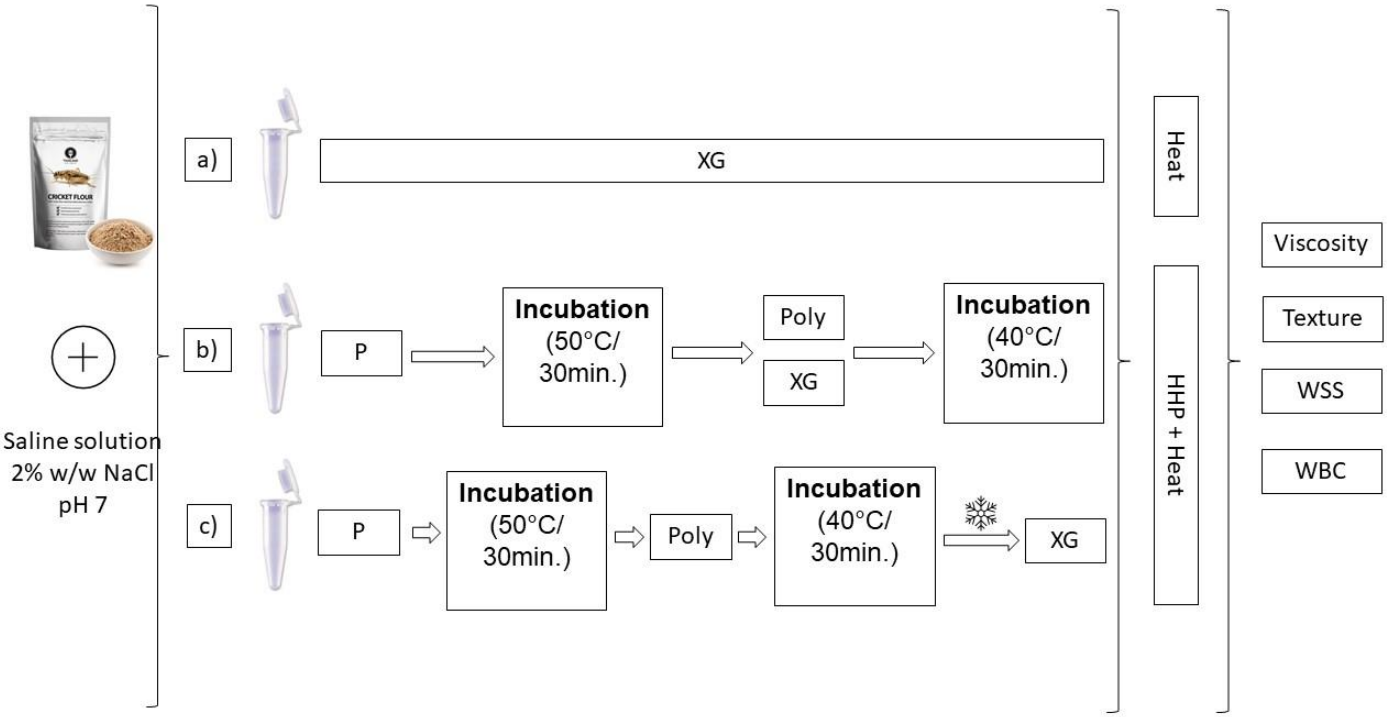

Figure 13. Xanthan gum essays with and without enzymatic treatment. **XG:** xanthan gum; **P:** protease; **Poly:** polymerase; **HPP:** high hydrostatic pressure; **WSS:** water-soluble solids; **WBC:** water binding capacity.

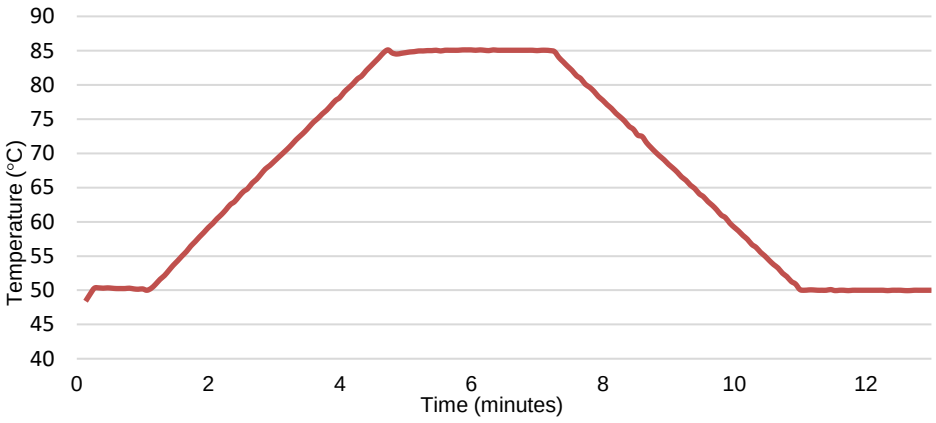

Figure 14. Temperature evolution over time during the RVA 4500 heating process.

4.4.3 Texture analyses

The samples for the viscosity test were poured in a 6 well culture plate, rested for 1 h and then the texture was measured with a texture TA.XT. Plus (Stable Micro Systems, Godalming, England) where a compression test was performed. The probe used was cylinder probe (p/25) with a cell load of 30 kg. Every sample was measured in duplicate. The process is schematized in figure 15.

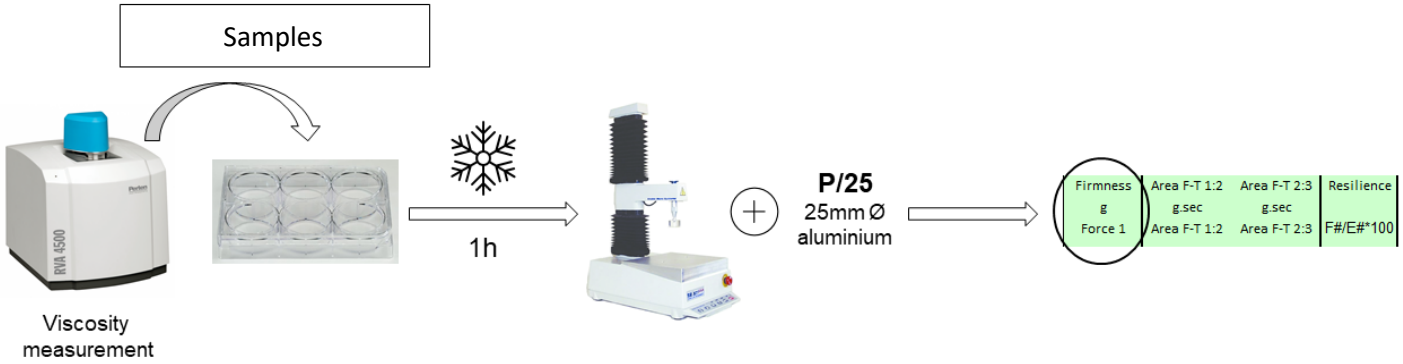

Figure 15. Texture measurement scheme.

4.4.4 Water- soluble solids analyses

These analyses were performed as described in 4.3.4. (figure 12).

4.4.5 Water-binding capacity analyses

These analyses show the capacity that the solution has, after centrifugation, to retain the water inside the network. The higher the WBC, the higher the gelation capacity because, a gel is formed when the water gets trap in the network formed. The quantification of this physical parameter was performed based in Ribotta *et al.* (2012) methods with some changes (figure 16). The eppendorf tubes were weighted with no liquid inside. After viscosity and texture analysis (figure 15) the mixture was poured in the tubes and submitted to a centrifugation process with an eppendorf 5702 R centrifuge at 13,200 RFC, at 4ºC for 5 min. Finally, the supernatant was used for water-soluble solids analyses and the tube with the pellet was weighted. The water binding capacity was given by the weight of the pellet and two replicates for each sample were made.

4.5 Statistical analysis

The analyses were performed in excel (office365) for data exposure, mean and standard deviation calculations and graphics formation (bar charts and simple or combined scatter plots with smooth lines). The ANOVA one-way analyses performed to understand if there were significant differences between means with a confidence level of 95% or 99% and the post-hoc analyses performed were the tukey-HSD. These were performed in IBM SPSS Statistics 25 (2017, NY).

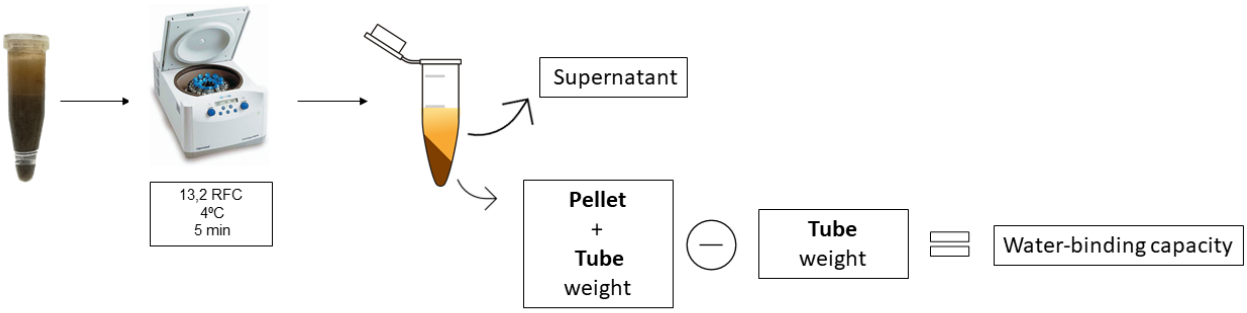

Figure 16. Water-binding capacity quantification scheme.

5. Results and discussion

5.1. Studies of physical and chemical processing methods on the gelation capacity of *Acheta domesticus* powder

In literature it is expressed that the gelation process can be triggered by numerous factors that can rather be physical (heat-gelation, pressure gelation, etc.), chemical (enzymes) or a combination of both (Totosaus et al., 2002). Totosaus et al. (2002) tested both methods in the *A. domesticus* powder to understand if the gelation could occur under the conditions stablished. Some studies already tested these methods but always in proteins extracted from insect powder (Ndiritu et al., 2019; Yi et al., 2013). Though, in the food industry the search for more sustainable and easy ways of production is important and so, the removal of a time and energy consuming part of the production chain is imperative.

The objective of this phase is to assess which processing methods can be more eligible for the increase of gelation capacity of house cricket powder.

4.1.1 Physical processing methods on the gelation capacity of Acheta domesticus powder.

The approached physical methods included heat, highly used in the gelation of biopolymers, the standard treatment used in this part of the work, and high hydrostatic pressure (HHP) at 2 different pressures (300 MPa and 500 MPa). Figure 17 shows the visual results from the application of each of the physical methods on the cricket powder combined with the saline solution (pH 7).

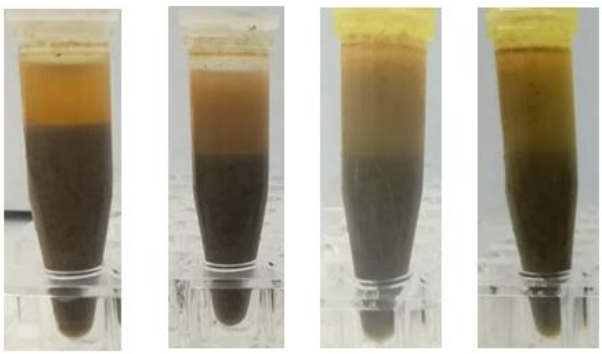

Figure 17. Physical gelation methods: Visual analysis. From left to right: Control, Heat, 300 MPa and 500 MPa.

From this figure, it is possible to understand that no gelation had occurred but some changes in the consistency of the *Acheta domesticus (AD)* powder solutions are evident.

As the quantity and the consistency of the solution were not suitable for viscosity and texture measurements, the quantification of the WSS was the more reliable method to withdraw viable conclusions (figure 18). By analysing the WSS in figure 18, it is possible to conclude if the solutes are retained in the network that is formed after each physical treatment or if they continue in the liquid phase of the solution. When a network forms, the solutes get held in the new bonds and disappear from the liquid phase of the tube, consequently, a low content of WSS implies some gelation capacity (Chen *et al.*, 2017).

Accordingly with the literature, under certain values of high pressure treatments a positive effect in the gelation capacity and physical characteristics of myofibrilar proteins gels can be verified, however, over 300 MPa this positive effect can have opposite results (Z. Guo et al., 2019; Z. Li et al., 2019). Results shown in figure 18 indicate that from the two pressure treatments tested, the 300 MPa is the one with the lower values, but without significant differences with 500 MPa. In a general point of view, the two pressure treatments have lower values when compared with control and heat treatments. However, between the control sample and the other samples physical changes have occurred (figure 17) and no significant differences between the WSS values were seen (FIGURE 18) and so, the studies with the different physical approaches needs to be continued in order to understand its behaviour when combined with other factors.

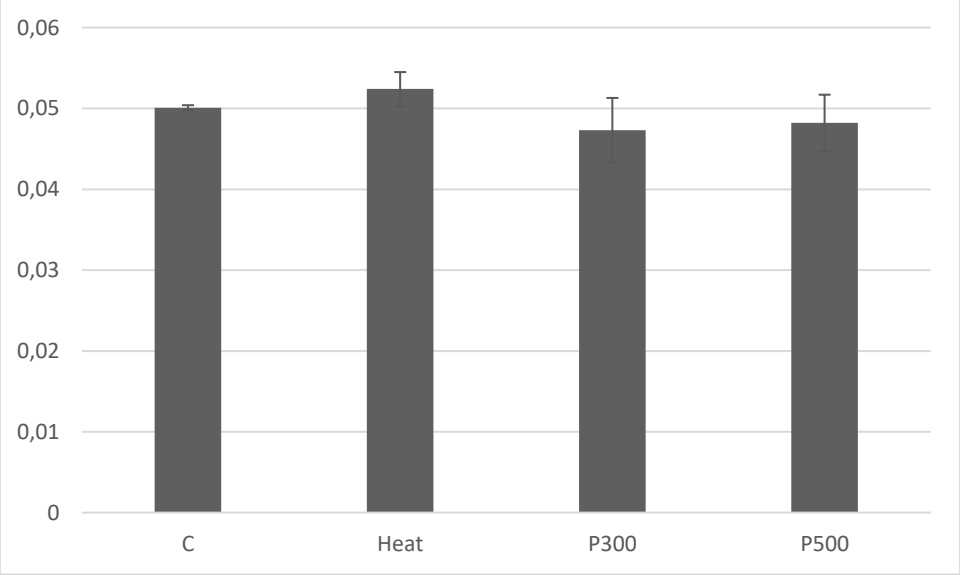

Figure 18. Water soluble solids quantification of the physical methods: no treatment (C), heat and pressure levels applied.

5.1.2 Enzymatic processing methods on the gelation capacity of *Acheta domesticus* powder

Enzymes are used in gelation processes with the intent to change the conformity of the molecules which will lead to a modification of its functional properties. For this purpose, enzymes with opposite catalytic activity are used. In one hand degrading enzymes that can be used to modify the structure of the food elements, and in the other hand, crosslinking enzymes can be used to enhance the networking capacity of the molecules and thus, increase the gelation properties (Zeeb et al., 2017). In this work, two types of enzymes with different functions were used: Proteases (degrading enzymes) and polymerases (crosslinking enzymes).

5.1.2.1 Effect of proteases on the increase of protein solubility

The myofibrilar proteins are the main proteins present in the house cricket and, consequently in the powder used in this work (Urbina, 2018). This kind of protein presents low water solubility, which can lead to difficulties on its gelation capacity, because the molecules would not be available to rebound and change their conformity. Likely, the hydrophobic nature of these proteins is responsible for their low water solubility. Proteases are enzymes that hydrolyse peptide bonds within protein structure, which allows opening the quaternary structure, and might turn them more hydrophilic and in consequence increase their water solubility. They could be used as a pre-treatment to prepare the proteins to form new covalent and non-covalent intermolecular bounds. Besides, depending on the protease and the concentration applied, the solubility response of the proteins could be different. Therefore, in this work three different proteases: Alcalase (A), Flavourzyme (Fla) and iZyme BA (BA), at different concentrations (1% and 5%), were used to understand how the solubility of the proteins could be increased.

The WSS presented a good measure, indicating the effect of enzymes on the *AD* powder because, if the WSS are high it means that the proteins became more soluble in the water phase, confirming the hydrolysis of the protein chains, leading to soluble peptides. Table 3 and figure 19 show the pre-treatments and the WSS data from those pre-treatments, respectively.

Table 3. Pre-treatments applied to the *Acheta domesticus* powder solutions.

Protease	Concentration (% v/w)	Label
Alcalase (A)	1	A1
	5	A5
Flavourzyme (Fla)	1	Fla1
	5	Fla5
iZyme BA (BA)	1	BA1
	5	BA5

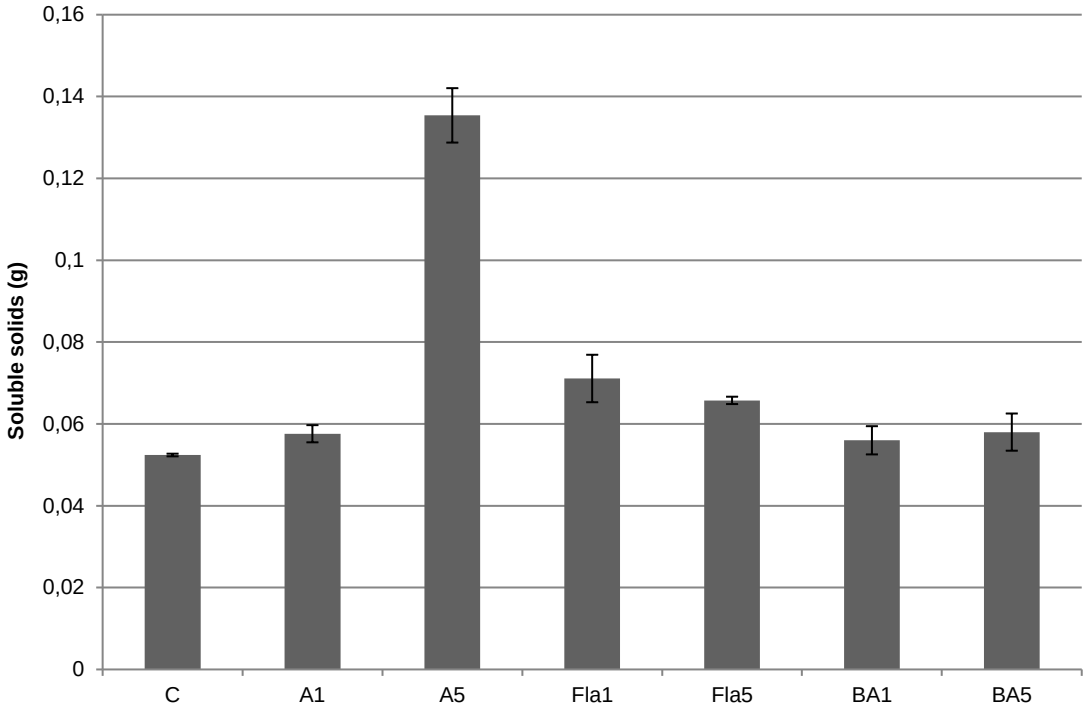

Figure 19. Water soluble solids quantification from the protease treatments. C (Heat control); A1 (alcalase 1%); A5 (Alcalase 5%); Fla1 (Flavourzyme 1%); Fla5 (Flavourzyme 5%); BA1 (iZyme BA 1%); BA5 (iZyme 5%).

Figure 19 shows that the application of proteases increases WSS compared to the control (C), although these increases are not always statistically significantly ($p>0.05$). Although for iZyme BA (BA) and flavourzyme (Fla) there are not significantly differences in WSS between 1 and 5% concentration ($p>0.05$), alcalase (A) at 5% increases significantly ($p<0.05$) the WSS. Therefore, this concentration was considered the best for the protease pre-treatment. These results are congruent with findings of Yoon *et al.* (2019) that characterized protein hydrolysed from three different species of edible insects

with A and Fla. They measured the resulting solubility when proteases were combined with edible insect proteins and assessed a visible increase on the solubility of the proteins. Those results, besides SDS-PAGE of the hydrolysed proteins, confirmed that Alcalase, among all the proteases, showed better hydrolysis efficiency, which agrees with the results present in this chapter of the work.

5.1.2.2. Combined effect of proteases and polymerases in the gelation capacity of Acheta domesticus powder

Polymerases have the ability to catalyse the polymerization of proteins from amino acids or small chain proteins (Buchert et al., 2010). The polymerases used in this study were glucose oxidase (GO) and transglutaminase (TGase) at 1% w/w and 2% w/w concentration, respectively, as both have been used in the food industry as natural crosslinking agents. In this part of the work were select the most suitable enzyme concentration for the enhancement of the gelation capacity of the cricket powder as its explained next. WSS was used as an indirect measurement of crosslinking. If this parameter decreases it means that the molecules, peptides, or soluble protein chains, initially present in the water phase are now retained in the network, since they are no longer available as a WSS. Results from figure 20 show that GO is the polymerase that leads the lower WSS when combined with a previous protease treatment at 5% w/w, therefore to a higher degree of crosslinking. Same conclusion can be withdrawn from table 4, where WSS for different protease-polymerase combination are shown. The effect of transglutaminase is rather variable, inducing an increase of the WSS when combined with flavourzyme or iZyme, and some reduction is observed when added to the powder treated with alcalase. In the case of polymerization with GO, the reduction of WSS was more significant with alcalase when compared with the other two proteases, followed by flavourzyme, and finally it is barely noticeable the changes observed in the presence of iZyme BA.

Different oxidases started to be more broadly studied as a food network catalyst. Jiang *et al.* (2017) studied the effect of adding glucose oxidase, glucose, and horseradish peroxidase to soybean protein isolate, to understand how they influence the crosslinking. It was assessed that when these enzymes were added, the solubility of the solution decreased when compared with the control, as it was seen in the present work. Therefore, from the results shown it is possible to conclude that the treatment with GO at 1% would be more interesting, taking in the account the objective of the study.

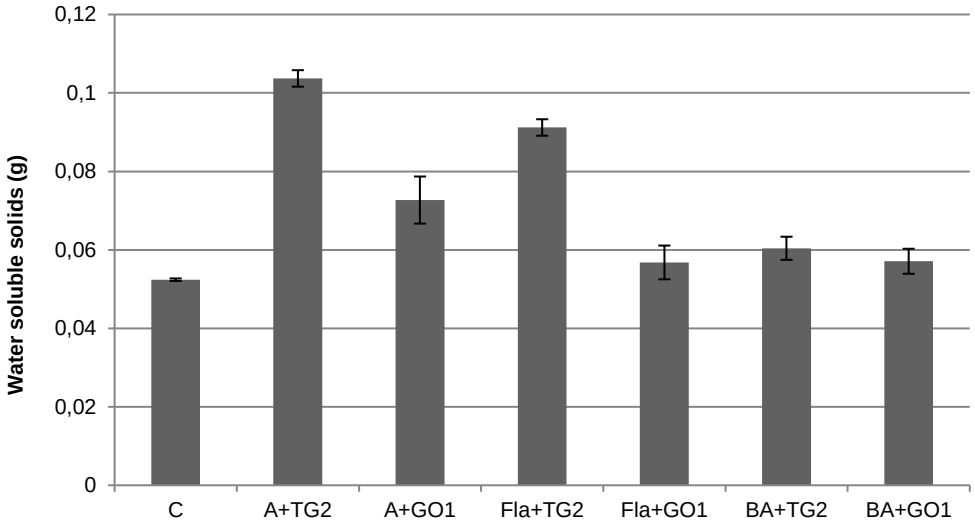

Figure 20. Water-soluble solids of the samples treated with the proteases at 5% w/w and the polymerases: Tranglutaminase (2% w/w) and Glucose oxidase (1% w/w).

Table 4. Water-soluble solids from the pre-treatments alone (proteases) and combined with polymerases.

Pre-treatment	Polymerase	Label	WSS (g)	Standard deviation
Alcalase	-	A5	0,1354	±0,0058
	TGase	A+TGase2	0,1037	±0,0060
	GO	A+GO1	0,0727	±0,0021
Flavourzyme	-	Fla	0,0658	±0,0034
	TGase	Fla+TGase2	0,0912	±0,0043
	GO	Fla+GO1	0,0568	±0,0029
iZyme BA	-	BA5	0,0580	±0,0036
	TGase	BA+TGase2	0,0604	±0,0032
	GO	BA+GO1	0,0571	±0,0015

5.1.2 Effect of high hydrostatic pressure (HHP) combined with enzymes on the gelation capacity of Acheta domesticus powder

From the above results (figure 20), the most favourable enzymatic treatments (proteases at 5% w/w and GO at 1% w/w) were chosen to be combined with pressure treatments. The behaviour of the sample of house cricket powder when combined with chemical and physical gelation methods were evaluated.

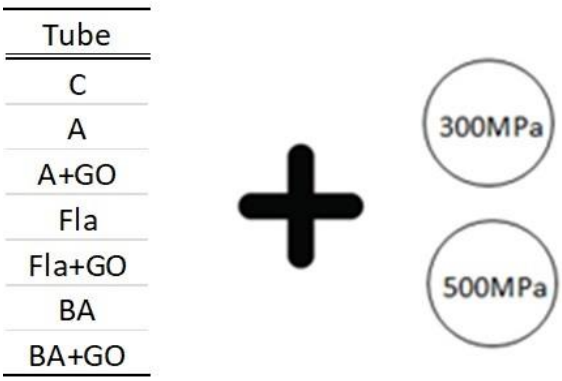

Figure 21. Enzymatic and pressure treatments combined. **C:** No enzymes, **A:** Alcalase (5% w/w), **A+GO:** Alcalase (5% w/w) + GO (1% w/w), **Fla:** Flavourzyme (5% w/w), **Fla+GO:** Flavourzyme (5% w/w) + GO (1% w/w), **BA:** iZyme BA (5% w/w), **BA+GO:** iZyme BA (5% w/w) + GO (1% w/w).

In the literature, pressure treatments were studied for the gelation of various materials among them, myofibrilar proteins. The application of high pressure treatments can lead to an improvement of the gel characteristics depending on the pressure applied (Mirmoghtadaie *et al.*, 2016). Having this into account, and accordingly with the results above, the samples chosen to be combined with this physical process were the ones shown in figure 21: a sample with no enzymes to assess the effect of pressure by itself, a sample with the pre-treatment (protease at 5% w/w) to analyse if the increase of the solubility is sufficient to increase the gelation capacity when combined with pressure, and finally samples with both enzymes (protease at 5% w/w and GO at 1% w/w) combined with pressure. Again, low values of WSS mean that the protein molecules are retain in a network formed because of the applied treatment. The results are displayed in figure 22. For the pressure level applied, in a general way, the values of WSS were lower at 300 MPa, which is congruent with the bibliography where Z. Guo et al. (2019) analysed the physicochemical properties of golden threadfin bream myosin. Therefore, at 300 MPa lower number of soluble compounds are obtained, thus initially soluble compounds are integrated in the polymer structure.

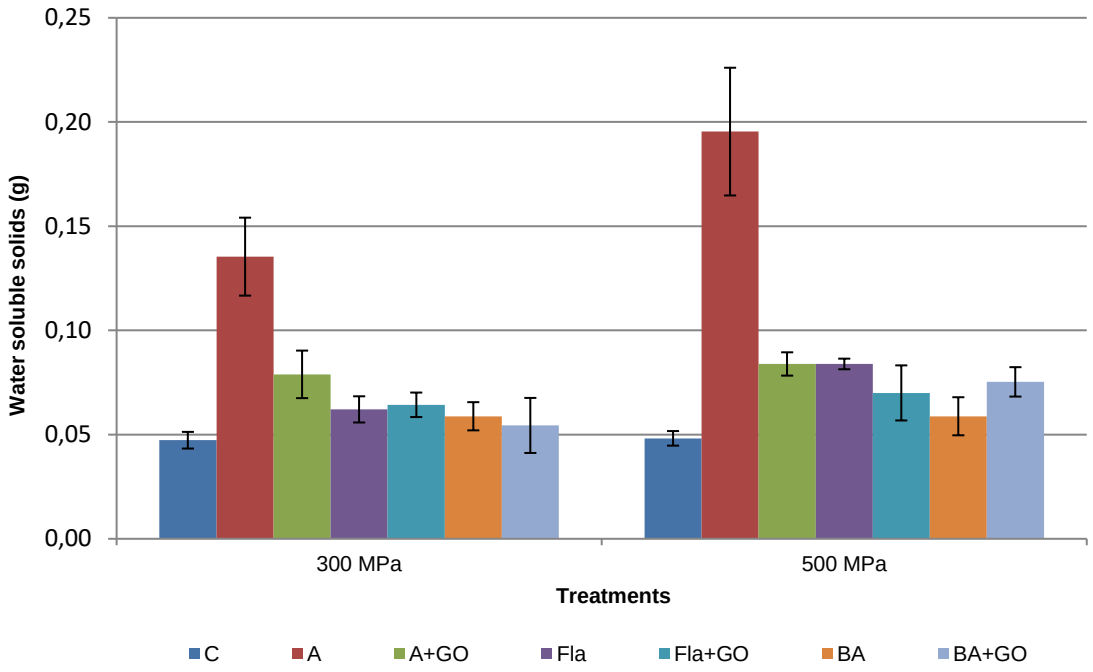

Figure 22. Combination of enzyme and pressure treatments on the WSS quantification.

Considering the enzyme or mix of enzymes used, alcalase shows very high values of WSS, those values can be explained by the enhancement of the hydrolysis of the protein when this enzyme is combined with a pressure treatment. The results, agree with the findings reported by Dong *et al.* (2019). When the alcalase is combined with the polymerase the reduction of WSS was notable, however the values remain higher than those for the other enzyme combinations. The remaining treatments, with and without GO, show very similar results between them and significantly lower WSS values when compared with alcalase treated with or without polymerase at 300 MPa. The impact of the use of transglutaminase and high pressure treatments on the physicochemical properties of myosin gels was already explored by other authors (Ye *et al.*, 2019; Zhu *et al.*, 2014). In that study, the combination of enzyme treatment and medium pressure (200 MPa – 300 MPa) enhanced the gel characteristics (Ye et al., 2019; Zhu et al., 2014). In opposition, in the present study, when samples are treated with TG, results indicate lower gelation capacity than those with GO. In consequence, GO was the choice for the pressure treatments performed, even though this enzyme is less studied in literature.

5.1.3 Comparison between all the physical and enzymatic gelation methods on the gelation capacity of *Acheta domesticus* powder

The objective was to compare all the physical treatments (heat and HHP) when combined with the enzyme treatments. This will show the information about the most appropriate physical treatment (figure 23) that will be used further in the investigation. From figure 23, it is possible to perceive that 500 MPa is the treatment that leads to higher WSS values, therefore, to an increase on the solubility which could lead to a lower gelation capacity of house cricket powder. On the other hand, a reduction of solubility (lower WSS values) is found in both, heat and 300 MPa treatments. The reduction of solubility of proteins when treated with high pressure and wet-heat was reported by Y. Zhang *et al.* (2016) in almond proteins. The present study with house cricket powder shows that similar results to those indicated above could be obtained when treating animal proteins.

Although, between the two best treatments, the temperature shows lower WSS values in all the samples studied (figure 23), both treatments (85 ºC and 300 MPa) are chosen for further investigations as gelation mechanism differs for thermal and HPP treatment and different behaviour could be found between samples.

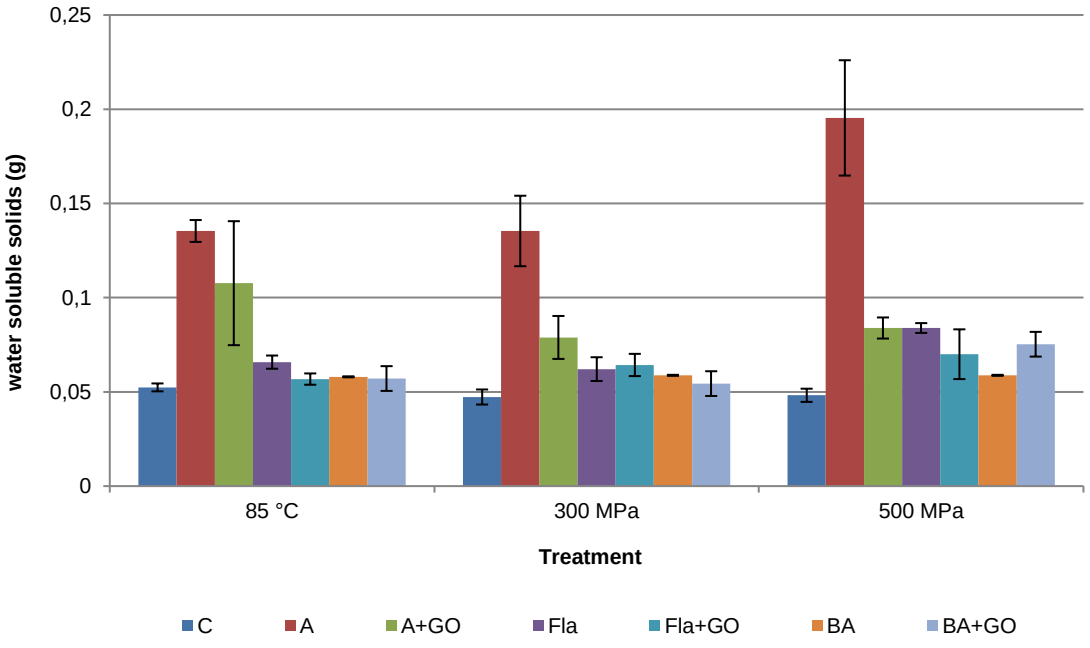

Figure 23. Comparison of the physical treatments when combined with enzymatic treatments.

The enzymatic treatment that behaves better in every conditions considered in this first part of the study will be the one that will be studied in more detail in the second part of the work and this analysis is shown in figure 24. Accordingly, with figure 24 it is possible to assess that the lowest values of WSS in all the physical treatments are the enzymatic treatments performed with iZyme BA with and without GO addition. In other hand, the enzymatic treatments with alcalase with and without GO show the highest values of WSS, meaning that, although alcalase appears to be the best pre-treatment because it shows high values of WSS in the solubility tests, and a further network formation is formed when using a polymerase like GO, values of WSS are still higher than those obtained with the other proteases. To reinforce the data shown above, a one-way ANOVA and tukey HSD post-hoc analyses were performed and demonstrate that there are significantly differences ($p \leq 0.10$) among the 6 treatments at a 90% confidence (Table 5). Table 5 shows that iZyme BA (BA) is the sample that shows the lowest WSS values independently on the physical treatment applied, with no significant difference with BA+GO combination values. Therefore, the enzymatic treatment that will continue in the next part of the work will be the iZyme BA alone and with GO because it also has low WSS values. Since the polymerase can have a different behaviour when combined with the hydrocolloid it is decided that will be used in the next part of the work.

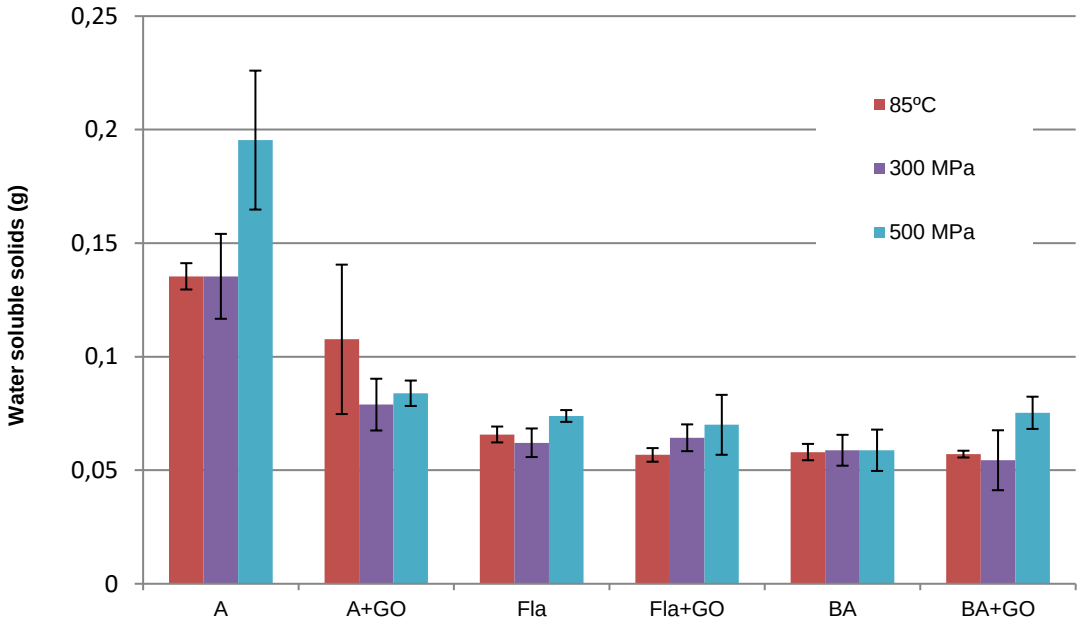

Figure 24. Comparison between all the enzymatic treatments.

Table 5. ANOVA groups for all the enzymatic treatments combined with each of the physical treatments ($p \leq 0, 10$). (post-hoc tukey HSD analyse)

Treatment	Mean WSS(g) ± standard deviation
A	0,1554 ±0,0379^{c}
A+GO	0,7960 ± 0,0098^{b}
Fla	0,0676 ± 0,0074^{a,b}
Fla+GO	0,0645 ± 0,0133^{a,b}
BA	**0,0586 ± 0,0079^{a}**
BA+GO	**0,0643 ± 0,0142^{a,b}**

^{a,b,c} – Homogenous groups within each analyse according to the tukey HSD test as 90% confidence level.

5.2. Studies of the addition of xanthan gum on the gelation capacity of enzyme treated *Acheta domesticus* flour submitted to different physical processing methods: Temperature and Pressure

On the first part of the dissertation, the assessment of the better enzymatic and physical processing method to enhance the gelation capacity of *Acheta domesticus* flour was studied. From this study the best enzymatic treatments appear to be the pre-treatment, iZyme BA (BA) with and without Glucose oxidase (GO) and the best physical treatments were temperature (85 ºC) and pressure (300 MPa). Despite the enhancement of the gelation capacity, a strong gel was never formed. In that way, the combination of the *Acheta domesticus* (AD) flour with a well-known hydrocolloid, XG at different concentrations was performed and texture, WSS and WBC tests were performed.

5.2.1 Assessment of the more suitable enzyme treatment to *Acheta domesticus* flour

In order to assess the best enzyme or combination of enzymes (iZyme BA alone or iZyme BA with Glucose oxidase) when combined with XG, various analyses were performed and the results are displayed in figure 25 and figure 26. For these preliminary tests, xanthan gum at 1% w/w was chosen because it is a widely used concentration in food production, to give strength and consistency to gels. Besides that, is more convenient, taking in account the conditions of the study. Analysing figure 25, the higher viscosity

value appears when the xanthan is used alone which indicate that the enzymes are changing the conformity of the protein molecules. The XG was added to the insect powder suspension after being enzymatically treated with individual addition of the protease (BA) or the combined action of protease and polymerase (BA+GO). Results in Figure 26 show better results adding both enzymes and blending them with xanthan gum. Specifically, this combination led to a gel with increased firmness, lower number of WSS and increased WBC. Consequently, this enzymatic treatment shows better physical behaviour, good capacity to retain the liquids and higher gelation capacity which are important features for gel formation. Taking this in account, the combined action between protease and polymerase (BA+GO) was used in the rest of the experimental work. In the next step, different concentrations of XG were used to assess the effect of this hydrocolloid in the enzymatically treated solution of *Acheta domesticus* flour.

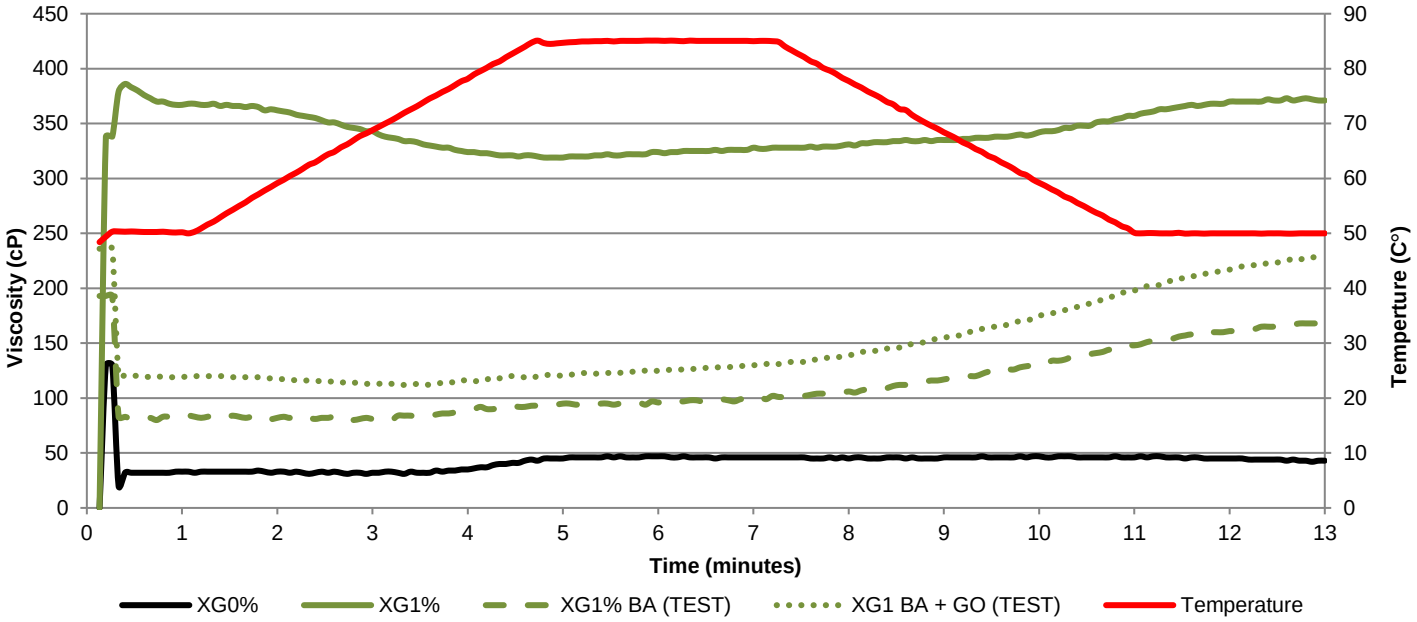

Figure 25. Viscosity analyse of both enzymatic treatments in combination with Xanthan gum at 1% w/w.

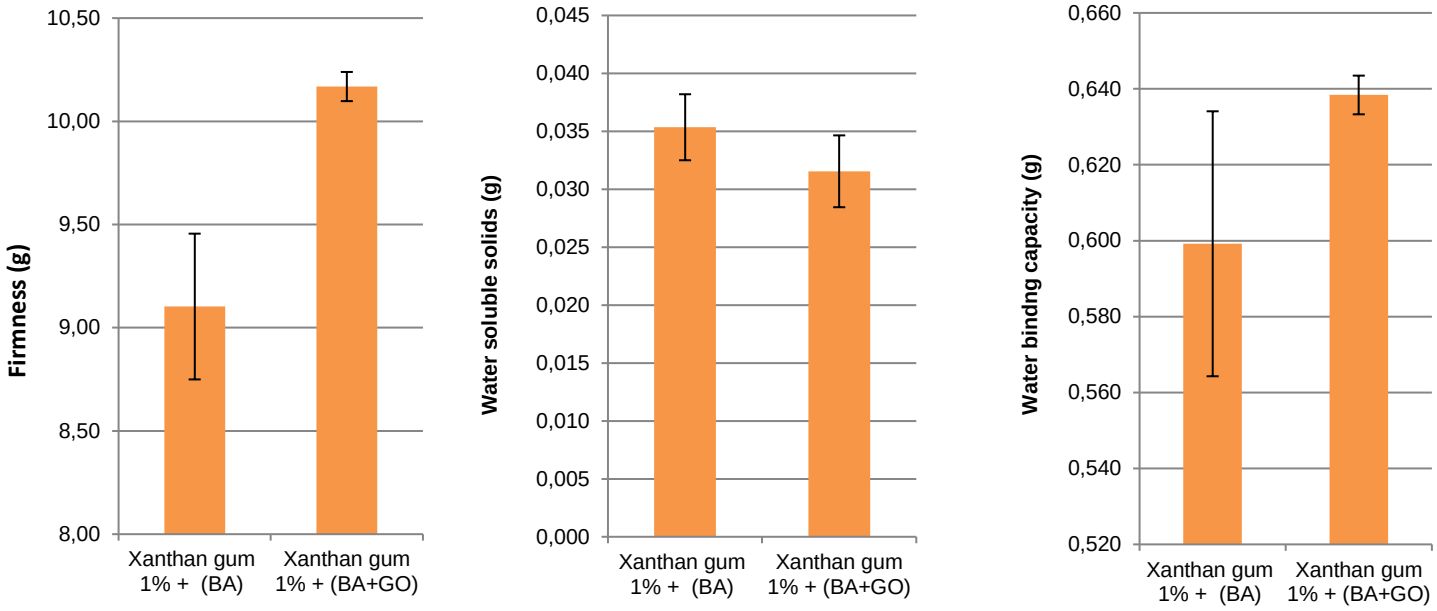

Figure 26. Firmness, water soluble solids and water binding capacity analyses of enzyme treatments combined with xanthan gum.

5.2.2 Assessment of the gelation capacity of Acheta domesticus flour combined with Xanthan gum and temperature

At this point of the work, three different concentrations (% w/w) of XG where tested (Figure 27). All the samples were subjected to a heating-cooling cycle with the RVA recording the apparent viscosity. Resulting gels were tested for texture, Water Binding Capacity (WBC) and Water-soluble solids (WSS). The results are present in figure 28, figure 29, figure 30 and figure 31. Xanthan gum was used alone and in combination with the enzymes chosen in chapter 5.1.3 (BA and GO). In the last case, to test the impact of possible xanthan inhibition of the enzymatic activity, xanthan gum was added after enzymatic treatment or during it (figure 27). So, for the analyse of the results three variables need to be considered:

- The concentration of XG used.
- the use or no use of the enzymes.
- the order of addition of the XG in the solution.

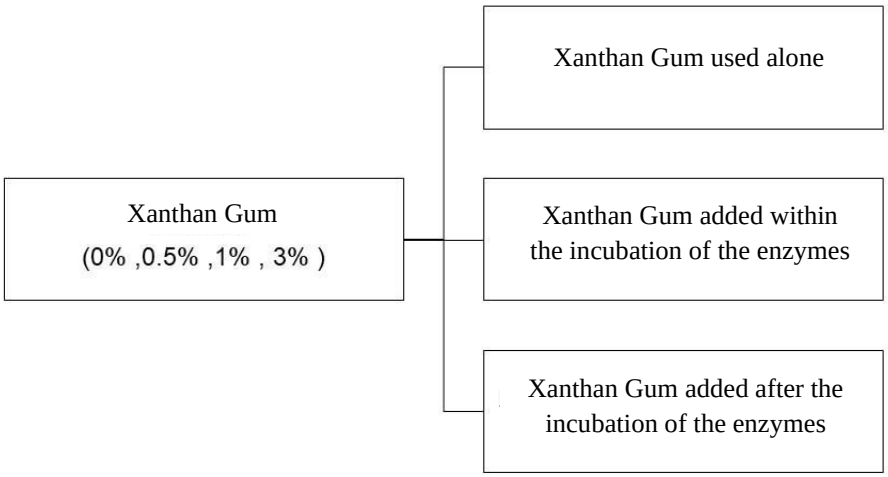

Figure 27. Xanthan gum treatments: concentration, presence of enzymes and moment of addiction.

Figure 28 shows the viscosity recorded during the heating-cooling treatment of all the samples. Accordingly, to the first factor (xanthan gum concentration) in study, viscosity plots show that the addition of xanthan gum to the insect flour suspension increased the viscosity during heating-cooling process. Plots also show that the viscosity decreased during heating, which might indicate lower stability of the gel during heating. The extent of the viscosity increase was dependent on the concentration of xanthan gum. The same was reported by Graça *et al.* (2020) in the study of the effect of Xanthan gum in extruded snacks coating.

Through the second factor (xanthan gum addition at the incubation of enzymes) (Figure 28, dotted lines), it was observed that when xanthan gum was added with the enzymes and the suspension was subjected to the heating-cooling cycle a decrease in the apparent viscosity was observed up to 1% xanthan level, although the viscosity pattern are very low. This might be due to the concentration of the hydrocolloid is so low that could not overtake the changes induced by the enzymes. Conversely, that trend was not observed when xanthan gum was added at 3%, likely the hydrocolloid inhibits the enzymatic action or overtakes the physical changes conferred by them, leading to no change in viscosity. In the case of the third factor, when xanthan gum was added after enzymatic treatment (Figure 28, dashed lines), a similar trend as the one described for the hydrocolloid addition before enzymatic incubation was observed. Again, the addition of 3% xanthan gum was an exception, but in this case a decrease of the viscosity was

observed although it increased during the cooling stage, leading to high viscosity gels. Therefore, heat is a very important step for the visible effect of XG. Those decreases in the apparent viscosity show that the enzymes are responsible for microstructural changes of the molecules present in the solution as explained in the review work of Sun and Holley (2011).

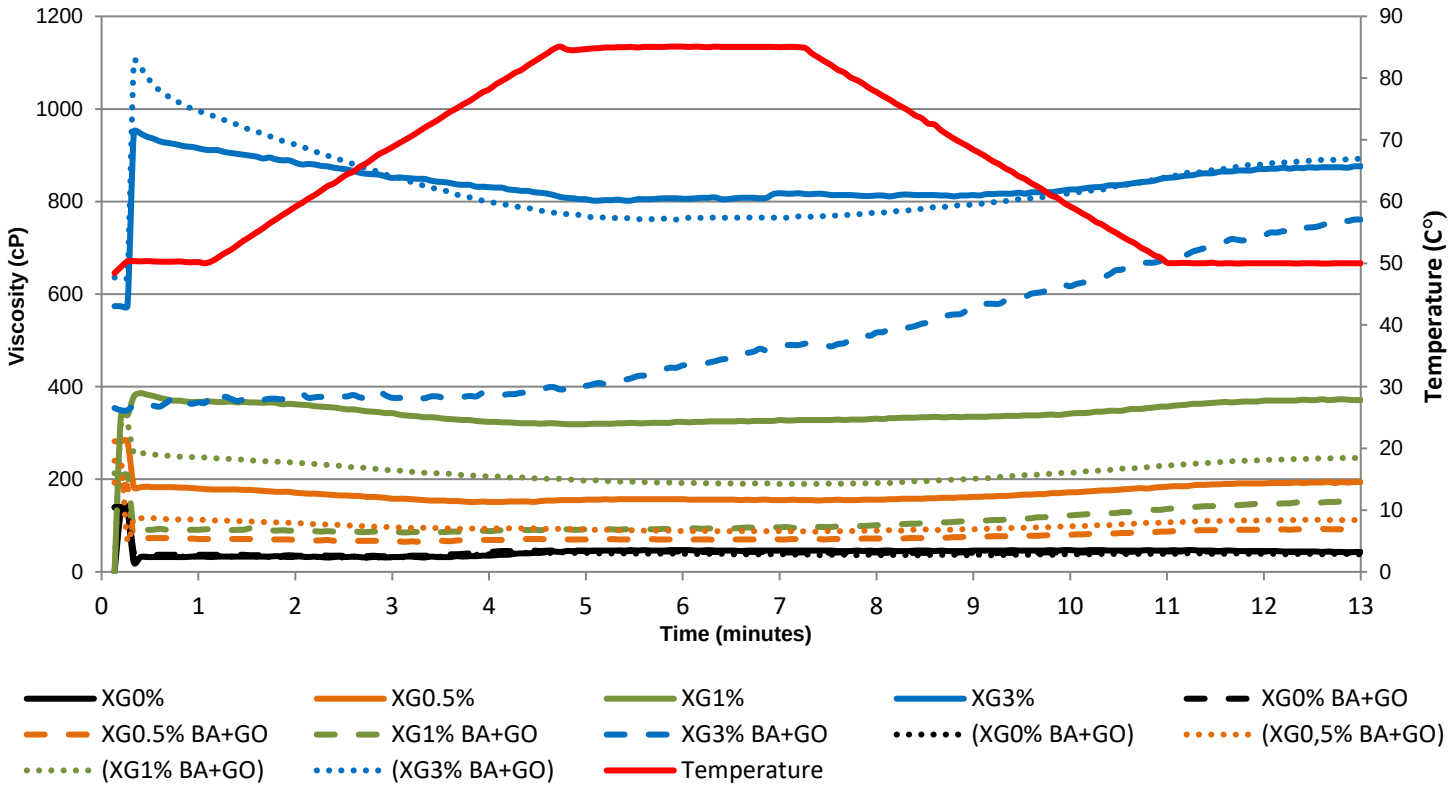

Figure 28. Viscosity analyses of Xanthan gum at different concentrations (0; 0,5; 1,0 and 3,0% w/w) samples with and without enzymatic treatment (BA+GO).

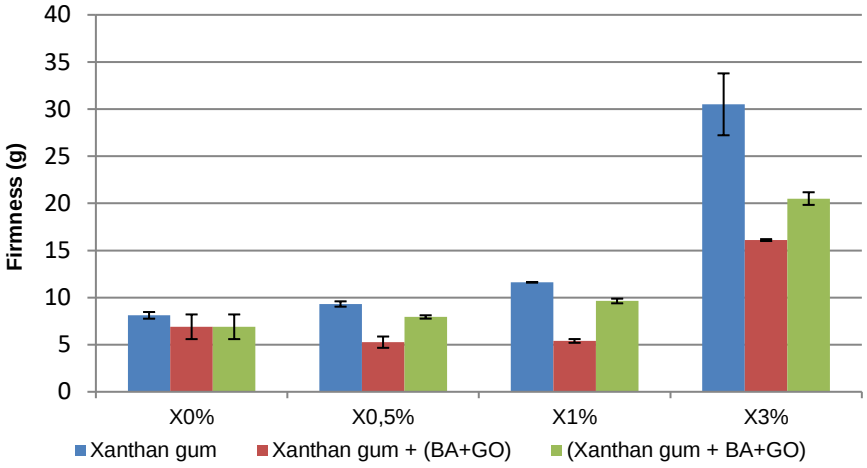

Figure 29. Firmness of the heated samples.

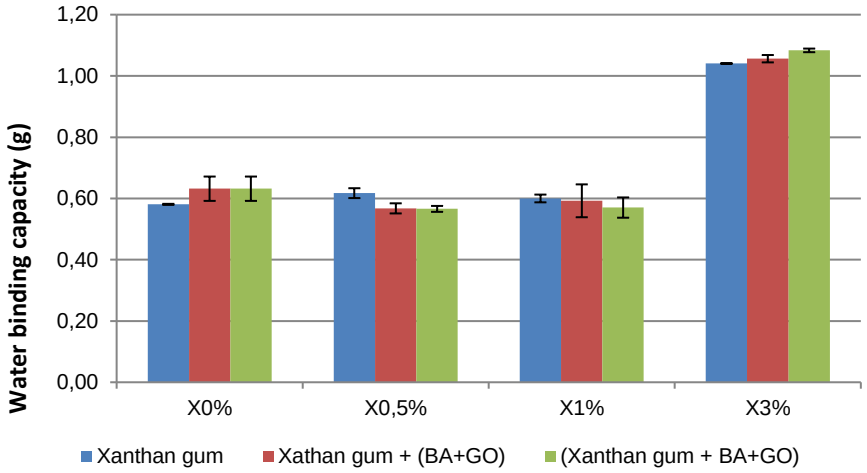

Figure 30. Water binding capacity analyses of the heated samples.

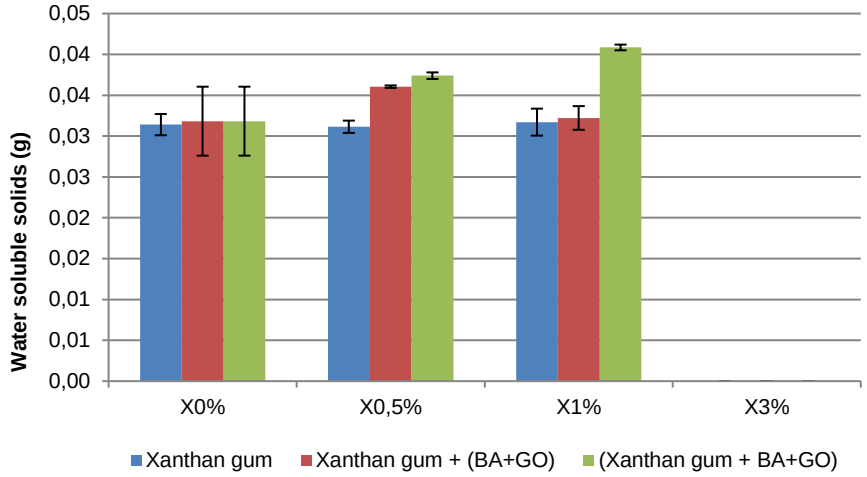

Figure 31. Water soluble solids analyses of the heated samples.

In figure 29, the values of firmness of the gels obtained after the respective treatments show very low values, in all cases. However, it is possible to see a certain pattern. In the lower concentrations of XG the values between them are almost the same (between 5 and 12 g). In 3% w/w of XG, an increase to values between 15 and 30 g was observed. Xanthan gum is often related with weak gels and so, low values of firmness were expected, as well as the slightly increased in firmness already reported for yellow layer cakes that were firmer in the presence of XG (Gómez *et al.*, 2007). The use of enzymes when related with this parameter reflects lower values of firmness which is congruent with the results of viscosity. When enzymes are used, the addition of XG during the incubation of the enzymes have higher values of firmness, which can be related with the benefit of heat exposure in samples with XG.

Analysing the WBC in figure 30, information is given about the capacity that the solution displays to retain the water in the network formed after the treatment. This parameter can be related with the syneresis, that is the liberation of water from a gel. So, higher values of syneresis reflect lower values of WBC. Mohanan *et al.* (2020) assessed foam stabilization on pulse protein-Xanthan gum complexes and observed an increase on syneresis in the absence of XG, that means a reduction on WBC. So as expected, the presence of XG at 3% w/w reflected the higher WBC of the four different concentrations. The presence of enzymes does not present significant differences between the samples, which indicate that the main responsible for the liquid retention is the hydrocolloid at the proper concentration. The order of the addition of XG neither has any effect in this parameter.

Finally, in figure 31, also shows that at 3% w/w XG concentration no water-soluble solids are present which is a very interesting result. This happens because, after the centrifugation, there were no supernatant in the tube which means that the concentration of XG added in this samples was sufficient to trap all the water present in the solution and give origin to a gel-form solution. Though, this only happened after the centrifugation process. WSS were used in this work as a reflection of soluble solids present in the supernatant. This means that the solids present can be free molecules like amino acids that did not get trapped within the gel formation, so higher the WSS lower the gelation capacity. Taking this in account, other parameters can be related with this as, protein solubility or increase of disulphide and non-disulphide bonds within the gel. H. Zhang *et al.* (2019) appraised that the presence of XG in egg-white heat induced gels could lead to a decrease in sulphydryl molecules that are no longer available in the aqueous solution, reflecting a reduction of WSS, which is congruent with our results. With no

enzyme, the WSS until the 1% w/w XG concentration do not show any visible change. With the presence of enzymes till the 1% w/w concentration there is an increase in the water-soluble solids and so, a decrease in the gelation capacity of this solution. This fact is important because it shows that the enzymes change the internal conformity of the solution. The order of the addiction of the XG appears to show slightly higher WSS when it is added during the incubation process.

Taking in account the three variables studied, concentration, use of enzymes and moment of addition of the enzymes, only concentration showed visible differences. So, a one-way ANOVA was performed for the concentration variable to assess if there were significant differences between the different concentrations. There were significant differences between the XG concentration. Knowing that a tukey HSD post-hoc test was performed to see which were the differences between the groups in study (Table 6).

Table 6. ANOVA groups from the concentration variable using the post-hoc tukey HSD test.

		Firmness (g) ± standard deviation	WSS (g) ± standard deviation	WBC (g) ± standard deviation
Concentration (% w/v)	0,0	7,3894±1,3199[a]	0,0319±0,0039[b]	0,6156±0,0446[a]
	0,5	7,5219±1,8788[a]	0,0349±0,0030[b]	0,5838±0,0305[a]
	1,0	8,8852±2,8479[a]	0,0349±0,0048[b]	0,5887±0,0429[a]
	3,0	22,4323±7,0169[b]	0,000±0,000[a]	1,0601±0,0212[b]

[a,b] - Homogenous groups within each analyse according to the tukey HSD test as 95% confidence level.

It is important to understand that this work is preliminary and that the *Acheta domesticus* flour used is a no well-known raw material in the food industry. Consequently, the main goal is not to have the firmest gel but to understand the effect of different additives and treatments in the physical characteristics of the solutions obtained, to extend possible different applications of this product. For example, analysing all the parameters studied in this chapter, we saw that all the treatments at 3% w/w of XG do not present WSS reflecting the best gelation capacity obtained until this point. Besides that, they all present different patterns of viscosity and texture. So, gels with the addiction of enzymes combined with XG have good gelation capacity but softer texture. Knowing that, the

enzymes could be a good additive if the final product would be something with good form but a weak internal structure to be easy to swallow.

5.2.3. Assessment of the gelation capacity of *Acheta domesticus* flour combined with Xanthan gum, pressure, and temperature

Results from the first part showed that better performance with 300 MPa pressure treatment, because of that this pressure level was selected to assess the impact when combined with the hydrocolloid chosen.

Accordingly, with the results from the chapter 5.2.2 the concentration of XG at 3% w/w was the only concentration with significant differences in the results for texture, WSS, and WBC ($p \leq 0,05$) (table 6). Like so, all the samples with this concentration were exposed to a 300 MPa pressure treatment and after that the viscosity during heating-cooling was recorded (figure 32). At the same time, the assessment of the gel firmness (figure 33), WSS and WBC (figure 34) were performed. As the other variables have already been analysed in this chapter the only variable taken in account was the effect of the pressure treatment.

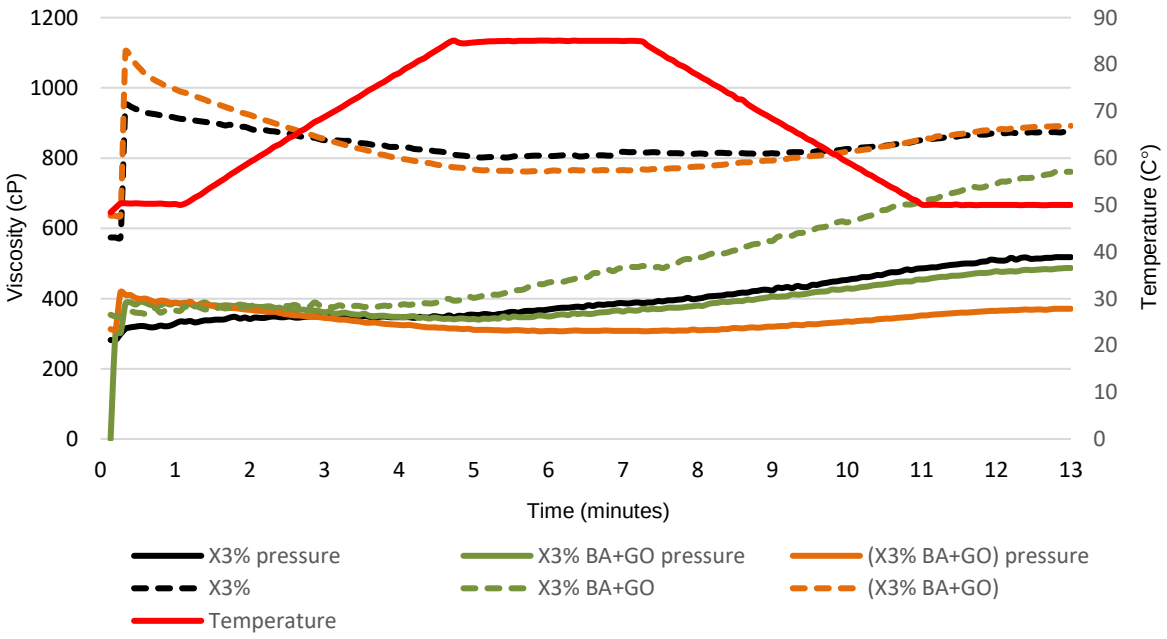

Figure 32. Viscosity analyses of the pressured and no pressured Xanthan gum treatments. Xconcentration (XG alone); Xconcentration BA+GO (XG added after the incubation of the enzymes); (Xconcentration BA+GO) (XG added within the incubation of the enzymes).

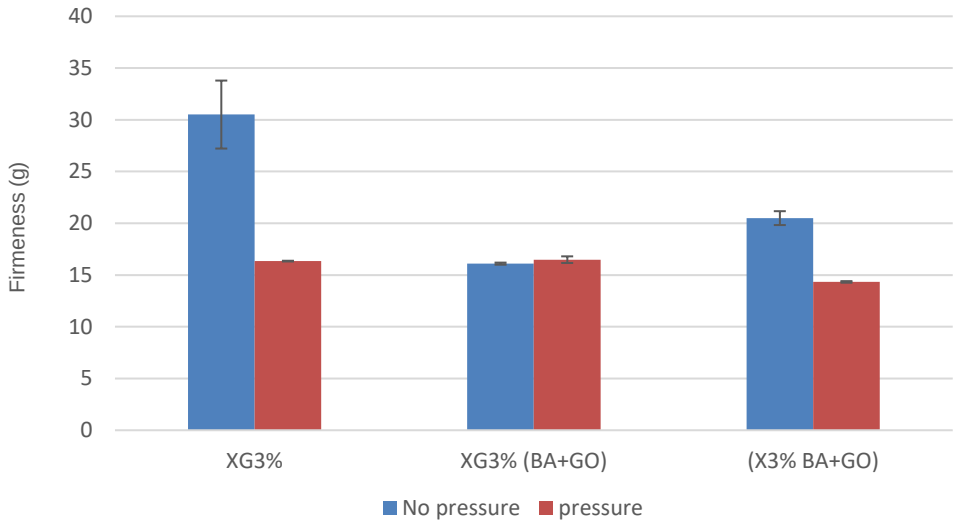

Figure 33. Firmness analyses of the samples treated with pressure and temperature.

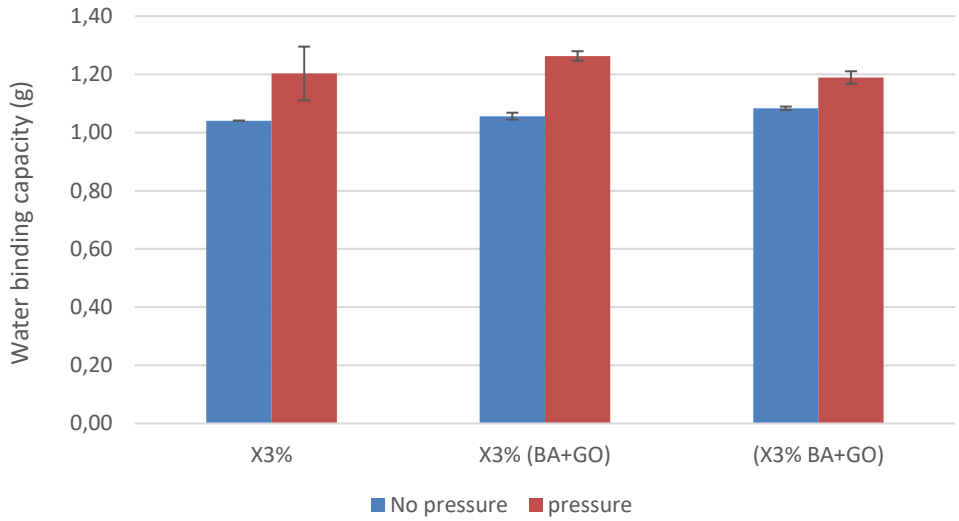

Figure 34. Water binding capacity of the samples submitted to pressure and temperature.

According to figure 32, it is possible to assess that the samples with the pressure treatment have lower values of viscosity when compared with the ones without this treatment. This could mean that the pressure treatment affects the internal structure of the solution in a much bigger way than the use of only enzymes. As it is possible to see, the XG at 3% w/w is sufficient to increase the viscosity of the samples with no pressure treatment but it is not for the samples with the treatment. This was reported in various studies of the effect of high-pressure treatments in xanthan gum solutions. Laneuville *et al.* (2013) and Eren *et al.* (2015) observed an increasing degradation of the network

structure of the gel which lead to lower viscosity on the presence of pressure as assessed in this work.

For firmness (figure 33), there is an expected decrease in this parameter when the samples underwent a pressure treatment since the viscosity decrease and the internal network is highly affected (Eren et al., 2015). This was precepted in XG3% and (XG3% BA+GO) because the firmness decreases in the samples treated with pressure. In the samples where Xanthan Gum was added after the incubation, XG3% (BA+GO), the firmness was almost the same with and without pressure treatment. This might be explained because, as said before, with no pressure, the viscosity of that sample only increased after the temperature exposure, on RVA. In figure 32 , it was assessed that the pressure treatment changes the conformity of the solution more widely than only the enzymes, and that this sample, XG3% (BA+GO), did show the increase of viscosity after the temperature exposure. The late increase of viscosity can lead to less firm solutions, explaining the same results with and without pressure.

HPP treatment does not modified the WSS, therefore, 3% XG seems to be the major responsible for the water retaining of the solution. All the water was trapped in the solid solution leaving no supernatant to evaluate. When a HHP treatment is performed, the system ionic interactions appear to increase which can be related with better gelation capacity (Chattong *et al.*, 2015)

Lastly, in figure 34 it is possible to evaluate the WBC of the solutions. A slight increase in this parameter was observed which appears to have something to do with the possible conformational change that might lead to a better water imprisonment. This WBC can reflect the water retention capacity (WRC) of a solution as well. So, Chattong et al. (2015) observed that XG could increase the WRC with the addition of XG and a pressure treatment supporting our results.

Future studies must be performed to assess more precisely the results of this work. Microstructural three-dimensional analyses, for example confocal laser scanning microscopy (CLSM), would be interesting, for the assessment of the conformational changes that seem to happen in the analyses of the parameters of viscosity, texture, WSS and WBC studied. In deep analyses of physical and chemical parameters would be interesting to the possible incorporation of the *Acheta domesticus* flour as an ingredient in a food matrix.

6. Conclusion

Results from this study allows concluding:

- Physical (heat and high hydrostatic pressure) and biochemical (proteases and polymerase) treatments affect the gelation properties for the *Acheta domesticus* flour, being promising methods for extending the application of this flour in food processing.
- The treatment of insect flour with proteases increases WSS, confirming the hydrolysis of the protein chains, likely leading to soluble peptides.
- Conversely, the addition of polymerases decreases the WSS, specifically the greatest effect was observed with Alcalase (protease) and GO (polymerase).
- The combination of iZyme BA (protease) with GO (polymerase) leads the lowest values of the WSS indicating that proteins breakage followed by crosslinking promotes greatest structural changes enhancing strength.
- Regarding physical treatments, the heat treatment is the most effective for reaching gelation.
- The addition of Xanthan Gum at 3% w/w leads to house cricket flour gels, with higher viscosity profiles, increased texture, and water binding capacity and zero water-soluble solids after centrifugation.
- The addition of Xanthan Gum together with the action of enzymes results in less viscous, weaker gels with lower water holding capacity, probably due to a change in the internal conformation of the solution.
- HPP treatments in insect flour gels with xanthan gum lead to lower viscosity, firmness, and higher WBC, probably due to the gel network structure degradation.

By the application of different physical and/or biochemical strategies, insect flour gels/solutions with different physical characteristics can be obtained. Therefore, it is necessary to go deeper into this type of studies to be able to know the different possibilities that could be obtained with insect flour to formulate foods with “taylor-made” texture.

Besides that, this was a very preliminary study so more investigation needs to be performed with this food product specially on structural and sensorial characteristics and consumer acceptance of this kind of product.

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