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Growth of honey bee bacteria in whey-based medium

Master´s thesis

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Sustainable Agriculture and Food Security

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This master's thesis is submitted as a requirement for the Master´s degree in Sustainable Agriculture and Food Security at the Czech University of Life Sciences Prague and the Master´s degree in Biosafety and Food Quality at the University of Pisa. The submission is made concurrently to both universities under the double degree program agreement.While the substantive content of this thesis remains consistent for both submissions, there are variations in the title page and the roles of the supervisor and co-supervisor, adhering to the respective guidelines of each university.The dual submission of this thesis is a reflection of the academic collaboration and educational innovation fostered by the double degree program, aiming to broaden the academic and cultural perspectives of its participants.

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ABSTRACT

The *Apis mellifera* pollinator influences genomic diversity in plant communities and contributes to ecosystem structure. Concerns have arisen from the disappearance of bee colonies due to pollution, biocides and diseases. The use of probiotics in the bee diet can improve the gastrointestinal microbiota, promoting metabolic balance and fortifying colonies against stressors and diseases.

Six beneficial strains, isolated from the gastrointestinal tract of worker bees, were identified based on the 16S rRNA gene as *Lactobacillus melliventris, Lb. apis, Lb. helsingborgensis, Lb. kimbladii* and *Bifidobacterium asteroides*. A whey-based medium was prepared by lactose hydrolysis using *β*-galactosidase, given the indigestibility of lactose by the beneficial strains and also by bees. Two different whey samples i.e. a fresh whey sample and a whey sample frozen for 24 hours were used. Conditions of using galactosidase were optimized, identifying the products by proton nuclear magnetic resonance (^{1}H NMR). Considering the percentage of converted lactose,-the fresh whey-based medium was selected and inoculated with the strains. The bacterial growth in the fresh whey-based medium, leading to the release of metabolites such as organic acids (acetic, lactic and succinic acid), was analyzed by the quantitative ^{1}H NMR, at 24 and 48 hour. Whey is a suitable product for industrial growth of potential honey bee probiotic bacteria. Spectrum of organic compounds produced during the fermentation suggests a possible use as a drink suitable for human consumption. This research represents a basis for cheap large scale production of these bacteria.

Abbreviations: NMR, Nuclear Magnetic Resonance; LAB, lactic acid bacteria; AMPs, antimicrobial peptides; GI, gastrointestinal; GRAS generally recognized as safe; GC, guanine and cytosine; HMOs, human milk oligosaccharides; SCFAs, short chain fatty acids; F6PPK, fructose-6-phosphate-phosphoketolase; M.R.S., de Man, Rogosa and Sharpe; TPY, Trypticase Phytone Yeast.

1 AIM OF THE THESIS

The objective of this thesis is to optimize a whey-based medium for the growth of beneficial strains isolated from worker bee gastrointestinal tract.

2 INTRODUCTION

The social honey bee, *Apis mellifera,* plays a crucial role as an important pollinator, exerting a profound impact on genomic diversity within plant communities and contributing to the overall structure of ecosystems. Beyond their ecological importance, bee products are commonly used by humans around the world.

The alarming problem of disappearing bee colonies is attributed to various factors, including environmental pollution, the presence of biocides and the susceptibility of bees to diseases. An encouraging strategy to tackle this issue entails harnessing the potential of probiotic bacteria. These probiotics could enhance the composition of the bee natural microbiota, thereby fostering metabolic balance. Strengthening bee colonies with sufficient probiotics can enhance their resilience against environmental stressors and potential diseases, ultimately securing the crucial role they play in pollination and maintaining ecological balance (Nowak *et al.*, 2021).

2.1 Gut microbiota composition of honey bees

There is compelling evidence highlighting the significant role of gut microorganisms in supporting various aspects of the immune system, influencing epithelial homeostasis, promoting lifespan and larval growth during food scarcity. A well-balanced intestinal microbiota acts as an effective barrier against pathogen colonization, generates essential metabolic substrates such as vitamins and short-chain fatty acids, and engages in active communication with the host to activate and instruct mucosal immunity (Gaggìa, Baffoni and Alberoni, 2018).

Experiments comparing worker bees with and without a normal gut microbiota reveal that the presence of a healthy gut microbiota provides numerous benefits, such as protection against bacterial pathogens, enhanced appetite, intestinal development, weight gain, and an increased production of enzymes capable of neutralizing food toxins (Motta *et al.*, 2022).

Knowledge of microbiota can be used in strategies to strengthen the immunity of *Apis mellifera* (Hroncova *et al.*, 2019).

Specific bacterial symbionts adapted to host niches play a crucial role in defense, nutrition, and physiology. Most intestinal commensal bacteria are essential for maintaining homeostasis and health in both individual insects and the hive. In social

insects like bees, symbiosis is particularly significant, as microbial and host elements collaborate synergistically for proper nutrition, health, and immunity (Gaggìa, Baffoni and Alberoni, 2018). Bee health is therefore closely linked to a balanced intestinal microbiota (Tootiaie, Moharrami and Mojgani, 2021). In addition, the variation of lactobacilli and bifidobacteria reflects seasonal changes and the flowers visited by bees, serving as an indicator of the bee colony's health status (Gaggìa, Baffoni and Alberoni, 2018).

The adult bee's alimentary canal consists of four distinct sections: the rectum, ileum, midgut, and honey crop. Each of these compartments hosts unique microbial populations adapted to their respective niches. The midgut and hindgut (comprising the ileum and rectum) of adult worker bees serve as the main sites for colonization by a highly specialized collection of bacteria, including a cluster composed of *Bacillota*.

The persistent presence of these unique phylotypes in individual bees suggests that bacteria and bees have evolved in a symbiotic relationship and that these phylotypes play an essential role in host health (Ye *et al.*, 2023).

Apis mellifera are host to a distinct microbiota which is similar across honey bee species and continents, and where *Actinomycetota*, *Bacillota*, *Bacteroidia* and *Pseudomonadota* represent the four main dominant phyla (Hroncova *et al.*, 2015, 2019; Niode *et al.*, 2020; Ye *et al.*, 2023). Several species among them belong to the lactic acid bacteria (LAB).

However, the microbiome of honey bee larvae shows considerable variability and presents a different profile compared to adult bees. For instance, in some larvae of the 5th instar, *Bacillota* were seen to be predominant, while, following larval defecation and during pupation, their amount reduced, favoring *Gammaproteobacteria*. A deeper comprehension of innate bee immunity and the mechanisms that enable them to adjust to environmental stress is crucial, as evidenced by the recent losses of *Apis mellifera* and the possible correlation between these decreases and a variety of infectious pathogens (Hroncova *et al.*, 2015).

2.1.1 Lactic acid bacteria and their utilization as probiotics in bees

Like other animals, LAB are an integral part of the microbiota in bees. Microaerophilic conditions that dominate the digestive system of bees, the temperature of 35°C and the presence of sugars from the nectar are ideal conditions for LAB (Nowak *et al.*, 2021).

Of the several symbiotic LAB strains that have been isolated from the bees' digestive tracts, a few have demonstrated promising outcomes for possible probiotic development (Niode *et al.*, 2020; Tootiaie, Moharrami and Mojgani, 2021).

Some bacteria, such those belonging to *Lactobacillus* genus, the most significant representative of the LAB, have been connected to defense and immunity against pathogens also in honey bees. Especially in the larval stages, when several diseases harm the brood, pathogen defense is essential (Hroncova *et al.*, 2015).

LAB can produce various bioactive compounds such as organic acids (lactic acid, acetic acid and formic acid), hydrogen peroxide (H_2O_2), ethanol, enzymes, antimicrobial peptides (AMPs), free fatty acid, and volatile compounds that act synergistically as antimicrobials against various pathogens. Formic and lactic acid could decrease the environmental pH of wounds, thus preventing the growth of pathogenic microbes. Some of the bioactive components of LAB isolated from the bee gut are antimicrobial peptides (AMPs), a very important part of the bee immune system that also represents a promising alternative to current antibiotic treatment in humans or prevention of microbial infections (Niode *et al.*, 2020).

The use of LAB could represent a way to mitigate the harmful effects of pesticides and pathogens. The integration of these, in fact, can reduce the absorption of pesticides following their degradation or sequestration. Regarding the inhibition of pathogens, it has been seen that LAB can stimulate the production of AMPs, which increase the survival of bees during infection by *Paenibacillus larvae* for example. Isolates of *Apilactobacillus kunkeei,* on the other hand, have been seen to inhibit *Vairimorpha ceranae, Paenibacillus larvae*, and *Serratia marcescens*, as well as producing biofilms, thus favoring its vertical transmission from one generation to the next.

In addition to these two benefits, LAB supplementation leads to the production of more honey, pollen reserves, and broods.

These positive effects are attributed both to the production of organic acids and to the restoration of the microbiota; in fact, it has been seen that thriving colonies have higher levels of *Lactobacillus* and *Bifidobacterium* than non-thriving ones.

As regards application techniques, dried LAB supplements can be used by sprinkling them on the hive, or via sucrose-based syrups with the bacteria added. However, these two methods have drawbacks; powders are subject to humidity, while syrups do not always favor the survival of bacteria and would also attract other insects. The use of

pollen balls supplemented with beneficial bacteria represents a valid solution (Chmiel *et al.*, 2020).

2.1.2 Beneficial or probiotic bacteria

Lactobacilli and bifidobacteria, extensively studied for their probiotic properties in animals and humans, have found commercial applications in the food, feed, and pharmaceutical markets. The identification of these genera in the intestinal microbiota of bees has heightened scientific interest in drawing parallels with well-researched probiotic bacteria in humans and other animals. Known for producing AMPs, lactobacilli and bifidobacteria show potential as natural protective symbionts for bees.

Referring to these microorganisms as "probiotics," the transfer of the probiotic concept from vertebrates to invertebrates necessitates further exploration and debate.

Experiments involving the feeding of beneficial bacteria to bees aim to combat widespread pathogens which affect both larvae and adults. In larvae, the integration of various beneficial bacteria has shown a significant reduction in mortality levels, particularly in those infected with *Paenibacillus larvae*. A mixture of beneficial bacteria, including *Lactobacillus kimbladii, Lb. helsingborgensis, Lb. melliventris, Lb. apis*, and *B. asteroides*, demonstrated efficacy in reducing mortality from 70% to 22% in infected larvae (Gaggìa, Baffoni and Alberoni, 2018).

For adult bees, the emerging pathogen *Vairimorpha ceranae*, impacting bee health, has been targeted with beneficial bacteria. While reductions in *V. ceranae* were observed, the effectiveness was limited as the number of spores remained high, leading to bee mortality. However, a protective effect was noted in bees with low natural infection levels, suggesting a potential role in containing the spread of infection.

Controversially, the administration of non-native probiotic strains, including those for animals and humans, has shown mixed results, with some causing an increase in *Vairimorpha* spp. infection and mortality. The preference is for using microorganisms from the bee intestine due to their extensive gene pool for host interaction.

Research in this area, although promising, is still inconclusive regarding the effectiveness of beneficial microorganisms in limiting pathogen spread and supporting bee health and hive productivity. The key question revolves around understanding how the modulation of the intestinal microbiota influences both its composition and the host's immunity and physiology (Gaggìa, Baffoni and Alberoni, 2018).

2.2 Probiotic bacteria

The term “probiotic”, which means “for life”, is currently used to name microorganisms, primarily bacteria and yeasts, associated with beneficial effects for humans and animals. Probiotics are defined by the Food and Agriculture Organization and the World Health Organization as “live microorganisms which, when administered in adequate amounts, confer health benefits on the host” (Joint FAO WHO Working Group on Drafting Guidelines for the Evaluation of Probiotics in Food, 2006).

The first observation of the beneficial role of some bacteria is attributed to Eli Metchnikoff, a Russian Nobel Prize winner who worked at the Pasteur Institute in the early twentieth century. He affirmed that “the dependence of intestinal microbes on the food makes it possible to adopt measures to modify the flora of our body and to replace the harmful microbes by useful microbes”. During the same period, French pediatrician Henry Tissier noticed that children with diarrhea had fewer bacteria with an odd Y-shaped morphology in their stools, conversely, healthy children had high concentrations of these “bifid” bacteria (Pokusaeva, Fitzgerald and Van Sinderen, 2011; fao.org accessed on 28 December 2023).

Over the years, the term “probiotic” has taken on different meanings, arriving at the term still in use today.

2.2.1 Beneficial effects on the host’s immune system by probiotics

All mammals, including humans, come into contact with a wide variety of microbes which reside in their gut, and which represent those bacteria that have been selected by evolution based on their ability to endure and multiply in the intestinal environment (Mazziotta *et al.*, 2023). All these bacteria constitute the intestinal microbiota that plays a very important role in the gut homeostasis (Min and Rhee, 2015).

It is known that commensal bacteria and immune cells in the human gut constantly interact and respond to each other in a stable environment to maintain appropriate immunological activities. Specific immunological functions and immune homeostasis can be modulated by probiotic bacteria through interactions and stimulation of commensal microflora and intestinal immune cells. An increasing body of research demonstrates the significant immunomodulatory and health-promoting effects of probiotic bacteria, which could therefore represent a valid strategy to improve the functions of the immune system (Mazziotta *et al.*, 2023).

Probiotics taken orally have been shown to have a number of positive effects on intestinal homeostasis, including improving innate and adaptive immune responses, improving the bioavailability of specific natural or metabolic components and essential nutrients, and reducing food intolerance in predisposed individuals (Mazziotta *et al.*, 2023).

The activities of probiotics have been explained by different methods and, probably, in most cases, various mechanisms operate simultaneously (Kligler and Cohrssen, 2008).

Most of the identified beneficial effects of probiotics relate to gastrointestinal (GI) conditions, including antibiotic-associated diarrhea, acute infectious diarrhea, and irritable bowel syndrome (Kligler and Cohrssen, 2008).

A beneficial effect has also been seen in children suffering from atopic dermatitis where the mechanism is probably linked to the effect of probiotics on the early development of immune tolerance during the first year of life. Probiotics may help reduce the excessive immune response against foreign antigens that leads to atopy and may also contribute to the systemic downregulation of inflammatory processes. Probiotics have been shown to reverse the increased intestinal permeability characteristic of children with food allergies, as well as enhance specific serum immunoglobulin A (IgA) responses that are often defective in these children (Kligler and Cohrssen, 2008).

2.2.2 Criteria that a probiotic microorganism must satisfy

Before a probiotic can benefit host health, several criteria must be satisfied.

Specifically, it must have good technological properties so that it can be manufactured and incorporated into food products without losing viability and functionality or producing off-flavors or textures, and it must survive the passage through the upper GI-tract and arrive alive at its site of action. To study the probiotic strain in the GI-tract, molecular techniques must be established to distinguish the selected and ingested probiotic strain from the other bacterial strains that make up the gastrointestinal ecosystem. In addition, techniques are required to establish the effect of the probiotic strain on other members of the intestinal microbiota and on the host. Finally, not only do beneficial aspects have to be considered, but it also has to be demonstrated that the bacteria don't cause any deleterious effects (Saarela *et al.*, 2000).

2.2.3 Bacteria species belonging to the probiotics category

In the category of probiotics, various species were discovered - however, not all strains within these species necessarily serve as probiotics. The health advantages of probiotics vary depending on the strain, thus it's critical to consider strain-specific effects when choosing or researching probiotic supplements, but they also vary by host (Musa *et al.*, 2009).

Among these species there are *Lactobacillus acidophilus*, *Lb. reuteri*, *Lacticaseibacillus casei, Lacticaseibacillus rhamnosus, Lactiplantibacillus plantarum, Limosilactobacillus fermentum*, *Bifidobacterium bifidum*, *B. longum*, *B. breve*, *B. animalis*, *Streptococcus thermophilus*, *Enterococcus faecium*, *Bacillus licheniformis, Bacillus subtilis*, *Clostridium butyricum* (Musa *et al.*, 2009).

2.3 Use of probiotics in animals and insects

The use of probiotics to protect health is not limited to humans; favorable benefits have also been shown in farmed animals and insects. For both, well-being is a prerequisite that should not be overlooked in order to obtain high quality products and avoid the onset of diseases that would lead to the subsequent use of antibiotics (Musa *et al.*, 2009; Arsène *et al.*, 2021; Savio, Mugo-Kamiri and Upfold, 2022).

2.3.1 Probiotics in animal feeding

Probiotic research has also been applied to pets, horses, and farm animals, with most studies focusing on chickens and pigs. The claims made for microbial products include enhanced performance and feed conversion for target species, reduced morbidity or mortality, and advantages for customers due to improved product quality. Less recent research has found that probiotics help minimize leg weakness in broiler chickens, prevent starving infertility in young sows, while, in ruminants, they have been demonstrated to regulate ruminal pH and lactate, enhance food absorption, and have a growth-promoting impact comparable to antibiotic treatment (Musa *et al.*, 2009; Arsène *et al.*, 2021). Furthermore, probiotics improve animal development rates and increase milk, meat, and egg production (Musa *et al.*, 2009).

The favorable effects of probiotics in animal production have been linked to various mechanisms of action. The improvement in production performance of all poultry species fed probiotics is mostly due to probiotics' ability to boost metabolic processes of digestion

and nutrient use. The improvement in metabolic processes observed after probiotic supplementation is thought to be due to improved gut development and an increase in the height of the microvilli, which leads to the expansion of the microvilli's absorption surface and allows for optimal nutrient utilization (Ezema, 2013).

The most used probiotics bacteria in animal breeding belong to strains of *Lacticaseibacillus rhamnosus, Lacticaseibacillus casei*, *Bifidobacterium pseudolongum, B. animalis, B. longum*, *Bacillus licheniformis*, *Enterococcus faecium*, and *Lactococcus lactis* (Arsène *et al.*, 2021).

2.3.2 Probiotics' maintenance of insect health

Insect farming is becoming increasingly important in the livestock market as it is evolving into a kind of intensive production with a high density of individuals kept in controlled habitats. Among the negative aspects, there is an increased risk of transmission of infections and nutritional deficits with consequent lowering of reproductive performance and growth. The use of antimicrobials to mitigate these problems could lead to the development of resistant bacteria; for this reason, the path has moved towards the use of probiotics. These probiotics could improve growth and reproductive performance, and minimize the occurrence of diseases due to bacteria, viruses, fungi, protists, and nematodes in insects. However, their use depends on the interaction between the host and its intestinal bacteria, so the acquisition of the microbiota becomes important. This idea is particularly important for social insects, which maintain a constant core microbiota throughout the year and across geographic regions. It has been shown that insects can acquire their microbiota horizontally from the environment, mainly through diet, capable of influencing both the composition and resilience of intestinal ecosystems, but can also be acquired through social interaction, as well as vertical transfer from part of the parents. These microbes can adhere to the intestinal lumen and, after successful colonization, can take part in various activities such as the breakdown of indigestible polysaccharides of plant origin, which lead to the formation of short-chain fatty acids that affect microbe and host nutrition, helping to maintain the integrity of the intestinal barrier, and detoxify plant defense compounds and pesticides (Savio, Mugo-Kamiri and Upfold, 2022).

Some of the probiotics that could be used include strains belong to *Lactobacillus acidophilus*, *Lacticaseibacillus casei, Lacticaseibacillus rhamnosus, Lactiplantibacillus plantarum, Bifidobacterium bifidum*, *Bacillus subtilis*, *Enterococcus faecalis* (Savio, Mugo-Kamiri and Upfold, 2022).

2.4 Fermentation profile of probiotic bacteria

The most commonly employed probiotic strains belong to *Lactobacillus acidophilus, Lb. delbrueckii subsp. Bulgaricus*, *Lacticaseibacillus casei*, *Limosilactobacillus reuteri*, *Limosilactobacillus fermentum, Levilactobacillus brevis, Lactiplantibacillus plantarum, Latilactobacillus curvatus*, *Bifidobacterium bifidum, B. adolescentis, B. animalis, B. longum, B. thermophilum*, and Gram-positive cocci (*Streptococcus thermophilus, S. intermedius, Lactococcus lactis subsp. Cremoris, Lactococcus lactis subsp. Lactis, Enterococcus faecium*). Furthermore, several strains belonging to the *Lactobacillus* genus and Gram-positive cocci belong to LAB, with the first being the main representatives (Killer *et al.*, 2014).

2.4.1 LAB metabolism

The LAB group is currently classified as part of the Bacillota phylum, Bacilli class, and Lactobacillales order. They are Gram positive, non-sporulating, anaerobic or facultative aerobic *cocci* or rods, with low guanine and cytosine (GC) content. Their name lactic acid bacteria is derived from the fact that 50% of them can produce lactic acid. They are present in vegetables, in the digestive tract of some mammals, in milk and its derivatives (Quinto *et al.*, 2014). They are extensively utilized in the food and feed industries, are generally recognized as safe (GRAS) by the Food and Drug Administration (Ye *et al.*, 2023), and certain species having the potential to be developed into probiotics (Niode *et al.*, 2020). Several genera belong to the LAB, the biggest of which being *Lactobacillus*.
LAB can be divided into homofermentative and heterofermentative, based on their ability to ferment carbohydrate (Widyastuti, Febrisiantosa and Tidona, 2021).
The metabolic product of homofermentative is only lactic acid – specifically, two molecules of lactic acid and two molecules of ATP are produced for each molecule of glucose fermented (Pokusaeva, Fitzgerald and Van Sinderen, 2011). Homofermentative use glycolysis and once pyruvate is reached, lactate dehydrogenase intervenes which produces lactate, so fructose-1,6-diphosphate aldolase is the key enzyme (Laëtitia, Pascal and Yann, 2014).
Instead, heterofermentative produce CO_2, one molecule of lactic acid, one molecule of acetic acid or ethanol, according to the NADH:NAD ratio, and one molecule of ATP. In this case, the pentose phosphate path is taken, with phosphoketolase as the main enzyme (Pokusaeva, Fitzgerald and Van Sinderen, 2011; Laëtitia, Pascal and Yann, 2014).

Strains from the same genus can have various metabolisms, for example, in the *Lactobacillus* genus, there are obligate homofermentative, obligate heterofermentative, and facultative heterofermentative which use hexoses like obligate homofermentative and pentoses like obligate heterofermentative.

2.4.2 Bifidobacteria metabolism

The genus *Bifidobacterium* belongs to the Actinomycetota philum and class of *Actinomycetes*, order *Bifidobacteriales*. They are characterized as Gram-positive, heterofermentative, non-motile, anaerobes, non-spore-forming, with high GC content. They can be found as components of the gastrointestinal microbiota where they are believed to exert health-promoting actions, such as protective activities against pathogens through the production of antimicrobial agents (e.g. bacteriocins) and/or blocking pathogen adhesion, and modulation of the immune response (Pokusaeva, Fitzgerald and Van Sinderen, 2011; Quinto *et al.*, 2014).

Because they are saccharolytic organisms, *bifidobacteria* mainly depend on their capacity to utilize the complex carbohydrates that are present in this environment in order to colonize the gastrointestinal tract (Egan and Van Sinderen, 2018).

Indigestible complex sugars, such as human milk oligosaccharides (HMOs) for neonatal development, fructo-oligosaccharides, and galacto-oligosaccharides, act as prebiotics, in particular *Bifidobacterium*. The fermentation of these substances produces short chain fatty acids (SCFAs) such as acetic acid, and lactic acid, which promote the development and protection of the immune system (Hundshammer and Minge, 2020).

These carbohydrates are able to pass practically intact into the colon, resisting the acidic environment and the action of salivary and digestive enzymes. Once in the colon, they are able to promote intestinal health by stimulating the growth of microbes, in particular to *bifidobacteria* (Hundshammer and Minge, 2020).

The genus *Bifidobacterium* possess the fructose-6-phosphate-phosphoketolase (F6PPK) pathway to ferment carbohydrates. In the end, for each molecule of glucose degraded, we will have the theoretical ratio of lactic acid: acetic acid is 1.0:1.5 and the energy yield is 2.5 molecules of ATP. In other words, starting from two molecules of glucose, three molecules of acetic acid, two of lactic acid and five of ATP are obtained (Fushinobu, 2010). Unlike heterofermentative LAB, *bifidobacteria* do not produce CO_2 during fermentation (Sela, Price and Mills, 2010).

2.5 Media and conditions used for lactobacilli and bifidobacteria growth

Lactobacilli and bifidobacterial strains are commonly used to make probiotics.
In response to the growing demand for probiotics and their entry into new food markets, the industries produce significant quantities of stable probiotic cultures.

2.5.1 Lactobacilli medium

De Man, Rogosa and Sharpe (M.R.S.) agar is a selective medium used for *Lactobacillus* sp. Isolation and cultivation from clinical samples, foods, and dairy products.
Inside this medium, the enzymatic digestion of casein, glucose, beef extract, and yeast extract ensure the nutritional component; sorbitan monooleate, magnesium, and manganese sulphate provide the growth stimulation; ammonium citrate and sodium acetate provide the selectivity against streptococci and molds ('de man, rogosa and sharpe (MRS) agar', 2003).
MRS agar has limits because it is characterized as a growth medium suitable for laboratories, which would be prohibitively expensive (Miloud, Halima and Nour-Eddine, 2017) for commercial production of probiotics and might involve unapproved components like food additives (Bolivar-Jacobo *et al.*, 2023).
Several studies have attempted to develop new medium that would boost the growth of lactobacilli by combining technology and by-products. For example, the use of milk as a culture medium would be less expensive, safer to eat, and appropriate for the development of probiotics that may be used in the creation of functional foods. In this instance, it is vital to employ high-intensity ultrasound to hydrolyze milk proteins that would otherwise be unavailable for the growth of probiotics (Bolivar-Jacobo *et al.*, 2023).
Yeast extract is the primary source of nitrogen, however it might be replaced with whey proteins, which are also sources of this component (Miloud, Halima and Nour-Eddine, 2017).

2.5.2 Bifidobacteria medium

Initially, in the search for a selective culture medium for these bacteria, a medium containing many factors that stimulate the growth of lactobacilli was used. It was found that in a relatively simple, cysteine-containing medium, these strains used ammonium salts as a nitrogen source. The presence of cysteine has been shown not to be able to be

replaced by methionine, homocysteine or other compounds. As for B vitamins, only biotin and calcium pantothenate were required (Poupard, Husain and Norris, 1973).

Trypticase Phytone Yeast (TPY) is a recommended medium for the growth of bifidobacteria. It contains trypticase, phyton, glucose, yeast extract, L-cysteine, magnesium chloride, zinc sulfate, calcium chloride and ferric chloride. Trypticase is made up of soy peptone and meat extract which provide nitrogen, vitamins, minerals and amino acids essential for growth, while yeast extract is a source of vitamins, especially of group B (Nguyen *et al.*, 2019).

To increase bifidobacterial growth, oligosaccharides can be used. N-acetyl-D-glucosamine-containing saccharides were identified as growth factors of this microorganism because they are a substrate for cell wall synthesis (Poupard, Husain and Norris, 1973).

2.6 Novel generation of fermented drinks and their potential effect

Fermentation has been a simple and natural process used to generate a variety of meals and drinks since the Neolithic era and has been passed down from generation to generation (Boukid *et al.*, 2023; Tomar *et al.*, 2023).

All over the world it is known that people with different ethnic groups produce fermented beverages using a variety of substrates depending on the abundance and availability of substrates in the region. These traditional fermented drinks, not only have a profound bond with rituals but they are also consumed for nutrients and therapeutic purposes (Tomar *et al.*, 2023).

2.6.1 Fermented products

Nowadays, a vast array of fermented plant and animal products are produced utilizing a variety of raw ingredients, microbes, and production methods.

Traditionally produced all over the world, fermented meat products like salami, ham, and sausages now hold a unique place in the gastronomic trade of meat products. Common foods like cheese and yogurt that have undergone fermentation are known to include LAB, which may have probiotic properties. Fermentation, however, has not been limited to foods of animal origin but has also been applied to plant sources such as coffee, bread, chocolate and wine, in order to improve their nutritional value, aroma and taste, texture and stability (Boukid *et al.*, 2023).

Among the oldest fermented beverages are wines, which are primarily enjoyed in Europe and the Mediterranean region. However, the earliest records of wine consumption date back to China, where rice was utilized as a substrate along with honey and various fruits (Tomar *et al.*, 2023).

Overall, the flavor, aroma, and texture of fermented foods make them an essential component of the human diet (Boukid *et al.*, 2023). In addition, there are the benefits that these types of products give to health and which can be expressed directly through the interactions of live microorganisms ingested with the host, as a probiotic effect, or indirectly through the ingestion of microbial metabolites synthesized during fermentation (Widyastuti, Febrisiantosa and Tidona, 2021).

To produce fermented food, LAB species are most frequently utilized as starter cultures (Alexandre *et al.*, 2023).

2.6.2 LAB activity in fermented products

The bioconversion of carbohydrates, lipids, proteins, phenolics, and certain vitamins and minerals into organic acids, such as lactic, acetic, propionic, and butyric acids, is LAB's area of expertise for LAB. This process lowers the pH of fermented food, extending its shelf life by preventing the growth and survival of numerous pathogenic bacteria. Furthermore, the production of volatiles, exopolysaccharides, and bacteriocins (peptides), in addition to the direct competition of microorganisms, all contribute significantly to the antimicrobial action of various food products (Alexandre *et al.*, 2023). These extend shelf life and improve the techno-functional characteristics, nutritional quality, and bioactive properties of fermented dairy products thanks to the generation of numerous low molecular weight substances (also called metabolites) during fermentation.

The most often utilized LAB cultures for the regulated fermentation of dairy products are *Streptococcus thermophilus*, *Lactococcus lactis*, *Leuconostoc* spp., and *Lactobacillus* spp. Nonetheless, the genera *Weissella*, *Oenococcus*, and *Pediococcus* are equally crucial to the advancement of fermented beverages made from plants (Alexandre *et al.*, 2023; Sharma *et al.*, 2023).

2.6.3 Whey as a by-product used in the production of innovative fermented drinks

To be accepted by modern customers, a beverage must meet at least some of the primary success factors: appealing sensory quality, thirst-quenching effectiveness, a reasonable price, and a positive 'health image' (Chavan *et al.*, 2015).

Innovation in fermented beverages is shifting toward the utilization of by-products that are generated from fruit, vegetable, and cereal manufacturing industries. This can be a great substitute for customers who follow vegan or vegetarian diets or who have a lactose intolerance; in addition to helping to reduce food waste. Furthermore, because these by-products' phytochemical profiles differ from those of other fruit components, they might include bioactive chemicals with higher antioxidant activity (Alexandre *et al.*, 2023).

Whey is a by-product that has considerable nutritional content and has been shown to be an ideal medium for the growth of some probiotics. Thanks to these proprieties and its liquid nature, whey has become an ideal alternative for the creation of a variety of innovative food products which include unfermented and fermented beverages (Elkot *et al.*, 2024).

Whey represents approximately 85–95% of the milk volume and retains 55% of milk nutrients; it is nothing other than the liquid remaining after the removal of fat and caseins. Among the nutrients within whey are lactose, soluble proteins, lipids and mineral salts. Whey also contains appreciable quantities of other components, such as lactic and citric acids, non-protein nitrogen compounds (urea and uric acid) and B group vitamins (Guimarães, Teixeira and Domingues, 2010).

Whey proteins have a variety of therapeutic features, including antioxidant activity, antibacterial actions, immunological stimulation, potential anti-cancer capabilities, the ability to control blood pressure, lower the risk of cardiovascular disease and osteoporosis, and regulate satiety (Elkot *et al.*, 2024).

For this reason, these are often the favored source for ready-to-drink protein beverages due to their great nutritional characteristics, bland flavor, ease of digestion, and unique functioning in beverage systems. In terms of nutritional value, whey proteins rank second only to egg protein among all accessible protein sources (Chavan *et al.*, 2015).

Whey has been shown to be an effective medium for the growth of yogurt bacteria and probiotic bacteria, such as *Lactobacillus* spp. And *Bifidobacterium* spp., because its components not only promote the growth and maintenance of beneficial bacteria, but also increase the viability of LAB in the digestive tract (Elkot *et al.*, 2024)

2.7 ^{1}H NMR as analytical tool for assessment of bacterial metabolites

One of the most effective methods for analyzing sample components, figuring out the structures of substances and researching chemical reaction processes is nuclear magnetic resonance (NMR) spectroscopy, reported for the first time in 1963 by Jungnickel and Forbes. NMR is a quick and non-invasive analytical technique that was initially employed to examine food moisture and the chemical composition of a product (Qu and Jin, 2022).

2.7.1 NMR spectroscopy

NMR spectroscopy is an analytical technique that provides precise information on the structure of molecules by examining the behavior of atomic nuclei in a magnetic field.
After immersing the understudied molecule in a strong magnetic field, the absorption of radio frequency radiation (from 100 to 1000 MHz) is measured which causes nuclear spin transitions in particular atoms such as ^{1}H or ^{13}C.
Only nuclei with a spin nuclear magnetic moment can be seen with NMR because they orient themselves in an applied magnetic field like a compass needle.
Knowing that the NMR machine generates a uniform magnetic field, the proton immersed in this field will tend to align the projection of its magnetic moment to the external magnetic field. There will be an imbalance between the protons that orient themselves parallel to the field (slightly lower energy), and those that orient themselves antiparallel to the field (energy will be higher), since the former will be in greater numbers.
Another quantum phenomenon also intervenes, known as Larmor frequency, which describes the movement of the magnets, called precession motion (it can be imagined as a rotation), which occurs with a frequency proportional to the energy difference between protons oriented parallel to the field and those oriented in the opposite direction. As the applied magnetic field increases, the Larmor frequency increases and therefore the energy difference increases.
This rotation can be amplified by providing energy to the system through an electromagnetic wave.
If the sample is irradiated with electromagnetic radiation of a frequency equal to the Larmor frequency, there is an interaction of the magnetic component of the radiation with the nuclear magnetic moments (also oscillating at the Larmor frequency). The energy of the radiation can thus be transferred to the nuclei. Each absorption of radiation causes a change in the orientation of the nuclear spin.

The magnetic field generated by all the spins that add up can be calculated by exploiting the phenomena of electromagnetic induction, passing the field through circuits and then measuring the current induced by the magnetic fields.
As radiation is introduced from the outside, the spins will come to have a projection towards one direction, and this will give a peak.
Since all hydrogen atoms have the same magnetic dipole, they should all resonate at the same frequency in a given magnetic field. Instead, various hydrogen atoms absorb at slightly different frequencies from each other depending on their chemical environment. Depending on the electronegativity of the atom to which it is bonded, the hydrogen atom can be more or less immersed in the bonding electron cloud.
Hydrogens bonded to more electronegative atoms are de-shielded, so they experience a stronger effective magnetic field and undergo the transition to higher frequencies and, in the spectrum, are located further to the left, with a higher chemical shift (Macomber, 1998; Keeler, 2010).

2.7.2 NMR use in food field

Dairy research has long employed NMR to investigate the physical characteristics of liquid milk. The primary benefit of using NMR to estimate the characteristics of liquid milk is that no sample preprocessing is necessary. It's easy to show the distinctions between various kinds of cheese by demonstrating how water protons interact with milk proteins (Sharma *et al.*, 2023).
Reduced levels of arginine and citrulline during ripening were seen in aqueous extracts analyzed in another study investigating possible metabolites as molecular markers for cheese ripening. It was discovered that mature cheese was linked to tyrosine and lysine, but young cheese's sensory qualities were linked to β-galactose and glycerol.
In addition to characterization, NMR analysis has also been used to explain degradation changes or variations in bioactive components during fermented dairy product preservation. Italian soft cheese storage was associated with an increase in signals related to glucose, phosphocholine, creatine, glycerophosphocholine, and lactate. Using an NMR metabolite profile, the authenticity of mozzarella cheese could also be verified based on the place of origin. In comparison to new samples, samples that were two days old contained higher quantities of lactic acid, acetic acid, and isobutyric acid, which could protect consumers from fraud (Sharma *et al.*, 2023).

Key metabolites such lactate, citrate, phospholipids, and amino acids were shown to be related to the sensory quality after 52 cheese samples were separated, based on NMR metabolomics and intensities associated with sensory qualities. As a result, research suggests that NMR may be used to track modifications during the ripening, renneting, and storage processes, as well as, more generally, to understand the intricate processes involved in the production of fermented dairy products (Sharma *et al.*, 2023).

3 MATERIALS AND METHODS

Bacterial strains were isolated from the intestines of some worker bees and cultivated on specific media, with the aim of isolating the DNA and analyzing the 16s rRNA gene to allow their identification.

In this study, two whey samples preserved in various ways, whey sample and whey sample frozen for 24 hours were used. For each of them eighteen NMR tubes were prepared for analysis, for a total of thirty-six samples.

During sample preparation, the addition of the enzyme β-galactosidase and its subsequent deactivation by heat, after a certain period of time, represents an important step in obtaining a lactose-free medium. NMR has been the method of choice for quantifying metabolism in media.

3.1 Molecular identification of bacteria isolated from honey bees' GI-tract

The bacterial strains used in the experiments were obtained from the Czech Academy of Life Sciences, Department of Physiology and Genetics.

3.1.1 Isolation of bacteria from honey bees' GI-tract

The digestive tracts of 3–6 bee workers were meticulously excised using sterile tweezers and promptly transferred into Hungate tubes pre-filled with 1.8 mL of sterile anaerobic TPY broth supplemented with glass particles. Following this, 0.1 mL of the suspension was carefully applied to the base of 90 mm Petri dishes and overlaid with specialized media designed for the isolation of distinct bacterial groups (refer to Table 1). To maintain an anaerobic environment, the media was thoroughly saturated with CO_2.

Subsequently, the Petri dishes were incubated in anaerobic conditions, utilizing an Anaerobic chamber (Oxoid, 3.5 L) equipped with an Anaerogen 3.5 L production system, at 37 °C for a duration of 72-96 hours. Individual bacterial colonies were meticulously selected using a sterile bacteriological loop and transferred to Hungate tubes containing 6-9 mL of anaerobic TPY/Rogosa/MRS. Cultivation was continued at 37 °C for 24-48 hours to foster optimal growth and development.

	TPY	M.R.S.	Rogosa
Isolation of Composition (g/L):	Bifidobacteria	Lactic acid bacteria	Lactobacilli
Peptone	10	10	mixture in a container (82 g / L)
Glucose	10	20	
Fructose	-	-	
Yeast extract	5	5	
Soybean peptone	5	5	
Beef extract	2	-	
NaCl	-	6	
Natrium acetate	-	5	
KH_2PO_4	2	2	
K_2HPO_4	0.5	-	
$MgCl_2$ x $6H_2O$ Agar	1 (15)	1 (15)	

3.1.2 Genomic DNA extraction

DNA was extracted using "PrepMan Ultra" (Applied Biosystems) according to the manufacturer's protocols. In particular, fresh (24 h) cultures (isolates) in the quantity of 1.0 – 1.5 mL underwent centrifugation at 9000 rpm for 6 minutes. The resulting pellet was carefully resuspended in 80 µL of PrepMan ultra sample preparation reagent and subsequently subjected to boiling for a duration of 10 minutes. Following the cooling period of 2 minutes, the samples underwent a second centrifugation at 13200 rpm for 3 minutes.

In preparation for further processing, Rnase-free microtubes were utilized, into which 60 µL of the supernatant and 1.5 mL of Dnase were meticulously added.

3.1.3 Amplification of 16S rDNA of bacterial isolated

For the amplification of the 16S rRNA gene, three primer pairs were employed: fP1-rP2 (Weisburg et al., 1991); 616V-630R (Loy et al., 2002), and 27F-1541R (Galkiewicz JP, Kellogg, 2008). The PCR mix, totaling 30 µL, was prepared with 15 µL Ready Mix, 12 µL pcrH_2O, 1 µL of each primer P1 and P2, and 1–2 µL of DNA (concentration 10–1000 ng/ µL) for all primer pairs. The amplification protocol involved an initial denaturation step at 95 °C for 4 minutes, followed by 35 cycles of denaturation at 95 °C for 1 minute, annealing at 52 °C for 90 seconds, extension at 72 °C for 2 minutes, and a final extension step at 72 °C for 5 minutes.

To verify successful amplification, gel electrophoresis was conducted using a 1.5% agarose gel at 120 V for 35 minutes, alongside a DNA marker ranging from 200 to 1500

base pairs. The resulting amplicons were purified using the Monarch PCR & DNA Cleanup Kit (New England BioLabs, United Kingdom) following the manufacturer's instructions. Subsequently, the purified amplicons were sequenced bidirectionally (using both primers) at SeqMe company (Czechia).
For sequence analysis, ChromasLite 2.1 (Technelysium, USA) and BioEdit 7.1 were employed to check and assemble the sequences. The assembled sequences were then entered into the database to determine the percentage match to the most closely related taxon based on the 16S rRNA gene ID.

3.2 Whey-based medium preparation

Before usage, frozen whey sample was thawed by soaking in room-temperature water.
As initial sample preparation step, 50 mL tubes were filled with whey and centrifuged for 10 minutes at 11500 rpm. This step was carried out with the aim of separating the big solid particles from the liquid.
Exactly 40 mL of the supernatant were collected and transferred in a new 50 mL tube.

Because the whey is rich in lactose, a compound not fermented by *Lactobacillus* species to which belong the isolated strains and indigestible for bees, it has undergone a targeted treatment to promote its hydrolysis through the use of the enzyme β-galactosidase.
For this reason, samples were warmed in a warm bath at 37°C for 10 minutes to establish optimal working conditions for *β*-galactosidase.
For each sample, 40 µl of *β*-galactosidase were added in each 50 mL tube and mixed using vortex (3000 rpm, 5 sec).
After that, 1 mL of whey has been picked up from the tube and put in a microtube to measure the amount of lactose at zero time point.
Throughout the entire process, the two 50 mL tubes were left in the warm bath at 37°C, allowing *β*-galactosidase to work better, and shaked every 20 minutes to maintain homogeneity.
Each aliquot collected at time 0, 1 minute, 30 minutes, 1- , 2- and 4-hours (three replicates for each time) had to be warmed up in the dry bath at 100°C for 10 minutes to deactivate *β*-galactosidase.
Once the deactivation time was completed, the microtube was transferred to the centrifuge for 5 minutes at 13300 rpm.

3.3 ^{1}H NMR analysis of the whey-based medium to evaluate lactose content

All chemical reagents used for sample preparation for analysis were of analytical grade. NaN_3 (≥99.5%), 3-(trimethylsilyl)-2,2,3,3-tetradeuteropropionic acid (99.9% TSP), KH_2PO_4 (99%), NaH_2PO_4 (99%), D_2O (99.9% D_2O) and NaOH (36.5–38.0) were purchased from VWR (Radnor, PA, USA).

To each 720 µl of sample, 80 µl of phosphate buffer solution (1.5 M K_2HPO_4 / 1.5 M NaH_2PO_4, 5 mM TSP + D_2O, 0.2% NaN_3, pH 7.4) was added and mixed using vortex. The total 800 µl volume of prepared sample was centrifuged for 5 minutes at 13300 rpm, after that, 600 µl of supernatant was transferred to a 5-mm NMR tube and subsequently analyzed.

3.3.1 NMR data acquisition

All spectra were recorded on a Bruker Avance III spectrometer equipped with a broad band fluorine observation (BBFO) SmartProbeTM with z-axis gradients (Bruker BioSpin GmbH, Rheinstetten, Germany) operating at a proton NMR frequency of 500.18 MHz. The temperature was set to 298 K (25 °C). ^{1}H NMR spectra were acquired and processed under the same conditions. The Bruker pulse sequence (noesypr1d) was applied to suppress the residual water signal at 4.696 ppm. For each sample, 32 scans and 4 dummy scans were collected as 64 K data points using a spectral width of 8 K Hz, receiver gain of 8, relaxation delay of 1 s, acquisition time of 4.00 s, and mixing time of 0.1 s. The total acquisition time was 3 min. Tuning, lock, gain, 90° pulse calibration, and shimming were calibrated automatically for each sample by the standard module routine developed by Bruker (atma, lock, pulsecal and topshim). The free induction decay (FID) was multiplied by 0.3 Hz line broadening prior to Fourier transformation.

3.3.2 Metabolite profiling

The ^{1}H NMR spectra were phased, and baseline corrected in MestReNova ver. 14.1.0 (Mestrelab Research, Santiago de Compostela, Spain). Annotion and quantification of Lactose, Glucose, Galactose was carried out using Chenomx NMR suite 8.5 software, professional edition (Chenomx Inc., Edmonton, AB, Canada) comprising the Reference Library 10 and an in-house library.

3.4 ^{1}H NMR analysis of the whey-based media after growth of isolated strains

After enzymatic treatment, the whey solution was subjected to sterilization at a relatively low temperature of 105°C for a duration of 50 minutes. This careful sterilization process was implemented to ensure the preservation of the integrity of the bacterial strains and their potential antagonist activity while eliminating unwanted contaminants. The pH of the whey-based medium was measured before sterilization, after sterilization but before inoculation of the isolated strains and 48 hours after their inoculation.

The initially classified bacterial strains were grown in the medium for 24 and 48 hours, after which the NMR analyses were repeated following the same procedure previously described.

4 RESULTS

4.1 Identification of bacterial strains

The sequence homology results showed that the strains isolated from gastrointestinal tract of the honey bees belong to the following bacterial species:

Table 1 Identification bacterial strains isolated from the intestine of honey bees.

BACTERIAL STRAINS	IDENTIFICATION BY 16S RRNA
D1-RO2	*Lb. Melliventris*
D1-MR10	*B. asteroides*
P1-MR12	*Lb. Apis*
VT5	*Lb. Apis*
VSM-RO4	*Lb. Helsingborgensis*
VU-RO6	*Lb. Kimbladii*

These bacterial species, although belonging to the Lactobacillus genus, have the peculiarity of not producing products from lactose (Zheng *et al.*, 2020).

4.2 Effect of β -galactosidase and freezing on lactose concentration

The technique outlined above was carried out on both whey samples preserved in various ways. After adding β-galactosidase and analyzing with NMR, the final samples exhibit varying degrees of lactose content.

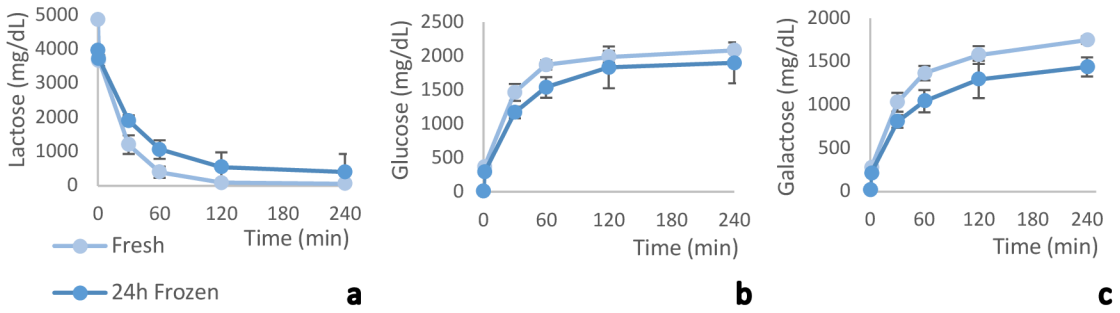


Figure 1 β-galactosidase activity was measured in two whey samples. Graphs a, b and c respectively show the time trend of the concentrations of lactose, glucose, and galactose in both whey samples.

Graph (a) depicts the variation in lactose concentration over time for the two samples. Notably, a significant reduction is observed, approaching zero, in the fresh whey sample, while the whey sample frozen for 24 hours maintains slightly higher concentrations.

Graph (b) shows the trend of glucose concentration for the two samples over time. The increase in glucose concentration corresponds to the hydrolysis of lactose by β-galactosidase.

Graph (c) illustrates the evolution of galactose concentration for the two samples over time. Similar to glucose, galactose concentration increases with lactose hydrolysis. Both samples exhibit an upward trend, signifying lactose hydrolysis.

The percentage of converted lactose, i.e. the percentage of converted lactose, was calculated and, based on the results obtained, the fresh whey sample was selected. In fact, the percentage of converted lactose in fresh whey sample turned out 98,72%, while the one in whey sample frozen for 24 hours turned out 74,48%.

4.3 Metabolite profile of the whey-based medium pre and post strains growth

According to the percentage of converted lactose, the fresh whey sample was found more suitable than the other sample. Therefore, following a mild sterilization, the isolated bacterial strains were inoculated in the selected medium.
The pH value before sterilization at 105°C for 50 minutes corresponded to 5.75, while once the treatment took place the pH reached the value of 5.5. By measuring the pH of the different whey-based media 48 hours after the inoculation of the bacterial strains, the following values were obtained:

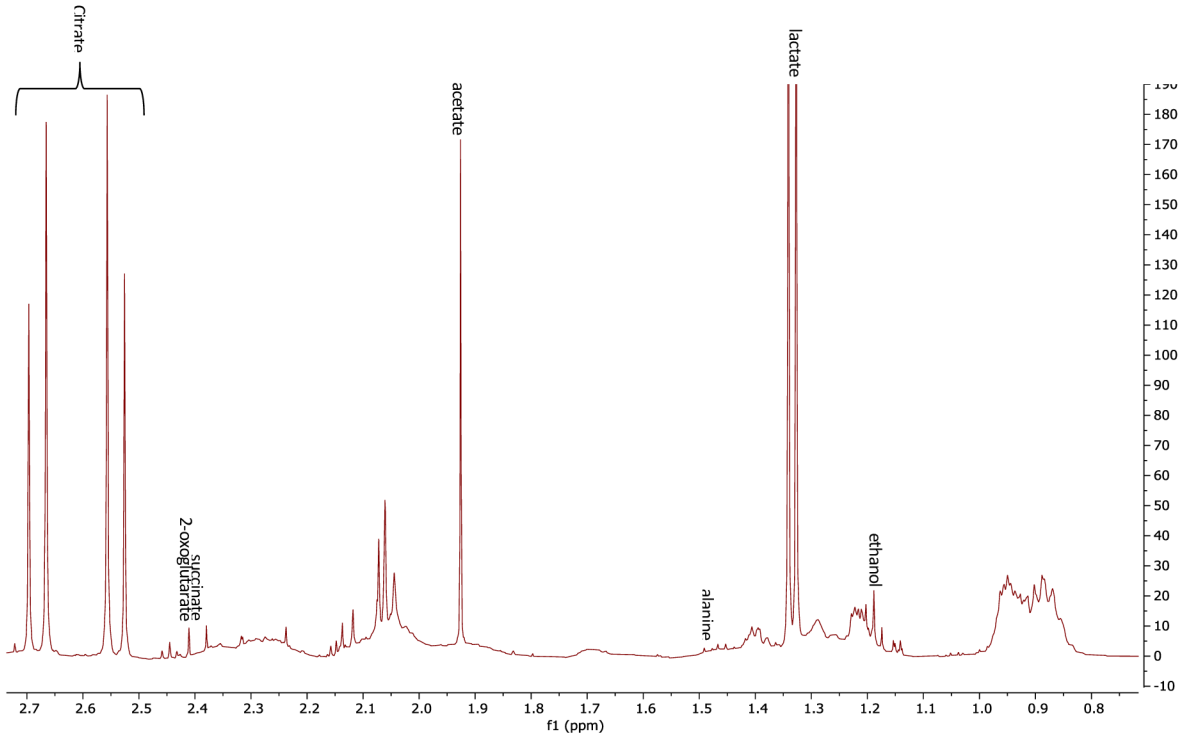
BACTERIAL STRAINS	PH
D1-RO2	4,3
D1-MR10	3,53
P1-MR12	4,2
VT5	4,33
VSM-RO4	4,2
VU-RO6	3,63

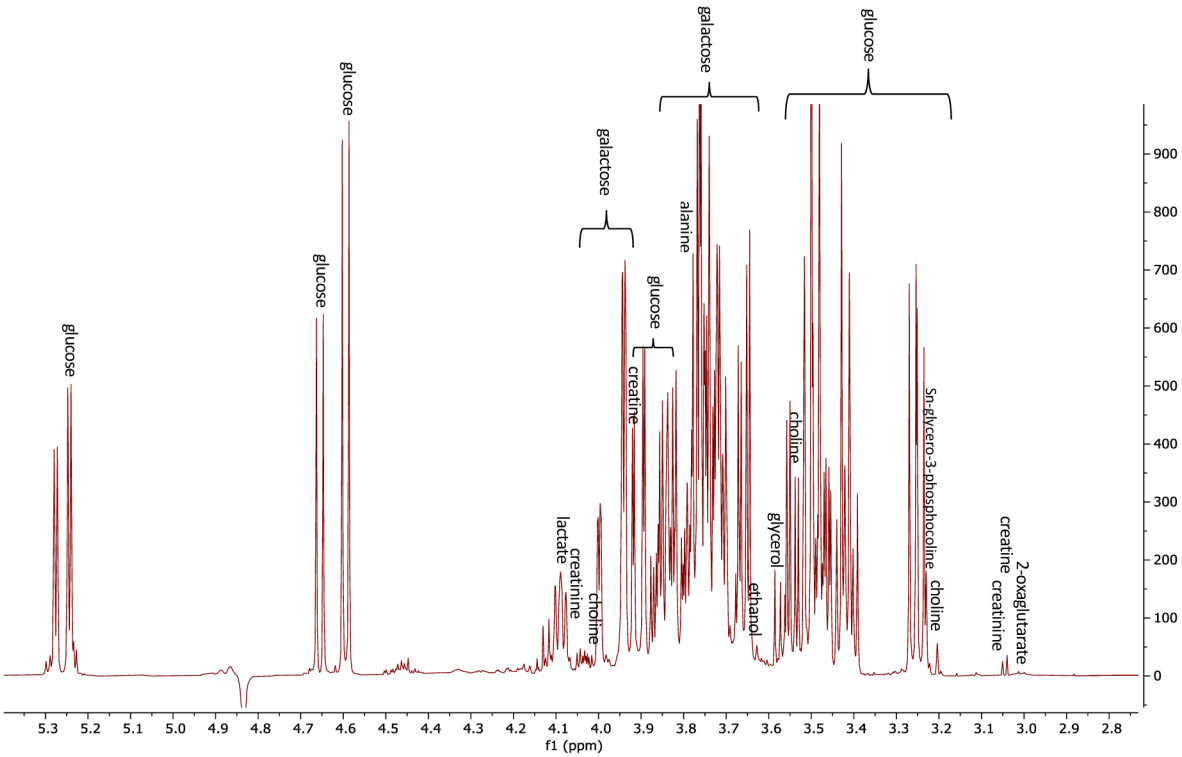
The more acidic pH of whey-based media after 48 hours from the inoculation of the isolated bacterial strains indicates the production of organic acids.

A total of fifteen compounds were searched for by NMR analysis. Using NMR analysis, lactose, glucose and galactose molecules were quantified. Furthermore, other metabolites were researched with the aim of being able to compare this whey-based medium with the media resulting from bacterial growth.

A representative ^{1}H NMR spectrum acquired from a sample of whey-based medium is shown in Figure 2.

^{1}H NMR spectroscopy is based on the nuclear magnetic resonance properties of the hydrogen nucleus. Each hydrogen nucleus within a molecule experiences a slightly different magnetic field because of its distinct chemical environment and absorbs energy at slightly different frequencies. Different molecular structures were assigned and identified based on the analysis of 1D and 2D NMR spectra (Table 2).


Citrate
acetate
lactate
2-oxoglutarate
succinate
alanine
ethanol
f1 (ppm)


glucose
glucose
glucose
galactose
galactose
glucose
alanine
glucose
creatine
choline
Sn-glycero-3-phosphocoline
lactate
creatinine
choline
glycerol
ethanol
choline
creatine
creatinine
2-oxoglutarate
f1 (ppm)

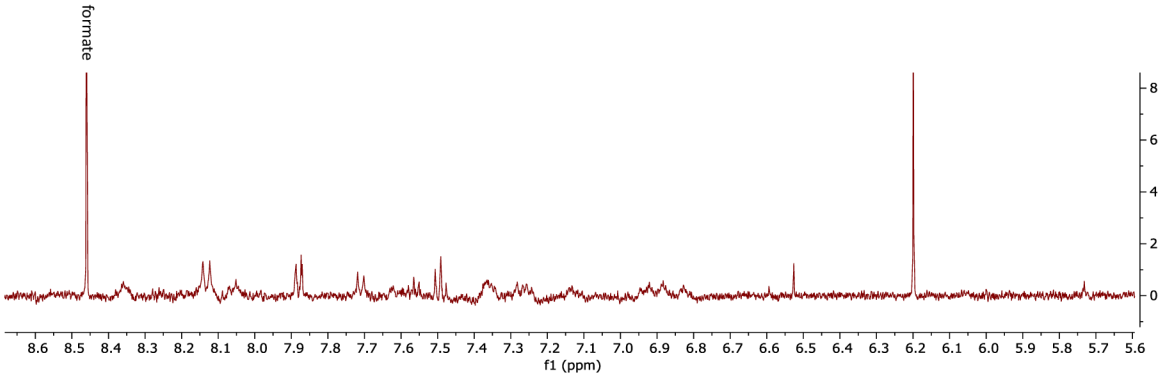


Figure 2 ^{1}H NMR spectrum of whey sample-based medium. f1 on the horizontal axis represents the chemical shift (ppm), while the vertical axis represents the signal intensity.

Table 2 A list of identified metabolites with the assignment from Chenomx Profiler. Peak multiplicities in parentheses: s, singlet; d, doublet; t, triplet; dd, doublet of doublets; tt, triplet of triplets; q, quartet; and m, multiplet.

COMPOUND	δ_H
2-OXOGLUTARATE	2.99 (t) 2.43 (t)
ACETATE	1.91 (s)
ALANINE	3.77 (q) 1.47 (d)
CHOLINE	4.05 (m) 3.51 (dd) 3.19 (s)
CITRATE	2.68 (d) 2.52 (d)
CREATINE	3.92 (s) 3.02 (s)
CREATININE	4.04 (s) 3.03 (s)
ETHANOL	3.65 (q) 1.17 (t)
FORMATE	8.44 (s)
GALACTOSE	5.26 (d) 4.07 (t) 3.98 (d) 3.92 (d) 3.82 (m) 3.72 (m) 3.64 (dd) 3.48 (dd)
GLUCOSE	5.24 (d) 4.64 (d) 3.90 (dd) 3.84 (m) 3.82 (m) 3.75 (dd) 3.71 (dd) 3.70 (dd) 3.52 (dd) 3.48 (t) 3.46 (m) 3.40 (t) 3.39 (m) 3.23 (dd)
GLYCEROL	3,55 (m) 3.65 (m) 3.66 (tt)
LACTATE	4.10 (q) 1.32 (d)
SUCCINATE	2.39 (s)
SN-GLYCERO-3-PHOSPHOCHOLINE	3.20 (s)

For each sample of whey-based medium in which a bacterial strain was cultivated, NMR analysis was conducted, and the same fifteen metabolites were identified and quantified. This allows to compare the various media to each other as well as to the control whey-based medium.

In Table 3 are shown the concentrations in mg/dL of the fifteen metabolites for each medium where a different bacterial strain grew for a period of 24 and 48 hours.

Table 3 Concentration (mg/dL) of the different metabolites in each medium after bacterial growth.

Sample	***2-Oxoglutarate***	***Acetate***	***Alanine***	***Choline***	***Citrate***	***Creatine***	***Creatinine***	***Ethanol***
Control	2,64	11,87	0,71	3,74	156,56	5,03	4,29	2,40
D1-RO2 24h	1,08	117,98	6,73	3,38	0,81	4,82	4,14	2,16
D1-RO2 48h	0,99	121,18	7,31	3,75	0,72	4,15	4,66	3,33
D1-MR10 24h	1,08	247,61	4,43	2,65	0,93	4,74	4,08	7,70
D1-MR10 48h	1,38	363,87	4,27	3,92	0,58	4,55	5,37	9,06
P1-MR12 24h	1,05	116,42	6,34	3,40	0,91	4,82	3,93	2,39
P1-MR12 48h	1,28	125,93	7,07	3,98	0,78	4,91	3,97	2,61
VT5 24h	1,17	38,84	5,60	3,97	129,30	4,70	4,04	2,48
VT5 48h	1,06	44,38	6,59	3,55	109,48	4,65	4,22	2,71
VSM-RO4 24h	0,84	113,26	8,43	3,78	0,84	5,02	3,85	2,41
VSM-RO4 48h	0,94	124,09	9,38	3,31	0,60	4,72	3,89	2,98
VU-RO6 24h	2,46	189,12	7,51	3,70	0,45	4,59	3,85	5,06
VU-RO6 48h	1,68	299,09	7,58	4,04	0,60	4,55	4,69	5,96

Sample	***Formate***	***Galactose***	***Glucose***	***Glycerol***	***Lactate***	***Lactose***	***Succinate***
Control	3,62	1649,9	2429,2	85,05	111,12	73,46	0,92
D1-RO2 24h	3,57	2116,0	1852,3	93,89	279,58	65,60	67,28
D1-RO2 48h	3,70	2192,9	2083,5	103,70	302,57	76,25	66,05
D1-MR10 24h	9,44	2041,8	1526,7	146,93	371,98	58,35	57,19
D1-MR10 48h	5,39	2195,3	1510,1	117,06	610,62	57,72	60,47
P1-MR12 24h	3,38	2081,8	2054,7	90,89	266,87	62,91	75,07
P1-MR12 48h	3,36	2039,9	1780,0	98,96	301,96	68,57	74,24
VT5 24h	3,63	2116,7	1932,5	103,08	210,14	68,83	6,21
VT5 48h	3,33	2100,9	2243,2	92,91	233,14	55,30	9,33
VSM-RO4 24h	3,48	2283,2	2242,7	108,53	277,89	67,65	77,18
VSM-RO4 48h	3,52	2256,0	2202,3	107,67	313,09	70,56	76,53
VU-RO6 24h	6,56	2068,0	1427,4	104,17	299,71	70,19	64,26
VU-RO6 48h	3,86	2056,2	1554,0	98,27	519,49	55,38	67,40

Sample	*sn-Glycero-3-phosphocholine*
Control	31,01
D1-RO2 24h	28,78
D1-RO2 48h	31,47
D1-MR10 24h	29,77
D1-MR10 48h	34,33
P1-MR12 24h	31,51
P1-MR12 48h	30,40
VT5 24h	29,70
VT5 48h	30,45
VSM-RO4 24h	29,89
VSM-RO4 48h	31,58
VU-RO6 24h	31,70
VU-RO6 48h	29,08

The two most representative metabolites are galactose and glucose, while the concentration of the other metabolites is more variable, especially the concentration of acetate, citrate, lactate and succinate.

In the following graph each column represents a compound, identified by a specific color, of a different medium where a different bacterial strain was grown. A control whey-based medium sample, where no microorganisms were grown, was also added.

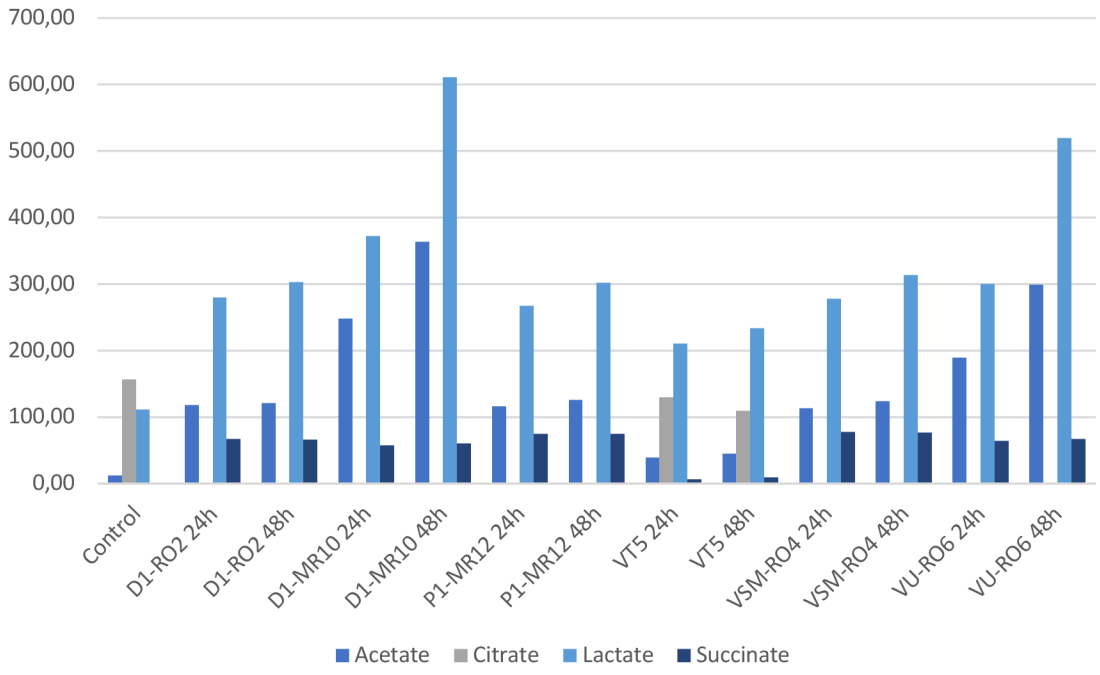


Figure 3 Acetate, citrate, lactate, and succinate represent the metabolites with the most variable concentrations between the different media. An elevation in the concentrations of acetate, citrate, and succinate within strains affiliated with the Lactobacillus genus suggests the presence of oxygen, indicating

the initiation of aerobic metabolism. Notably, these organic acids are also generated by bifidobacteria, as evidenced by the levels observed in the D1-MR10 strain sample belonging to the Bifidobacterium genus. In the case of the D1-MR10 strain, the concentration of acetate surpasses that found in other media. This aligns with the expected metabolic pathway of glucose in bifidobacteria, where 1.5 molecules of acetic acid are produced for each glucose molecule. This stands in contrast to Lactobacillus genus, where only one molecule of acetic acid is produced from a glucose molecule. Therefore, bifidobacteria exhibit a propensity for higher acetate production.

Lactate production is a common feature across all bacterial strains and tends to increase over time. The D1-MR10 strain showcases the highest concentration of lactic acid. This nuanced understanding of the metabolic activities of these bacterial strains sheds light on their adaptive responses and highlights the intricate interplay between oxygen availability, metabolic pathways, and the resultant organic acid profiles.

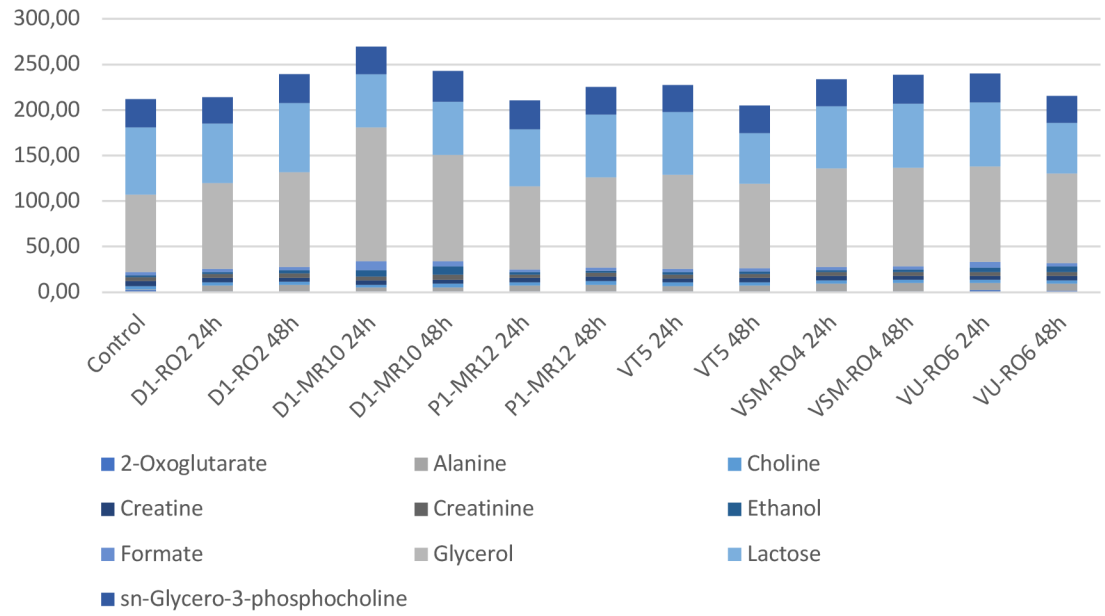


Figure 4 The other metabolites analyzed are present with a similar concentration between the different media but are less representative. Among these ten compounds, 2-oxoglutarate and ethanol are potential products of microbial activity. Specifically, 2-oxoglutarate, also recognized as α-ketoglutarate, is a byproduct of the Krebs cycle. On the other hand, ethanol is generated by heterofermentative lactobacilli, for each molecule of glucose there is the production of one molecule of ethanol. The increase in alanine concentrations could be attributed to its de novo synthesis originating from α-ketoglutarate, facilitated by microbial enzymes such as glutamate dehydrogenase and alanine aminotransferase. The reduction in lactose concentration in the growth medium, as compared to the control medium, indicates its enzymatic hydrolysis by microbial β-galactosidases. Choline, creatine, creatinine, formate, glycerol and sn-Glycero-3-phosphocholine remain with similar concentrations to the control sample.

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4.4 Fermentation profiles of selected strains using ^{1}H NMR

A dendrogram is a two-dimensional diagram representing a tree of relationships, whatever their nature. It is a particularly useful tool for representing a taxonomic system when used in conjunction with a cluster analysis to examine the composition of the associated operational taxonomic units. A similarity matrix, with its entries representing estimations of the similarities between the respective units and its rows and columns denoting the operational taxonomic units, is typically the foundation for a cluster analysis (CaliŃski, 2014).

The dendrogram (Figure 5) showed two main clusters. The first cluster showed two subclusters which grouped the whey-based control medium and the media with *Lb. apis* VT5, *Lb. melliventris* D1-RO2, *Lb. helsingborgensis* VSM-RO4 and *Lb. apis* P1-MR12. Also the second cluster showed two subclusters which grouped *B. asteroides* D1-MR10 and *Lb. kimbladii* VU-RO6 after 24 hours growth and *B. asteroides* D1-MR10 and *Lb. kimbladii* VU-RO6 after 48 hours growth. From the diagram can be observed how these last two strains are very distant from the whey-based medium, more similar to the other strains. In a 48-hour growth period, the sample with *B. asteroides* D1-MR10 exhibits greater similarity to the medium containing *Lb. kimbladii* VU-RO6 measured at the same point time, compared to the sample with the same strain but after 24 hours of growth.

The media into which *B. asteroides* D1-MR10 and *Lb. kimbladii* VU-RO6 were inoculated are those that contain a higher concentration of organic acids (lactic, acetic and succinic acid), especially after 48 hours of incubation.

Constellation plots are a general methodology that combine many complementary molecular representations to improve the information presented in a chemical space analysis and visual depiction. The approach combines a "classical" coordinate-based representation of chemical space with a sub-structure-based description and classification of molecules. One unique result of the procedure is the production of molecular groups, or "constellations," in chemical space as a result of arranging the compounds in analog series (Naveja and Medina-Franco, 2019).

Also, in this representation (Figure 6) it is possible to observe the two clusters. The first consisting of *B. asteroides* D1-MR10 and *Lb. kimbladii* VU-RO6 strains, while the second includes the control whey-based medium and the media with *Lb. apis* VT5, *Lb. melliventris* D1-RO2, *Lb. helsingborgensis* VSM-RO4, and *Lb. apis* P1-MR12.

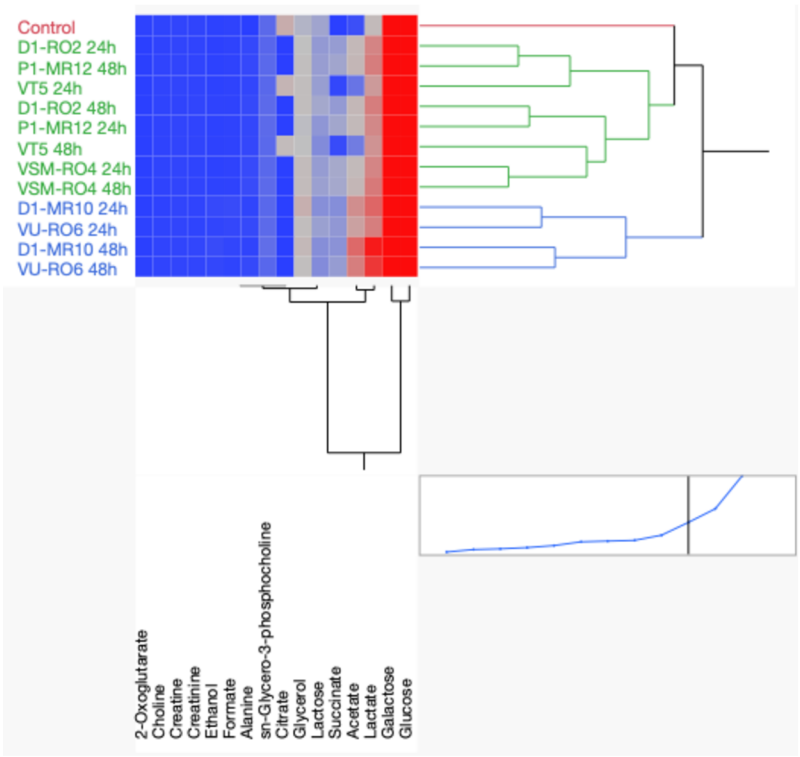

Figure 5 The dendrogram obtained by the Ward Method, Non-standardized and Robust, using JMP pro 17 software. Colors correspond to normalized mean data levels from low (blue) to high (red).

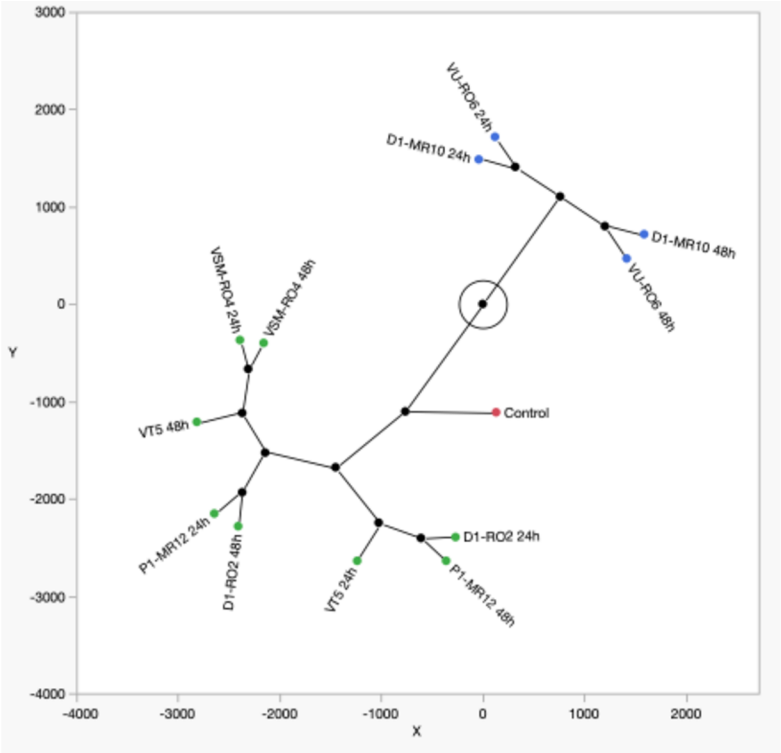

Figure 6: Constellation plot obtained using JMP pro 17 software.

5 DISCUSSION

The different strains, isolated from the GI-tract of worker bees, developed on a lactose-free whey-based medium. The breakdown of lactose is important since this compound cannot be fermented by isolated bacterial strains (Zheng *et al.*, 2020), as well as being indigestible for honey bees (Standifer, 2003).

For this reason, 0,1% of *β*-galactosidase was added and left to act for different lengths of time, before being deactivated by heat, with the aim of understanding what was the minimum time in which the action of the enzyme allowed the decrease in lactose levels leading to this concentration level.

β-galactosidase is a hydrolytic enzyme that catalyzes the hydrolysis of terminal non-reducing *β*-D-galactose residues in *β*-D-galactosides, such as lactose (Fekete *et al.*, 2007). The hydrolysis of this sugar, by the enzyme, leads to obtaining its two constituent monosaccharides, glucose and galactose. From this it can be understood that where *β*-galactosidase has acted effectively, the concentration of lactose present will plummet while that of glucose and galactose will increase. Most commercial *β*-galactosidases derives from the dairy yeast *Kluyveromyces lactis and K. marxianus*; while other *β*-galactosidases obtained from, for example, *Niallia circulans* or *Aspergillus oryzae*, are less suitable for producing lactose-free dairy products because of the different pH or temperature optimums (Dekker, Koenders and Bruins, 2019).

In the following study, two different whey samples were used to understand if there was any difference in using one rather than another.

The most suitable sample is represented by fresh whey, where the percentage of converted lactose turned out 98,72%; in fact, levels of lactose decreased significantly after 4 hours from the addition of the enzyme (Figure 1.a).

In the sample stored in the freezer for 24 hours, the lactose concentration reached, after the maximum time studied, are higher than for the fresh-whey sample (Figure 1.a).

Graphs in Figures 1.b and 1.c show the concentration of lactose constituents, glucose and galactose. It can be noted how, in both samples, while the concentration of lactose decreases over time, the concentration of the two monosaccharides increases.

Calculating the lactose balance in the two samples, a higher percentage was found for the sample made up of fresh whey (98.72%). For this reason, the fresh whey sample was selected as growth medium for the bacterial strains isolated from the GI- tract of bees. These bacterial strains have demonstrated the ability to grow on this medium.

The whey-based media in which strains were grown were analyzed by NMR, and by comparing them a different concentration of organic acids (acetate, lactate and succinate) was noted (Table 3).

The first is a product of heterofermentative LAB under aerobic conditions, as well as representing a SCFA that can be produced by bifidobacteria. Lactate is produced by both heterofermentative and homofermentative lactic acid bacteria following the action of the enzyme lactate dehydrogenase, which acts on pyruvate that will be reduced to lactate thanks to the presence of the reducing agent NADH. Lactate is also produced by bifidobacteria following the fermentation of carbohydrates. Succinate is a product of aerobic metabolism; in fact, it represents a metabolite of the Krebs cycle.

By comparing the control sample - consisting of a whey-based medium in which no strains were grown - with the different media where strains growth occurred, it can be observed how the concentration of some components remained almost unchanged, while the levels of other components such as acetate, citrate, lactate, and succinate are changed (Figure 3).

Similarities and contrasts between the media can be seen by creating a dendrogram (Figure 5) and a constellation plot (Figure 6), which group the analyzed media into two clusters. Thanks to these graphic representations it is possible to understand which bacterial strains could be the most promising given the greater production of organic acids. *Bifibacterium asteroides* D1-MR10 and *Lactobacillus kimbladii* VU-RO6 are the strains that produced a higher concentration of organic acids (lactic, acetic and succinic acid).

6 CONCLUSION

In this study, a nutrient-rich by-product such as whey was used to prepare a lactose-free medium for the growth of beneficial strains isolated from the gastrointestinal tract of worker bees with the aim of creating a probiotic product to be administered to bees.

These preliminary studies will need to be followed by further studies to understand how this probiotic product can be administered to bees, whether they ingest it and what benefits they derive from it. It would be interesting to grow multiple bacterial strains within the same medium, thus creating bacterial mixtures to compare with products consisting of a single beneficial strain and select the best.

At the same time, the same whey-based growth medium could be consumed as a fermented drink. By carrying out more in-depth analyzes of the compounds present in the whey-based medium, carrying out microbiological safety studies, and finally adding any flavorings, colorings and carrying out sensory analysis tests it would be possible to obtain a fermented beverage suitable for human consumption.

7 REFERENCES

Alexandre, E.M.C. *et al.* (2023) 'Properties of Fermented Beverages from Food Wastes/By-Products', *Beverages*, 9(2). Available at: https://doi.org/10.3390/beverages9020045.

Arsène, M.M. *et al.* (2021) 'The use of probiotics in animal feeding for safe production and as potential alternatives to antibiotics', *Veterinary world*, 14(2), p. 319.

Bolivar-Jacobo, N.A. *et al.* (2023) 'Culture Age, Growth Medium, Ultrasound Amplitude, and Time of Exposure Influence the Kinetic Growth of *Lactobacillus acidophilus*', *Fermentation*, 9(1). Available at: https://doi.org/10.3390/fermentation9010063.

Boukid, F. *et al.* (2023) 'Fermentation for Designing Innovative Plant-Based Meat and Dairy Alternatives', *Foods*, 12(5). Available at: https://doi.org/10.3390/foods12051005.

CaliŃski, T. (2014) 'Dendrogram', in R.S. Kenett et al. (eds) *Wiley StatsRef: Statistics Reference Online*. 1st edn. Wiley. Available at: https://doi.org/10.1002/9781118445112.stat05624.

Chavan, R. *et al.* (2015) 'Whey based beverage: its functionality, formulations, health benefits and applications', *Journal of Food Processing & Technology*, 6(10), p. 1.

Chmiel, J.A. *et al.* (2020) 'Understanding the Effects of Sublethal Pesticide Exposure on Honey Bees: A Role for Probiotics as Mediators of Environmental Stress', *Frontiers in Ecology and Evolution*, 8, p. 22. Available at: https://doi.org/10.3389/fevo.2020.00022.

'de man, rogosa and sharpe (MRS) agar' (2003) in *Progress in Industrial Microbiology*. Elsevier, pp. 511–513. Available at: https://doi.org/10.1016/S0079-6352(03)80066-8.

Dekker, P.J.T., Koenders, D. and Bruins, M.J. (2019) 'Lactose-Free Dairy Products: Market Developments, Production, Nutrition and Health Benefits', *Nutrients*, 11(3). Available at: https://doi.org/10.3390/nu11030551.

Egan, M. and Van Sinderen, D. (2018) 'Chapter 8 - Carbohydrate Metabolism in Bifidobacteria', in P. Mattarelli et al. (eds) *The Bifidobacteria and Related Organisms*. Academic Press, pp. 145–164. Available at: https://doi.org/10.1016/B978-0-12-805060-6.00008-9.

Elkot, W.F. *et al.* (2024) 'Development and characterization of a novel flavored functional fermented whey-based sports beverage fortified with Spirulina platensis', *International Journal of Biological Macromolecules*, 258, p. 128999. Available at: https://doi.org/10.1016/j.ijbiomac.2023.128999.

Ezema, C. (2013) 'Probiotics in animal production: A review', *Journal of Veterinary Medicine and Animal Health*, 5(11), pp. 308–316.

Fekete, E. *et al.* (2007) 'Induction of extracellular β-galactosidase (Bga1) formation by d-galactose in Hypocrea jecorina is mediated by galactitol', *Microbiology*, 153(2), pp.

507–512. Available at: https://doi.org/10.1099/mic.0.2006/001602-0.

Fushinobu, S. (2010) 'Unique Sugar Metabolic Pathways of Bifidobacteria', *Bioscience, Biotechnology, and Biochemistry*, 74(12), pp. 2374–2384. Available at: https://doi.org/10.1271/bbb.100494.

Gaggìa, F., Baffoni, L. and Alberoni, D. (2018) 'Probiotics for Honeybees' Health', in D. Di Gioia and B. Biavati (eds) *Probiotics and Prebiotics in Animal Health and Food Safety*. Cham: Springer International Publishing, pp. 219–245. Available at: https://doi.org/10.1007/978-3-319-71950-4_9.

Guimarães, P.M.R., Teixeira, J.A. and Domingues, L. (2010) 'Fermentation of lactose to bio-ethanol by yeasts as part of integrated solutions for the valorisation of cheese whey', *Biotechnology Advances*, 28(3), pp. 375–384. Available at: https://doi.org/10.1016/j.biotechadv.2010.02.002.

Hroncova, Z. *et al.* (2015) 'Variation in honey bee gut microbial diversity affected by ontogenetic stage, age and geographic location', *PloS One*, 10(3), p. e0118707. Available at: https://doi.org/10.1371/journal.pone.0118707.

Hroncova, Z. *et al.* (2019) 'In-hive variation of the gut microbial composition of honey bee larvae and pupae from the same oviposition time', *BMC Microbiology*, 19(1), p. 110. Available at: https://doi.org/10.1186/s12866-019-1490-y.

Hundshammer, C. and Minge, O. (2020) 'In Love with Shaping You—Influential Factors on the Breast Milk Content of Human Milk Oligosaccharides and Their Decisive Roles for Neonatal Development', *Nutrients*, 12(11), p. 3568. Available at: https://doi.org/10.3390/nu12113568.

Joint FAO WHO Working Group on Drafting Guidelines for the Evaluation of Probiotics in Food (ed.) (2006) *Probiotics in food: health and nutritional properties and guidelines for evaluation ; report of a Joint FAO/WHO Expert Consultation on Evaluation of Health and Nutritional Properties of Probiotics in Food including Powder Milk with Live Lactic Acid Bacteria, Córdoba, Argentina, 1 - 4 October 2001 ; report of a Joint FAO/WHO Working Group on Drafting Guidelines for the Evaluation of Probiotics in Food, London, Ontario, Canada, 30 April - 1 May 2002. Expert Consultation on Evaluation of Health and Nutritional Properties of Probiotics in Food including Powder Milk with Live Lactic Acid Bacteria*, Rome: Food and Agriculture Organization of the United Nations [u.a] (FAO food and nutrition paper, 85).

Keeler, J. (2010) *Understanding NMR spectroscopy*. 2nd ed. Chichester, U.K: John Wiley and Sons.

Killer, J. *et al.* (2014) 'Lactobacillus apis sp. nov., from the stomach of honeybees (Apis mellifera), having an in vitro inhibitory effect on the causative agents of American and European foulbrood', *International Journal of Systematic and Evolutionary Microbiology*, 64(Pt 1), pp. 152–157. Available at: https://doi.org/10.1099/ijs.0.053033-0.

Kligler, B. and Cohrssen, A. (2008) 'Probiotics', *American Family Physician*, 78(9), pp. 1073–1078.

Laëtitia, G., Pascal, D. and Yann, D. (2014) 'The Citrate Metabolism in Homo- and Heterofermentative LAB: A Selective Means of Becoming Dominant over Other Microorganisms in Complex Ecosystems', *Food and Nutrition Sciences*, 05(10), pp. 953–969. Available at: https://doi.org/10.4236/fns.2014.510106.

Macomber, R.S. (1998) *A complete introduction to modern NMR spectroscopy*. New York, NY: Wiley (A Wiley-Interscience publication).

Mazziotta, C. *et al.* (2023) 'Probiotics Mechanism of Action on Immune Cells and Beneficial Effects on Human Health', *Cells*, 12(1), p. 184. Available at: https://doi.org/10.3390/cells12010184.

Miloud, B., Halima, Z.-K.A. and Nour-Eddine, K. (2017) 'Development of a sweet whey-based medium for culture of Lactobacillus', *African Journal of Biotechnology*, 16(30), pp. 1630–1637. Available at: https://doi.org/10.5897/AJB2017.16088.

Min, Y.W. and Rhee, P.-L. (2015) 'The Role of Microbiota on the Gut Immunology', *Clinical Therapeutics*, 37(5), pp. 968–975. Available at: https://doi.org/10.1016/j.clinthera.2015.03.009.

Motta, E.V.S. *et al.* (2022) 'Prospects for probiotics in social bees', *Philosophical Transactions of the Royal Society B: Biological Sciences*, 377(1853), p. 20210156. Available at: https://doi.org/10.1098/rstb.2021.0156.

Musa, H.H. *et al.* (2009) 'The potential benefits of probiotics in animal production and health', *J. anim. vet. adv*, 8(2), pp. 313–321.

Naveja, J.J. and Medina-Franco, J.L. (2019) 'Finding Constellations in Chemical Space Through Core Analysis', *Frontiers in Chemistry*, 7, p. 510. Available at: https://doi.org/10.3389/fchem.2019.00510.

Nguyen, B.T. *et al.* (2019) 'Probiotic beverage from pineapple juice fermented with *Lactobacillus* and *Bifidobacterium* strains', *Frontiers in nutrition*, 6, p. 54.

Niode, N.J. *et al.* (2020) 'Lactic Acid Bacteria from Honey Bees Digestive Tract and Their Potential as Probiotics', in *Proceedings of the International Conference and the 10th Congress of the Entomological Society of Indonesia (ICCESI 2019). International Conference and the 10th Congress of the Entomological Society of Indonesia (ICCESI 2019)*, Kuta, Bali, Indonesia: Atlantis Press. Available at: https://doi.org/10.2991/absr.k.200513.041.

Nowak, A. *et al.* (2021) 'Characterization of Apis mellifera Gastrointestinal Microbiota and Lactic Acid Bacteria for Honeybee Protection—A Review', *Cells*, 10(3), p. 701. Available at: https://doi.org/10.3390/cells10030701.

Pokusaeva, K., Fitzgerald, G.F. and Van Sinderen, D. (2011) 'Carbohydrate metabolism in Bifidobacteria', *Genes & Nutrition*, 6(3), pp. 285–306. Available at:

https://doi.org/10.1007/s12263-010-0206-6.

Poupard, J.A., Husain, I. and Norris, R.F. (1973) 'Biology of the bifidobacteria', *Bacteriological reviews*, 37(2), pp. 136–165.

Qu, Q. and Jin, L. (2022) 'Application of nuclear magnetic resonance in food analysis', *Food Science and Technology*, 42, p. e43622. Available at: https://doi.org/10.1590/fst.43622.

Quinto, E.J. *et al.* (2014) 'Probiotic lactic acid bacteria: a review', *Food and Nutrition Sciences*, 5(18), p. 1765.

Saarela, M. *et al.* (2000) 'Probiotic bacteria: safety, functional and technological properties', *Journal of Biotechnology*, 84(3), pp. 197–215. Available at: https://doi.org/10.1016/S0168-1656(00)00375-8.

Savio, C., Mugo-Kamiri, L. and Upfold, J.K. (2022) 'Bugs in Bugs: The Role of Probiotics and Prebiotics in Maintenance of Health in Mass-Reared Insects', *Insects*, 13(4). Available at: https://doi.org/10.3390/insects13040376.

Sela, D.A., Price, N. and Mills, D.A. (2010) 'Metabolism of bifidobacteria', *Bifidobacteria: genomics and molecular aspects*, pp. 45–70.

Sharma, H. *et al.* (2023) 'Impact of lactic acid bacteria and their metabolites on the techno-functional properties and health benefits of fermented dairy products', *Critical Reviews in Food Science and Nutrition*, 63(21), pp. 4819–4841. Available at: https://doi.org/10.1080/10408398.2021.2007844.

Standifer, L. (2003) 'Honey bee nutrition supplemental feeding', *Beekeeping in the United States Agriculture Handbook*, 335, pp. 39–45.

Tomar, S. *et al.* (2023) 'Unravelling the hidden ethnic fermented treasure of the Himalayas - A review on the traditionally fermented beverages of the Northwest Indian Himalayan Region', *Food Chemistry Advances*, 2, p. 100254. Available at: https://doi.org/10.1016/j.focha.2023.100254.

Tootiaie, S., Moharrami, M. and Mojgani, N. (2021) 'Honeybee Gut: Reservoir of Probiotic Bacteria', in N. Mojgani and M. Dadar (eds) *Probiotic Bacteria and Postbiotic Metabolites: Role in Animal and Human Health*. Singapore: Springer Singapore (Microorganisms for Sustainability), pp. 221–236. Available at: https://doi.org/10.1007/978-981-16-0223-8_9.

Widyastuti, Y., Febrisiantosa, A. and Tidona, F. (2021) 'Health-Promoting Properties of Lactobacilli in Fermented Dairy Products', *Frontiers in Microbiology*, 12, p. 673890. Available at: https://doi.org/10.3389/fmicb.2021.673890.

Ye, M. *et al.* (2023) 'Beneficial bacteria as biocontrol agents for American foulbrood disease in honey bees (Apis mellifera)', *Journal of Insect Science*, 23(2), p. 6. Available at: https://doi.org/10.1093/jisesa/iead013.

Zheng, J. *et al.* (2020) 'A taxonomic note on the genus *Lactobacillus*: Description of 23 novel genera, emended description of the *genus* Lactobacillus Beijerinck 1901, and union of Lactobacillaceae and Leuconostocaceae', *International Journal of Systematic and Evolutionary Microbiology*, 70(4), pp. 2782–2858. Available at: https://doi.org/10.1099/ijsem.0.004107.