Creating a Chicken Analogue with Mycoprotein

Master's thesis

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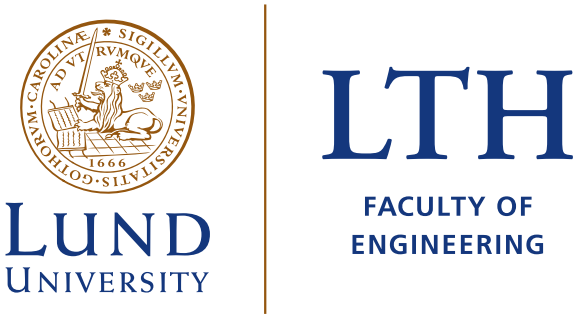

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Abstract

To meet the growing demand of meat alternatives, this project was dedicated to creating a chicken analogue prototype with mycoprotein. Within the project, two approaches of alginate gelation, external (diffusion) crosslinking and internal crosslinking, were studied in this particular food matrix.

Using the external crosslinking method, the effects of major ingredients on the texture, structure and taste of the product were studied. Biomass is the major structure forming ingredients, and alginate, citrus fiber, salt and oil contribute to increasing the water holding capacity and modifying the firmness of the sample. The effects of different concentration of calcium salt solutions on the rate of crosslinking were investigated. The crosslinking rate from high to low is: 2.5% (w/w) $CaCl_2$, 5% calcium lactate, 1% $CaCl_2$, and 2% calcium lactate. Several heat treatment approaches were investigated to eliminate the astringency and off-taste generated by the inclusion of oil in the formulation. And heating the mycoprotein and water mixture was found to be the most suitable method.

Two approaches of internal crosslinking were studied. While the combination of sodium alginate solution, D-glucono-δ-lactone, and $CaCO_3$ did not lead to any desirable results, a sample with balanced texture between tenderness and chewiness was obtained with the combination of sodium alginate powder and $CaSO_4$.

The fiber alignment of the prototype was created through simplified extrusion, and the presence of alignment was examined by running a texture analysis. The significant difference ($p<0.05$) in fracture force and hardness of sheared samples in perpendicular and parallel directions verified the existence of aligned fibers in one general direction.

Overall, the objectives of this project were achieved and a chicken analogue prototype with aligned fibrous structure was obtained.

Preface

This master thesis project was fully carried out at the facilities of the company Mycorena AB at Gothenburg. I would like to express my great appreciation for my supervisors Sicong Zhu and Paulo Teixeira, and all the colleagues at the company, who have given me guidance, support and many valuable feedbacks throughout the project. It was overall a very pleasant experience that I learned a lot from.

I would also like to thank my supervisor Lars Nilsson and my examiner Björn Bergenståhl from LTH for the academic support and valuable inputs on this project.

Popular Science Summary

This project made use of the fibrous nature and taste-neutral characteristic of fungi mycelia, and developed a chicken alternative with this alternative protein.

In recent years, the demand for protein from non-animal sources has been ever-growing due to the increasing population and environmental consciousness. Besides plant-based protein, fungi as a protein source have received high interest in the industry. Mycelia, which can be considered as the root of fungi, contains more than 50% of protein on a dry mass basis. In addition, its color and taste neutral features, and fibrous texture makes it a material with high potential for constructing meat alternatives.

Gelling agents are usually needed for meat alternatives to act as a glue holding proteous materials together. Alginate is a natural gelling agents derived from algae, and is often used in the food industry. When in contact with Ca^{2+}, alginate forms a stable gel that withstand a wide temperature range. In this project, mycelia, alginate, together with oil are used to create an alternative protein food product that highly resembles chicken.

The effects of ingredients on product texture and structure were studied to aid the formulation process of the current and future projects. The gelation of alginate can be approached by either soaking the food matrix in calcium solution bath, or pre-mixing calcium that can be slowly released into the matrix. Both approaches were studied. Different calcium solution bathes were tried out to see their effects on the gelation rate and $CaCl_2$ was found to be more effective than calcium lactate. Adding vegetable oil was found to increase the tenderness and juiciness of the product. However, it brings about undesirable mouthfeel and odor, and different oils were found to generate these effects to different extends. By adding additional heat treatment after mycelia and water was mixed can effectively avoid the unpleasant attributes. Mixing a premade powder of sodium alginate and $CaSO_4$ into the mycelium matrix gave good results in terms of a texture and mouthfeel similar to chicken. The fibrous muscle strand-like structure of the product was created by simply squeezing the paste through a small outlet. The aligned fibers could not only be seen visually, they were also detected by texture analyzer.

Overall, this project successfully created a chicken alternative with a neutral taste, meaty texture and fibrous appearance.

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1. Introduction

1.1 Background

Proteins, due to their significant effects on human health, are a crucial part of our diet. Despite the predicted increase in animal-derived protein demand, the trend for consumers to include more alternative proteins in their diet has also become increasingly evident in recent years (Henchion et al., 2017). 59 % of the people surveyed in a study in the United Kingdom in 2013 indicated that they had reduced or were willing to reduce their meat consumption (Vegetariansociety, 2013). The act of animal product avoidance could derive from ethical considerations, nutritional concerns, or lifestyle choices, etc. (Rosenfeld and Burrow, 2017). Sustainability is also one of the major driving forces on promoting a protein shift. By 2050, the global population is expected to grow to 9.7 billion, presenting a huge challenge to the world agricultural system to provide enough of nutritious food to meet the demand of the growing population (Fao, 2018). Concerns over food security and environmental issues arise along with the increasing demand.

In order to create a more sustainable future, the world seeks solutions in various alternative protein sources. Alternative proteins, obtained from plants, insects, or single cells (such as algae and fungi), are used to replace conventional animal-based sources, such as meat, dairy, and eggs (Bashi et al., 2019). Plant-based proteins sourced from cereals and pulses are the most well-established alternative proteins nowadays (Sha and Xiong, 2020). However, plant cultivation uses vast resources of land and water, and its sustainability is further compromised when international transportation is required for its usage (Balogh and Jámbor, 2020, Jaeger et al., 2016). Other environmental issues such as deforestation, loss of biodiversity, and fertilizer-caused pollutions can also be connected with certain crop cultivation (Boerema et al., 2016). The incomplete amino acid profiles of plant proteins and the anti-nutrients existing in them further challenge its replacement of meat products (Sha and Xiong, 2020). The application of insect proteins currently lies mainly on consumer acceptance and food safety issues (Zhang et al., 2022). Cultured-meat, not conventionally defined as an alternative protein as it is obtained from growing animal tissue cells in culture media, is another promising meat-replacement approach (Henchion et al., 2017). But there are still major technical challenges to be overcome for cultured-meat to become a readily available commercial

product.

In recent years, a more renewable and sustainably protein producing method that is not limited by geographic or climatic conditions has received more and more attention. Single cell protein (SCP), often produced by yeast, bacteria, microalgae, or fungi in bioreactors, is also referred to as bioprotein, microbial protein or biomass (Sharif et al., 2021). Compared with plant proteins, production of SCP does not have as intensive demand on water or land (Nyyssölä et al., 2022). The production life cycle can be as short as several hours (Sharif et al., 2021). SCP has a long history of being produced and used as both animal feeds and human foods. The first commercial microbial feed, Pruteen, was launched as early as the 1970s by Imperial Chemical Industries (Westlake, 1986). The most well-known, also currently the only commercially available single cell protein food on the market is Quorn. Quorn foods, containing mycoprotein derived from the fungus *Fusarium venenatum*, was first launched in 1985 by Marlow Foods (Quorn, 2019). Its success has inspired many other companies in the world to explore the development of novel meat alternative food products from mycoprotein.

1.2 Aim

This thesis project aims to develop a shear-induced structuring method and formulation that is suitable for creating a chicken analogue with aligned fibrous structure using mycoprotein. In order to achieve the final goal, several aspects were studied in the product development process:

- The effects of fungi biomass, sodium alginate, citrus fiber, oil, and salt on the final product texture and mouthfeel;
- Applicability of external (diffusion) and internal setting method of ionotropic gelation of alginate in this product formulation, and factors influencing the gelation rate and end product texture;
- The influences of fat on the product sensory profiles;
- Alignment of fibers and its relationship to extrusion;
- Obtain a prototype of chicken analogue using fungi biomass;

1.3 Mycoprotein and mycelium-based meat alternatives

Mycoprotein was introduced to the world as a food source over 50 years ago as a result

of the green revolution in the UK to find a new sustainable protein source (Finnigan, 2011). In 1985, the results of a ten-year assessment of the safety of the *Fusarium venenatum* strain earned the approval from the UK Ministry of Agriculture, Fisheries and Food to sell mycoprotein as food (Ahmad et al., 2022). And the US Food and Drug Administration recognized mycoprotein as "generally recognized as safe" (GRAS) in 2002. Mycoprotein is generally produced in liquid media in fermentation tanks. After "harvesting", a series of downstream processes, such as washing, heating, pressing, and freezing, are carried out to make the mycoproteins ready to be used for food production (Finnigan, 2011). The fermentation stage is crucial for the efficient growth of the fungi and only the targeted strain of fungi. The downstream processing is also detrimental to the safety of consumption of the mycoprotein. The whole production life cycle of fungi is very short. The process from pre-culture with spores to the final harvest takes a matter of days (Barzee et al., 2021). Within a week, the fungi derived food product would be ready to be shipped. This production is much more efficient compared to both animal products and plant-based alternatives in terms of time, land use, and energy use (Hashempour-Baltork et al., 2020). It is also possible to use side streams from other food industries as a substrate for the fermentation of the fungi, which helps to reduce wastage and step closer to circular agriculture (GmoserLennartsson and Taherzadeh, 2021).

Besides the environmental benefits of mycelium-based foods, mycoproteins also have many other prominent features that make it an excellent alternative protein source. Mycoproteins are a low-fat (very low in saturated fatty acids), high-protein (high in protein quantity, quality, and bioavailability), and high-fiber material with a variety of vitamins and minerals (Ahmad et al., 2022). Their flavor and color can be manipulated through the fermentation process to mimic one particular target food product or be very neutral (Barzee et al., 2021). The texture of fungi-based food is also unique. The natural texture of the mycelium, especially in filamentous morphology, is described to be fibrous, which could mimic the myofibrils of the animal muscle tissue. Assisted with texturization techniques, mycoprotein can form meat alternative products with visibly aligned structure (Miri et al., 2005).

1.4 Texturization method for meat alternatives

One of the biggest hurdles for producing meat analogue is to imitate the characteristic texture and structure of meat with non-animal derived materials. The structuring

techniques can be divided into 2 categories: top-down strategies and bottom-up strategies (DekkersBoom and Van Der Goot, 2018). Bottom-up strategy puts together individual structural elements that have been constructed first into a larger body. It includes cell culturing, wet spinning, and electrospinning. While the top-down approach only mimics the structure in a macroscopic view. Techniques such as extrusion, freeze structuring, and shear cell technology belong to this category.

Extrusion is the most commonly used technique for the commercial production of meat analogues (Dekkers et al., 2018). Plant protein extrusion can be conducted under various different moisture and temperature conditions. During the extrusion process, materials are conveyed, mixed, sheared, and cooked (Ek and Ganjyal, 2020). A series of physical and chemical changes induced by the extrusion process results in end products with very different textural properties. A typical food extruder consists of: feeding system, preconditioner, extruder barrel, and knife cutter (Riaz, 2013). However, any process where "molten material is forced through a die to produce components of a fixed cross-sectional area" can be considered as extrusion (Hill, 2005). Therefore, complex machinery is not always necessary to apply extrusion.

Though many studies and industrial applications have been done on the extrusion of plant proteins, the published works on the aligning effects of shear on mycoprotein is rather limited. Miri et al. (2005) have shown that extrusion process subjected on mycoprotein paste could result in a high proportion (~80-90 %) of fiber alignment, indicating that extrusion technique could be potentially used to create structurally aligned meat analogue with mycoprotein.

1.5 Alginate

Alginate is a natural occurring polysaccharide in some algae and bacterial cell walls (Helgerud et al., 2010). Alginic acid, and several alginate salts are recognized as additives safe for food use in the USA and in Europe. It has a wide range of application in the field of food, biomedicine, pharmaceutics, and 3D printing, etc. (Hu et al., 2021). Due to its ability to form a hydrogel, it is widely used as stabilizer, emulsifier, thickener, and gelling agent in food applications (Ahmad RausWan Nawawi and Nasaruddin, 2021). Upon the encounter of multivalent ions, G-blocks (regions on the alginate molecule that consists of only guluronic acid units) of alginate molecules interact and form junctions that link molecules closely together. This interaction is commonly described as the "egg-box model" (Figure1-1). The gel formed by alginate is thermo-

irreversible, which is not common for food grade hydrocolloid gels (Helgerud et al., 2010).

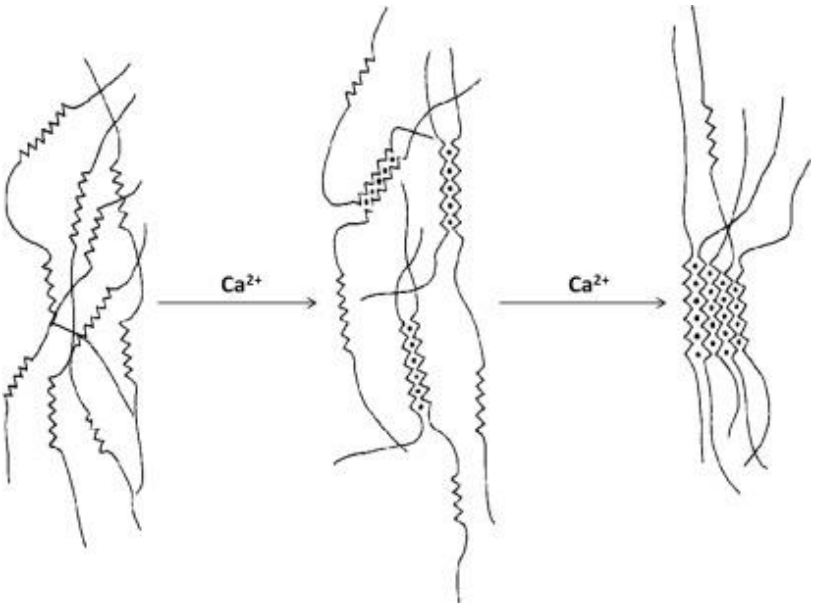

Figure 1-1 The egg-box model of alginate gel formed with Ca^{2+} (Petzold et al., 2019)

Alginate form gels under low pH environment (pH<4.0, and preferably around pH 3.4) and with divalent or trivalent cations (Helgerud et al., 2010). In the food industry, calcium is the most commonly used cation for alginate gelation. The ionotropic gelation of alginate can be divided into: external setting, when alginate containing matrix is submerged into a soluble calcium salt bath for the Ca^{2+} to diffuse into the matrix for crosslinking; and internal setting, when Ca^{2+} is slowly released into the matrix from its insoluble or chelated form (Gurikov and Smirnova, 2018).

1.5.1 External crosslinking

Alginate can crosslink with any divalent metal ions except magnesium and mercury to form hydrogels (Hu et al., 2021). In external crosslinking method, the gel formed has an inhomogeneous structure as the rapid gelation of the outmost layer hinders the diffusion of calcium ions into the core of the alginate matrix (Ramdhan et al., 2019). There is a concentration gradient in thickness for calcium ions in the formed gel. While the outer layer of has formed a firm gel, the central part could remain un-crosslinked. This would cause problems and limitations in food applications when alginate is the sole gelling agent. The dimension of the product is therefore restricted by the diffusion

capability of Ca^{2+}. While higher concentration of the crosslink cations can shorten the gelation time and increase gel strength, it also induce a higher degree of syneresis, which is water loss that can considered as a quality defect in the final product (Ramdhan et al., 2019). Ramdhan et al. (2019) also pointed out that the pH of the alginate solution could have an effect on the final gel strength and syneresis. Lower pH leads to increased gel strength and syneresis.

1.5.2 Internal crosslinking

The slow and gradual release of cations in the internal crosslinking method is often attributed to chemical reactions. The combination of $CaCO_3$ and a slow hydrolyzing acid D-glucono-δ-lactone (GDL) is the most commonly used. While this method avoids the calcium concentration gradient throughout the matrix as $CaCO_3$ particles are evenly dispersed, the diffusion of the cations will be somewhat restricted when a film of gel is formed around the embedded $CaCO_3$ particles (Hu et al., 2021). However, compared with external crosslinked gels, gels obtained through the internal crosslinking method has better homogeneity and the gelation thickness will no longer by restricted. Despite the higher homogeneity, internal crosslinked gels have higher permeability as small cavities were formed upon the release of CO_2 when $CaCO_3$ is dissolved by acid (ChanLee and Heng, 2006).

1.6 Astringency

Astringency is defined as “the complex of sensations due to shrinking, drawing or puckering of the epithelium as a result of exposure to substances such as alums or tannins” (Astm, 2009) . As clearly opposed to taste, astringency is a sensation and feeling. The most straightforward reflection of the occurrence of astringency, is the increase in friction between the oral mucosal surfaces (Ranc et al., 2006). However, the mechanism behind astringency is more complex than lubrication. Many components in the oral cavity, such as cell membrane proteins, epithelial cells, receptors of mechanical and chemical signals, take part in the interactions with astringent compounds, resulting in the perception of astringency (Rossetti et al., 2008). On popular hypothesis is that astringent is able to induce precipitation of proteins, thereby break the continuity of salivary pellicle which reduce oral lubrication (Gibbins and Carpenter, 2013). Some astringent compounds are able to get through the pellicle layer and be detected by the mechano and chemoreceptors underneath, further stimulating more mechanical

perceptions. The interaction between protein in the saliva, mucosa, or cells and food components is the most studied mechanism. Astringency is most often associated with polyphenolic compounds. Foods such as tea, red wine, dark chocolate, and some fruits that are rich in polyphenols often cause a drying, puckering mouthfeel (Pires et al., 2020). However, some other compounds including organic and inorganic acids, dehydrating agents, multivalent salts, and proteins may also exert astringency in the oral cavity (Biegler et al., 2016). While in some cases, astringency is more tolerable or even considered as a desirable feature as it provides a sense of cleanness in the mouth, under many circumstances it is perceived as an unpleasant sensation (Des Gachons et al., 2012). Therefore, it is very important for food developers to identify and somewhat quantify this particular mouthfeel.

As a sensation comprised of complex reaction in the oral environment, it is very difficult to accurately quantify the degree of astringency. There are a series of methodologies that can be used to measure astringency. The methods include monitoring or predicting of interactions, indirect measurements of salivary complexes and direct measurements (Pires et al., 2020). Direct measurements are carried out by sensory evaluation of panelists or by observation of animal preference. Sensory analysis, though as one of the most used methods of evaluation, comes with many complications. There are many influencing factors on the perception of astringency. Oral pH, temperature, saliva composition, other food components such as polysaccharides can all alter the perception of astringency (GuinardPangborn and Lewis, 1986, LawlessHorne and Giasi, 1996, OzdalCapanoglu and Altay, 2013, BajecPickering and Decourville, 2012, Lamy et al., 2017, OzawaLilley and Haslam, 1987). Other taste, texture profiles and sensations generated by the particular food product also play a part in the perceived astringency. As some astringents possess both astringency and bitterness, there could be a confusion in differentiating the intensity of these 2 attributes when they coexist (Lee and Lawless, 1991). The same situation also applies for sourness (Laaksonen, 2011). There are some sensorial properties, such as sweetness and fattiness can suppress or mask the astringent sensation. Due to the nature of astringency perception, in complex food systems, it is very difficult to evaluate the level of astringency even with trained panelists. Nevertheless, in order to have a better evaluation and control of this sensation, it would be very helpful and important to identify the astringent compound in the food matrix.

1.7 Enzymes in mycoprotein

In order to grow and multiply, mycelia need to have the ability to capture and utilize nutrients from the surrounding environment. This is a process that involves various intracellular and extracellular enzymes. Enzymes that have been detected in mycelia are amylase, lipase, cellulase, pectinase, xylanase, protease, and catalase etc. (Nadim et al., 2015). Extracellular enzyme activities are important to the growth and development of fungi as they not only are nutrient acquisition pathways, but also act as defense mechanism against environmental stress. For example, mycelia living in aerobic environment often exhibit high antioxidant enzyme activities, protecting themselves from oxidative stress (Ahmad et al., 2022). Fungal enzymes have also been utilized by researchers for applications in various fields, including the food industry, animal feed, paper industry, biofuel production, medical industry and bio-waste treatment, etc. (Gupta et al., 2016, Li et al., 2022). In the food industry, extracted fungal enzymes have been used as bread dough strength conditioner, beer brewing aid, and cheese ripening accelerator etc. (Saxena et al., 2001). *Pleurotus albidus* mycoprotein flour has been incorporated into cookies to boost the nutritional profile and bioactivity (Stoffel et al., 2021). Antioxidant enzymes existing in the mycoprotein was shown to largely increase the antioxidant activity of the cookies made. While in many cases, the enzyme activities provided by the fungi is beneficial and desirable, sometimes the enzymes could be problematic. Extracellular enzymes present in the biomass is easy to get rid off or deactivate by thermal treatment, but intracellular enzymes, protected by the cell structures, are more difficult to be influenced. Unwanted, and unpredictable enzyme activities could lead to unfavorable changes in food product quality.

2. Materials and Methods

2.1 Materials

Sodium alginate (The Kitchen Lab); table salt (FALKSALT); fungi biomass; rapeseed oil (Brakes); olive oil (Zeta); coconut oil (Neutral kokosolja from Kung Markatta); shea butter (AAK); peanut oil (Zeta); sunflower seed oil (Coop); hydrogenated rapeseed oil (AAK); citrus fiber (Culinar); Easy Binder (The Kitchen Lab); GDL (Roquette); $CaCO_3$ (Kalcipos), $CaCl_2$, calcium lactate (The Kitchen Lab)

2.2 Biomass acquisition

Filamentous fungi were washed by water and heat treated to deactivate its RNA after being harvested from the bioreactors. Excessive liquid was removed from the biomass by filter and belt press or decanter. The remaining biomass was grinded to fine granules using a spice grinder (MultiTalent 8, BOSCH) and stored in -20 ℃. The biomass was defrosted to room temperature before use. In total of 8 batches of biomass were obtained and used during the process of this project. The batches are numbered and the characteristics of each batch are summarized and listed in Table 2-1. Filamentous refers to the type of fungi morphology where they grow as freely dispersed mycelia (Figure 2-1 a). Pellet refers to the densely packed biomass morphology (Figure 2-1 c). Filamentous-pellet refers to the type of morphology that is in intermediate states (Figure 2-1 b). Batch 1 to 6 were used to conduct experiments with the external crosslinking method. Batch 7 and 8 was used for trials with the internal crosslinking method. The change between different batches of biomass was due to availability of supply.

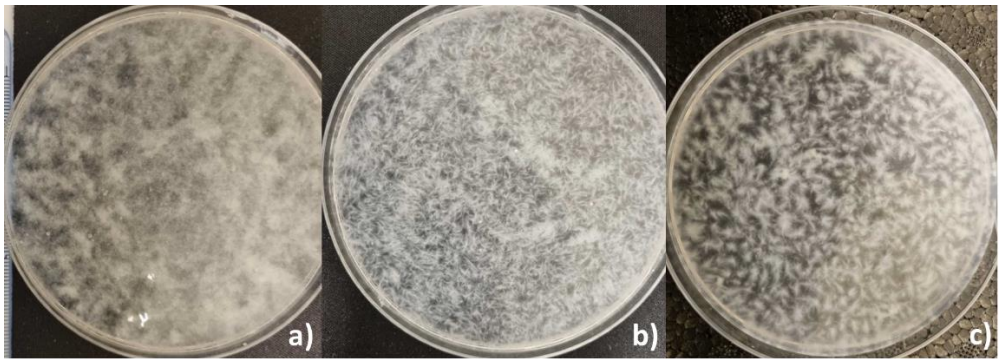

Figure 2-1 Different morphologies of the fungi biomass. a) filamentous; b) filamentous-pellet; c) pellet

Table 2-1 Information of different batches, including batch number, batch code, the morphology and downstream processing method.

Batch No.	Batch code	Morphology	Downstream processing method
1	210825-0900	filamentous	belt press
2	211104-1100	filamentous-pellet	decanter
3	211105-0745	filamentous-pellet	decanter
4	220317-0900	filamentous-pellet	decanter
5	220204-0745	filamentous-pellet	belt press
6	220324	pellet	decanter
7	220330	filamentous	belt press
8	220330	filamentous	decanter

2.3 Formulation setup

The fungi biomass provided by the collaboration company would be the major solid content and the only protein source of the final product. Other ingredients included, such as water, oil, gelling agent, and salt, are generally used in meat alternatives to provide structure or flavor. In this project, the major gelling agent studied was sodium alginate. In addition, citrus fiber may be added into the formulation to increase the water and oil holding capacity.

2.4 External crosslinking

2.4.1 Sample preparation

According to the formulation, the corresponding amounts of biomass and water were mixed together first using a spice grinder (MultiTalent 8, BOSCH). The rest of the ingredients in the formulation were mixed together and then added to the biomass. All the ingredients were blended in the spice grinder until a homogenous and smooth paste was obtained. The resulting paste was extruded using a pastry bag with an outlet diameter of 1 cm or 1.5 cm into a calcium solution. The samples were kept in the solution for approximately 5 h until the crosslinking process was complete, i.e., the overall sample was in a gelled state. The obtained samples were boiled in water or 1 % salt water for 2 min before they were evaluated by tasting.

2.4.2 Effects of major ingredients on texture and structure

To study the effects of major ingredients, flavoring agents were excluded in this phase as it was expected to contribute little to the texture or structure of the product. The concentrations of other major ingredients, including fungi biomass, water, sodium alginate solution, table salt, and rapeseed oil, are shown in Table 2-2. All percentages indicated in the table refers to weight percentage (w/w).

Table 2-2 Formulations of samples used to study the effects of major ingredients

	Concentration of ingredients (%)					
No.	**Fungi biomass**	**Water**	**Sodium alginate solution (4%)**	**Salt**	**Rapeseed oil**	**Citrus fiber**
1	40.0	47.5	12.5	-	-	-
2	40.0	45.5	12.5	2.0	-	-
3	40.0	33.0	25.0	2.0	-	-
4	40.0	37.5	12.5	-	10.0	-
5	40.0	25.0	25.0	-	10.0	-
6	40.0	32.5	12.5	-	15.0	-
7	40.0	47.5	12.5	-	-	1.0
8	40.0	47.5	12.5	-	-	2.0
9	50.0	32.5	17.5	-	-	1.0
10	45.0	32.5	17.5	-	5.0	1.0
11	45.0	32.5	17.5	-	5.0	-
12	50.0	27.5	17.5	-	5.0	1.0
13	50.0	22.5	17.5	-	10.0	1.0

2.4.3 Effects of calcium solution on the crosslinking speed and product quality

Cylindrical samples with the diameter of 1 cm were soaked in 1 % and 2.5 % $CaCl_2$, and 2 % and 5 % calcium lactate solutions. All concentrations here refer to weight concentration (w/w). Pieces of samples were taken at 30 min, 1 h, 2 h, 3 h, 4 h, and 5 h after the samples were put into the calcium solutions, and the measurements of thickness of the gelled layer were recorded. When the gelled thickness reached 5 mm which was the radius of the cylindrical sample, the crosslinking was considered to be complete. The weight concentration of calcium lactate solution is doubled the weight concentration of $CaCl_2$ solution to make sure the concentration of calcium ions in the solutions are approximately equal. The rate of crosslinking is reflected by the time or expected time needed for the sample to fully convert to solid. Rates of crosslinking by different types of calcium solutions and the effects of calcium ion concentration on the crosslinking rate were studied.

2.4.4 Effects of fat type on sensory perceptions

10 g of 7 types of oil was incorporated in the formulation respectively to see its influences on the final product sensory profiles. The oils used were rapeseed oil, olive oil, coconut oil, hydrogenated rapeseed oil, shea butter, sunflower seed oil, and peanut oil. Batch 1 biomass was used for this set of experiments. Major aspects of the sensory profile evaluated by tasting were astringency and off-flavor. Off-flavor here is defined as any additional flavor compared with samples made without oil. A score of 1 to 10 (1 being the weakest, and 10 being the strongest) was given by the subjective opinions of the taster. Addition notes may be recorded for samples that presented distinct features.

2.5 Heat treatment

In order to completely deactivate lipase, an extra step of heat treatment was added before the final cooking step before consumption. The heat treatment could take place in 5 major processing steps during the mixing of ingredients. They are demonstrated as marked red arrows in Figure 2-2. Method 3 could be divided more specifically by applying heat treatment after adding part of the ingredients in this stage while adding the rest after the heating was complete (Table 2-3).

The formulation used in this stage of trials was 50 g biomass, 26.5 g water, 17.5 g 4 % sodium alginate solution, 10 g rapeseed oil and 1 g citrus fiber. The extruded samples were soaked in 0.5 % (w/w) $CaCl_2$ solution for 5 h before cooking in boiling salt water (1 % w/w) for 2 min. Heat treatments 1 to 4 were applied by steaming (Steamer, Russell Hobbs) the samples for 10 min. Heat treatment 5 was carried out by submerging the extruded samples in 0.5 % hot $CaCl_2$ solution (85 °C) for 10 min before transferring the samples to a cold solution. The effects of the heat treatments were evaluated by visual evaluations and tasting of the samples.

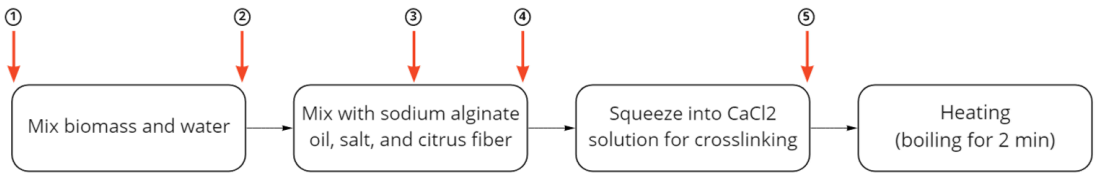

Figure 2-2 Possible approaches for adding heating treatment in the sample preparation process (① to ⑤ refers to the different heating methods)

Table 2-3 Ingredients added when heat is applied in heating method 3 ("x" marks the ingredients that has been added before heating)

Sample No.	Fungi biomass	Sodium alginate	Citrus fiber	Oil
1	x	x		
2	x		x	
3	x			x
4	x	x	x	
5	x		x	x
6	x	x		x

2.6 Internal crosslinking

2.6.1 Sodium alginate solution-GDL-$CaCO_3$ combination

$CaCO_3$ powder was obtained by manually grinding the calcium carbonate tablet and sieving. The obtained calcium powder was dispersed in water using Homogenizing system X 120 (CAT). GDL solution was prepared right before use. The ratio of ingredients and the mixing procedure varies. The detailed formulation setup is demonstrated in Table 2-4.

For trial 1 to 7, sodium alginate solution and calcium suspension were mixed till homogenous before GDL is added into the mixture. The obtained mixture is then added to the biomass and water mixture. From trial 8 to 12, the sodium alginate-calcium mixture was first blended into the biomass and water mix (oil and citrus fiber were optional). GDL was blended in at the end of the process. The obtained paste was extruded into a chocolate mold (SILIKOMART) with the dimension of 10×2.5×1.5 cm using a pastry bag. The samples were placed in the fridge overnight for the complete formation of the gel. On the following day, the samples were cooked for 15 min in a steamer before being evaluated by tasting.

Table 2-4 Recipes for internal crosslinking samples with sodium alginate solution-GDL-$CaCO_3$

Trial No.	Ingredients (g)								
	Fungi biomass	Water	Sodium alginate solution (4%)	CaCO3 dispersion		GDL solution		Oil	Citrus fiber
				CaCO3 powder	Water	GDL powder	Water		
1	45	15	17.5	0.5	10	0.15	1	10	1
2	24	6.5	8	0.5	4.5	0.2	0.9	4	0.4
3	10	3	8	0.5	4.5	0.2	0.9	2	0.2
4	20	10	8.75	0.5	5	0.25	1	-	-
5	20	10	5.5	0.3	3	0.25	1	-	-
6	10	5	8.75	0.5	5	0.15	1	-	-
7	10	5	17.5	1	10	0.15	1	-	-
8	25	10	6	0.5	1.2	0.3	2	-	0.5
9	25	10	6	0.5	1.2	0.3	2	-	-
10	25	10	9	0.7	1.8	0.3	2	-	-
11	25	10	6	0.5	1.2	0.3	2	-	-
12	25	10	6	0.5	1.2	0.3	2	3	-

2.6.2 Sodium alginate powder-$CaSO_4$ combination

A commercial powder (Easy Binder, The Kitchen Lab) which is a mixture of sodium alginate, $CaSO_4$ and stabilizers (pentasodium triphosphate, tetrasodium diphosphate) was used for this particular method of internal crosslinking. Easy Binder was added to the rest of the ingredients either as a 10 % or 8 % dispersion in water or as a powder. The effects of the pH value on gel formation were studied by observing the final product properties over a pH range of 5.9, 6.4, 6.7, 7.0, and 7.6 (FiveEasy Plus pH meter, Mettler Toledo). The specific formulations are listed in Table 2-5. Sheared samples were squeezed using a pastry bag with and outlet diameter of 1.5 cm. Non-sheared samples were obtained by manually filling of the mycoprotein paste into a tube with 1.5 cm diameter. The samples were placed in the fridge overnight and cooked in the steamer for 15 min before being evaluated by tasting.

Table 2-5 Formulation and pH values for internal crosslinking sample with Easy Binder

Trial No.	pH	Ingredients (g)						
		Fungi biomass	Water	Easy Binder			Oil	Citrus fiber
				10% dispersion	8% dispersion	Powder		
1	7.6	20	10	30	-	-	-	-
2	7.6	20	10	10	-	-	-	-
3	7.6	21	9	-	10	-	-	-
4	7.6	21	8	-	10	-	-	1
5	5.9	20	15	-	-	1	10	-
6	5.9	20	15	-	-	2	10	-
7	6.4	20	15	-	-	1	10	-
8	6.7	20	15	-	-	1	10	-
9	7.0	20	15	-	-	1	10	-
10	7.5	20	15	-	-	1	10	-

2.7 Texture analysis (TA)

A cutting test was conducted on 4 cylindrical samples (specifications of the samples are shown in Table 2-6) with Stable Micro Systems (TA. XT plus C). Sample 1 and 3 were made with formulation no. 7 from Table 2-5. Formulation no. 13 from Table 2-2 was used to make sample 2 and 4. The samples used for TA had a diameter of 1.5 cm and a length of 1.5 cm. The TA was done using the cutting blade to cut the samples both parallel and perpendicular to the extrusion direction (Figure 2-3). The pre-test speed was set at 1 mm/s, and the test speed and post-test speed were set at 5 mm/s. The target distance of the test was 20 mm, and the test was triggered by 5 g of force. Measurements were done in triplicates for each sample in both cutting direction. Fracture point (the first peak force taken from force-distance curve) and hardness (the highest force throughout the cutting test) were taken for statistical analysis. An independent samples t-test with a confidence interval of 95 % was carried out using SPSS (IBM) to determine significant differences in the force in both cutting directions between samples structured with different crosslinking method (internal and external crosslinking), and samples structured with or without extrusion process.

Table 2-6 TA sample specifications

Sample No.	pH	Crosslinking method	Extrusion
1	6.4	Internal	Yes
2	-	External	Yes
3	6.4	Internal	No
4	-	External	No

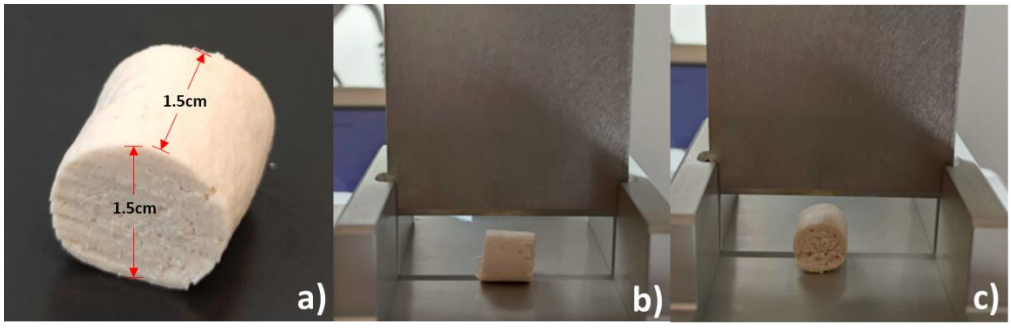

Figure 2-3 a) parameters of the TA samples; b) cutting test in parallel direction to potential fiber alignment; c) cutting test in perpendicular direction to potential fiber alignment

3. Results and Discussion

3.1 Batch variations of biomass

Batch 1 to 6 were used for trials within the external crosslinking method. However, different batches behaved differently when mixed with other ingredients. Whether the biomass is considered to be applicable for this particular product development was evaluated by if the biomass could form a smooth and homogenous paste when other ingredients were added. The comparison between how different batches behaved is demonstrated in Figure 3-1. As it can be seen from the pictures, only batch 1 displayed a smooth, viscous and cohesive paste-like texture with some visible fibrous strands, while batch 2 to 6 appeared coarse and grainy to different degrees and lacked cohesiveness. Such inhomogeneous mixture was tested to be unsuitable to form a product with homogenous texture or aligned fibrous structures. Thus, batch 2 to 6 were deemed inapplicable for further use in this project. A color difference in the biomass mixture was also observed.

The different batches were produced at different times and were processed with 2 different types of downstream processing machineries, decanter and belt-press. While both equipment is designed for getting rid of excessive liquid in the harvested biomass, decanter uses centrifugal force to achieve solid-liquid separation (Leung, 2020), and belt-press utilizes simple horizontal forces to squeeze the liquid out and collect the solid dewatered press-cake (Pierson, 1990). Compared with belt press, decanter generates much higher shear force during the dewatering process, which could result in structural disruption or destruction of mycoprotein, further causing the changes in its functionalities.

Another potential contributing factor to the batch variations is the difference in morphology of the fungi. Only batch 1 out of the 6 batches belongs to the filamentous morphology, which is a highly dispersed form that could be more easily spread out evenly as a matrix for other ingredients during the mixing procedure. The more densely packed colonies existing in other more flocculated morphologies could increase the difficulties in blending of the materials, hindering the incorporation of other food ingredients into the fungal matrix. The more granular texture for the paste made with biomass batch 2 to 6 could be a reflection of the existence of undispersed biomass flocculates.

Even though the fermentation and downstream processing procedure for biomass production is standardized, practical conditions during the fermentation could have variations from batch to batch. Therefore, resulting in differences in the biomass obtained. As fungus is a living organism, its growth, to a certain extent, is not fully controllable. From the existing results, it cannot be deducted which factor is detrimental for the biomass applicability in this product. However, the speculation that both morphology and downstream processing method play important roles in the functional properties and application of mycoprotein can be made from the observations.

Figure 3-1 Status of the pastes made with different batches of biomass (numbers in the bottom right corner indicate the batch code corresponding to Table 2-1)

3.2 Effects of major ingredients on texture and structure

The effects of the major ingredients in the formulation are mainly evaluated through 3 aspects: 1) firmness, the resistance of the food against deformation (Chen and Rosenthal, 2015); 2) water holding capacity, the ability of the food to hold liquid in the matrix upon deformation (Gyawali and Ibrahim, 2016); 3) structure here refers to the visually clear alignment of fibers. The effects of the 5 major ingredients on these 3 aspects are summarized in Table 3-2. Citrus fiber has no obvious influence on the firmness of the samples.

Table 3-2 Effects of major ingredients on the firmness, structure, and water holding capacity of the sample. "+" and "–" refers to strengthening or weakening effects of the ingredient toward the corresponding textural or structural feature. More signs signify stronger effects

	Firmness	Structure	Water holding capacity
Fungi biomass	+	++	+
Sodium alginate	++	-	++
Salt	-	-	+
Citrus fiber	+/-	-	++
Oil	--	-	+

NB: In this stage of the study, the concentration (w/w) range of the ingredients are: fungi biomass 40-50 %, sodium alginate solution (4 %) 12.5-25 %, salt 0-2 %, rapeseed oil 0-15 %, citrus fiber 0-2 %. Specific formulations studied can be found in Table 2-2.

The fungi biomass, which has been shown to form fiber alignment when subjected to extrusion, provides the major structure of the sample (Miri et al., 2005). However, it has poor water holding capacity, i.e., liquid leached out when chewed on the sample with high biomass content. The resulting mass after a few times of chewing was dry, fibrous, and impossible to break by teeth. This could be due to the presence of high amounts of insoluble glucans and chitins in the fungal cell wall (Ruiz-Herrera and Ortiz-Castellanos, 2019). Both citrus fiber and sodium alginate has great capacity to absorb and hold water, while alginate can form hydrogels upon contact with divalent ions (Lee and Mooney, 2012, ZhangLiao and Qi, 2020). Therefore, there is no surprise that the addition of sodium alginate or citrus fiber could largely improve the water holding capacity of the final product. However, increase in alginate content could at the same time largely increase the firmness of system, resulting in a brittle gel texture that is far away from meat. Though citrus fiber did not lead to obvious changes in the overall firmness, it was observed that higher fiber content increases the visually perceived density of the product, making it more difficult for individual fiber strands to separate. This also decrease the degree of resemblance of the product to meat. Therefore, in order to improve the coherency of the product and achieve a desirable meaty texture, alginate and citrus fiber are to be added in moderate amounts.

Salt, other than contributing to the taste of food, has also been shown to enhance the tenderness and juiciness of the samples. Salt impacts the texture of foods through

alterations of protein structures and on the interactions of protein with other components (Cargill, 2010, Stieger and Van De Velde, 2013). It was also noted in the trials that 1% of salt addition in the formulation gives lower saltiness and less distinguishable effects on the texture than adding the same concentration of salt in the final boiling water. This could be due to that salt added in the formulation was largely lost during the long soaking process in the calcium solution and rinsing of the samples before cooking. Thus, if salt is necessary to contribute to an overall better accepted product, it would be more efficient to add it at the final cooking step.

Oil decreases the firmness and disrupt the fibrous structure of the product as it is present as dispersed droplets in the system, disrupting the continuous protein-hydrocolloid network. It also improves the perceived water holding capacity. Liquid seemed to be more slowly released when chewed and the samples tended to retain moisture for a longer period of time in the mouth while giving a desirable fatty mouthfeel and taste. The moistness of the food is not simply dependent on the water content, presence of fats also plays an important role (Chen and Rosenthal, 2015). Especially to meat and meat alternative products, when an appropriate amount of fats is present, the overall texture and mouthfeel can be improved.

Some other observations were also noted during the experimental process. Higher oil content prolonged the crosslinking time needed which could be attributed to the dispersed oil droplets in the matrix hindering the formation of a gelled network. More viscous paste resulted in more organized alignments of the fibers which might be a result of the increased shear stress when the rate of shear is unchanged and viscosity increases (Esin, 2021).

Fungi biomass is the only ingredient in the formulation that is capable of forming the muscle fiber-like aligned structure. Other compositions either forming a complex with the biomass or dispersed in the food matrix disrupt the aligned structure to some extent. It was also difficult to set a standardized line for to which extent the fiber alignment was still desirable and acceptable. In addition, it should be pointed out that all the textural and structural aspects were evaluated only by objective observations and perceptions of a small number of untrained persons in an uncontrolled environmental setup. Food texture and structure are complex, abstract and difficult to quantify. Even though only 3 aspects were the focus of the evaluation, other sensory attributes inevitably influence the evaluation. However, the goal of this stage of experiments,

which was to give the product developer a general idea of the effects of major ingredients in this formulation, was achieved.

3.3 Effects of calcium solution

Diffusion of Ca^{2+} from the solution into the center of the sample matrix induce crosslinking of alginate and thereby the gelation of the sample. The unset part of the sample was gently removed by rinsing a small piece of the sample under running water and the remaining part was considered to be the gelled layer (Figure 3-2). The thickness of the gelled layer reflects the crosslinking process. The gelled thickness was plotted against time (Figure 3-3). As shown in the figure, samples soaked in 2.5 % $CaCl_2$ solution was the fasted to complete the crosslinking process, followed by samples in 5% calcium lactate, 1 % $CaCl_2$, and lastly 2 % calcium lactate. Higher concentrations of the calcium ions effectively decrease the time needed for crosslinking. When calcium ion concentrations are the same, $CaCl_2$ induced faster crosslinking than calcium lactate. The rate and extent of crosslinking is dependent on pH, the concentration of free Ca^{2+} in the solution (HengChan and Wong, 2003). The ionization constant and diffusivity of the calcium salt also play a part in the amount of free Ca^{2+} present. $CaCl_2$ can dissociate completely while this is not the case for calcium lactate. As lactic acid is a weak acid, its conjugated base, lactate, is a strong conjugated base according to the Brønsted-Lowry acid-base theory (House, 2020). It has a stronger tendency to capture protons from the aqueous environment. When the environmental pH is high, the dissociation of lactate from calcium lactate is not favored. Therefore, the concentration of free Ca^{2+} in calcium lactate solution may be lower than that in $CaCl_2$ resulting in the lower rate of crosslinking.

$CaCl_2$ exerts primarily a bitter taste if present in high concentration in food (Lawless et al., 2004). A high concentration of $CaCl_2$ would not always be suitable for achieving a shorter crosslinking time. Bitterness could be detected in samples prepared in 1 % $CaCl_2$. Therefore, if $CaCl_2$ solution were to be used in food production, the concentration needs to be further decreased to avoid off-taste. Calcium lactate though gives a much more neutral taste, is not as effective in the crosslinking of alginate. When a higher concentration (5 %) of calcium lactate was used, an off-taste in the product was also detected. In addition, oversaturation of Ca^{2+} could lead to too tight crosslinking, causing water expulsion from the gel structure, and resulting in increased gel shrinkage and weeping (Growney Kalaf et al., 2016).

In order to develop a meat alternative that is reproduceable and applicable for large-scale production, lower off-taste and shorter crosslinking time are desired. Additionally, due to practical and hygienic considerations, putting the product in a water bath over extended hours does not seem to be the appropriate solution for industrial-scale production of food. Therefore, the external crosslinking method would only be suitable for lab-scale research, and 0.5 % $CaCl_2$ solution was selected for further studies within the external crosslinking method.

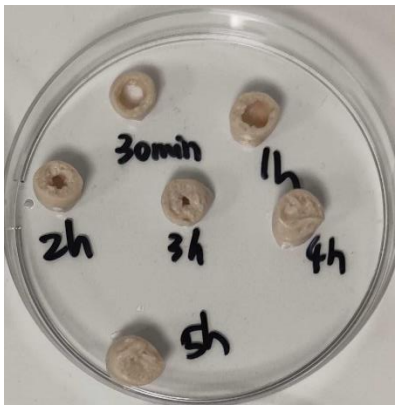

Figure 3-2 Thickness of the gelled layer at time intervals of 30 min, 1 h, 2 h, 3 h, 4 h, and 5 h

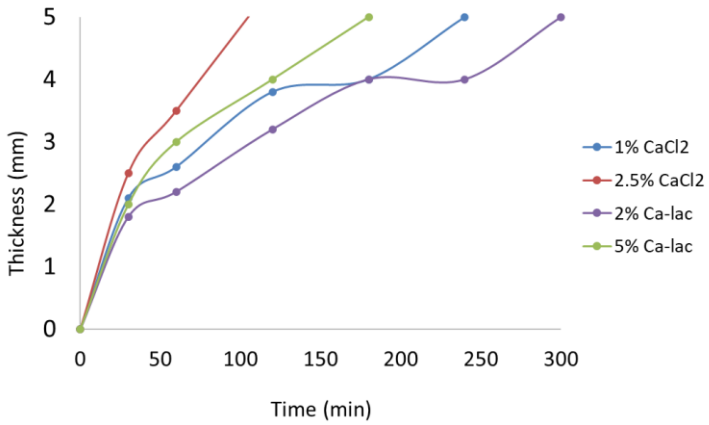

Figure 3-3 Time needed for the gelled layer to reach a thickness of 5 mm for samples in 1 % $CaCl_2$, 2.5 % $CaCl_2$, 2 % calcium lactate, and 5 % calcium lactate solutions, respectively

3.4 Fat induced sensorial changes

When using different types of oil in the formulation, the sensory profile of the final product varies. The resulting score of astringency and off-flavor were summarized in

Table 3-3. Generally, the taste and aroma of the oil itself were more pronounced when it was incorporated in the formula than when it was consumed alone. Olive oil, which already possesses a strong characteristic flavor by itself, resulted in a sample with overpowering olive taste. The magnifying effects for flavors were more obvious for oils that originally have very faint flavors, such as peanut oil and sunflower seed oil. It is worth pointing out that despite the coconut oil used in the experiment was deodorized, the samples made with it displayed very strong rancid coconut aroma and taste. The hydrogenated rapeseed oil sample, though did not presented strong astringency nor off-flavor, left a distinct greasiness and mouthcoating feeling in the oral cavity after chewing. The hydrogenated rapeseed oil used in the trials had a slip melting point of 53 ℃, which is much higher than the normal temperature in the oral cavity, thus the fat crystals may not melt but form a fatty layer in the mouth, causing the greasy sensations.

It can be speculated that the astringency generated in samples with included fat arose from free fatty acids broken down by lipase. Free fatty acids, different from triglycerides, are perceived as irritants rather than fatty (Delompre et al., 2019). A few examples that are perceived as astringents are oleic acid, linoleic acid and linolenic acid. The contents of these 3 fatty acids in each oil (except for hydrogenated rapeseed oil) were listed in Table 3-4. The total amount of these 3 types of fatty acid in the oil approximately corresponds to the level of astringency perceived. As for the emphasized off-flavors, it could also be speculated to be attributed to enzymatic reactions. However, due to the limited availability of equipment, the hypothesis for the presence of lipases has not been tested directly. In the following section, additional heating intended to fully deactivate enzyme activities was applied. The changes induced in sensory attributes could provide more argumentation on this hypothesis.

Table 3-3 Sensory profile of samples with different types of fat in the formulation (score 1 means the weakest sensation, and 9 means the strongest)

Fat type	Astringency	Off-flavor
Coconut	1	10
Shea	2	4
Hydrogenated rapeseed	2	1
Peanut	3	2
Olive	3	7
Sunflower seed	6	3
Rapeseed	8	1

Table 3-4 Oleic acid, linoleic acid and α-linolenic acid contents in rapeseed oil, olive oil, sunflower seed oil, peanut oil, coconut oil, and shea butter (UgeseBaiyeri and Mbah, 2010, Orsavova et al., 2015)

	Rapeseed oil	Olive oil	Sunflower seed oil	Peanut oil	Coconut oil	Shea butter
Oleic acid	61%	55-83%	19.50%	46.50%	6%	40-60%
Linoleic acid	21%	3.5-21%	65.70%	31.40%	-	3-11%
α-linolenic acid	9-11%	0-1.5%	-	-	-	1%

3.5 Effects of additional heat treatment on product flavor and texture

The appearance of the mixture right after heating, the appearance, taste and texture of the finished final product corresponding to each heating approach are summarized in Table 3-5. Except for approach 1, all samples formed with additional heat treatment did not possess any astringency or off-flavor. The 6 different mixing steps (Table 2-3) within method 3 did not make a distinguishable difference in the final product.

After the biomass has been treated by method 1, it could no longer form a smooth, homogenous, and cohesive paste when mixed with other ingredients. Therefore, the experiment did not proceed to make a final product out of it. The explanation behind this phenomenon could be that the mycoprotein is more susceptible to heat damage under this approach. When the protein is completely thermally denatured, the aggregated protein has lost its functionalities (Wijayanti et al., 2019). Therefore, it was no longer able to interact with other ingredients to form a homogenous network. But for method 2 and 3, protein was dissolved or dispersed in an aqueous environment before being heated, it was able to form a weak gel upon thermal heat denaturation (Van Kleef, 1986). Though the protein gel structure was broken when it was blended with the remaining ingredients in the formulation, it was still able to form a rather smooth pasty mixture in the end, enabling a smooth homogenous final product to be obtained. For method 4, as all ingredients have been combined before the heating was applied, the heated mixture was directly extruded into $CaCl_2$ solution. Since the mixture was already partially gelled, the flowability of mixture was reduced. Therefore, the air bubbles that were trapped inside during blending had more difficulties getting out. Some bubbles were positioned on the surface of the formed samples, and led to an uneven and rough appearance. Reduced flowability also hindered the alignment of fibers during the extrusion process, resulting in poor structure. The heating method of

treatment number 5 is more different from other ones. As the extruded samples were put in hot $CaCl_2$ solutions, elevated temperature promoted the Brownian motion of the calcium ions, and accelerated the crosslinking process (Chang, 2016). As mentioned before in section *3.3 Effects of calcium solutions*, a tight crosslinking results in loss of liquid and a tough and dry product texture.

Overall, all approaches of heat treatment could effectively eliminate or rather prevent the generation of astringency and off-taste, which supported the hypothesis of lipase activities. Both method 2 and 3 resulted in samples with desirable texture and taste. While considering the applicability and convenience in industrial production, approach 2, which heats the biomass and water mixture, would be the most suitable solution.

Table 3-5 Summarized results of different heat treatment approaches

Heat treatment method	Appearance right after heating	Appearance of the final product	Taste	Texture
1	Biomass turned to a darker yellowish color			
2	A weak gel was formed	Smooth glossy surface, no difference from non-heated samples	No astringency, mild and neutral taste	Tender and juicy with well aligned meat-like texture
3	A weak gel was formed	Smooth glossy surface, no difference from non-heated samples	No astringency, mild and neutral taste	Tender and juicy with well aligned meat-like texture
4	A weak gel was formed	Rough uneven surface, many bubbles within the structure	No astringency, mild and neutral taste	Clumpy, unhomogenous, and no obvious fiber alignment
5	Surface well-crosslinked, rough surface	Rough uneven surface, many bubbles within the structure	No astringency, mild and neutral taste	Clumpy, unhomogenous, and very drying tough texture

3.6 Internal crosslinking

All samples that used the alginate-$CaCO_3$-GDL combination for internal crosslinking did not obtain satisfactory results. The structure of the samples was too weak for all samples despite different ratios of the components were tried out. They were very easily deformed when put under pressure. The CO_2 cavities generated when the insoluble $CaCO_3$ dissolved under the work of acid might not visible to human eyes, but were disruptive to the gel network (Chan et al., 2006). Therefore, the tensile strength of the

overall matrix was compromised.

Samples made with Easy Binder can be categorized into 2 main types, samples made with Easy Binder dispersion, and with Easy Binder powder. When Easy Binder was made into 10 % and 8 % dispersions, which was according to instructions given by the producer (Thekitchenlab, 2022), the obtained dispersions were very viscous with chunks of particles that were very difficult to fully disperse distributed within the fluid. The solid particles remained in the final product which could be considered as a quality defect. Sample 1 with higher amount of Easy Binder dispersion formed a firm and brittle gel which was similar to the texture of a pure alginate gel, did not display enough chicken-like characteristics. Sample 2, 3, and 4 had weak structures that were very easily deformed. In addition, they also gave a “slimy” mouthfeel when chewed. The sliminess might be attributed to fungal cell wall components, such as polysaccharides, glycoproteins and glycolipids, that form a loosely packed matrix that is perceived as slimy (Op De BeeckPersson and Tunlid, 2021). The slimy and slippery mouthfeel might have been amplified by the particular alkaline pH conditions of the samples. The weak structure could be due to the lack of binding ability from alginate in the formulations in the presence of excessive water.

Sample 5 to 10 were prepared by adding in Easy Binder powder directly. The preliminary concern with adding in the powder form of Easy Binder directly was that the alginate would be instantly hydrated on the spot of contact with the biomass mixture, thereby wouldn’t get well dispersed. Though the viscosity of the mixture was drastically increased when Easy Binder powder was added, it was possible to get a homogenous mixture in the end that was suitable for further extrusion process. Sample 6 compared with 5 had double the amount of alginate, thereby the fully crosslinked sample was very firm and gel-like, lacking the features of meat. With increasing pH, the final samples had weaker structures that dissembled more easily. The slimy mouthfeel was not detected for samples with pH values lower than 7.0. In conclusion, sample 5 with pH value of 5.9, 20 g biomass, 15 g water, 1 g of Easy Binder powder, and 10 g rapeseed oil was considered to be the formulation resulting in the most promising texture for chicken analogue.

3.7 Texture analysis

The facture force and hardness of the samples when being cut in parallel (denoted as “//”) and perpendicular (denoted as “⊥”) directions to the cylinder were plotted in

Figure 3-3 and Figure 3-4. Abbreviations for samples cut in different method were used: SPara, sheared parallelly cut; SPer, sheared perpendicularly cut; NSPara, non-sheared parallelly cut; NSPer, non-sheared perpendicularly cut. All data tables for t-tests can be found in Appendix.

For all samples that have been extruded (denoted as "sheared" in the figures), there are significant differences in both the fracture force and the hardness when the samples were cut in 2 different directions ($p<0.05$). On the contrary, no significant differences in either the fracture force or the hardness were shown for the non-sheared samples when cut in 2 directions ($p>0.05$). The difference in the cutting forces needed for sheared samples in different directions are speculated to be the effects of fiber alignment. Higher forces are needed to break the fibers (perpendicular cutting) than separating them along their aligned direction (parallel cutting). It can be concluded from the TA results that extrusion is crucial for the formation of fiber alignment. The textural characteristics of externally and internally crosslinked samples revealed by this TA test follow the same pattern, which shows that the extrusion method works on both crosslinking approaches to create aligned fibers.

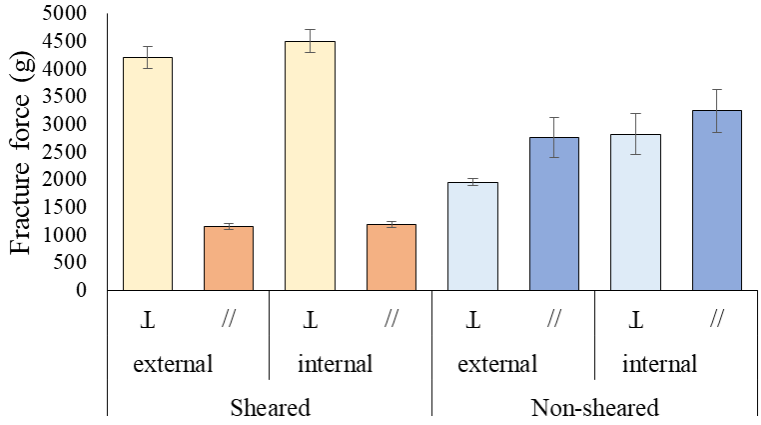

Figure 3-4 Fracture force of externally and internally crosslinked SPara, SPer, NSPara, and NSPer samples

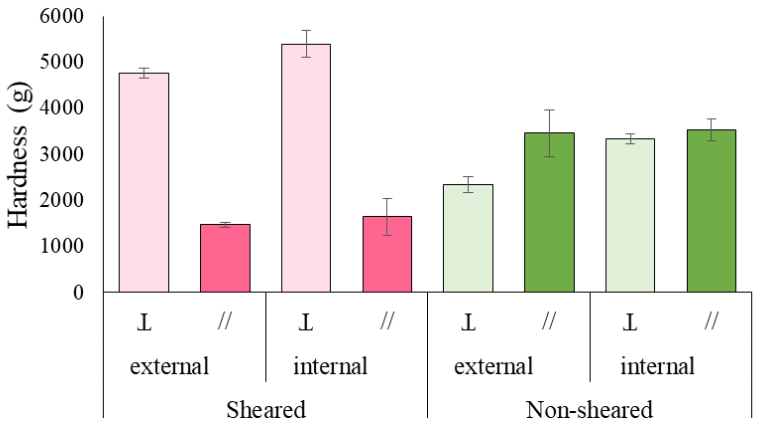

Figure 3-5 Hardness of externally and internally crosslinked SPara, SPer, NSPara, and NSPer samples

4. Conclusion

This project has developed a prototype for chicken analogue with fibrous structure using mycoprotein with simplified extrusion. The effects of salt, biomass, alginate, calcium solution, and fat on sensory attributes of the final formulated product were studied. The gelling agent used in the formulation was sodium alginate. Both external and internal crosslinking method for gelation were explored during the product development process. The type of calcium source and the concentration of the solution are crucial to the crosslinking process of externally crosslinked alginate gels. After testing the effects of 1 % and 2.5 % $CaCl_2$, 2 % and 5 % calcium lactate solutions, it can be concluded that $CaCl_2$ of a weight concentration lower than 1 % is suitable for use in this product development, as it both ensures the crosslinking efficiency and avoids the generation of bitter off-taste. In the study of internal crosslinking method, 2 different approaches were used. The alginate-GDL-$CaCO_3$ solution combination cannot form a stable gel with the biomass, while the alginate-$CaSO_4$ powder combination (Easy Binder) gave more promising results.

Fat, as an important ingredient in the formulation, does not only contribute to the nutritional content and texture, might also has complex interactions with the enzymes and other active compounds in the formulation, resulting in the occurrence of astringency and off-tastes. A hypothesis for the fat induced sensorial changes was brought up and tested out by implementing additional heat treatment to the biomass to deactivate enzyme activities. While all heating approaches worked on eliminating astringency and off-tastes, some induced alterations in undesirable appearance or texture. Heating the biomass and water mixture before the incorporation of other ingredients was considered to be the most suitable approach for obtaining a chicken analogue.

TA was conducted on both externally and internally crosslinked samples that were extruded and non-extruded. Differences in the forces between the parallel and perpendicular cut of the extruded samples reflected the alignment of mycelium strands into one general direction, and that it is induced by extrusion. And this method of alignment creation works on both crosslinking approaches.

Overall, biomass, water, oil, and Easy Binder together when extruded together can form a prototype with a neutral taste and a texture, mouthfeel and structure that highly

resembles chicken. Therefore, this formulation has high potentials to create a chicken analogue with aligned structures.

5. Future perspective

In general, the goal to develop a chicken analogue using mycoprotein was achieved. The obtained prototype has a texture that is balanced between tenderness and chewiness, and a juicy mouthfeel. Though receiving promising results in this project, the prototype obtained in the end is still far from being ready to be commercialized in the market. In order to make a reproducible formulation, further research is needed to figure out the reasons behind the batch variations of biomass and how it impacts its application. The solution for creating a reproducible, scalable product is to either find out the exact type of biomass that is applicable for this application or to find a formulation that flexibly works for all sorts of biomass. Even though a visibly well aligned fibrous structure was obtained squeezing the paste through pastry bag outlet, which acted as a simplified extruder, the mechanism behind the alignment is scarcely understood. Many studies have been done on the extrusion technique for plant proteins, but little is known for mycoprotein. As a material with its unique characteristics, more research on flow induced fiber alignment of mycelium should be conducted. Parameters such as the ratio between die and barrel diameter, extrusion speed, and paste viscosity are interesting aspects that could be looked into.

The astringency issue solved by applying additional heat treatment was based on the hypothesis that the problem was caused by enzyme activities. However, the presence of enzyme has not been tested in a direct approach. In order to target the problem more accurately, the presence and the type of enzymes need to be confirmed. More studies on the exact deactivating conditions of the enzymes are needed to be done to ensure the quality of the product and an energy efficient production process.

The prototype obtained does not include salt or other flavoring agents. To make a more appealing commercial product, it is common practice for meat alternatives to be sold marinated. In further work on the development of the chicken analogue, flavoring agents such as salt, spices, and other natural or synthetic flavors should be included to boost the “meaty” flavors. It was presumed in this project that flavors do not contribute to textural changes. However, marination with or inclusion of spices and salt might have influences on the textural profiles. Salt have been shown to influence the solubility and promote proteolysis of myofibrillar which leads to increased tenderness of meat (Madhusankha and Thilakarathna, 2021). It might have similar effects to mycoproteins. Spices often contains active antioxidant compounds which could result in differences

in texture and shelf life (Câmara and Pollonio, 2018). Spice granules are mostly insoluble. When added in the formulation, the dispersed particles could interfere with the protein-hydrocolloid network formation, also contributing to a texture shift.

The evaluation of the sensorial profile of the prototype in this project was very limited. All sensorial aspects were evaluated by the objective opinion of a few persons. The opinions aid the product development process, but is not valid to reflect the real profiles of the product, or the opinion of the general public. A well-designed sensory evaluation trial with a larger board of panelists is needed to obtain representative data. It would be valuable to compare the prototype with similar commercial products to see the acceptance and preference of consumers.

Overall, this chicken analogue prototype has great potentials to be converted to a marketable product. However, more work on adapting the process to industrial scale and studies on shelf-life of the product are needed to be done.

6. References

Ahmad, M. I., Farooq, S., Alhamoud, Y., Li, C. & Zhang, H. 2022. A review on mycoprotein: History, nutritional composition, production methods, and health benefits. *Trends in Food Science & Technology,* 121(14-29. doi: https://doi.org/10.1016/j.tifs.2022.01.027

Ahmad Raus, R., Wan Nawawi, W. M. F. & Nasaruddin, R. R. 2021. Alginate and alginate composites for biomedical applications. *Asian Journal of Pharmaceutical Sciences,* 16(3): 280-306. doi: https://doi.org/10.1016/j.ajps.2020.10.001

Astm, I. 2009. Standard terminology relating to sensory evaluations of materials and products. *West Conshohocken, PA: ASTM International. p. E253-209a,* doi:

Bajec, M. R., Pickering, G. J. & Decourville, N. 2012. Influence of Stimulus Temperature on Orosensory Perception and Variation with Taste Phenotype. *Chemosensory Perception,* 5(3): 243-265. doi: 10.1007/s12078-012-9129-5

Balogh, J. M. & Jámbor, A. 2020. The Environmental Impacts of Agricultural Trade: A Systematic Literature Review. *Sustainability,* 12(3): doi: 10.3390/su12031152

Barzee, T. J., Cao, L., Pan, Z. & Zhang, R. 2021. Fungi for future foods. *Journal of Future Foods,* 1(1): 25-37. doi: 10.1016/j.jfutfo.2021.09.002

2019. *Alternative proteins: The race for market share is on*. https://www.mckinsey.com/industries/agriculture/our-insights/alternative-proteins-the-race-for-market-share-is-on#:~:text=Alternatives%20are%20protein%2Drich%20ingredients,based%20sources%20(Exhibit%203). (Accessed

Biegler, M., Delius, J., Käsdorf, B. T., Hofmann, T. & Lieleg, O. 2016. Cationic astringents alter the tribological and rheological properties of human saliva and salivary mucin solutions. *Biotribology,* 6(12-20. doi: https://doi.org/10.1016/j.biotri.2016.03.002

Boerema, A., Peeters, A., Swolfs, S., Vandevenne, F., Jacobs, S., Staes, J. & Meire, P. 2016. Soybean Trade: Balancing Environmental and Socio-Economic Impacts of an Intercontinental Market. *PLOS ONE,* 11(5): e0155222. doi: 10.1371/journal.pone.0155222

Câmara, A. & Pollonio, M. A. 2018. The effects of herbs and spices on the sensory and physicochemical properties of healthier emulsified meat products: Adding value by natural antioxidant claims.

2010. *Food processing salt functionality beyong flavor*. https://www.cargill.com/salt-in-perspective/function-of-salt-in-food#:~:text=Texture%20Enhancer&text=Salt%20alters%20the%20structure%20of,breads%20can%20be%20more%20firm. (Accessed 2022-May. 3).

Chan, L. W., Lee, H. Y. & Heng, P. W. S. 2006. Mechanisms of external and internal gelation and their impact on the functions of alginate as a coat and delivery system. *Carbohydrate Polymers,* 63(2): 176-187. doi: 10.1016/j.carbpol.2005.07.033

Chang, Q. 2016. Chapter 2 - Diffusion and Brownian Motion. *In:* CHANG, Q. (ed.) *Colloid and Interface Chemistry for Water Quality Control.* Academic Press.

Chen, J. & Rosenthal, A. 2015. 1 - Food texture and structure. *In:* CHEN, J. & ROSENTHAL, A. (eds.) *Modifying Food Texture.* Woodhead Publishing.

Dekkers, B. L., Boom, R. M. & Van Der Goot, A. J. 2018. Structuring processes for meat analogues. *Trends in Food Science & Technology,* 81(25-36. doi: https://doi.org/10.1016/j.tifs.2018.08.011

Delompre, T., Guichard, E., Briand, L. & Salles, C. 2019. Taste Perception of Nutrients Found in Nutritional Supplements: A Review. *Nutrients,* 11(9): doi: 10.3390/nu11092050

Des Gachons, C. P., Mura, E., Speziale, C., Favreau, C. J., Dubreuil, G. F. & Breslin, P. a. S. 2012. Opponency of astringent and fat sensations. *Current Biology,* 22(19): R829-R830. doi: 10.1016/j.cub.2012.08.017

Ek, P. & Ganjyal, G. M. 2020. Chapter 1 - Basics of extrusion processing. *In:* GANJYAL, G. M. (ed.) *Extrusion Cooking.* Woodhead Publishing.

Esin, A. 2021. Chapter 7 - Fluid mechanics in food process engineering. *In:* JAFARI, S. M. (ed.) *Engineering Principles of Unit Operations in Food Processing.* Woodhead Publishing.

Rome, Italy. 2018. *The future of food and agriculture - Alternative pathways to 2050*. https://www.fao.org/publications/card/en/c/I8429EN/ (Accessed

Finnigan, T. J. A. 2011. 13 - Mycoprotein: origins, production and properties. *In:* PHILLIPS, G. O. & WILLIAMS, P. A. (eds.) *Handbook of Food Proteins.* Woodhead Publishing.

Gibbins, H. L. & Carpenter, G. H. 2013. Alternative Mechanisms of Astringency – What is the Role of Saliva? *Journal of Texture Studies,* 44(5): 364-375. doi: https://doi.org/10.1111/jtxs.12022

Gmoser, R., Lennartsson, P. R. & Taherzadeh, M. J. 2021. From surplus bread to burger using filamentous fungi at bakeries: Techno-economical evaluation. *Cleaner Environmental Systems,* 2(doi: 10.1016/j.cesys.2021.100020

Growney Kalaf, E. A., Flores, R., Bledsoe, J. G. & Sell, S. A. 2016. Characterization of slow-gelling alginate hydrogels for intervertebral disc tissue-engineering applications. *Materials Science and Engineering: C,* 63(198-210. doi: https://doi.org/10.1016/j.msec.2016.02.067

Guinard, J.-X., Pangborn, R. M. & Lewis, M. J. 1986. Preliminary studies on acidity-astringency interactions in model solutions and wines. *Journal of the Science of Food and Agriculture,* 37(8): 811-817. doi: https://doi.org/10.1002/jsfa.2740370815

Gupta, V. K., Kubicek, C. P., Berrin, J.-G., Wilson, D. W., Couturier, M., Berlin, A., Filho, E. X. F. & Ezeji, T. 2016. Fungal Enzymes for Bio-Products from Sustainable and Waste Biomass. *Trends in Biochemical Sciences,* 41(7): 633-645. doi: https://doi.org/10.1016/j.tibs.2016.04.006

Gurikov, P. & Smirnova, I. 2018. Non-Conventional Methods for Gelation of Alginate. *Gels,* 4(1): doi: 10.3390/gels4010014

Gyawali, R. & Ibrahim, S. A. 2016. Effects of hydrocolloids and processing conditions on acid whey

production with reference to Greek yogurt. *Trends in Food Science & Technology,* 56(61-76. doi: https://doi.org/10.1016/j.tifs.2016.07.013

Hashempour-Baltork, F., Khosravi-Darani, K., Hosseini, H., Farshi, P. & Reihani, S. F. S. 2020. Mycoproteins as safe meat substitutes. *Journal of Cleaner Production,* 253(119958. doi: https://doi.org/10.1016/j.jclepro.2020.119958

Helgerud, T., Gaserod, O., Fjæreide, T., Andersen, P. & Larsen, C. 2010. 4. Alginates. *Food Stabilisers, Thickeners and Gelling Agents; Wiley-Blackwell: Oxford, UK,* 50-72. doi:

Henchion, M., Hayes, M., Mullen, A. M., Fenelon, M. & Tiwari, B. 2017. Future Protein Supply and Demand: Strategies and Factors Influencing a Sustainable Equilibrium. *Foods (Basel, Switzerland),* 6(7): 53. doi: 10.3390/foods6070053

Heng, P. W. S., Chan, L. W. & Wong, T. W. 2003. Formation of alginate microspheres produced using emulsification technique. *Journal of Microencapsulation,* 20(3): 401-413. doi: 10.3109/02652040309178078

Hill, R. G. 2005. 4 - Polymers. *In:* HENCH, L. L. & JONES, J. R. (eds.) *Biomaterials, Artificial Organs and Tissue Engineering.* Woodhead Publishing.

House, J. E. 2020. Chapter 9 - Acid–base chemistry. *In:* HOUSE, J. E. (ed.) *Inorganic Chemistry (Third Edition).* Academic Press.

Hu, C., Lu, W., Mata, A., Nishinari, K. & Fang, Y. 2021. Ions-induced gelation of alginate: Mechanisms and applications. *Int J Biol Macromol,* 177(578-588. doi: 10.1016/j.ijbiomac.2021.02.086

Oregon, USA. 2016. *Agriculture Land & Water Use*. https://inr.oregonstate.edu/book/export/html/1301 (Accessed

Laaksonen, O. 2011. *Astringent food compounds and their interactions with taste properties.* Doctoral, University of Turku.

Lamy, E., Rodrigues, L., Louro, T. & Capela E Silva, F. 2017. The role of saliva in food sensory perception: relevant knowledge to design healthy foods.

Lawless, H., Rapacki, F., Horne, J., Hayes, A. & Wang, G. 2004. The taste of calcium chloride in mixtures with NaCl, sucrose and citric acid. *Food Quality and Preference,* 15(83-89. doi: 10.1016/S0950-3293(03)00099-5

Lawless, H. T., Horne, J. & Giasi, P. 1996. Astringency of Organic Acids is Related to pH. *Chemical Senses,* 21(4): 397-403. doi: 10.1093/chemse/21.4.397

Lee, C. B. & Lawless, H. T. 1991. Time-course of astringent sensations. *Chemical Senses,* 16(3): 225-238. doi: 10.1093/chemse/16.3.225

Lee, K. Y. & Mooney, D. J. 2012. Alginate: properties and biomedical applications. *Progress in polymer science,* 37(1): 106-126. doi: 10.1016/j.progpolymsci.2011.06.003

Leung, W. W.-F. 2020. 5 - Decanter Centrifuge. *In:* LEUNG, W. W.-F. (ed.) *Centrifugal Separations in Biotechnology (Second Edition).* Butterworth-Heinemann.

Li, X., Dilokpimol, A., Kabel, M. A. & De Vries, R. P. 2022. Fungal xylanolytic enzymes: Diversity and applications. *Bioresource Technology,* 344(126290. doi: https://doi.org/10.1016/j.biortech.2021.126290

Madhusankha, G. D. M. P. & Thilakarathna, R. C. N. 2021. Meat tenderization mechanism and the impact of plant exogenous proteases: A review. *Arabian Journal of Chemistry,* 14(2): 102967. doi: https://doi.org/10.1016/j.arabjc.2020.102967

Miri, T., Barigou, M., Fryer, P. J. & Cox, P. W. 2005. Flow induced fibre alignment in Mycoprotein paste. *Food Research International,* 38(10): 1151-1160. doi: https://doi.org/10.1016/j.foodres.2005.04.005

Nadim, M., Deshaware, S., Saidi, N., Abd-Elhakeem, M. A., Ojamo, H. & Shamekh, S. 2015. Extracellular Enzymatic Activity of <i>Tuber maculatum</i> and <i>Tuber aestivum</i> Mycelia. *Advances in Microbiology,* 05(07): 523-530. doi: 10.4236/aim.2015.57054

Nyyssölä, A., Suhonen, A., Ritala, A. & Oksman-Caldentey, K.-M. 2022. The role of single cell protein in cellular agriculture. *Current Opinion in Biotechnology,* 75(102686. doi: https://doi.org/10.1016/j.copbio.2022.102686

Op De Beeck, M., Persson, P. & Tunlid, A. 2021. Fungal extracellular polymeric substance matrices – Highly specialized microenvironments that allow fungi to control soil organic matter decomposition reactions. *Soil Biology and Biochemistry,* 159(108304. doi: https://doi.org/10.1016/j.soilbio.2021.108304

Orsavova, J., Misurcova, L., Ambrozova, J., Vicha, R. & Mlcek, J. 2015. Fatty Acids Composition of Vegetable Oils and Its Contribution to Dietary Energy Intake and Dependence of Cardiovascular Mortality on Dietary Intake of Fatty Acids. *International Journal of Molecular Sciences,* 16(12): 12871-12890. doi: 10.3390/ijms160612871

Ozawa, T., Lilley, T. H. & Haslam, E. 1987. Polyphenol interactions: astringency and the loss of astringency in ripening fruit. *Phytochemistry,* 26(11): 2937-2942. doi: https://doi.org/10.1016/S0031-9422(00)84566-5

Ozdal, T., Capanoglu, E. & Altay, F. 2013. A review on protein–phenolic interactions and associated changes. *Food Research International,* 51(2): 954-970. doi: https://doi.org/10.1016/j.foodres.2013.02.009

Petzold, G., Rodríguez, A., Valenzuela, R., Moreno, J. & Mella, K. 2019. Chapter 12 - Alginate as a versatile polymer matrix with biomedical and food applications. *In:* GRUMEZESCU, V. & GRUMEZESCU, A. M. (eds.) *Materials for Biomedical Engineering.* Elsevier.

Pierson, H. G. 1990. Part II – Horizontal Vacuum Belt Filters. *In:* SVAROVSKY, L. (ed.) *Solid–Liquid Separation (Third Edition).* Butterworth-Heinemann.

Pires, M. A., Pastrana, L. M., Fucinos, P., Abreu, C. S. & Oliveira, S. M. 2020. Sensorial Perception of Astringency: Oral Mechanisms and Current Analysis Methods. *Foods,* 9(8): doi: 10.3390/foods9081124

2019. *Quorn foods history*. https://www.quorn.co.uk/company (Accessed 2022-Mar. 28).

Ramdhan, T., Ching, S. H., Prakash, S. & Bhandari, B. 2019. Time dependent gelling properties of cuboid alginate gels made by external gelation method: Effects of alginate-CaCl2 solution ratios and pH. *Food Hydrocolloids,* 90(232-240. doi: https://doi.org/10.1016/j.foodhyd.2018.12.022

Ranc, H., Elkhyat, A., Servais, C., Mac-Mary, S., Launay, B. & Humbert, P. 2006. Friction coefficient and wettability of oral mucosal tissue: Changes induced by a salivary layer. *Colloids and Surfaces A: Physicochemical and Engineering Aspects,* 276(1): 155-161. doi: https://doi.org/10.1016/j.colsurfa.2005.10.033

Riaz, M. N. 2013. Chapter 16 - Food Extruders. *In:* KUTZ, M. (ed.) *Handbook of Farm, Dairy and Food Machinery Engineering (Second Edition).* San Diego: Academic Press.

Rosenfeld, D. L. & Burrow, A. L. 2017. Vegetarian on purpose: Understanding the motivations of plant-based dieters. *Appetite,* 116(456-463. doi: https://doi.org/10.1016/j.appet.2017.05.039

Rossetti, D., Yakubov, G. E., Stokes, J. R., Williamson, A. M. & Fuller, G. G. 2008. Interaction of human whole saliva and astringent dietary compounds investigated by interfacial shear rheology. *Food Hydrocolloids,* 22(6): 1068-1078. doi: https://doi.org/10.1016/j.foodhyd.2007.05.014

Ruiz-Herrera, J. & Ortiz-Castellanos, L. 2019. Cell wall glucans of fungi. A review. *The Cell Surface,* 5(100022. doi: https://doi.org/10.1016/j.tcsw.2019.100022

Saxena, R. K., Gupta, R., Saxena, S. & Gulati, R. 2001. Role of fungal enzymes in food processing. *In:* KHACHATOURIANS, G. G. & ARORA, D. K. (eds.) *Applied Mycology and Biotechnology.* Elsevier.

Sha, L. & Xiong, Y. L. 2020. Plant protein-based alternatives of reconstructed meat: Science, technology, and challenges. *Trends in Food Science & Technology,* 102(51-61. doi: https://doi.org/10.1016/j.tifs.2020.05.022

Sharif, M., Zafar, M. H., Aqib, A. I., Saeed, M., Farag, M. R. & Alagawany, M. 2021. Single cell protein: Sources, mechanism of production, nutritional value and its uses in aquaculture nutrition. *Aquaculture,* 531(735885. doi: https://doi.org/10.1016/j.aquaculture.2020.735885

Stieger, M. & Van De Velde, F. 2013. Microstructure, texture and oral processing: New ways to reduce sugar and salt in foods. *Current Opinion in Colloid & Interface Science,* 18(4): 334-348. doi: https://doi.org/10.1016/j.cocis.2013.04.007

Stoffel, F., Santana, W. D. O., Fontana, R. C. & Camassola, M. 2021. Use of Pleurotus albidus mycoprotein flour to produce cookies: Evaluation of nutritional enrichment and biological activity. *Innovative Food Science & Emerging Technologies,* 68(102642. doi: https://doi.org/10.1016/j.ifset.2021.102642

2022. *Easy Binder - Special Ingredients*. https://www.kitchenlab.se/p/easy-binder-special-ingredients/ (Accessed 2022-May 6).

Ugese, F., Baiyeri, P. & Mbah, B. 2010. Fatty acid profile of Shea tree (vitellaria paradoxa C. F. gaertn.) seeds from the Savanna of Nigeria. *Forests, Trees and Livelihoods,* 19(doi:

10.1080/14728028.2010.9752680

Van Kleef, F. S. M. 1986. Thermally induced protein gelation: Gelation and rheological characterization of highly concentrated ovalbumin and soybean protein gels. *Biopolymers,* 25(1): 31-59. doi: https://doi.org/10.1002/bip.360250105

2013. *Statistics-facts and figures*. https://vegsoc.org/info-hub/facts-and-figures/ (Accessed 2022-Mar. 24).

Westlake, R. 1986. Large-scale Continuous Production of Single Cell Protein. *Chemie Ingenieur Technik,* 58(12): 934-937. doi: https://doi.org/10.1002/cite.330581203

Wijayanti, H. B., Brodkorb, A., Hogan, S. A. & Murphy, E. G. 2019. Chapter 6 - Thermal Denaturation, Aggregation, and Methods of Prevention. *In:* DEETH, H. C. & BANSAL, N. (eds.) *Whey Proteins.* Academic Press.

Zhang, C., Guan, X., Yu, S., Zhou, J. & Chen, J. 2022. Production of meat alternatives using live cells, cultures and plant proteins. *Current Opinion in Food Science,* 43(43-52. doi: https://doi.org/10.1016/j.cofs.2021.11.002

Zhang, Y., Liao, J. & Qi, J. 2020. Functional and structural properties of dietary fiber from citrus peel affected by the alkali combined with high-speed homogenization treatment. *LWT,* 128(109397. doi: https://doi.org/10.1016/j.lwt.2020.109397

7. Appendix

7.1 T-test results for TA data

Table A1 Coding for output sample data in TA

Sample No.	pH	Crosslinking method	Extrusion	Cutting direction
1	6.4	Internal	Yes	Perpendicular
2	6.4	Internal	Yes	Parallel
3	-	External	Yes	Perpendicular
4	-	External	Yes	Parallel
5	6.4	Internal	No	Perpendicular
6	6.4	Internal	No	Parallel
7	-	External	No	Perpendicular
8	-	External	No	Parallel

Table A2 Raw data of fracture force and hardness for both externally and internally crosslinked sheared samples

	Fracture (g)		Hardness (g)	
	Perpendicular	Parallel	Perpendicular	Parallel
External	4231	1173	4543	1372
	4538	1224	4870	1448
	3862	1057	4883	1581
Internal	4842	1133	5167	1133
	4529	1292	5983	1378
	4135	1137	5040	2410

Table A3 Raw data of fracture force and hardness for both externally and internally crosslinked non-sheared samples

	Fracturen (g)		Hardness (g)	
	Perpendicular	Parallel	Perpendicular	Parallel
External	2066	2900	2334	3160
	1835	2080	2655	2772
	1955	3293	2047	4454
Internal	2383	2867	3255	3241
	2523	2845	3215	3329
	3552	4010	3552	4010

Table A4 T-test result for the fracture force of TA sample 1 and 2

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
fracture	Equal variances assumed	2.453	.192	15.176	4	.000	3059.00000	201.56774	2499.35822	3618.64178
	Equal variances not assumed			15.176	2.255	.003	3059.00000	201.56774	2279.15973	3838.84027

Table A5 T-test result for the fracture force of TA sample 3 and 4

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
fracture	Equal variances assumed	2.505	.189	15.700	4	.000	3314.66667	211.13134	2728.47208	3900.86125
	Equal variances not assumed			15.700	2.261	.002	3314.66667	211.13134	2499.65041	4129.68292

Table A6 T-test result for the fracture force of TA sample 5 and 6

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
fracture	Equal variances assumed	5.168	.085	-2.216	4	.091	-805.66667	363.49385	-1814.88738	203.55405
	Equal variances not assumed			-2.216	2.139	.149	-805.66667	363.49385	-2276.05775	664.72442

Table A7 T-test result for the fracture force of TA sample 7 and 8

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
fracture	Equal variances assumed	.018	.899	-.791	4	.473	-421.33333	532.76845	-1900.53569	1057.86902
	Equal variances not assumed			-.791	3.993	.473	-421.33333	532.76845	-1901.60968	1058.94301

Table A8 T-test result for the hardness of TA sample 1 and 2

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
hardness	Equal variances assumed	2.340	.201	25.993	4	.000	3298.33333	126.89541	2946.01520	3650.65147
	Equal variances not assumed			25.993	3.106	.000	3298.33333	126.89541	2902.14978	3694.51688

Table A9 T-test result for the hardness of TA sample 3 and 4

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
hardness	Equal variances assumed	.462	.534	7.661	4	.002	3756.33333	490.29538	2395.05514	5117.61153
	Equal variances not assumed			7.661	3.721	.002	3756.33333	490.29538	2353.86209	5158.80457

Table A10 T-test result for the hardness of TA sample 5 and 6

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
hardness	Equal variances assumed	4.188	.110	-2.076	4	.107	-1116.66667	537.95797	-2610.27744	376.94411
	Equal variances not assumed			-2.076	2.470	.149	-1116.66667	537.95797	-3056.28292	822.94959

Table A11 T-test result for the hardness of TA sample 7 and 8

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
hardness	Equal variances assumed	3.865	.121	-.701	4	.522	-186.00000	265.22988	-922.39620	550.39620
	Equal variances not assumed			-.701	2.738	.538	-186.00000	265.22988	-1077.59043	705.59043