Microbiological characterisation of fermented soybean product with pro-prebiotic potential for honeybee

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Abstract

Due to the problem of honeybee's exposure to glyphosate and pesticides, gut bacteria and their bioactive metabolites are disturbed, which may affect their effect on the host. However, there are not enough studies about dysbiosis in honeybees, and further investigations are needed. Therefore, the main objective of this project was to analyse and evaluate the microbiological status and quality of a specific fermented food product intended to improve the gut microbiota of bees. For this purpose, the pH, protein content, and the colony forming units in different culture media (PCA, MRS+Cys, MRS, CD EC, SBD), were analysed in three different product batches at different time points.

Colony counts and contaminants in the different batches before fermentation (T1) were low. Large variability in the results between batches (bacterial counts, protein content, pH) was observed after the first fermentation. In the final product (T4), many colonies were observed in the different culture media for the batches 1 and 3, and coliforms contaminations in the batches 2 and 3. The pH values obtained at time two after the first fermentation were low acid values (above 5), although in the case of time 3, values closer to the usual ones (between 4 and 4.6) were obtained in batches 2 and 3.

We conclude that, given the significant variation in the results obtained at different times (different number of colonies, pH, and quantity of proteins), it is necessary to optimize the preparation of the fermented soybean product and improve its fermentation process.

Keywords: Gut microbiota, honeybee, fermented soybean, microbiological quality

Index

1. Importance of microbiota

The microbiota plays an essential role in host physiology and metabolism. It is a potent regulator of immune response regulation, a critical factor in developing certain human diseases and processes such as metabolic, autoimmune, and liver diseases[1,2].

The different functions of the gut microbiota are highly conserved between individuals. Still, each person's gut microbiota has a specific combination of bacterial species due to intra-individual and inter-individual variations that humans have modified throughout life[3,4]. The microbiota composition has different species of microorganisms, including yeasts, viruses, and bacteria. In taxonomy, bacteria are classified according to phyla, classes, orders, families, genera, and species. The most common intestinal microbial phyla are Bacillota (formerly known as Firmicutes), Bacteroidota (formerly known as Bacteroidetes), Pseudomonadota (formerly known as Proteobacteria), Actinomycetota (formerly known as Actinobacteria), together these phyla account for approximately 90% of the intestinal microbiota (**Figure 1**)[4,5].

Regulation of the microbiota may be of great importance in humans and certain animals[1]. This aspect may have some repercussions, such as an economic impact on livestock farming. There could be an improvement in health due to treatment with probiotics and prebiotics, which may prevent diseases related to intestinal pathologies, avoiding possible treatment and health costs[1,2]. Nowadays, many scientific articles discuss the effects of diet, specifically the inclusion of fermented foods, on gut microorganisms and their interactions with hosts (gut microbiota), impacting disease and health[1].

Food components, such as probiotics and prebiotics influence gut microbiota. In research and dietetics, probiotics have gained interest as a potential product to modulate the gut microbiota and improve human health[1,2].

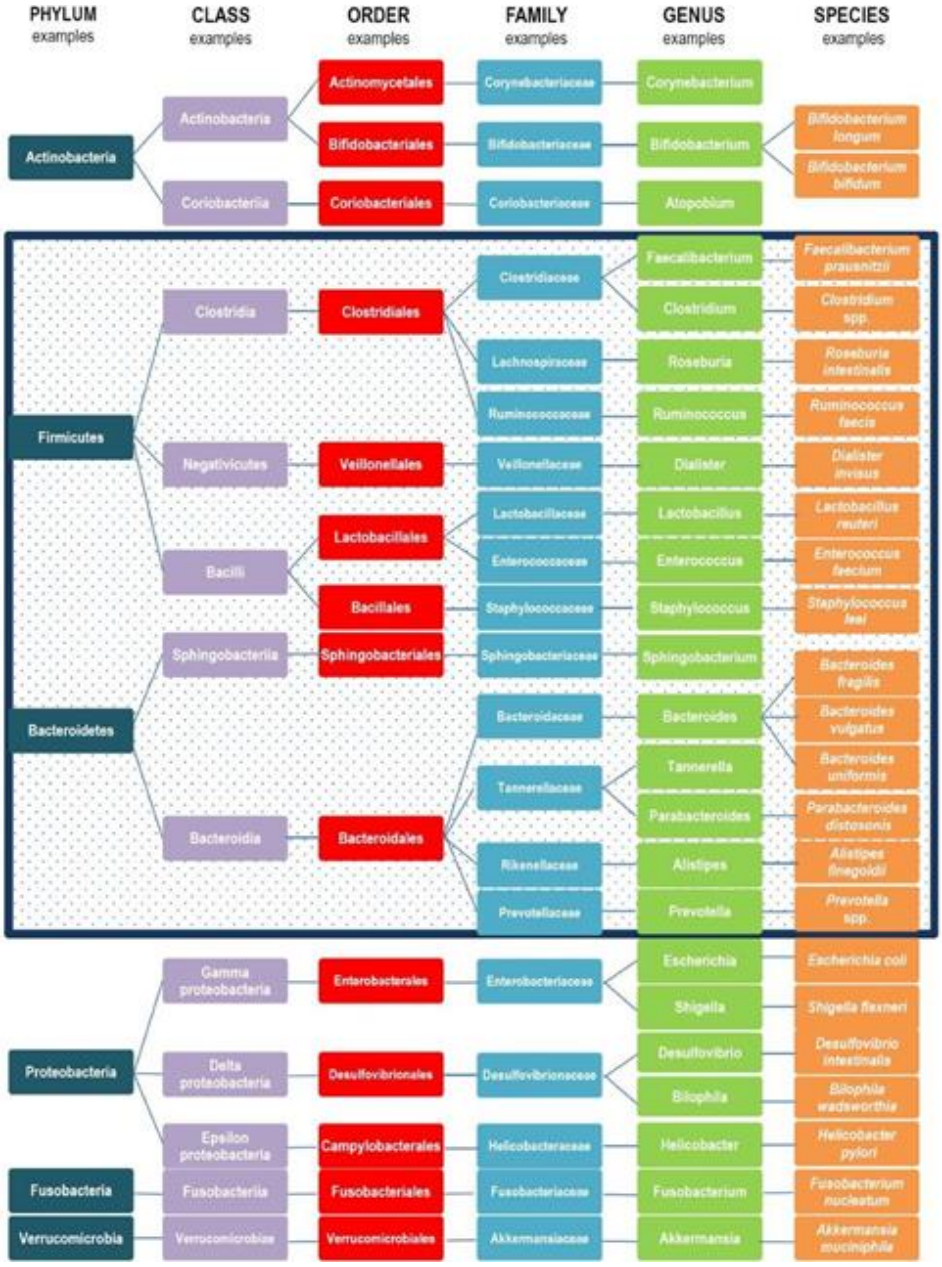

Figure 1: Taxonomic composition of microbiota[4]. At present, there are phylums that have changed the name; Actinobacteria, currently named Actinomycetota; Firmicutes, currently named Bacillota; Bacteroidetes, currently named Bacteroidota; Proteobacteria, currently named Pseudomonadota. And in the case of a very common genus, *Lactobacillus*, currently named *Limosilactobacillus*[5].

Typically, most scientific studies focus on the analysis of probiotics and prebiotics and their relationship to changes in human gut microbiota composition or animal models, mainly mammals like rodents[1,2]. However, there is currently no comprehensive knowledge about their effects on the microbiota of insects and the potential for their ability to prevent pathologies in these organisms. Among insects, honeybee (Apis mellifera) are an interesting group, as bees are the world's main pollinators, which are exposed to high amounts of glyphosates and pesticides, so studies focusing on the microbiota of bees have shown the possible impact of certain pesticides on the composition of the microbiota and, consequently, on their health and the way they relate to each other[3].

2. Gut microbiota of insects

Insects are a different and very diverse group of animals worldwide. The gut microbiota of insects can play an essential role in other functions of their organism, e.g., in complementing the host's nutrition, in symbiotic processes, and in facilitating protection against external agents such as pathogens[6,7].

Colonization of the digestive tract is the main factor in establishing symbiosis between the intestinal microbial population and its host[6]. There are a variety of mechanisms that limit or facilitate intestinal colonization, reflecting different levels of specificity in the symbiotic relationships and involving various factors in both the microbial population and the host[6,7].

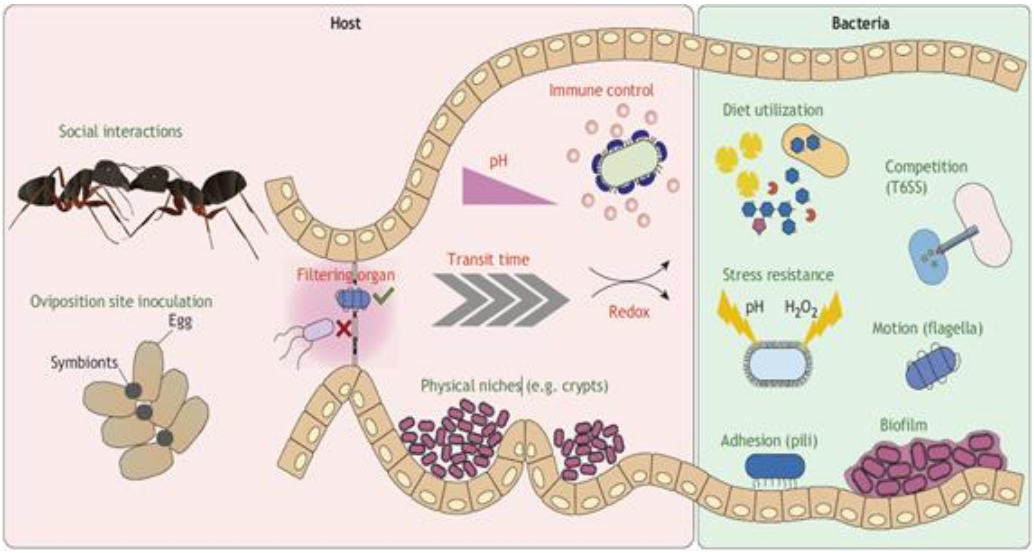

Figure 2. Summary of the different mechanisms that limit or facilitate the colonization of insects in the gut by bacteria[6].

(**Figure 2**) shows the mechanisms that facilitate (green) or limit (red) gut colonization. The factors are separated on the left side of the image into host factors (pink background) and bacterial factors (green background). Host factors that facilitate gut colonization include egg inoculation and social interactions[6]. In addition, physical niches can stimulate a protected microenvironment for microbes. Host factors that limit or hinder intestinal colonization include pH (a very acidic pH limits intestinal growth and colonization), immune effectors, which due to immune-driven reactions may delay intestinal colonization, or short transit time[6,7,8]. Among the properties of bacteria that facilitate gut colonization are diet utilization (by metabolization and digestion of different compounds), interbacterial competition, stress resistance, biofilm formation and host adhesion[6,7].

In insects, their digestive system has a very complex defence system, which is probably a factor in developing microbial populations in the gut. Different aspects of this defence system contribute to the host's additional resistance and tolerance properties towards the bacterial populations present in the gut[6,7,8]. Tolerance can reduce the adverse effects of a given bacterial load on the host, and resistance can minimize the bacterial load so that with this balance, the host's health is not affected[9].

2.1. Gut microbiota of honeybee

Honeybees are interested in research as they are essential agricultural pollinators. Thus, honeybees development, physiology, and behaviour are relatively well understood. Regarding the gut microbiota of honeybees, different studies have shown its great importance as it affects endocrine signalling, nutrition, weight gain and immune function in this insect[10,11,12]. Therefore, studying the gut microbiota in the honeybee (Apis mellifera) is essential. It allows us to understand how gut communities affect the bee and observe the processes that define the dynamics and composition of its gut community.

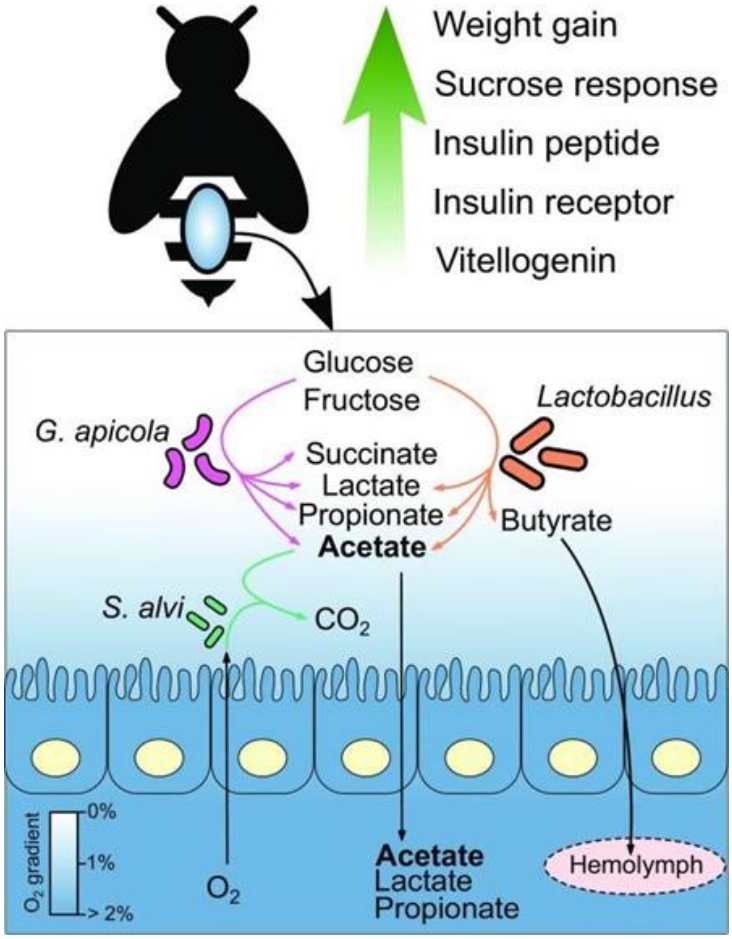

Figure 3: Overview of the effects of the honeybee gut microbiota on gut microbial metabolism and on the honeybee itself[13].

A small number of bacterial genera that dominate the gut microbiota of honeybees, such as *Lactobacillus* and Bifidobacterium[10,11]. The most common microorganisms in the ileum and rectum (*Giliamella apicola* and *Lactobacillus sp*.) ferment sugars and polysaccharides ingested by the host (honey and pollen) into short chain fatty acids (SCFAs) (**Figure 3**)[10,13]. In addition *Snodgrassella alvi* species are also abundant. These bacteria form a layer attached to the inner wall of the intestine, maintaining an anoxic intestinal environment as they can consume O2. This is remarkable because alteration of the gut microenvironment can significantly affect metabolic activities[10,13].

Gut microorganisms associated with humans and other animals in which plants form part of their diet can use complex carbohydrates and glycans as a source of energy for optimal growth[10,14]. In the honeybee, pollen is a significant component of their diet and their source of amino acids, vitamins, and minerals, and fat[15]. Most of these nutrients are absorbed and metabolized by the host gut microbiota, except for the more difficult to digest compounds such as pollen cell wall, hemicellulose, cellulose, and pectin, which are broken down by the microbial community in the hindgut[14,15].

A study comparing conventional honeybees with germ-free honeybees has shown that gut microbiota is necessary for weight regulation[13]. The effect on weight gain is associated with changes in endocrine signalling and gene expression, insulin signalling, and changes in vitellogenin concentration, which are responsible for regulating nutritional status in honeybees[10,13]. Vitellogenin governs the development of social behaviour in honeybees, suggesting an essential role of the gut microbiota in influencing social behaviour in honeybees[10].

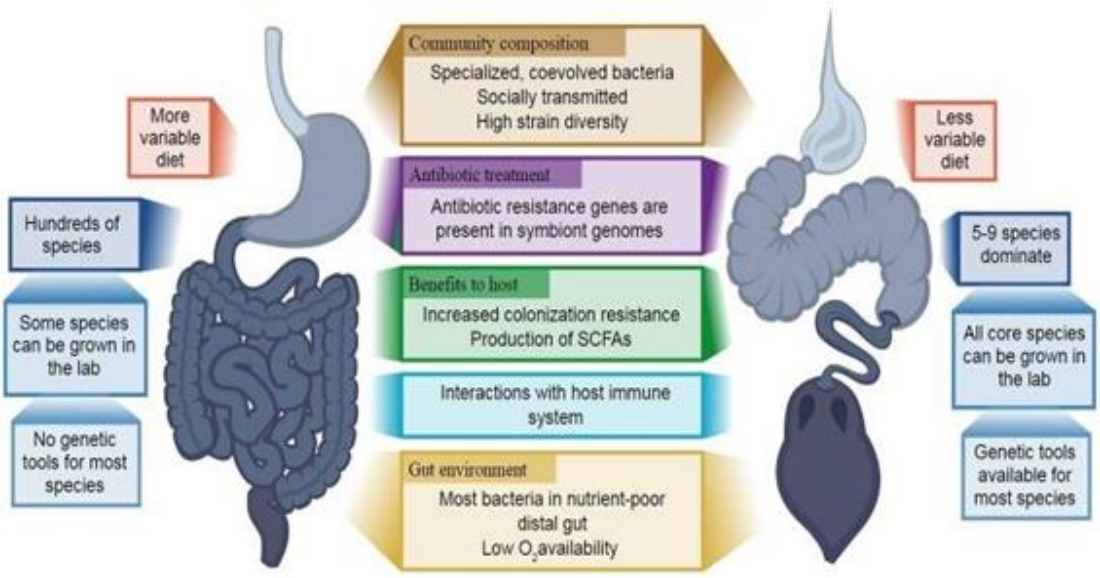

Figure 4. Dissimilarity and similarity in the gut microbiota of human and bees[10]. SCFA, short chain fatty acids.

In addition to the interest in honeybee microbiota given its effects on the health of this insect, its study offers certain advantages as it has several parallels with the human microbiota. Thus, the human and honeybee microbiota have some differences. In the case of honeybees, they have a less variable diet. This fact also leads to a smaller number of species, unlike the human microbiota, with many species and a much more varied diet. In the microbiota of honeybees, all the main species can be grown in the laboratory, and different genetic tools are available for most species, allowing the genome of these organisms to be modified, for example, to modify metabolic pathways obtain metabolites, etc. In the case of the human microbiota, some species can be grown in the laboratory, and there are no genetic tools for most gut microbial species (**Figure 4**).

There are also several similarities between the human and bee microbiota, such as the composition of the microbial community; within the community, there are

specialized bacteria, which have co-evolved with the development of each microbiota (human and bee)[10].

They have similarities in the production of SCFAs, metabolites that exert benefits in the host, such as regulation of glucose metabolism[10]. Moreover, in the enhancement of gut epithelial barrier functions, which is involved in the secretion of antimicrobial substances and intestinal motility[10,13].

2.1.1. Effects of dysbiosis in honeybee microbiota.

In the last years, bee populations have considerably reduced, mainly in the most developed countries, which causes a shortage in the pollination process[16]. This is a significant problem as bees are major pollinators and vital to cultivated and wildflowers. Nowadays, several causes of stress to which bees are exposed are being identified, some caused by environmental degradation and others caused by humans, such as pesticides and glyphosate[16,17]. These can affect their microbiota, leading to dysbiosis and altering the production of bacteria-derived bioactive compounds such as SCFAs. This dysbiosis may affect some bacterial populations that are very important in the defence against pathogens, growth, metabolism and immunity[10,16]. Therefore, alterations in the gut microbiota of this insect may have different effects on immunity, as it would favour infection by specific pathogens[16,18]. In addition, it would affect the responsiveness of bees and may lead to a decreased response to specific environmental stressors[11,17].

Indeed, gut microorganisms dysbiosis induced by antibiotics or other disruptors has led to alterations in its functionality. Therefore, different studies have analysed the effects of tetracycline, a broad-spectrum antibiotic, on the composition of the gut microbiota of honeybees[10,19]. Importantly, none of the dominant members of the microbiota were eliminated after antibiotic treatment. However, the change in the composition of the gut microbiota was detrimental even without the presence of opportunistic microorganisms[19]. Thus, conventional bees showed increased mortality after antibiotic treatment, even under sterile laboratory conditions, compared to antibiotic-treated microbiota-free bees[10,19]. Another study observed that disruption of the gut microbiota of worker bees with antibiotics resulted in decreased immune responses and increased susceptibility to a specific pathogen (Nosema ceranae)[10,20].

Regarding pesticides, the presence of glyphosate has been extensively investigated in recent years[12]. Glyphosate is the most widely used pesticide on earth to control weed growth. This massive use is linked to the alteration of the gut microbiota of honeybees and its different functions[12,16].

In one study, it was even observed that during the transport of nectar by bees to the hive, the nectar contains traces of glyphosate, so the effect of glyphosate on the larvae in the hive was observed[21]. It was concluded that the larvae developed more slowly (up to 40%) than bees not exposed to glyphosate in the nectar. Although there is no direct link, this effect could be associated with a reduced ability of the bees to produce honey[21].

3. Modulation of the microbiota: prebiotics, probiotics, and fermented products.

3.1. Probiotics

The relationship between insects and microbiota is very relevant in the evolution of many types and families of insects. The insect-microbe interaction is mutual and both exposure to pathogens and social relationships in insects can influence the population of microorganisms in the microbiota. Probiotics are *"live microorganisms which when administered in adequate amounts confer a health benefit on the host"*[22].

The main probiotics are Lactic Acid Bacteria (LAB), mostly belonging to the ancient genus *Lactobacillus*[23]. They are mainly noted for their health-promoting properties, as they improve the health of the microbiota and consequently the suitability of the intestinal environment[24,25].

The mechanisms of action of probiotics include increased adhesion to the intestinal mucosa, enhancement of the epithelial barrier and inhibition of pathogen adhesion, production of antimicrobial substances, competitive exclusion of pathogenic microorganisms and modulation of the immune system[26].

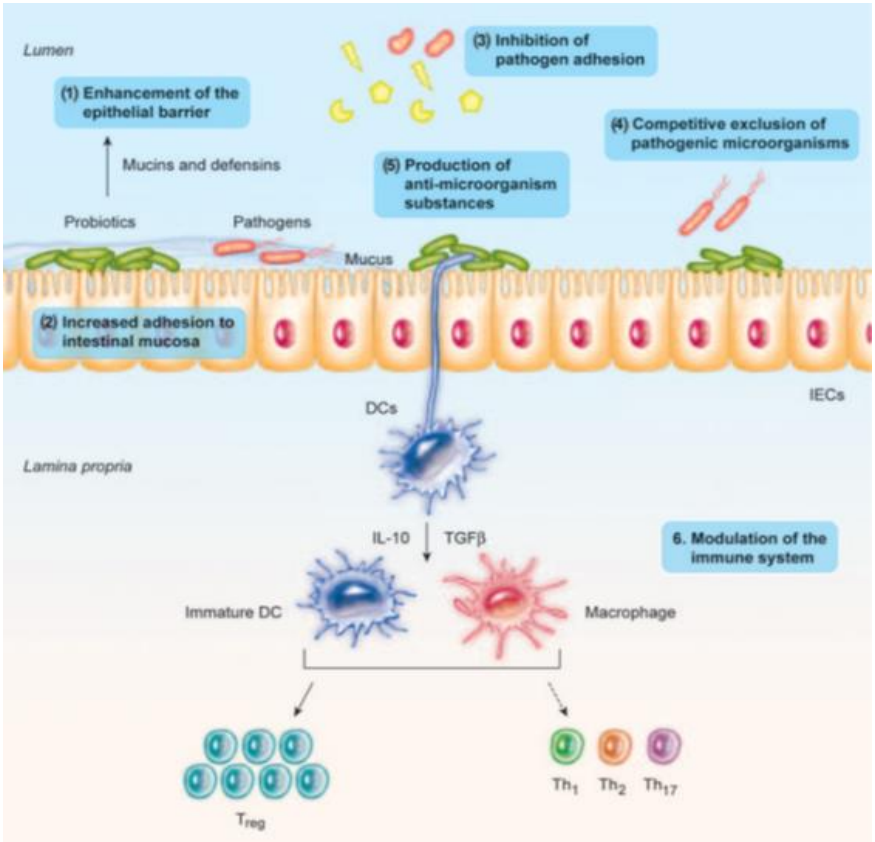

Figure 5: Probiotics, different mechanisms of action[27]. (1) Enhancement of the epithelial barrier; (2) increased adhesion to the intestinal mucosa; (3) inhibition of pathogen adhesion; (4) competitive exclusion of pathogenic microorganisms; (5) production of antimicrobial substances; (6) regulation of the immune system. DC, dendritic cell; IECs, Intestinal epithelial cells; IL-10, interleucine 10; TGF-β, Transforming growth factor beta; T reg, regulatory T cel; Th1,Th2, Th17, Type 1 helper T cells, Type 2 helper T cells, Type 17 helper T cells, respectively.

In (**Figure 5**) we can see the different mechanisms of action of probiotics. The improvement of the epithelial barrier is caused by mucins and defensins, stimulated by the presence of probiotics. In addition, it increases intestinal mucosal adhesion and inhibits the adhesion of pathogens. Another effect of probiotics is the production of antimicrobial substances, such as bacteriocins, which inhibit the growth of pathogenic microorganisms[26,27,28].

Probiotics also act on intestinal epithelial cells through competitive exclusion, this mechanism prevents the colonization of certain microorganisms, as two species occupying the same niche cannot coexist in the long term as they would compete for resources[27,28]. Hence, probiotics such as *Lactobacillus* and *Bacillobacterium* enhance this effect. Finally, probiotics stimulate the production of dendritic cells. These dendritic cells activate TGF-β and IL-10, which causes the maturation of the same dendritic cells (i.e., macrophages), macrophages can activate different helper cells (Th1, 2 and 17), which, when activated, cause further production and maturation of the same macrophages[27,28].

Non-pathogenic bacteria may contribute to improving intestinal barrier function, and probiotic bacteria are widely studied for their involvement in maintaining this barrier. However, the specific mechanisms by which probiotics enhance intestinal barrier function are not fully understood. Some studies suggest that increased expression of specific genes in tight junction signaling is a mechanism that enhances the integrity of the intestinal barrier[26].

There are many in vitro tests in which it has been shown that the administration of specific probiotics (such as *Lactobacillus* and *Bifidobacterium*, *Brevibacillus*) inhibited the growth of pathogenic micro-organisms such as *Paenibacillus larvae* or *Ascosphaera apis*[29,30].

However, there are few in vivo studies in which probiotics have been administered to honeybee larvae. Among them, there are two in which, by ingestion of larval food together with *Lactobacillus* and *Bifidobacterium* strains, a reduction in *Paenibacillus larvae* infection was also observed[29,31,32]. This effect is due to the antagonistic activity of the strain's, formation of bacteriocins of lactic acid. This response indicates an indirect response of probiotics for the immune response in the bee due to the mechanism of competitive exclusion of native microorganisms present in the microbiota, which is closely related to the microbiota of the honeybee[33].

3.2. Prebiotics

In 2016, (ISAPP), the International Scientific Association for Probiotics and Prebiotics reviewed the concept of prebiotics by experts from different scientific fields, such as nutrition, microbiology, and clinical research. Based on the developments analysed by scientists, the definition of a prebiotic was determined by consensus:

It is defined as a *"nondigestible compound that, through its metabolization by microorganisms in the gut, modulates composition and/or activity of the gut microbiota, thus conferring a beneficial physiological effect on the host"*[34].

There are foods rich in prebiotics, such as artichokes, bananas, legumes, potatoes, sweet potatoes, garlic, onions and leeks, wheat, oats, and barley, but there are prebiotics that can be produced artificially. Among these probiotics are galactooligosaccharides(GOS), maltooligosaccharides (MOS), and fructooligosaccharides (FOS)[35].

There is also the possibility of introducing these types of prebiotics into food to improve the nutritional properties of the food itself[34,35]. According to the international scientific association for probiotics and prebiotics (ISAPP) definition, prebiotics can be classified according to the following table (**Figure 6**).

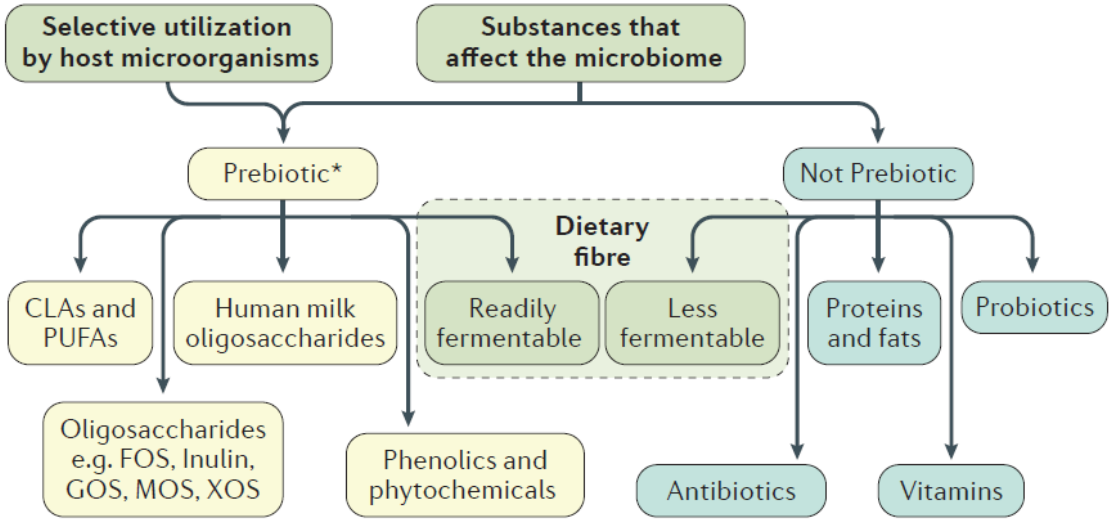

Figure 6. Classification of prebiotics according to the new consensus definition[34]. *The figure shows accepted prebiotics in which the levels of evidence may currently change. PUFA, polyunsaturated fatty acid; CLA, conjugated linoleic acid; GOCs, galactooligosaccharides; FOS, fructooligosaccharides; XOS, xylooligosaccharide MOS, mannanoligosaccharide;

Prebiotics are carbohydrates that are metabolised in the intestinal tract, with the ability to stimulate the maturation and development of micro-organisms and their metabolic activity[36,37]. The gut microbiota of the honeybee is composed of a diversity of microorganisms; the lactic acid bacteria (LAB) of the genus *Lactobacillus* and those of the genus *Bifidobacterium* are the most common[36].

The mechanisms of action of prebiotics are multiple but are usually direct (e.g., carbohydrate structures like host glycans can block adhesion to host cells, thus causing bacterial proliferation. These bacteria release metabolites that affect the gut microbiota (e.g., the effect of short-chain fatty acids on intestinal pH and modulation of immune function) and the effect of prebiotics on intestinal microbiota (e.g., the development of short-chain fatty acids on intestinal pH and modulation of immune function)[36,37].

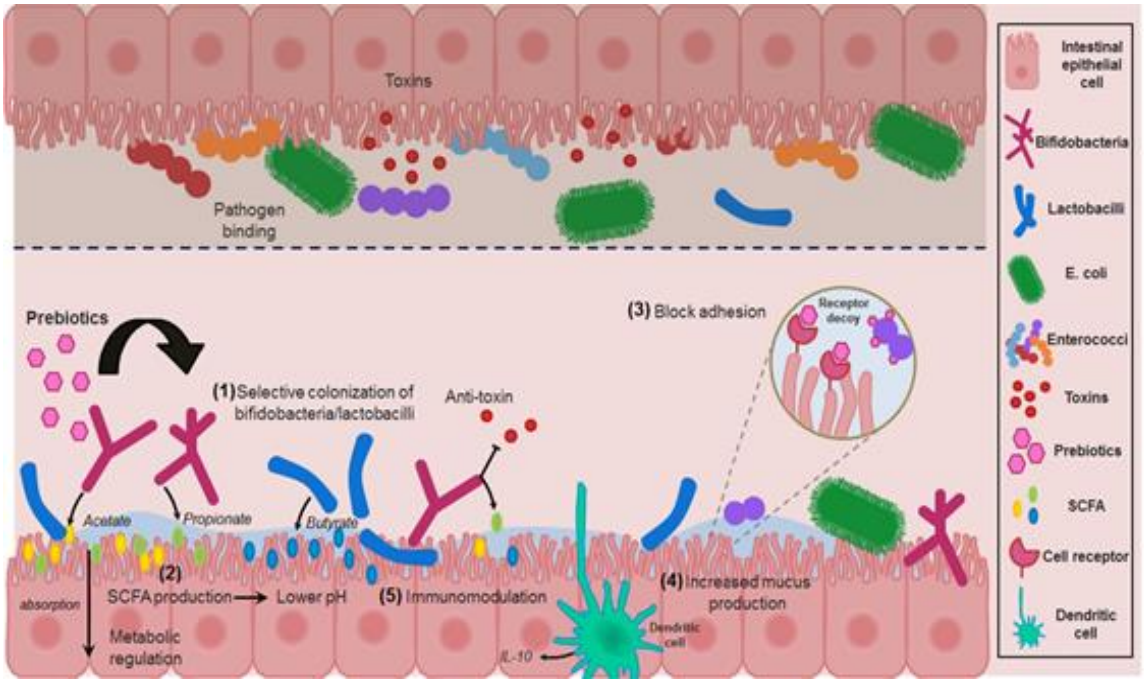

Figure 7: Summary showing mechanisms of prebiotics and their role on gut microbiota[36].

The upper part of the picture describes the intestinal epithelium and the different compounds and microorganisms involved in the mechanisms of action of prebiotics (**Figure 7**). Below the dashed line, the effects of prebiotics are shown, selective colonization of two probiotic bacteria (*Lactobacilli and Bifidobacteria*) by competition with the pathogenic strains by the prebiotics, production of short-chain fatty acids (SCFA), butyrate, propionate and acetate, these SCFA cause a decrease in pH, preventing the binding of pathogens to epithelial cells and intervention of components of the immune system (e.g., dendritic cells), thereby modulating the immune response[36,38]. Several studies and articles have examined the intake of prebiotic dietary fibre (such as the feeding of yeast derived from 1,3-1,6-glucan) supplementation in honeybees with associated beneficial effects, such as significantly reduced infections with deformed wing virus and increased bee survival[39,40].

3.3. Fermented products: Soybean

Soybean is a food of vegetable origin that is rich in protein and widely present in the human diet. Many fermented soy-based foods, including miso, tempeh, natto, and tamari, are known.

The effects of fermented soy-based foods or preparations have been extensively demonstrated in different studies and articles, highlighting their various health-promoting properties in humans[41,42]. Studies define the health benefits of soy and soy foods as the ability to protect against cardiovascular disease, diabetes, and related complications and potentially to benefit cognitive and immune function[41,42,43]. Fermented soy foods, with *Lactobacillus* and *Enterococcus* species of bacteria, have been shown to help increase these bacteria in the gut[44]. Regarding the regulation of the intestinal microbiota, a study carried out using an in vitro digestion model has demonstrated that tempeh stimulates the growth of bacteria of the *Lactobacillus* genus and *E coli*[45]*.* Thus, showing the antimicrobial properties of bean tempeh in the gut microbiota.

In this work and project, a fermented soy-based product will be analysed. Currently, no studies have been found on the effects of soya or a fermented soy-based food on the microbiota of honeybees or insects, so the analysis of the microbiological quality of a fermented product for later supplementing or not supplementing bees will serve as a novelty to analyse and determine the possible effects of a fermented soy-based product.

4. Objectives and hypothesis

The gut microbiota is responsible for several fundamental functions in the honeybee. The increasing use of pesticides worldwide causes the continuous exposure of the honeybee to these compounds, which has harmful effects on the honeybee, both in its socialisation with the environment and in other aspects such as the regulation and homeostasis of the intestinal microbiota. This work hypothesizes that using a fermented soybean product with a potential pre- or probiotic effect can improve the intestinal microbiota of the honeybee and minimise the impact of pesticide exposure.

Therefore, this work aims to analyse and evaluate the microbiological status and quality of a specific fermented food product to improve the microbiota of bees. In addition, the specific objectives of the work are:

1. The microbiological characterization of different batches of a fermented soy-based product at other fermentation times.

2. Protein content analysis of the different batches.

5. Materials and methods

5.1. Soy-based product.

In this work we studied the microbiological composition of a fermented compound based on soya, which has as starters *Bifidobacterium lactis, Lactobacillus bulgaricus and Streptococcus thermophilus.* The starters are the microorganisms in charge of the fermentation of the product.

To analyse the product, different culture media have been used to identify different kind of microorganisms (**Table 1**):

Table 1: Different types of media and their corresponding microorganisms, incubation time and temperature

Plate	To study	Temperature	Growth time
PCA	Total aerobes	+37°C	1 day
MRS+Cys	Bifidobacterium sp.	+37°C	2 days
MRS	Lactobacillus sp.	+30°C	2 days
Compact Dry EC	Escherichia coli and Coliforms	+37°C	2 days
SBD/SDA	Yeasts and moulds	+25°C	2 weeks

PCA= Plate count agar; MRS+Cys= MAN, ROGOSA and Sharpe Agar + Cysteine ; MRS=MAN, ROGOSA and Sharpe Agar ; SBD/SDA: Saboroud Dextrose Agar

According to the (**Figure 8**), in the first step, the product compounds are added, the sweet syrup (composed of glucose, fructose, boiled and cooled water), the solid mixture (composed of defatted soy flour, wheat gluten, dried mint and cardamom), and finally the pH is adjusted using industrial vinegar.

Finally, it is mixed and sterilized at 90-100 degrees Celsius. Once the sample is sterilized, the yeast is added (together with sugar, salt, and hot water) for activation and fermentation (lasting three days), thus obtaining time 1. At T1 (time 1), the yeast is added and left to ferment for three days and, we get the product at T2. In the second part of the process, *Lactobacillus* colonies from a sour yoghurt are added after the first fermentation at time 2, and then the fermentation of this mixture is analysed in two weeks (T3). After the analysis of the second fermentation after two weeks, drying, grinding, and sieving (drying at 37 degrees Celsius) is carried out, and the final product is obtained (T4).

The last section of the diagram, T5, refers to analysing a sample of sweet syrup that is intended to be mixed with T4 before being it to the bees, where only its microbiological quality is analysed, examining the presence of aerobic micro-organisms, coliforms, and/or moulds and yeasts.

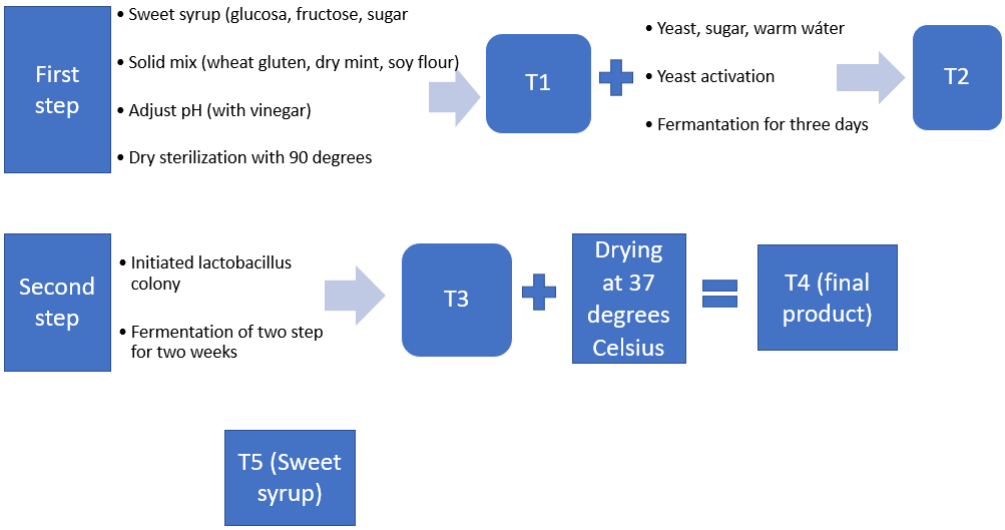

Figure 8: Diagram of the process of obtaining the fermented soybean product. The times T1, T2, T3 and T4 indicate the different stages of product processing at which the product was collected for analysis. T5 refers to the last analysis, the product to be analysed is only sweet syrup.

At time 1, samples are taken before fermentation in 3 different batches (B1.1, B2.1, B3.1), from here on all 3 batches are analysed at different times (**Table 2**).

Table 2: Different batches, times, and plate requirements

Timepoints	Day of collect from start of procedure	Batch information			Plate requirements
T1 (before the yeast fermentation)	1	B1.1	B2.1	B3.1	PCA
					Compact Dry EC
					MRS+Cys
					pH
T2(after yeast fermentation)	4	B1.2	B2.2	B3.2	SBD
					PCA
					pH
T3(two weeks after fermentation)	18	B1.3	B2.3	B3.3	Differential Lactobacilli (Gram stain)
					MRS
					MRS+Cys
					PCA
					pH
T4 Final product (after drying, milling and sieving)	20	B1.4	B2.4	B3.4	Compact Dry EC
					MRS+ Cys
					SBD
					MRS
					PCA
T5(sweet syrup)	20	B1.5	B2.5	B3.5	Compact Dry EC
					PCA
					SBD

PCA= Plate count agar; MRS+Cys= MAN, ROGOSA and Sharpe Agar + Cysteine; MRS=MAN, ROGOSA and Sharpe Agar; SBD/SDA =Saboroud Dextrose Agar; Compact Dry EC= Compact Dry E Coli and Coliforms.

5.1.1. Preparation of culture media

To analyse the microbiological quality of the product to be analysed, different culture media were used, allowing the identification of different classes of microorganisms.

-PCA: Total aerobic.

-Compact Dry EC: E Coli and coliforms.

-Agar Sabouraund: Yeasts and moulds.

-MRS agar: Lactobacillus.

-MRS + Cysteine: Bifidobacterium.

The amount of culture medium for varies according to the type of culture medium in the example, which is the preparation of Plate count agar (PCA). The grams of PCA for each litre is 23.5 grams, in total 94 grams.

In all the media prepared, in addition to PCA, there are Sabouraud Agar (SBD), MRS (MAN, ROGOSA, and Sharpe Agar), and MRS + Cysteine (MAN, ROGOSA, and Sharpe Agar + Cysteine). However, did not use the latter because there were already many plates prepared previously.

The autoclave is a pressure vessel that allows sterilisation processes with high pressure and steam. It is handy to kill different microorganisms, among which dangerous or pathogenic bacteria can be found.

Once the sample has been sterilised, the autoclave is opened and connected to the culture media dispenser. Once connected, the dispenser is activated, and agar starts to flow through the autoclave and can be poured into the Petri dishes. During pouring, the plates were put in groups of 5 for later storage in the fridge for convenience.

5.1.2. Analysis of the fermented product

First, the procedure is carried out in a fume hood. The material is placed inside the fume hood and then sterilised with ultraviolet light for 25 minutes, including all the material used (**Figure 9**).

- Scale.

- Beaker with Stomacher bag.

- Pipettes of 100 and 1000 µL.

- 1 ml graduated pipette.

- Pipette controller.

- Pipette tips.

- Test tubes with 9 ml peptone water (previously prepared).

- Vortex.

- Previously prepared culture media (PCA, MRS+Cys, MRS, SBD, Compact Dry).

- Sterilised spatula.

- Beaker with 90 ml of peptone water.

Figure 9: Material used for the analysis of the different samples of the fermented product.

After 25 minutes, weight 10 grams of product in the beaker into the stomacher bag, then add the 90 ml of peptone water (1/10 dilution) and transfer the bag to the stomacher for 30 seconds.

The sample is retaken to the fume hood, where 1 ml of sample is taken with the graduated pipette and added to one of the test tubes. In this case, this test tube will represent dilution 1/100. From here, 1 ml is added to the next one (dilution 1/1000) (in this case, the explanation is given by an analysis that was carried out up to dilution 1/100000).

Once dilutions are obtained (in this case, up to 1/100000), an extension is made in different culture media plates, except Compact Dry (in which 1ml is added), 100 µL of the dilution corresponding to the different media is added. In these cases, in duplicate. (Both PCA and MRS, CD, MRS + Cys, and SBD).

The plates are then incubated at the corresponding optimum temperature and the recommended time depending on the culture medium (**Table 1**). After incubation, colonies are counted.

5.1.3. Gram stain

Gram stain is a differential stain used in microbiology to visualise bacteria. It is used both to identify bacterial morphology and for bacterial differentiation. Gram-positive bacteria are visualised in purple, and Gram-negative bacteria are visualised in red or pink. The process steps in gram staining are detailed in the following protocol[46].

5.2. Protein Analysis

The Thermo Scientific Micro BCA Protein Assay Kit was used to determine the quantity of protein in the product. The method is based on bicinchoninic acid (BCA) as a reagent to identify Cu + 1. Cu + 1 is formed when Cu + 2 is reduced by proteins in an alkaline medium.1 A purple complex is formed by the chelation of two BCA molecules with a cuprous ion (Cu + 1) (**Figure 10**)[47].

This water-soluble complex has a strong absorbance at 562 nm that is proportional to increasing protein concentrations[47,48].

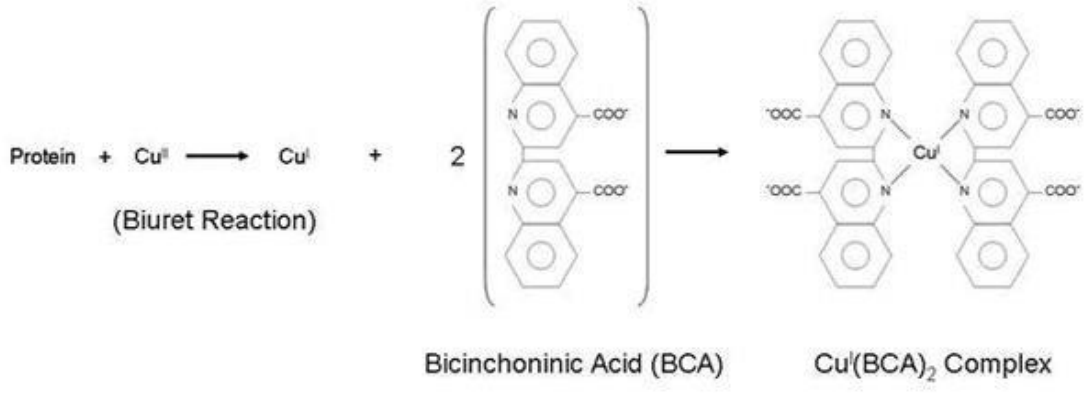

Figure 10: Micro BCA Protein Assay Kit Basics[49]**.**

To calculate the amount of total protein, first, it is necessary to perform the standard line according to different volumes indicated in the protocol (**Table 3**)[47].

Table 3. Development of Diluted Albumin (BSA) Standards[47]**.** The diluent is distilled water, as proteins are water-soluble.

Vial	Volume of Diluent	Volume and Source of BSA	Final BSA Concentration
A	4.5mL	0.5mL of Stock	200µg/mL
B	8.0mL	2.0mL of vial A dilution	40µg/mL
C	4.0mL	4.0mL of vial B dilution	20µg/mL
D	4.0mL	4.0mL of vial C dilution	10µg/mL
E	4.0mL	4.0mL of vial D dilution	5µg/mL
F	4.0mL	4.0mL of vial E dilution	2.5µg/mL
G	4.8mL	3.2mL of vial F dilution	1µg/mL
H	4.0mL	4.0mL of vial G dilution	0.5µg/mL
I	8.0mL	0	0µg/mL = Blank

Next, the required volume of Micro BCA Working Reagent (WR) is prepared. To calculate the volume of WR it is necessary to know the number of tubes to be prepared according to several factors using the following formula.

"(#standards + #unknowns) × (#replicates) × (volume of WR per sample) = total volume WR required"[47].

In this case, the number of standards or vials is 9 (vials A-I), three unknown samples are analysed (batch 1.4, 2.4, 3.4), and each batch is duplicated. Furthermore, the required volume of WR for each sample is 1 ml, so the formula is as follows:

(9 standards, vials A-I, + 3 unknowns) × (2 replicates) × (1mL WR for each sample) = 24mL WR (25 mL)[47].

The final volume required is 24 mL, but 25 mL of WR is prepared for a more accurate calculation. Once the volume of WR needed to perform the reaction has been calculated, the dilution required to prepare the 25 mL of WR is calculated.

According to the protocol, the ratio would be 25:24:1 (Reagent MA:MB:MC)[47]. Reagent MA:MB:MC is part of the protein determination kit. In these ratios, the WR reagent is formed.

5.2.1. Procedure Summary

The analysis procedure can be done in a test tube or a microplate. In this case, the process is carried out in test tubes due to greater convenience for the analysis with larger volumes[47]. The procedure to be followed by test tube analysis is as follows (**Figure 11**).

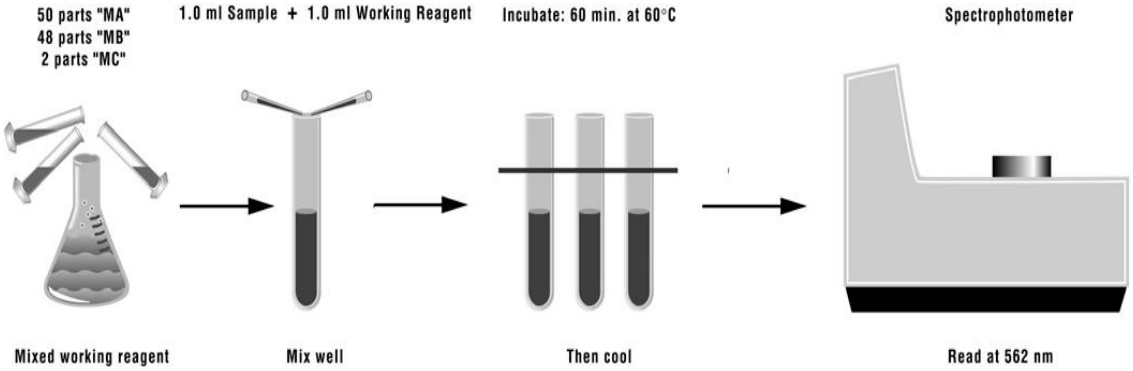

Figure 11: Summary of the protein analysis process[47]**.**

6. Results

6.1. Results fermented product

In the experimental part of the work, the different timepoints were analysed with the batches and their collection days.

6.1.1. Time 1: Before yeast fermentation

Table 4: Average colony forming units/g of sampe (CFU)

	B1.1	B2.1	B3.1
PCA	< 2,0E+04	2,7E+03	2,7E+03
CD EC	0	0	0
MRS	< 2,0E+04	2,4E+03	< 2,0E+04
SBD/SDA	0	0	0

PCA= Plate count agar; MRS=MAN, ROGOSA and Sharpe Agar; SBD/SDA: Saboroud Dextrose Agar; CD EC= Compact Dry E Coli and Coliforms

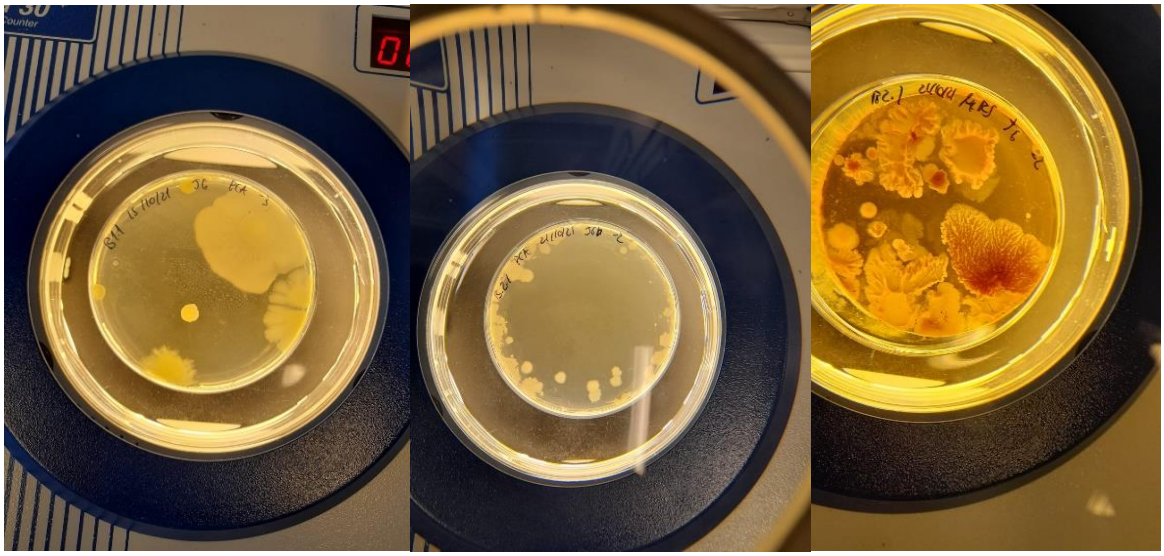

Figure 12: Growth of colonies on different culture media from time 1, (from left to right), colonies on PCA of B1.1, bacteria on MRS of 2.1 and growth on PCA of B3.1; PCA= Plate count agar; MRS=MAN, ROGOSA and Sharpe Agar

As can be seen in (**Figure 12**), in the different batches analysed (B1.1, B2.1, B3.1) very little bacterial growth is observed, both of total aerobic microorganisms (PCA) and *Lactobacillus* (MRS). This is because in the three different batches, they belong to time 1. The product has not undergone the first fermentation (fermentation with yeast) and the presence of total aerobes, except in fermented products, is an indicator of poor product quality and possible contamination[50,51,52]. Furthermore, as far as the growth of moulds and yeasts (SBD/SDA), Escherichia

coli and coliforms (CD EC) are concerned, there has been no growth in any of the two media (**Table 4**), this is indicative that in the different batches of the product in time 1, according to some articles and studies[51,52], they do not present any contamination.

6.1.2. Time 2: After yeast fermentation

Table 5: Average colony forming units/g of sample (CFU)

	B1.2	B2.2	B3.2
PCA	< 2,0E+03	9,6E+06	< 2,0E+04
SBD /SDA	0	0	0
pH	5,3	5,09	5,08

PCA= Plate count agar; SBD/SDA: Saboroud Dextrose Agar

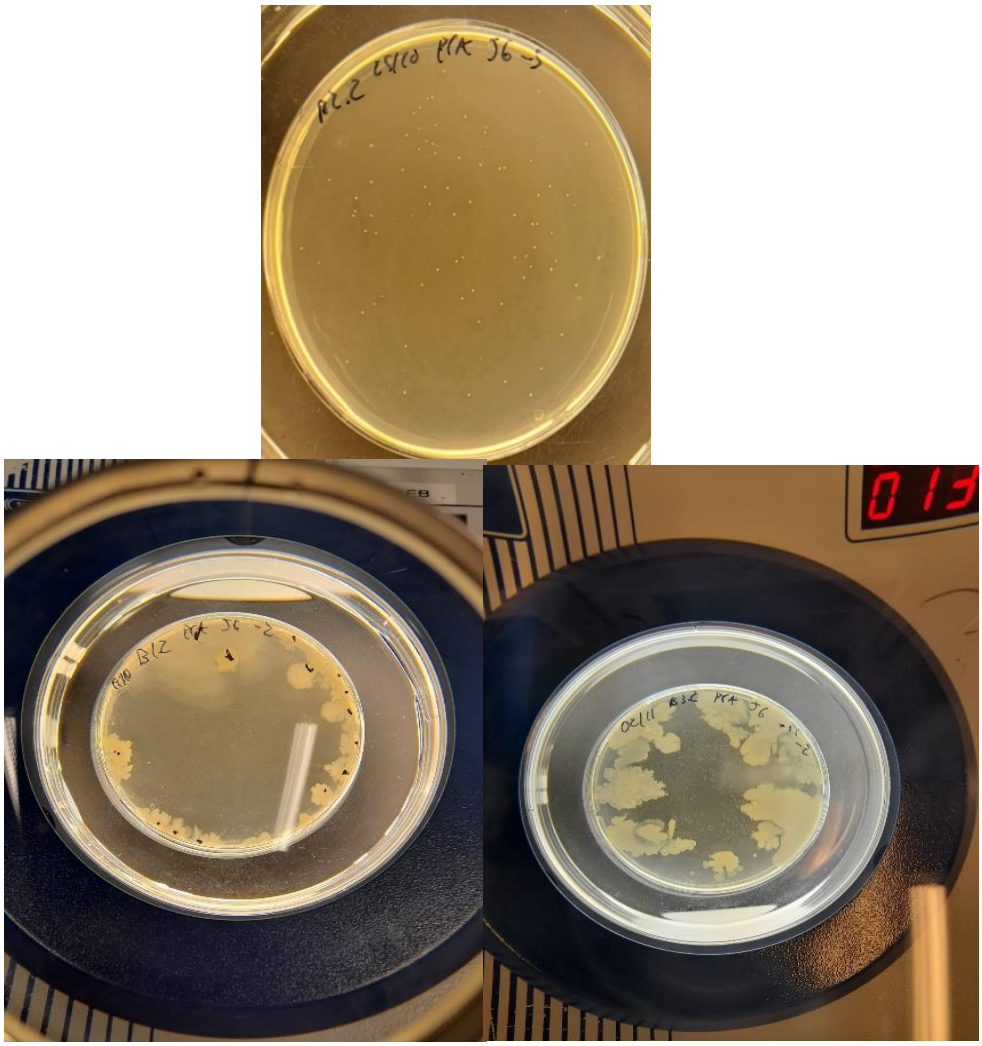

Figure 13. Colony growth at T2 in PCA culture medium. (From left to right), growth in B3.2, B1.2 and B2.2. PCA= Plate count agar

(**Figure 13**) shows the growth in the three aerobic colonies (PCA) batches after yeast fermentation. However, in batches 1 and 3, in very low amounts, compared to batch 2 there is a very high number of colonies (**Table 5**). At time 2, after yeast fermentation, a higher growth was expected in the different batches, especially in both B1.2 and B3.2, as the development and presence of total aerobes in samples where fermentation has taken place is a common characteristic[50,53,54], due to the presence of bacteria responsible for the fermentation of the product.

In the case of the results of pH in the batches (5.3, 5.09 and 5.08), are close to a pH of 5, the results are far from the expected, according to some articles[55,56,57], the most common pH in foods fermented by lactic acid bacteria (yoghurt, kefir, or fermented soya) is between 4 and 4.6. Some articles compared the fermentation in the case of the formation of yoghurt and fermentation of soy milk, and in both values of between 4 and 4.6 were obtained for 72 hours (in the case of yoghurt around 4, and soya around 4.5)[56], in another study, the pH of kefir was analysed, in this case, the final pH at 24h, after incubation at 30 degrees, the pH was around 4 and 4.4. So that the pH values obtained after the first fermentation of the yeast are far from the expected results based on the pH of different foods fermented by lactic acid bacteria (LAB)[57].

Finally, as regards yeast moulds, no presence was found, as mentioned in the previous analysis (after yeast fermentation), indicating the absence of contamination in the sample analysed.

6.1.3. Time 3: Two weeks after fermentation:

Table 6: Average colony forming units/g of sample (CFU)

	B1.3	B2.3	B3.3
PCA	**>** 2,0E+07	**<** 2,0E+03	**>** 2,0E+07
MRS	**>** 2,0E+07	**<** 2,0E+02	**>** 2,0E+07
MRS+CYS	**>** 2,0E+07	**<** 2,0E+02	**>** 2,0E+07
pH	5,02	4,35	4,52

PCA= Plate count agar; MRS+Cys= MAN, ROGOSA and Sharpe Agar + Cysteine; MRS=MAN, ROGOSA and Sharpe Agar

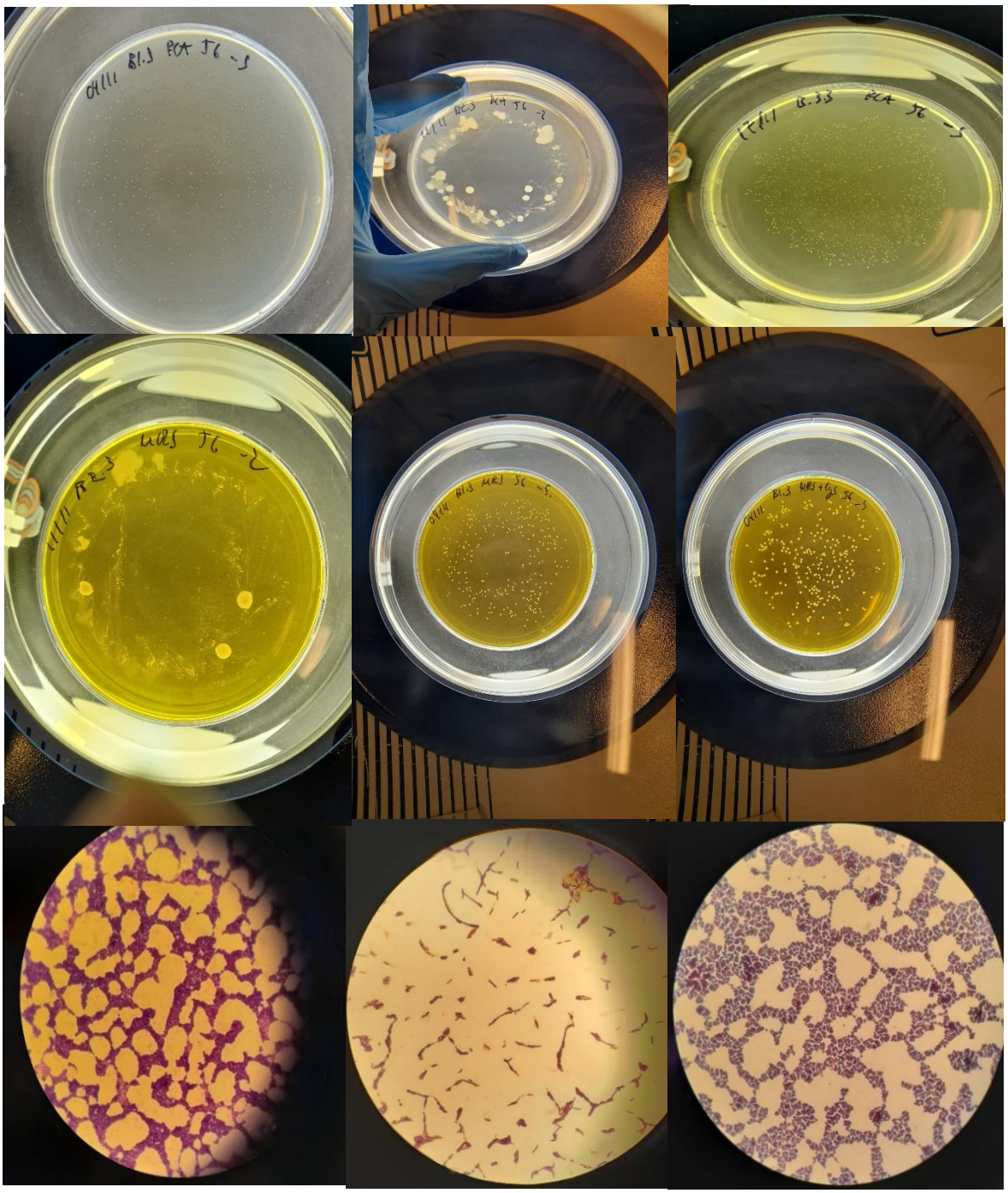

Figure 14. Colony growth in PCA culture medium at time 3, two weeks after fermentation, (from left to right at the top of the image), colony growth in MRS and MRS + Cys at time 3 (from left to right In the centre of the image) and Gram staining of the product in MRS medium at each batch, 1.3, 2.3, 3.3 (from left to right at the bottom of the image). PCA= Plate count agar; MRS=MAN, ROGOSA and Sharpe Agar

In the upper part a significant growth of colonies in the PCA medium, both in Batch 1.3 and 3.3, but in the case of 2.3, there was very little growth of total aerobes (**Table 6**) (**Figure 14**). In the central part, we observe, as in the case of the PCA analysis, high growth of *Lactobacillus* and *Bifidobacterium* (in MRS and

MRS + Cys, respectively), but in batch 2, there was very little growth in both media. According to (**Figure 14**), we have seen that batch 2.3 is the defective batch. After analysing the growth of the microorganisms in the different culture media in time 3 of the fermented product, it can be concluded that the data obtained are correct since, after a second fermentation, there is a high growth of the main starters of the fermented product (*Lactobacillus* and *Bifidobacterium*). These are the main ones responsible for the fermentation and an increased growth of total aerobes, indicative of the fermentation process.

In the pH analysis, we obtained different values depending on the batch, in the case of batch 1.3, the value is 5.02, in line with what was said in time 2. The value is higher than expected after the fermentation process. In this case, after the second fermentation, the value should be more acidic, due to the acidification process of the fermentation itself, and the result is very similar to the 5.3 of time 2. In the case of batches 2.3 and 3.3, pH values of 4.35 and 4.52, respectively, were obtained. According to the different references mentioned in time 2 (between 4 and 4.6) these values are correct [56.57,58], so that in the second fermentation of the compound the pH decreased to values typical of a fermented product based on BAL. The relationship between pH values and colony counts is not very clear because in the case of B1.3, the pH value is moderately acidic. There has been a high colony growth (in all the analysed media), while in B3.3, there has been a high growth but with a more acidic pH, and finally, in the case of B2.3, the colony count has been relatively low, and the most acidic pH was obtained. So, it seems that in the case of B2.3, the pH value influenced the growth of the colonies and fermentation could take place very early, thus avoiding the growth due to the acidic medium. Still, no conclusion can be drawn because this point is unclear, and there is no evidence.

During gram staining, the tested samples from the three batches (1.3, 2.3, 3.3) were observed, in each batch, staining was performed with 6 different colonies. Purple colonies were observed, indicating the presence of Gram-positive bacteria. Regarding the morphology, in the 6 replicates of batch 1 and 3 only cocci colonies were found, while in the case of batch 2 only bacilli were found in each one. Considering that the starters of the product are *Lactobacillus bulgaricus*, *Streptococcus thermophilus* and *Bifidobacterium lactis*, batch 1 and 3 may be *Streptococcus thermophilus* and batch 2 *Lactobacillus bulgaricus*, although it cannot be truly confirmed only with the gram stain and media growing.

6.1.4. Time 4: After drying, milling, and sieving (final product)

Table 7. Average colony forming units/g of sample (CFU)

	B1.4	B2.4	B3.4
PCA	> 2,0E+09	0	1,3E+08
CD EC	0	8,2E+03	2,5E+04
MRS	> 2,0E+09	0	1,8E+08
MRS+CYS	> 2,0E+09	0	2,0E+08
SBD/SDA	0	0	0

PCA= Plate count agar; MRS+Cys= MAN, ROGOSA and Sharpe Agar + Cysteine ; MRS=MAN, ROGOSA and Sharpe Agar ; SBD/SDA: Saboroud Dextrose Agar; CD EC= Compact Dry E Coli and Coliforms

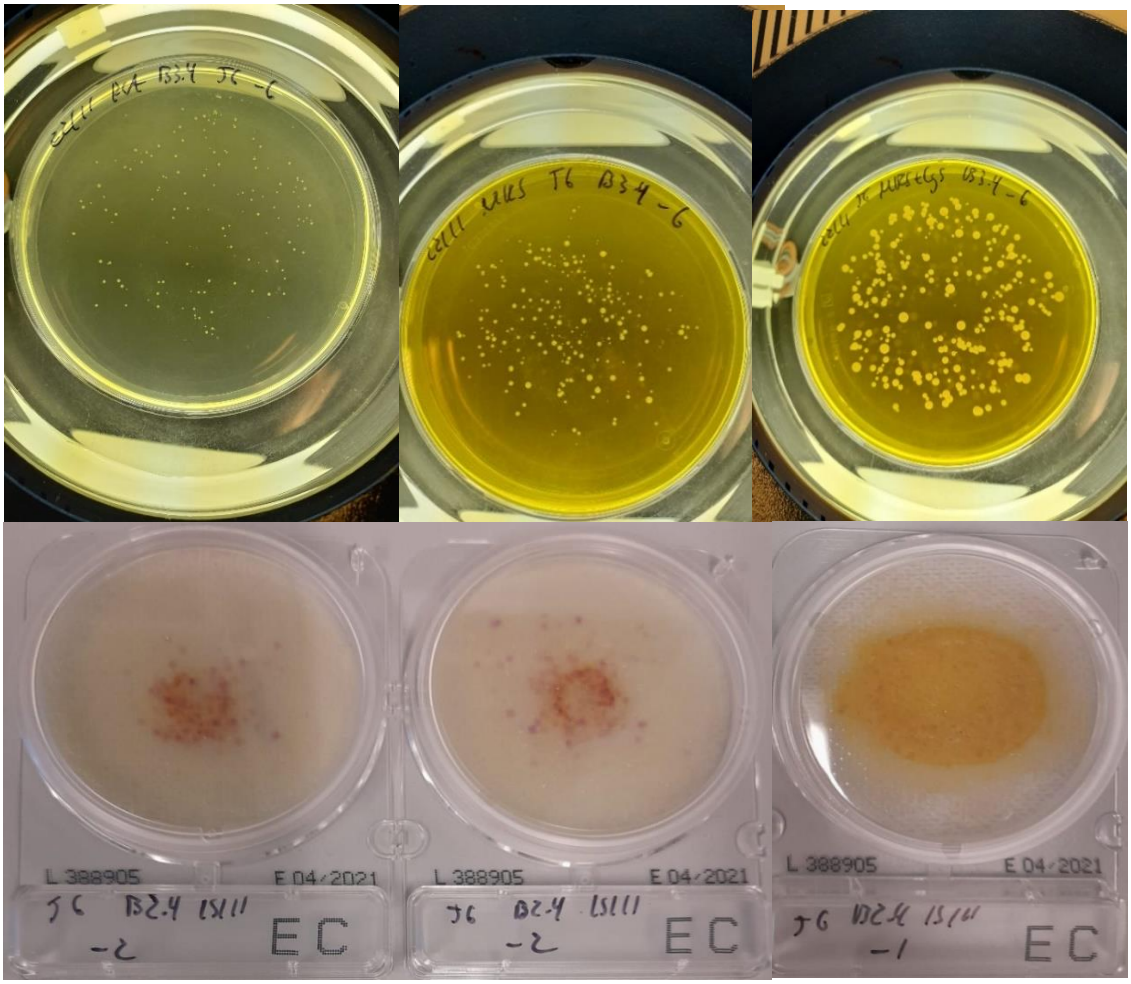

Figure 15: Growth at T4 on PCA, MRS and MRS+Cys media (from left to right at the top of the image) and growth of E coli and coliforms on Compact Dry EC (from left to right at the bottom of the image). PCA= Plate count agar; MRS+Cys= MAN, ROGOSA and Sharpe Agar + Cysteine; Compact Dry EC= E coli and Coliforms

At time 4, after drying, sieving, and milling at 37°C, the final product is obtained. According to the results, in case of batch 1.4, the number of colonies (in PCA, MRS and MRS+Cys) remains very high, even higher than the values of the same batch at time 3. After exposure to heat during the drying process, a similar occurrence happens with batch 3.4, in which a high colony count is also maintained after the thermal process (**Table 7**).

In batch B2.4, no growth of colonies was observed in any of the culture media (PCA, MRS and MRS+Cys), compared to batch 2.3 at time 3. In this case there is even less growth since at time 3 the colony count was meagre. We cannot conclude the reason for the lack of growth since, for example, the very presence of coliforms in this batch and B3.4 could indicate contamination of the sample that could cause the death or absence of the colonies. Still, in the case of B3.4, this growth is not altered, so it is not possible to know the reason for the inhibition in the growth of B2.4 (**Figure 15**).

There was growth of E. coli and coliforms (purple colonies) in B2.4 and B3.4 (CD EC). In both cases, it is most likely that the drying, milling, and sieving processes facilitated the growth of these microorganisms.

6.1.5. Time 5: Sweet syrup

Table 8. Average colony forming units/g of sample (CFU)

	B1.5(CFU)	B2.5 (CFU)	B3.5 (CFU)
PCA	0	0	3,0E+03
CD EC	0	0	0
SBD/SDA	0	0	0

PCA= Plate count agar; CD EC= Compact Dry E Coli and Coliforms; SBD/SDA: Saboroud Dextrose Agar

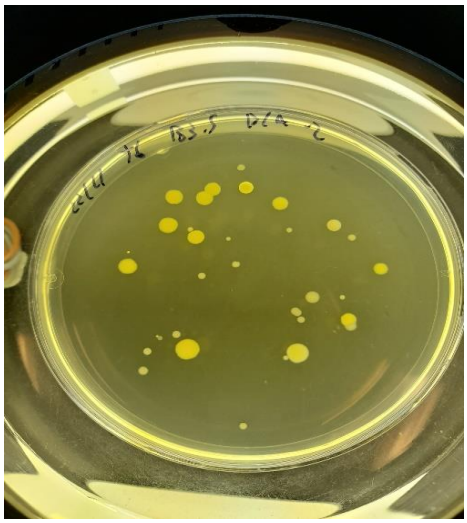

Figure 16: Colony growth at time 5 on PCA growth médium; PCA= Plate count agar

At time 5, the sugar syrup was analysed. We only have colony growth in the PCA medium of batch 3 (**Figure 16**) (**Table 8**). These results are to be expected, as there should not be any colony growth here, as the sample is sugar (although in batch 3 there is growth, it is not very high).

6.2. Protein analysis results

Table 9: Absorbance / [BSA]

[BSA]	Absorbance
0	0
0,5	0,02
1	0,039
2,5	0,125
5	0,206
10	0,449
20	0,753
40	1,585
200	5,8

[BSA]: Protein concentration (µg/ml)

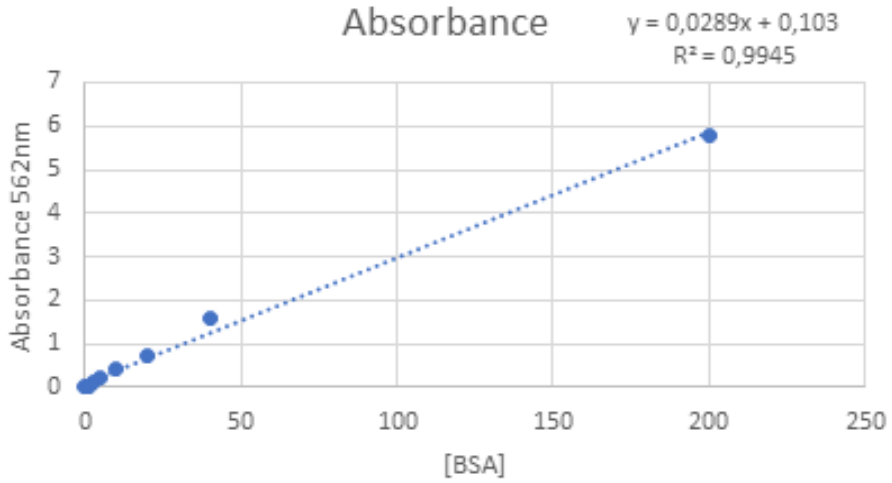

Figure 17: Standard line of Absorbance / [BSA] (µg/ml)

The calculation of the protein concentration was performed on the batches after drying (T4) (**Table 9**). When calculating the BSA (bovine serum albumin) concentration according to the amounts predetermined in the protocol the standard line is obtained, this line is not adequate, because between the different

points of the BSA concentrations of the calibration line there is not a similar distance between the concentration points (**Figure 17**).

Table 10: Different protein concentrations according to batches

Batch	[BSA]
B1.4	$5{,}9 \times 10^2$ mg/g
B2.4	$7{,}1 \times 10^2$ mg/g
B3.4	$6{,}4 \times 10^2$ mg/g

[BSA]: Protein concentration (mg/g)

The range of protein concentrations is between $5{,}9 \times 10^2$ and 7.1×10^2 mg/g between batches, and the average amount of protein is $6{,}4 \times 10^2$ mg/g (**Table 10**). Protein concentrations in soya products are highly variable, but according to some articles on the protein content of soybeans[58,59], the content is between 35 and 60 % of the dry weight. In the case of batch 1 (1.4), there is a concentration of about 60% of protein ($5{,}9 \times 10^2$ mg) as in the case of B3.4 ($6{,}4 \times 10^2$ mg), in the case of B2.4 ($7{,}1 \times 10^2$ mg), we find a somewhat higher amount of protein than the other two batches of about 70%, although the percentage is somewhat higher, it is considered as a correct concentration, since the average values are around 60%.

7. Conclusions

The fermentation process of the soybean fermented product needs to be optimized as it shows a great variability in the different studied parameters (bacterial counts, pH after fermentation and amount of protein) between the different batches and times. It would be worth exploring the fermentation process between T2 and T3 in more detail, due to the great variety of results between the three times in both periods.

Gram-positive bacteria were identified by gram staining in the 3 batches at T3 after the first fermentation (1.3, 2.3, 3.3). In terms of morphology, in the 6 replicates of batches 1 and 3, cocci colonies were observed, while in the case of batch 2 only bacilli were found. The number of coliforms is high in the final product (T4), and this should be controlled, as the presence of these microorganisms indicates contamination. However, the presence of these coliforms was not correlated with a negative effect on the growth of the colonies, as in B3.4 this was observed but in B2.4 it was not. Therefore, the presence of contamination may have originated from a problem during drying or milling.

No specific microbiological recommendations have been found for this type of fermented products, but according to a statement by the European Union for fermented soybean extracts the maximum limits should be[60]:

Total aerobes < 1000/g - the product is above the limits in batches 1 and 3. In other products that can serve as a reference, the accepted upper limit is 106, but we are still above the limits. E. coli - absence in 25g, coliforms < 30/g - the product is above the limits in batches 2 and 3, moulds and yeasts < 100/g.

8. Perspective of future

After the first preliminary results of the characterisation of the fermented soya-based product, further optimisation of the product is needed. The next step in the project is to test the product on bees, and to study the real effects on the microbiota of the bees and whether it can be useful as food to improve their health and minimise the impact of pesticides on the honeybee.

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Abbreviations

BCA: bicinchoninic acid

BSA: bovine serum albumin

CD EC: cell-detaching Escherichia coli

CFU: colony forming unit

CLA: conjugated linoleic acid

DC: dendritic cell

FOS: fructooligosaccharides

GOS: galactooligosaccharides

IEC: intestinal epitelial cells

IL-10: interleucine 10

ISAPP: international scientific association for probiotics and prebiotics

LAB: lactic acid bacteria

MOS: maltooligosaccharides

MRS: man, rogosa and sharpe agar

MRS+Cys: man, rogosa and sharpe agar and cysteine

PCA: plate count agar

PUFA: polyunsaturated fatty acid

SBD: sabouraud agar

SCFA: short-chain fatty acid

TGF-β: transforming growth factor β

Th1: t helper 1 lymphocytes

Th2: t helper 2 lymphocytes

Th17: t helper 17 lymphocytes

Treg: regulatory t cell

WR: working reagent

XOS: xylooligosaccharides