Instituto Tecnológico y de Estudios Superiores de Monterrey

Campus Monterrey

School of Engineering and Sciences

Interplay between kefir microbiota and berry by-products: evaluation of probiotic and prebiotic properties for functional food applications

A thesis presented by

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Submitted to the

School of Engineering and Sciences

in partial fulfillment of the requirements for the degree of

Master of Science

In Biotechnology

Monterrey Nuevo León, June 11th, 2021

Dedication

“One, remember to look up at the stars and not down at your feet. Two, never give up work. Work gives you meaning and purpose and life is empty without it. Three, if you are lucky enough to find love, remember it is there and don’t throw it away.”

— Stephen Hawking

To my parents and sister for accompanying me in this process, just as they have accompanied me in all situations throughout my life, always present and always encouraging me to pursue my dreams. Thank you for being an example of perseverance, tenacity and commitment. All that I am is thanks to you, I always learn from your example and I hope one day be able to give you back all what you have given to me.

To God, I thank Him and the universe infinitely for giving me the opportunity to do what I like, for giving me an incredible family, for allowing me to meet inspiring people and make valuable and true friends, I am because you are.

Acknowledgements

“Science is not only a disciple of reason but, also, one of romance and passion”.

— Stephen Hawking

I would first like to express my deepest gratitude to my advisor, Dr. Tomás García Cayuela, for being an inspiring person, an excellent advisor and above all an unconditional support and friend. For sharing his knowledge and passion for research, for his patience and for the teachings inside and outside the laboratory. I hope to continue learning and collaborating in the future! I would like to express my admiration and total gratitude to him for being an excellent example of mentor and researcher.

To my parents and sister for their love, support and for motivating me to be a better person, also for always concern about my education. To God, for blessing me with an amazing family, friends, abilities and opportunities to grow and improve as a person.

To Mariano del Toro, for all your unconditional support throughout this journey, I admire your intelligence and kindness. Thank you for being like a brother to me.

To Karla Martínez and Omar Pérez for kindly teaching me new techniques, for helping me with the experiments, and for their valuable time. To Diego Diaz, Food Biotech team and all my friends, for being with me throughout this journey, for make my days better, cheer me up and being there forever.

To Tecnológico de Monterrey, my alma mater since 2009 and to CONACyT for the scholarships and for they commitment to student and scientific development. The research presented in this document has been possible thanks to the funding from the Secretaría de Innovación, Ciencia y Tecnología del Gobierno de Jalisco, Consejo Estatal de Ciencia y Tecnología de Jalisco (COECYTJAL) and Tecnológico de Monterrey (Projects: PRODEPRO 7643-2019, PRODEPRO 7661-2019, FODECIJAL 7799-2019, and FODECIJAL 8245-2019).

Interplay between kefir microbiota and berry by-products: evaluation of probiotic and prebiotic properties for functional food applications

by

Alejandra Hurtado Romero

Abstract

A functional food is defined as a food that contains biologically active components with proven health benefits, offering the potential to reduce the risk of specific chronic diseases or beneficially affect target functions beyond its basic nutritional functions. Currently, some food industries re-process their by-products and use them as functional food ingredients, making sustainable and stable economic growth. Industrial by-products generated in Jalisco, including berries, leads to losses of food products that can represent up to 40% of the processed volume. Further, berries generate high amounts of by-products during industry manufacturing. In this context, these berry by-products could be recovered and used successfully for different industry purposes, such as production of prebiotic ingredients. Prebiotics are substrates selectively utilized by probiotic microorganisms conferring a health benefit. The potential prebiotic food ingredients obtained from plants or food by-products need to be characterized to determine their properties and the mechanisms by which they exert health benefits, as well as how they impact the gut microbiota composition. Therefore, different commercial probiotics and microorganisms isolated from an artisanal milk kefir beverage were evaluate along with the fermentative capacity of ingredients from industrial berry by-products. *Lactoccocus, Leuconostoc, Kluyveromyces* and *Lactobacillus* species were isolated and identified through MALDI-TOF analysis. Overall, *L. lactis* BIOTEC007, *K. lactis* BIOTEC009, *L. kefiri* BIOTEC014 and *L. pseudomesenteroides* BIOTEC012 species demonstrated desirable probiotic-related properties such as auto aggregation, co-aggregation with pathogens, antimicrobial activity related to the production of organic acids and resistance to *in vitro* gastrointestinal digestion conditions. Regarding the berry by- products, bagasse of berries were obtained and characterized, showing antimicrobial activity, high content of bioactive compounds and a high content of fiber, proteins, and carbohydrates. Hence, when fermented by kefir isolated microorganisms such as *L. lactis* BIOTEC007, *L. kefiri* BIOTEC014 and *L. rhamnosus* GG, maximum growth absorbance values in blueberry and

strawberry bagasse media were observed. Moreover, a substrate consumption assay showed a possible utilization of bagasse fibers and polyphenols. Finally, four synbiotic fermented beverages containing blueberry bagasse were formulated. The product fermented by *L. lactis* BIOTEC007 and *L. rhamnosus* GG were chosen as the best due to their physicochemical characteristics and sensorial attributes. The beverage also contained considerable levels of bioactive compounds including phenolic acids and anthocyanins. Finally, it was observed that this formulation with bagasse promoted the growth of microorganisms in the product and exerted in some way a protective effect, since they resisted the digestion stages in a better way than the isolates that were in a milk matrix with no bagasse as a control. These properties allow to confirm tha blueberry bagasse could be considered as a prospective prebiotic ingredient along with potential probiotic isolates in the formulation of functional fermented foods.

Interacción entre la microbiota del kéfir y los subproductos de las berries: evaluación de las propiedades probióticas y prebióticas para aplicaciones alimentarias funcionales

Por

Alejandra Hurtado Romero

Resumen

Un alimento funcional es aquel que contiene compuestos biológicamente activos con beneficios para la salud comprobados y que además ofrece el potencial de reducir el riesgo de padecer enfermedades crónicas. Actualmente, algunas industrias alimentarias reprocesan sus subproductos y los utilizan como ingredientes alimentarios funcionales, generando crecimiento económico sostenible. Los subproductos industriales generados en Jalisco, incluyendo las berries, generan pérdidas de productos alimenticios que pueden representar hasta el 40% del volumen procesado. Además, las berries generan grandes cantidades de subproductos durante su procesamiento industrial para jugos, puré, entre otros. En este contexto, los subproductos de las berries podrían recuperarse y utilizarse con éxito para diferentes fines industriales, como la producción de ingredientes prebióticos. Los prebióticos son sustratos utilizados selectivamente por microorganismos probióticos que confieren un beneficio para la salud. Tanto las cepas probióticas emergentes como los ingredientes prebióticos obtenidos de plantas o subproductos agroindustriales deben caracterizarse para determinar su potencial y dilucidar sus propiedades prebióticas y los mecanismos por los cuales ejercen beneficios para la salud, así como su impacto en el intestino y su efecto en la composición de la microbiota. Por lo tanto, en este estudio se evaluó el potencial probiótico de las cepas aisladas de la bebida fermentada casera kéfir y se determinó la capacidad prebiótica de los ingredientes de los subproductos industriales de berries. Se aislaron e identificaron las especies *Lactoccocus*, *Leuconostoc, Kluyveromyces* y *Lactobacillus* mediante el análisis MALDI-TOF. En general, las especies de *L. lactis* BIOTEC007, *K. lactis* BIOTEC009, *L. kefiri* BIOTEC014 y *L. pseudomesenteroides* BIOTEC012 demostraron propiedades deseables relacionadas con los probióticos, como la autoagregación, la coagregación con patógenos, la actividad antimicrobiana relacionada con la producción de ácidos orgánicos y la resistencia a condiciones de digestión gastrointestinal *in vitro*. En cuanto a los subproductos de las berries, se caracterizó el bagazo de fresas,

frambuesas, mora azul y zarzamora, que presentaron actividad antimicrobiana, una concentración considerable de compuestos bioactivos y un alto contenido en fibra, proteínas y carbohidratos. Por lo tanto, cuando los microorganismos aislados del kéfir llevaron a cabo la fermentación, las cepas *L. lactis* BIOTEC007, *L. kefiri* BIOTEC014 y *L. rhamnosus* GG, alcanzaron valores máximos de absorbancia de crecimiento en medios de bagazo de mora azul y fresa. Además, el ensayo de consumo de sustrato determinó una posible fermentación de la fibra presente en el bagazo, así como el aumento de la biodisponibilidad de polifenoles del bagazo. Finalmente, se formularon leches simbióticas fermentadas que contenían bagazo de mora azul. Las leches fermentadas por *L. lactis* BIOTEC007 y *L. rhamnosus* GG fueron elegidas como las mejores por sus características fisicoquímicas y atributos sensoriales. A su vez, presentaron niveles considerables de compuestos bioactivos, incluidos compuestos fenólicos y antocianinas, que debido a la fermentación pueden estar más biodisponibles para el consumidor. Finalmente, se observó que esta formulación con bagazo promueve el crecimiento de microorganismos en la bebida y ejerce cierto efecto protector sobre las cepas, ya que resistieron mejor las etapas de digestión que las cepas que se encontraban en una matriz de leche sin bagazo. Por lo tanto, estas propiedades permiten que el bagazo de mora azul pueda considerarse un potencial ingrediente prebiótico debido a la actividad observada *in vitro*, mientras que las cepas aisladas de kéfir, demostraron propiedades probióticas, lo que en conjunto permite la formulación de alimentos funcionales fermentados.

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CHAPTER 1. GENERAL INTRODUCTION

1.1. Introduction

Functional foods can promote health through prevention rather than treatment, hence these compounds have received much attention in recent years from the scientific community, food manufacturers and consumers. Currently, some food industries re-process their by-products and use them as functional food ingredients, making sustainable and stable economic growth (Bharat Helkar & Sahoo, 2016).

Mexico has positioned as one of the major berry-producing countries, exporting between 60% and 80% of its berry production, being Jalisco the second highest producing state. During the post-harvest and transformation stages, the agro industrial by-products generated in Jalisco, including berries, leads to losses of food products that can represent up to 40% of the processed volume. These wastes represent an available and renewable source of billions of tons of biomass per year, which can be used as a source of high added value compounds (Casas Godoy and Barrera Ramírez, 2021). Berries such as raspberry, strawberry, blackberry and blueberry have a significant content of antioxidant, phenolic acids, flavonoids, polyphenols and fibers, and generate high amount of waste during industry manufacturing (Ispiryan and Viškelis, 2019; Vázquez-González et al., 2020). In this context, these berry by-products could be recovered and used successfully for different industry purposes, such as production of prebiotic ingredients.

Functional food components with biological effects are susceptible to be metabolized by intestinal bacteria during the gastrointestinal passage, prior being absorbed. These complex bacterial community (more than 800 species) present in high concentrations in the colon is known as gut microbiota (Laparra and Sanz, 2010). Alterations in the composition and function of the gut microbiota is known as dysbiosis, and these leads to harmful effects on host health changing the metabolic activities of the microorganisms, or causing changes in their local distribution (Yoo et al., 2020). These changes in composition or function of gut microbiota may lead to acute or chronic disease states and syndromes such as acute diarrhea, irritable bowel syndrome, and inflammatory bowel diseases (Aziz et al., 2013).

Novel approaches have been suggested to maintain homeostasis; among them, the manipulation of intestinal microbiota through the use of probiotics and prebiotics for the regulation of immune and inflammatory responses that balance the microbial composition

(Maslowski and Mackay, 2011). Probiotics are defined as “live microorganisms that, when administered in adequate amounts, confer a health benefit on the host”. Several evidence suggests benefits often associated with probiotics are supporting a healthy digestive tract and a healthy immune system (Hill et al., 2014). Probiotics can settle and multiply into the gut through provision of prebiotic substrates such as soluble fibres, also administered as a combination of both probiotics and prebiotics, known as synbiotics; moreover, available as foods, dietary supplements or therapeutics (Vyas and Ranganathan, 2012). Probiotic organisms can be ingested in fermented foods and/or supplements. Fermented foods can be of both dairy and vegetable origin, while probiotic supplements consist of freeze-dried (lyophilized) bacteria in powder, capsule, or tablet form. For instance, kefir is a fermented milk drink with an acidic taste and creamy consistency produced by bacterial fermentation of kefir grains that has acquire attention due to the fact that it exhibits many health benefits owing to its antimicrobial, anticancer, gastrointestinal tract effects, gut microbiota modulation and anti-diabetic effects (Farag, Jomaa, Abd El-Wahed, & R. El-Seedi, 2020).

The current definition of prebiotic, proposed by The International Scientific Association for Probiotics and Prebiotics, is “a substrate that is selectively utilized by host microorganisms conferring a health benefit”. Not growth substrates like vitamins, minerals or antibiotics are not considered prebiotics, because they do not use mechanisms involving selective utilization by host microorganisms (Gibson et al., 2017). In order to provide consumers with products that contain meaningful levels of probiotics, requirements have to be fulfilled by probiotics such as reported abilities like viability and survival throughout the manufacturing process and colonization and immunogenicity properties, among others (Forssten et al., 2011).

Moreover, both emerging probiotic strains and prebiotic food ingredients obtained from plants or food by-products, need to be evaluated, in order to determine their potential and elucidate their specific prebiotic properties and the mechanisms by which they exert health benefits, as well as how they impact the gut microbiota composition (Senés-Guerrero et al., 2020). The analysis of probiotic properties can be used to design products that can serve a wider audience of people; further, research and characterization of the strain serves to easily incorporate it into food matrixes, depending the nature of the strain. Moreover, to promote synergy between probiotics and foods containing proteins, vitamins, minerals and bioactive compounds. The evaluation of potential prebiotic ingredients is essential to understand the digestion and fermentation. One of the most important aspects to validate a

food ingredient as prebiotic is its selective utilization by host microorganisms.

Providing bacteria with prebiotic ingredients allows the selective stimulation and enrichment of gut-beneficial bacterial strains. This is why the characterization of strains from fermented products and their application together with functional ingredients from agro-industrial berries by-products from the state of Jalisco is an interesting approach to develop functional foods.

1.2. Hypothesis

Ingredients obtained from berry by-products can stimulate the selective growth of lactic acid bacteria (LAB) and yeasts isolated from a fermented food, being novel sources of prebiotics with the potential of being used together with probiotics for the formulation of functional foods.

1.3. General Objective

Characterize the probiotic potential of microorganism isolated from an artisanal milk kefir beverage and the prebiotic capacity of ingredients from industrial berry by-products in order to formulate novel combinations of functional ingredients with potential health properties.

1.4. Specific Objectives

The specific objectives for the present thesis were the following:

1) Isolate and identify different LAB and yeasts from homemade milk kefir using MALDI-TOF mass spectrometry and characterize their probiotic properties such as auto and co-aggregation, antibiotic susceptibility, antimicrobial activity, and resistance to gastrointestinal conditions.

2) Characterize whole fruits of raspberry, strawberry, blueberry and blackberry determining its physicochemical properties (total weight, pH, soluble solids, titratable acidity); obtain the berry ingredients from by-products after juice

extraction and evaluate the content of nutritional facts (protein, fat, carbohydrates, fibre) and bioactive compounds (total phenolics and anthocyanins). Also, to assess the antimicrobial activity against pathogens of berry by-products extracts.

3) Evaluate the fermentation capacity and prebiotic potential of berry by-product ingredients through the monitoring of microbial growth using lyophilized berry bagasse as carbon source, as well as the analysis of cellular viability and carbon source consumption of microorganisms.

4) Formulate a synbiotic combination of the berry by-product ingredients with the selected kefir potential probiotic isolates in order to be used in a dairy fermented food model and determine in this product the physicochemical properties (pH, soluble solids, density, water holding capacity, viscosity), the bioactive content (phenolics and anthocyanins), the sensorial quality, and the growth and survival of the starter microorganisms after an *in vitro* digestion.

1.5. Thesis Structure

The present thesis is composed of 5 chapters. Chapter 1 presents a general introduction to the topic addressed in this thesis. Chapter 2 consists of a review of the current literature regarding functional foods, by-products as novel sources of functional ingredients, probiotics and prebiotics, classification and sources, its mechanisms, currents process of production, characterization and validation methods. Chapter 3 presents a study about the screening of autochthonous LAB and yeast isolates from homemade kefir, and the probiotic and prebiotic properties of these microorganisms through different assays. Chapter 4 addresses the characterization of the physicochemical properties, bioactive compounds, nutritional content and prebiotic potential of berry by-products. Chapter 5 presents a synbiotic formulation based on blueberry bagasse and kefir isolates and its characterization. Finally, based on the results from the present thesis, Chapter 6 summarizes a series of general conclusions and recommendations for future studies.

CHAPTER 2. LITERATURE REVIEW

2.1 Functional foods: trends and sources

Functional foods and ingredients can promote health through prevention rather than treatment, hence these products have received much attention in recent years from the scientific community, food manufacturers and consumers. Consumers interest is linked to perceived benefits and factors that influence purchase such as cost, taste and the product positioning platform used to market foods perceived as “pure,” “basic,” and “natural”. Moreover, some of the trends driving growth in the functional food market include: consumers interested in the prevention of health issues, the increasing cost of healthcare, the steady increase in life expectancy, and the desire of the aging for improved quality of life in their later years (Bogue, Collins, & Troy, 2017). In 2019, the global functional food market size was valued at USD 173.26 Billion and it is expected to reach USD 309.00 Billion by 2027, poised to grow at a compound annual growth rate of 7.5% during the forecast period 2020 to 2027 (Research, 2020).

A functional food is defined as a conventional food or similar in appearance (food matrix, beverage, dietary supplement) consumed on regular basis that contains biologically active components with proven health benefits, offering the potential to reduce the risk of specific chronic diseases or beneficially affect target functions beyond its basic nutritional functions (Doyon & Labrecque, 2008). There are a large number of studies reporting that diets dominated by fruits, vegetables, whole grains and dietary fibres (plant-based foods) prevent and reduce the risk to develop chronic diseases such as cancer (particularly epithelial cancers of the alimentary and respiratory tracts), diabetes and heart diseases. Other sources of functional foods include animals (fish), beverages such as tea, wine and dairy products (Gul, Singh, & Jabeen, 2016).

Some novel foods include foods and food ingredients with a new or intentionally modified primary molecular structure; consisting of or isolated from microorganisms, fungi or algae (Santeramo et al., 2018). The most common consumed items are yogurts (for digestive health), cereals, cholesterol-lowering margarine and butters (for heart health); shakes and bars to reduce hunger or increase energy; and health and energy beverages (Bogue et al., 2017). Most frequently mentioned functional foods are probiotics, prebiotics, soluble fibre, omega-3, polyunsaturated fatty acids, conjugated linoleic acid, plant

antioxidants, vitamins and minerals, some proteins, peptides and amino acids, as well as phospholipids. These foods include bioactive substances, foods supplemented with bioactive substances and derived food ingredients (Grajek, Olejnik, & Sip, 2005).

2.2 Functional foods in gastrointestinal health: Microbiota, probiotics and prebiotics

Functional food components with biological effects are susceptible to be metabolized by intestinal bacteria during the gastrointestinal passage, prior being absorbed. These complex bacterial community (more than 800 species) present in high concentrations in the colon (around 10^{12}-10^{14}) is known as gut microbiota (Laparra & Sanz, 2010). Gut microbiota and host act in a symbiotic manner; both microbiota and microbiome (its whole genome) provides humans with genetic and metabolic features they cannot develop by themselves (Cammarota, Ianiro, Bibbò, & Gasbarrini, 2014).

Microbiota composition is species-specific, and varies among individuals and within the same individual throughout life. These microbial species, dominated mostly by *Firmicutes* and *Bacteroidetes* are essential components to a healthy body-assisting digestion. Further, maintenance of a balanced microbial ecosystem is crucial for gut health as an ecological barrier to the external insults (Wan, Ling, El-Nezami, & Wang, 2019). The human gut has the dual function of nutrient absorption and protection against intestinal pathogens. Beneficial functions are ascribed to the microbiota in the human gut such as fermenting unused energy substrates, preventing growth of harmful, pathogenic bacteria, producing vitamins for the host (biotin, vitamin K) and producing hormones to direct the host to store fats. Moreover, gut microbiota competes for nutrients with potential pathogens and induces the secretion of antimicrobial peptides through interaction with intestinal epithelial cells. The gut microbiota can also stimulate the differentiation and proliferation of epithelial cells, which regulate intestinal homeostasis (Pagliari, Piccirillo, Larbi, & Cianci, 2015).

Regarding the fermentation of substrates, microbiota has enzymes that transform complex polysaccharides of the diet, into monosaccharides and short chain fatty acids (SCFAs), mainly acetic, propionic and butyric. The amount of SCFAs in the colon and in the blood is important for the immunoregulation of the host (Icaza-Chávez, 2013). Also, recent studies have shown that human gut microbiota is also involved in breakdown of various phenolic compounds or polyphenols, consumed in the diet and found in a variety of plants,

fruits and plant derived products. Polyphenols which usually remain inactive in diet are bio transformed to active compounds after removal of the sugar moiety by the gut microbiota, among other factors (Jandhyala, 2015).

Experiments conducted in germ-free animals demonstrate that microbiota colonization in early life is necessary for optimal development of the immune system. In the absence of microbiota, intestinal mucosal immunity is underdeveloped and animals present smaller mesenteric lymph nodes and reduced numbers of immune cells, resulting in a weakened capacity to fight off pathogenic bacteria. It is not clear exactly how microbial composition regulates immune homeostasis and mucus properties of the intestinal barrier. However, some studies show that the presence of specific bacteria species and the microbe stimulation of intestinal cells can shift immune responses, by favoring the development of certain subtypes of lymphocytes and increasing the production of proteins involved in host responses and proteins of core mucus (Takiishi, Fenero, & Câmara, 2017).

The balance of the microbial community can be disturbed by either loss of diversity, thriving of pathobionts, or withering of commensals. Gut dysbiosis refers to alterations in the composition and function of the gut microbiota that have harmful effects on host health changing the metabolic activities of the microorganisms, or causing changes in their local distribution (Yoo, Groer, Dutra, Sarkar, & McSkimming, 2020). These changes in composition or function of gut microbiota may lead to acute or chronic disease states and syndromes. Among the gastrointestinal conditions linked with altered gut microbiota are acute diarrhea, irritable bowel syndrome, and inflammatory bowel diseases (Aziz, Doré, Emmanuel, Guarner, & Quigley, 2013).

Environmental and host factors such as antibiotics, genetics and maternal transfer have a considerable effect on the gut microbial composition; diet itself is a factor that alters the composition of the gut microbiota, altering growth and/or metabolic activity of these bacteria in the colon thereby, its potential health effects on the organism. Novel approaches have been suggested to maintain homeostasis; the manipulation of intestinal microbiota through the use of probiotics and prebiotics can regulate immune and inflammatory responses that balance the microbial composition (Maslowski & Mackay, 2011).

2.3 Probiotics: definition, criteria and classification

Probiotics are defined as “live microorganisms that, when administered in adequate amounts, confer a health benefit on the host”. The most studied bacterial species as probiotics are *Bifidobacterium* (*adolescentis, animalis, bifidum, breve* and *longum*) and *Lactobacillus* (*acidophilus, gasseri, helveticus, johnsonii*), *Lacticaseibacillus* (*casei, paracasei*), *Limosilactobacillus fermentum, Lactiplantibacillus plantarum, Lacticaseibacillus rhamnosus and Ligilactobacillus salivarius*. Several studies suggest benefits often associated with probiotics are supporting a healthy digestive tract and a healthy immune system (Hill et al., 2014). Further, some of these microorganisms originate from the intestinal tract of humans or other animals and are used as starters in dairy products, providing fermented products with longer preservation time than the raw product and also adding beneficial nutritional and health effects on consumers. To provide these effects the probiotic product must contain a minimum level of probiotic bacteria (generally $1x10^6$ CFU/mL) (Drakoularakou, Rastall, & Gibson, 2011).

Probiotic strains must be sufficiently characterized and named according to the International Code of Nomenclature, since some probiotic activities might be strain specific. Proper strain designation is therefore composed of the official genus, species (and subspecies) names, followed by a strain designation which could be the catalog number of a recognized culture collection or a commercial strain designation. Also, probiotic strains need to be safe for the intended use, supported by at least one positive human clinical trial conducted according to generally accepted scientific standards and alive in sufficient numbers in the product at an efficacious dose throughout shelf life (Binda et al., 2020).

Some of the criteria considered useful for establishing safety of probiotics are: record the isolation history and taxonomic classification of the candidate probiotic, controls in industrial processing that eliminate contamination of the probiotic with microbes or other substances, dose administered, absence of allergenic material, transferable antibiotic resistance genes and absence of association of the probiotic with infectivity or toxicity, assessed at the strain level. Also, physiological status of the consuming population, special consideration must be made for use in vulnerable populations, including newborn infants and the critically ill (Sanders et al., 2010).

Probiotic organisms can be ingested in fermented foods and/or supplements. Fermented foods can be of both dairy and vegetable origin, while probiotic supplements

consist of freeze-dried (lyophilized) bacteria in powder, capsule, or tablet form. Also, as mentioned before, for clinical efficacy, products containing probiotic organisms must provide live organisms in sufficient numbers to exert therapeutic effects (Khalighi, Behdani, & Kouhestani, 2016). Dairy products such as yogurts, fermented sour milk and cheese remain at the forefront of probiotic food development at present. Another sources of probiotic foods available in the market at present and considered as non-dairy are soy products, cereal based products, fruit and vegetable juices, and fermented meat and fish (Ranadheera, Vidanarachchi, Rocha, Cruz, & Ajlouni, 2017).

2.4 Prebiotics: definition, criteria and classification

Probiotics can settle and multiply into the gut through provision of prebiotic substrates such as soluble fibres, also administered as a combination of both probiotics and prebiotics, known as synbiotics; moreover, available as foods, dietary supplements or therapeutics (Vyas & Ranganathan, 2012). The current definition of prebiotic, proposed by The International Scientific Association for Probiotics and Prebiotics, is “a substrate that is selectively utilized by host microorganisms conferring a health benefit”. Not growth substrates like vitamins, minerals or antibiotics are not considered prebiotics, because they do not use mechanisms involving selective utilization by host microorganisms (Gibson et al., 2017).

The criteria used to classify a food ingredient as a prebiotic are: (i) it has to resist gastric acidity, (ii) it neither be hydrolyzed by mammalian enzymes, nor absorbed in the upper part of the gastrointestinal tract; (iii) it has to be selectively fermented by intestinal bacteria, stimulated to grow and (iv) become metabolically active altering colonic microflora towards a composition associated with health and well-being (*HANDBOOK OF PREBIOTICS.*, 2019) (Kolida, Tuohy, & Gibson, 2002).The majority of identified prebiotics are carbohydrates, such as dietary fibre; evidence from *in vitro* and *in vivo* studies suggest oligosaccharides as the prebiotics with most fulfilling criteria, because of the extensive and advanced research (Rastall & Gibson, 2015). These includes fructooligosaccharides (FOS), galactooligosaccharides (GOS), isomaltooligosaccharides (IMO), xylooligosaccharides (XOS), transgalactooligosaccharides (TOS), and soybean oligosaccharides (SBOS) (Paulina Markowiak & Katarzyna Śliżewska, 2017)

Oligosaccharides are found in human breast milk and cow´s milk, as well as in several vegetables (asparagus, onions, garlic) as fructans and in soybeans as stachyose (Watson & Preedy, 2016). Lactulose is a semi-synthetic disaccharide that has a proven prebiotic effect by promoting the growth of beneficial bacteria and treating constipation (Pranami et al., 2017). Among prebiotic polysaccharides is pectin, several studies demonstrate that pectin from different sources such as seaweed, soy or citrus, is capable to be fermented and produce metabolites involved in gut health; being promising fibre sources and pectin a potential prebiotic ingredient (Tingirikari, 2019).

Other polysaccharide examples are glucans, guar gums and resistant starches; beta-glucans are soluble compounds located in the endosperm cell walls of oat, barley and other cereals, also mushrooms and have demonstrated beneficial effects to human health especially due to their immune-stimulatory effects (Lam & Chi-Keung Cheung, 2013). Guar gum is formed in the endosperm of the plant *Cyamopsis tetragonolobus*; resistant starches are not digested in the upper gastrointestinal tract, some of them are found naturally in foods, while others are synthetic (Carlson, Erickson, Lloyd, & Slavin, 2018).

From the redefinition of what a prebiotic is, other substances are contemplated, among them polyphenols: bioactive compounds abundant in plants, they constitute a family of over 8000 phytochemicals and they come from various plant sources including green tea, red wine, cranberry, blueberry, grape seed, aloe vera, agave, peaches, garlic, among others (Westfall, Lomis, & Prakash, 2018). They are classified into flavonoids and non-flavonoids (phenolic acids, stilbenes, and lignans) and when reaching the colon, they come in direct contact with the gut microbes, resulting in a complex and multidirectional interaction, showing potent prebiotic activity (Van Hul & Cani, 2019). Moreover, some substances like growth factors, proteins, polyunsaturated fatty acids (PUFA), organic acids and bacterial metabolites have been proposed as prebiotic substances because they can be used to potentiate the effect of probiotics (Bomba et al., 2002). However, further investigation needs to be done to achieve prebiotic criteria. The proposed prebiotic classification is illustrated in Figure 1.

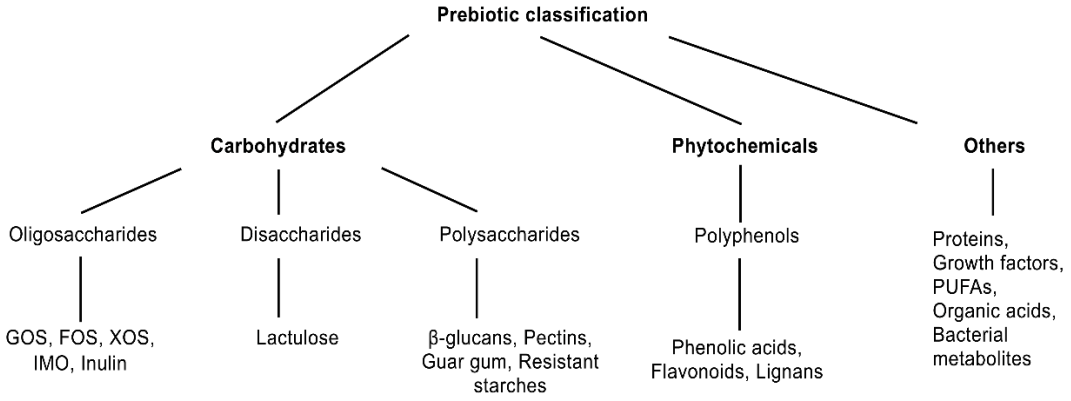


Figure 1. Prebiotic classification: carbohydrates, phytochemicals and others. GOS, galactooligosaccharides; FOS, fructooligosaccharides; XOS, xylooligosaccharides; IMO, isomaltooligosaccharides; PUFAs, polyunsaturated fatty acids. From Hurtado-Romero et al. (2020).

2.5 Probiotic and prebiotic mechanisms in human health and gut microbiome

2.5.1 Colonization, adhesion to intestinal mucosa and pathogen exclusion

Adhesion to intestinal mucosa is necessary for colonization of microorganisms and is important for the interaction between probiotic strains and the host, specifically, the epithelial cells (ECs) and mucus. ECs secrete mucin, a complex mixture of proteins that prevent the adhesion of pathogen bacteria. In addition, the mechanism of exclusion is the result of different properties of probiotics to inhibit pathogen adhesion, including the stimulation of ECs and the reduction of pH. Competitive exclusion by intestinal bacteria is based on a bacterium-to-bacterium interaction mediated by competition for available (Bermudez-Brito, Plaza-Díaz, Muñoz-Quezada, Gómez-Llorente, & Gil, 2012).

Several *Lactobacillus* proteins have been shown to promote mucous adhesion, bacteria display surface adhesins that mediate attachment to the mucous layer. Moreover, when lactobacilli are ingested, they compete for binding sites, leaving less binding sites open for pathogens, which pass through gut leaving the body sooner when no binding sites are available. These bacteria present in the gut also utilize more nutrients than pathogenic bacteria, which may suffer starvation, and not survive. Thus, the competitive exclusion also

takes place inhibiting the pathogens by consuming the nutrients and energy source which pathogens need (Bajaj, Claes, & Lebeer, 2015). For instance, *Lactobacillus reuteri* has shown to produce strain-specific adhesins including mucus-binding proteins and also other surface proteins that may contribute to the adhesion properties of the strain (Jensen et al., 2014).

Moreover, prebiotics are selectively fermented in the gut stimulating proliferation of *Lactobacillus* and *Bifidobacterium,* bacteria generally regarded as safe because they mainly ferment carbohydrates and are not pathogenic nor toxigenic; besides they have a role in colonization resistance (Steed & Macfarlane, 2009). For instance, bifidobacteria competes with pathogens for adhesion in intestinal epithelium; proliferation of species such as *Escherichia coli, Salmonella, Shigella, Campylobacter jejuni* and *Clostridium perfringens* are depressed by various strains of bifidobacteria (Khare et al. 2018), indicating that the decrease in the number of harmful bacteria is related to bifidogenic inhibition by lowering the luminal pH or producing bacteriocins. Recent studies of potential prebiotic polyphenols, particularly anthocyanins found in elderberry extracts, showed an inhibition of the pathogens *Bacillus cereus* and *L*isteria *monocytogenes* and also promote the growing of probiotic strains *Lactiplantibacillus plantarum* and *Lacticaseibacillus rhamnosus*; suggesting these compounds and their metabolites may exert a positive modulation on the intestinal bacterial population (Coman et al., 2018).

In addition, it has been found that some species of phylum *Firmicutes* found in the microbiota can utilize the galacturonide oligosaccharides derived from sugar beet pectin to produce anti-inflammatory compounds suggesting that it may have potent effects *in vivo* (Chung et al., 2017). With these mechanisms, prebiotics are believed to improve the intestinal barrier by protective bacteria that upregulate epithelial defense mechanisms that protect against intestinal inflammation *in vivo*, restoring the intestinal epithelial integrity and by increasing mucus production (Looijer–Van Langen & Dieleman, 2009).

2.5.2 Production of nutrients, enzymes and antimicrobial substances

Bacteria that colonize the intestine possess a far wider diversity of genes and a larger repertoire of degradative enzymes and metabolic capabilities than their hosts. In addition, the fermentation of complex carbohydrates in the intestine involves interactions between community members that include both nutritionally specialized and widely adapted species.

Bacteroidetes, a group of dominant species in the gut, possess very large numbers of genes encoding carbohydrate active enzymes allowing microorganisms to switch readily between different energy sources in the gut depending on availability (Flint, Scott, Duncan, Louis, & Forano, 2012). For instance, production of proteolytic enzymes by bifidobacteria and LAB is important for utilization of protein components within the nutrient media used for manufacturing probiotics. It was reported that *B. adolescentis, B. longum, B. bifidum*, and *Lactobacillus sp*. produced extracellular proteolytic enzymes active in hydrolyzing proteins under neutral, acid, and alkaline conditions. The increased production of proteolytic enzymes correlated also with a high growth activity (Novik, Samartsev, Astapovich, Kavrus, & Mikhalyuk, 2006). In addition, another study revealed that the potential probiotic*, Lactobacillus sp*. G3_4_1TO2 produced amylase enzyme, similar results were on different strains including *L. plantarum* strains and *L. fermentum* (Padmavathi, Bhargavi, Priyanka, Niranjan, & Pavitra, 2018).

Several probiotic bacteria produce a variety of antimicrobial compounds such as bacteriocins and short-chain fatty acids, that may enhance their ability to compete against other microbes. Bacteriocins are ribosomally synthesized antimicrobial peptides, they have a narrow spectrum of activity and they are bactericidal in nature. Its bactericidal mechanism of action is located in the cytoplasmic membrane region of receptor binding on bacterial surfaces. These bacteriocins are non-toxic peptides, sensitive to proteases compared to antibiotics and its defensive role helps probiotic bacteria to occupy a specifc niche and also limits the advancement of pathogens to neighboring cells (Indira, Venkateswarulu, Abraham Peele, Nazneen Bobby, & Krupanidhi, 2019).

Short-chain fatty acids (SCFAs) are metabolites produced by the fermentation of prebiotic functional ingredients by probiotic bacteria (along with ethanol and carbon dioxide). These SCFAs are: lactate, pyruvate and acetate, which are used by other colon bacteria as starting units for the synthesis of propionate and butyrate (Fernández et al., 2016). The most abundant SCFAs in gastrointestinal tract are acetate, propionate and butyrate. Acetate and propionate are mainly produced by *Bacteroidetes* whereas *Firmicutes* are the primary contributors for butyrate (Feng, Ao, & Peng, 2018). Some examples are shown in Figure 2.

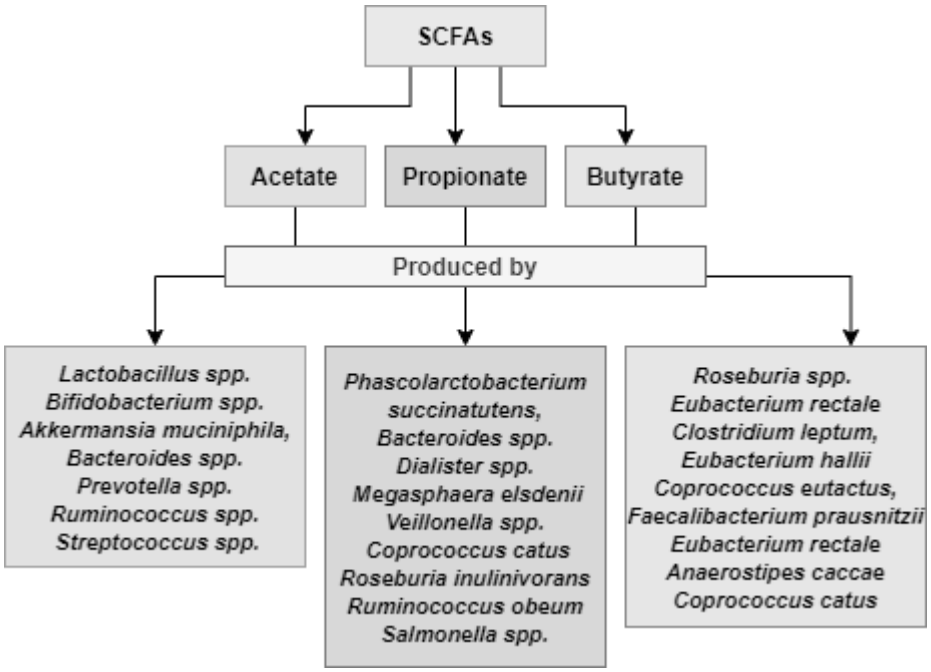


Figure 2. Most abundant short chain fatty acids (SCFAs) produced by principal colon microbiota microorganisms.

The concentration of SCFAs depends on the composition and size of the population of intestinal microorganisms, genetic factors, environmental factors and the diet conditioning access to appropriate substrates. SCFAs are as energy substrates for colonocytes and regulate intestinal barrier function (synthesis of mucin-MUC2) and immune system through G-protein-coupled receptors. Also, play a very important role in regulating pH, increasing the amount of mucus produced and the speed of blood flow; increasing the absorption of calcium, iron, as well as magnesium, being beneficial for glucose and protein metabolism in the liver (Markowiak-Kopeć & Śliżewska, 2020).

Among SCFAs, butyrate, primarily derived from the fermentation of dietary fibres and resistant starches by the colonic microbiota, is of special interest due to its ability to affect growth and differentiation of colonocytes and show beneficial effects by preventing colonic cancer (McNabney & Henagan, 2017). Another effect includes the induction of immunosuppressive molecules and apoptosis in tumor cells, overexpression of detoxifying enzymes, enhancement of mucosal barrier function and anti-inflammatory properties (Fernández et al., 2016).

Literature has reported effects of fibre supplements (resistant starches and inulin) on the structure and function of bacterial communities in the gut. These supplements led to an increase in *B. faecale/adolescentis/stercoris* and *R. bromii,* but the increase is not always associated with a change in fecal butyrate from bifidobacteria, suggesting bifidobacteria do not establish cross-feeding reactions with butyrogenic populations as readily as *Ruminococcus*, which yield higher butyrate concentrations, potentiated by butyrate-producing species in microbiota such as *Eubacterium rectale* (Baxter et al., 2019). A recent study suggests that low doses of 6 g per day of prebiotics (indigestible dextrin, α-cyclodextrin and dextran) can increase the generation of acetate and propionate in an *in vitro* system by the fermentation of bacteria belonging to the *Bacteroidetes* and *Actinobacteria* phylum, showing that prebiotics are capable of activating the metabolism of colonic microbiota even at low doses (Sasaki et al., 2018).

Moreover, it has been observed that intestinal degraded products of phenolic short chain fatty acids are capable of reducing colonic cancer risk. For instance, polyphenols and their metabolites by fecal bacteria, are potent in suppressing the growth of bacterial pathogen, such as *C. perfringens* and *Bacteroides* with a minimal effect on desirable *Bifidobacterium* and Lactobacillus strains (Thilakarathna, Langille, & Rupasinghe, 2018).

2.5.3 Modulation of immune system

As mentioned before, modulation of the immune response associated with consumption of specific probiotics can occur by different indirect mechanisms such as regulation of the intestinal epithelium and mucus secretion, competition within bacterial ecosystem via secretion of antimicrobial compounds but also via innate and adaptive immune system. These mechanisms can be easily assessed in *in vitro* setting and therefore represent valid tools to study the properties of newly discovered bacteria strains (La Fata, Weber, & Mohajeri, 2018).

The gut microbiota modulates the immune system producing molecules with immunomodulatory and anti-inflammatory effects due to the probiotic interaction with epithelial cells, dendritic cells and with monocytes/macrophages and lymphocytes. Cytokines stimulated by probiotic bacteria lead to the expression of Treg cells that maintain the immune homeostasis in the gut mucosa. After probiotic stimulation, macrophages distant from the gastrointestinal tract such as peritoneum and spleen, increase their functionality

reinforcing the innate immune response. Moreover, in malnutrition processes, the probiotic administration contributes to restore the thymus histology and stimulates the adaptative immune response (Maldonado Galdeano, Cazorla, Lemme Dumit, Vélez, & Perdigón, 2019).

For instance, different strains such as *B. breve*, *L. rhamnosus*, and *L. casei* induce different amounts of cytokine production in human and mouse primary immune cells. Moreover, several reports demonstrate production of interleukins, activation of toll-like receptors and regulation gene expression and signaling pathways in the host cells (Plaza-Diaz, Ruiz-Ojeda, Gil-Campos, & Gil, 2019).

Prebiotics influence the immune system directly by the metabolites that are products of fermentation or indirectly by altering microbial composition of the gut. An example of an indirect mechanism is the exposure to commensal microbes that rapidly expands the number of mucosal lymphocytes, also a significant increase in serum immunoglobulin levels and the induction of regulatory T cells in gut lymphoid follicles (Guarner, 2013). Directly, microbial products (SCFAs, defensins, bioactive peptides) interact with immunocompetent cells modifying their activity. For instance, studies have demonstrated that the fermentation of fructans in gut modulates immune response by the production of SCFAs; the fatty acids bind to SCFAs receptors on gut-associated lymphoid tissue immune cells, activating G protein-coupled receptors (GPR) and subsequently, affecting the recruitment of leukocytes to inflammatory sites and suppressing the production of proinflammatory cytokines and chemokines (Franco-Robles & López, 2015).

2.6 Probiotics in industry: characteristics, production and examples

In order to provide consumers with products that provide meaningful levels of probiotics, several requirements have to be fulfilled by probiotics selected for industrial production. One is the documented health benefits, reported abilities like viability and survival throughout the manufacturing process, colonization and immunogenicity properties, among others. Second, the technological properties of the strain such as: safety, genetic stability, ability to be produced at large scale, stability in the fermented product and neutral or positive contribution to flavor or taste (Forssten, Sindelar, & Ouwehand, 2011).

For a probiotic product to be effective, it must maintain the viability of the strain during its manufacture, distribution and storage. For instance, a person should consume at least 100 g (containing at least 10^8 to 10^9 viable cells) of probiotic cultures in order to meet the minimum required effective concentration (at least 10^6 CFU/g) to show beneficial health effects. Immobilization, encapsulation or microencapsulation of viable cells is an important technique for preserving the viability of probiotic bacteria, helping to protect the probiotic microbes from conditions such as changes in pH, temperature and various harmful microbial attacks (Dalli, Uprety, & Rakshit, 2017). Immobilization is defined as the process of attaching a cell or entrapping it within a suitable inert material (called a matrix), while encapsulation technology is based on packing solid, fluid or gas compounds in milli-, micro- or nano-scaled particles which release their contents upon applying specific treatments or conditions. For engineering probiotic containing capsules, a coating is usually employed which can withstand acidic conditions in the stomach and bile salts from the pancreas after consumption (Manojlović, Nedović, Kailasapathy, & Zuidam, 2010).

The manufacturing processes of probiotics have the following steps generally: a single pure strain is used in a limited number of sequential seed fermentations to achieve the desired inoculum volume, then is transferred to the main fermentation vessel for growth (a blend of water, nitrogen sources, carbohydrates, salts, and micronutrients). Then cells are concentrated by separating the cells from spent medium through centrifugation. Pellets are frozen and dried (trough lyophilization, freeze-drying or other) to a defined particle size, the milled material can then be used for blending with excipients to make finished formats such as capsules. Moreover, there are many food formats that can successfully incorporate probiotics and deliver them at the required dose and usually involve experimentation to find the optimal formulation and strain combination (Fenster et al., 2019).

2.6.1 Functional dairy and non-dairy products

Dairy products are the most common formats in which probiotic foods are available. Among dairy, the major products are fermented milk, yoghurts, buttermilk, and kefir. Kefir is a fermented milk drink with an acidic taste and creamy consistency produced by bacterial fermentation of kefir grains. The grains contain a mixture of LAB (*Leuconostocs, Lactobacilli, Streptococci, Lactococci, Enterobacter, Acinetobacter*, *Enterococcus, and Pseudomonas spp.),* acetic acid bacteria and yeasts (*Kluyveromyces, Candida, Torulopsis,*

Saccharomyces, Rhodotorula and Zygosaccharomyces), which coexist and interact to produce a unique fermented dairy product. The starter culture employed in kefir production exhibits a significant effect on its viscosity and on chemical composition (Farag et al., 2020).

During fermentation, the kefir grains increase in size and number, and are usually recovered from the fermented milk and reutilized, they are able to retain their activity for years if carefully preserved. The interaction between yeast and LAB can be stimulated or inhibited by the growth of one or both, in co-cultures. For instance, due to its high capacity to metabolize lactose the genus *Lactococcus* tends to grow faster than yeast in milk, hydrolyzing lactose, producing lactic acid and a making a suitable environment for yeast growth. Thus, yeasts are able to grow and synthesize vitamins and hydrolyze milk proteins (Leite et al., 2013). In kefir grains the main polysaccharide is kefiran, a heteropolysaccharide composed by equal proportions of glucose and galactose. Kefiran improves the viscosity and viscoelastic properties of acid milk gels and is able to form gels that have interesting viscoelastic properties at low temperatures. Compared with other polysaccharides, kefiran has outstanding advantages such as antitumor, antifungal, antibacterial properties (Prado et al., 2015).

Yogurt is a fermented milk product that has been prepared traditionally by allowing milk to sour at 40– 45° C, S. *thermophilus* and *L. bulgaricus* are used as starter cultures and exhibit a symbiotic relationship during the processing of yogurt. In recent years, yogurt products have been reformulated to include live strains of *L. acidophilus* and species of *Bifidobacterium* in addition to the conventional yogurt organisms, *S. thermophilus* and *L. bulgaricus*. Therefore, yogurt contains live probiotic microorganisms (Lourens-Hattingh & Viljoen, 2001).

Cheese is the generic name for a group of fermented milk-based food products, manufactured throughout the world in a great diversity of flavours, textures and characterized by the conversion of milk, into a solid material, the curd. Curd is modified by processes such as pressing, salting and ripening. Cheese has a dense matrix and relatively high fat content that may offer additional protection to probiotic bacteria in the stomach (Gomes da Cruz, Alonso Buriti, Batista de Souza, Fonseca Faria, & Isay Saad, 2009). Numerous strains of probiotic bacteria have been successfully added into different types of cheeses including lactobacilli (*Lactobacillus acidophilus, Lacticaseibacillus casei,*

Lacticaseibacillus paracasei, Lactiplantibacillus plantarum, Lacticaseibacillus rhamnosus, and Lactobacillus gasseri) and Bifidobacterium spp. *(Bifidobacterium animalis ssp. lactis, Bifidobacterium longum, Bifidobacterium bifidum, and Bifidobacterium infantis),* and to a lesser extent*, Propionibacterium freudenreichii ssp. Shermanii* (Karimi, Mortazavian, & Da Cruz, 2011).

Nondairy probiotics are available both in the form of beverages and fermented foods. Fermented vegetables include sauerkraut, kimchi and Suan Tsai or Suan Cai or Chinese fermented vegetables among others. Spontaneous fermentation of these products is carried out by natural microorganisms of cabbage, cucumber, radish or green onion and includes LAB species (*Leuconostoc*, *Lactobacillus*, and *Pediococcus*). Production of probiotic sauerkraut by incorporating *Leuconostoc mesenteroides* LMG 7954 and *Lactiplantibacillus plantarum* L4 probiotic cultures during controlled fermentation of cabbage has been reported. Also, novel probiotic species namely *Lactobacillus harbinensis* sp. nov. has been isolated from conventional “Suan cai” of China (Bansal, Mangal, Sharma, & Gupta, 2016).

Fruit juices have also been suggested as an ideal substrate for the development of non-dairy probiotic beverages due to the nutritional content including vitamins, antioxidants and polyphenols, they also offer several advantages for the growth and survival of probiotic microorganisms. Cereals like oats and rice have also been used for the production of probiotic beverages. LAB and *Bifidobacterium* convert starchy materials of rice to malto-sugars and enrich the final product with antioxidants and bioactive substances. Soy milk is the most commonly used alternative to milk, as a cheap substrate for the production of probiotic products (Kandylis, Pissaridi, Bekatorou, Kanellaki, & Koutinas, 2016).

The elaboration of bakery products including probiotics in their formulation, requires a different approach due to the high temperatures in which they are baked. An alternative to provide a benefit to its consumers is sourdough technology. Sourdough is as a mixture of wheat or rye flour and water that is fermented by LAB, with or without yeasts; benefits associated to these technology include a wider range of aroma, flavor, and texture in the product, increased shelf-life by a higher content of organic acids and enrichment with compounds originated from either biotransformation such as: proteins, essential amino acids or essential short chain fatty acids (Longoria-García et al., 2018).

In meat sector, development of probiotic meat products comprises the elaboration of probiotic sausages. Fermented sausages can be potential candidates for probiotics since they are subjected to mild heating, probiotic strains such as *Lactobacillus* or *Bifidobacterium* are used as started cultures and does not affect the flavor. Moreover, the meat matrix may enhance the survival of bacteria in the digestive system (Khan et al., 2011). Meat spread products fermented with probiotic LAB (*Lacticaseibacillus rhamnosus* FERM P-15120) and dry sausages such as salami have also been proposed due to its process of fermentation without heat treatment (Keizo Arihara, 2006). Applicability of strains like *L. gasseri* JCM1131 and *L. rhamnosus* GG, LC-705 and VTT-97800 was tested on sausage fermentation with the result that strains GG and E-97800 were found to be suitable for use as probiotic starter cultures in fermenting dry sausage (K. Arihara & Ohata, 2011).

Additionally, several applications for probiotic edible films such as fruits and vegetables, bakery and confectionery products, olives, dairy products, fishery products, cereal bars, and meat products have been studied being a promising strategy to enhance probiotics 'survival during storage time (Zoghi, Khosravi-Darani, & Mohammadi, 2020).

2.6.2 Isolation and characterization of probiotic microorganisms

Currently, significant development exists in the market of nutritional functional foods. The analysis of probiotic properties can be used to design products that can serve a wider audience of people; further, research and characterization of the strain serves to easily incorporate it into food matrixes, depending the nature of the strain. Moreover, to promote synergy between probiotics and foods containing proteins, vitamins, minerals and bioactive compounds. The development of modern probiotics requires several activities in different stages including sampling from a specific source, strain identification, growth capacity, resistance to the passage through the stomach and upper intestine, safety evaluation, *in vitro* and *in vivo* studies and clinical investigations (Del Piano et al., 2006).

A reliable probiotic product requires correct identification of the bacterial species used, strains should be identified using currently available methods, mainly divided in Polymerase Chain Reaction (PCR) based methods (Specific Polymerase Chain Reaction, Randomly, Amplified Polymorphic DNA, Real Time PCR, PCR-Denaturing Gradient Gel Electrophoresis, Multiplex PCR), non PCR-based techniques (DNA-DNA Hybridization,

Pulsed Field Gel Electrophoresis, Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis), and combination of two or more methods (Amplified Fragment Length Polymorphism and Restriction Fragment Length Polymorphism). In regard to growth capacity and physiological characteristics, carbohydrate fermentation and enzymatic activity profiles have been used widely to select the specific substrates or enzymatic activities relevant to the expected functional effects of the strain (Bagheripoor-Fallah, Mortazavian, Hosseini, Khoshgozaran-Abras, & Rad, 2015) (Gueimonde & Salminen, 2006).

Metabolically active bacteria must overcome the gastrointestinal barrier and transitorily persist to exert their beneficial effects. Most important characteristics are: tolerance to an extremely low pH (1·5–3·0), gastric enzymes, bile salts and other intestinal enzymes. Thus, various *in vitro* assays have been designed to mimic these stress conditions. *In vitro* systems such as controlled incubations in real or simulated gastric juices, chemical and/or enzymatic media (pH 1·0–4·0), have been preferentially used in the evaluation of new probiotic strains.

In the case of biliary salts resistance, which facilitate the digestion of lipophilic compounds and behave as an antimicrobial agent, in vitro assays are conducted in 0·3–0·7 % bovine bile (Oxgall) for 60–180 min. Probiotics show highly variable resistance to acid and bile salts, and this characteristic is both species and strain dependent (Fontana, Bermudez-Brito, Plaza-Diaz, Muñoz-Quezada, & Gil, 2013).

In regard of safety, although no universal international standard for safety evaluation of probiotics is available, in the United States, bacteria considered safe for human consumption are awarded as GRAS (Generally Recognized as Safe), status given by the Food and Drug Administration. Every probiotic supplement or product must contain probiotic strains that are GRAS. Moreover, the European Food Safety Authority has proposed an introduction on the “Qualified Presumption of Safety (QPS),” which could be applicable probiotics based on four considerations: taxonomic level or grouping for which QPS is sought; familiarity or knowledge enough to reach a decision on their safety and pathogenicity or information about virulence determinants or toxigenic potential to exclude pathogenic strains. Finally, whether viable organisms enter the food chain or whether they are used to produce other products (Shokryazdan et al., 2017). Various different in vitro and in vivo approaches have been used to measure the efficacy of probiotics and also prebiotics, so this approach is discussed in depth in later sections of this literature review.

2.7 Prebiotics in industry: characteristics, production and examples

Prebiotics are part of the trending of functional foods with additional new nutrients or components not normally found in a particular food (labeled enriched products) being inulin and oligofructose among the most studied and well established (Bigliardi & Galati, 2013). They are also used as components to fortify commonly consumed foods, they improve sensory features like freshness and provide a more well-balanced nutritional composition because it is often use as dietary fibre and added as a low energy ingredient; their solubility allows fibre incorporation to liquid systems such as drinks and dairy products. In addition, prebiotics have gelling properties that improve low fat foods maintaining the emulsion and water hold without any adverse effect on taste or texture (Al-Sheraji et al., 2013).

For prebiotics to serve as functional food ingredients, they must not affect negatively the organoleptic properties of the product and be chemically stable to food processing treatments, such as heat, low pH, and Maillard reaction conditions (Wang, 2009). If there is a chemical alteration and/or a degradation of the prebiotic into its mono and disaccharide components, it would not be available for bacterial metabolism nor provide selective stimulation of beneficial microorganisms or exert their mechanisms to improve health.

Currently, the production of prebiotic ingredients on a large scale is limited to extraction from plants by classical methods with enzymes, hot and supercritical water and chemical hydrolysis. The enzymatic hydrolysis is the most common method due to its reproducibility and high yield. However, a novel approach has been introduced recently to produce prebiotic ingredients with thermal and nonthermal innovative technologies such as high hydrostatic pressure, ultrasound, microwave, extrusion and drying. These technologies have minimal effects on the color, aroma, taste, and nutritional value of food products due to the use of a shorter processing time than the conventional showing a great potential to improve the extraction process, and to modify the extracted compounds (Hurtado-Romero, Del Toro-Barbosa, Garcia-Amezquita, & García-Cayuela, 2020).

Prebiotics incorporate to different products of the food industry such as bakery products, dairy foods, beverages, meat and livestock feed, among others. Many studies

have proven prebiotics add value and improve nutritional and sensory characteristics; also, they can remain in appropriate conditions during the processing and storage (Rolim, 2015).

Regarding bakery products, prebiotics act mainly as fat and sugar replacers in bread, biscuits, pasta, among others; they also have several effects on the rheology and baking quality. The addition of fibres like cellulose, wheat bran, and oat hulls for bread making has an impact on the rheological properties of the dough and the final product. The changes include: increase or decrease in water absorption, an increase in mixing time, a decrease in loaf volume, gritty texture, taste and mouthfeel, and a decrease in softness (S. & P., 2014).

Bran addition in high levels can be used in bread to give functionality while still achieving acceptable sensory results. Also, the addition of FOS to cookies for an enrichment and sugar replacement has demonstrated an improvement in the physical properties of the cookie, including the decrease in hardness and a higher concentration of dietary fibre(Longoria-García et al., 2018). Several pretreatments of β-glucan preparations can cause better technological properties of bread with β-glucan addition, such as texture, color, and rheology. The best performing method reported was boiling the β-glucan preparation following dough addition. The industrial application of boiling the β-glucan preparation before its use in bread making increases the fortified bread quality and has a higher content of β-glucan in the final product (Kurek et al., 2018).

In the dairy food sector, inulin has been widely used because of its textural properties, which are similar to the creaminess of fat. For instance, rheological and textural properties like the spreadability of processed cheese with low content of fat can be positively affected by inulin as a fat replacer, in addition to enhancing the beneficial health effects (L.L. Ferrão et al., 2016). It can also increase viscosity and stabilize emulsion systems, which is desirable for products like yogurt. The addition of prebiotics improves the chemical, microbiological, and organoleptic properties of yogurt. For instance, the literature reported a yogurt produced with raffinose. It preserved its physicochemical characteristics and maintained the viability of probiotic culture after four weeks of storage. Also, when combining yogurt with fruit, polyphenol-rich fruits can have prebiotic effects in yogurt, maintaining the survival of probiotic bacteria in yogurt and providing additional substrates for growing activity once in the colon (Pop et al., 2019).

Moreover, the addition of the prebiotics β-glucan and Hi-maize (resistant starch) at 1.5% in ABY-type probiotic yogurt (*Lactobacillus acidophilus* LA-5, *Bifidobacterium animalis* ssp. lactis BB-12 and yogurt bacteria), provided better rheological, syneresis and sensory properties; and also, enhanced the viability of probiotics immediately after fermentation and during the refrigerated storage (Heydari et al., 2018).

Inulin added to baby milk formula has shown positive results in the reduction of *Clostridium* bacteria and Gram-positive cocci, while increasing the number of bifidobacterial. This demonstrates a positive modulating effect on the intestinal ecosystem. Furthermore, short- and long-chain inulin have been added to flavored milk, optimizing consistency and sweetness, and leading to better acceptability of the product (Abed, S. M., Ali, A. H., & Noman, A., n.d.). Another example is the development of a new prebiotic dairy dessert containing oat, wheat and corn flour, inulin and stevioside, which exhibited appropriate sensory characteristics and a considerably lower content of sugar and fat, compared to the commercial ones (Ghanbari et al., 2017). Finally, the addition of XOS to cream cheese has been shown to improve the physical-chemical characteristics, reducing consistency, fluidity, viscosity, and grain size; increasing elasticity, firmness, and melting capacity. Improvements in sensorial characteristics, such as an increment of sweet and acid flavors, increase of homogeneity, and reduction of bitterness were also observed (Luana L. Ferrão et al., 2018).

Some prebiotics, mainly FOS, GOS, and inulin, are currently used in beverages (like tea, coffee, soft drinks, health drinks, powder beverages, and alcoholic and dairy beverages) to replace sugar or lipid functionalities in low-calorie beverages. They can be dissolved into the liquid matrix by mechanical mixing before or after any thermal treatment. An example of this is the powder beverages that require a spray-drying process. Carbohydrates, gums, or proteins are used as helpers to facilitate the drying, maltodextrin being one of the most used drying-aid agents (Sebastián, Carlos Ariel, & Carlos Eduardo, 2019). Soy-based synbiotic beverages made of a soy hydrosoluble extract containing FOS are a suitable substrate for the growth of probiotic bacteria. GOS is also used in beverages because it forms clear solutions and does not alter the viscosity of the product. Oligosaccharides sustain high processing temperatures and are also stable under low pH conditions (Singla & Chakkaravarthi, 2017).

Some examples of prebiotic components that can be applied in meat emulsions are: FOS, inulin, resistant starch, and polydextrose. Inulin has been added to products like sausages and meatballs and along with polydextrose shows good performance as a fat substitute due to their ability to form a gel and improving texture by replacing fat. Resistant starch can also be easily incorporated due to its microparticulate structure that does not affect the appearance of the final product. Another ingredient is FOS, which shows a neutral taste, stability over a wide pH and temperature range (Felisberto, Galvão, Picone, Cunha, & Pollonio, 2015).

Resistant starch can also be easily incorporated without affecting the appearance of the final product due to its microparticulate structure. Lastly, FOS shows a neutral taste, and stability over wide pH and temperature ranges (Felisberto et al., 2015). Resistant starch and β-glucan have proved to be an appropriate combination for sausage elaboration, increasing juiciness, soft texture, and resulting in a higher sensory acceptance (Amini Sarteshnizi, Hosseini, Bondarianzadeh, Colmenero, & khaksar, 2015). Moreover, (Glisic et al., 2019) replaced 16% of pork-back fat with inulin gelled suspension and inulin linseed oil gelled emulsion in dry-fermented sausages. All sensory attributes were acceptable and the reformulations led to a decrease in springiness, chewiness, and hardness and an increase in the adhesiveness of the sausages.

Recently, there is a novel approach to produce prebiotic ingredients with non-thermal technologies such as high hydrostatic pressure, ultrasound and microwave among others. Non thermal technologies are technologies that do not use heating to process ingredients, having minimal effects on physicochemical properties (color, aroma, taste) and nutritional value of food products. Other advantages are shorter treatment times, higher energy efficiency, higher levels of safety and a longer shelf-life. Moreover, these technologies can ensure shorter processing times and use lower temperatures that enhance food safety (Zhang, Wang, Zeng, Han, & Brennan, 2019).

2.7.1 Validation techniques: from food ingredient to prebiotic

The emerging prebiotic food ingredients obtained from plants, food by-products, and even the chemically synthesized ones, need to be evaluated, in order to determine their potential and elucidate their specific prebiotic properties and the mechanisms by which they

exert health benefits, as well as how they impact the gut microbiota composition (Senés-Guerrero, Gradilla-Hernández, García-Gamboa, & García-Cayuela, 2020). The evaluation of potential prebiotic ingredients is essential to understand the digestion, fermentation The potential prebiotic substance can have an impact on various sites that can directly affect the host body positively, while having different roles.

According to the prebiotic definition, one of the most important aspects to validate a food ingredient as prebiotic is its selective utilization by host microorganisms. Providing bacteria with prebiotic ingredients allows the selective stimulation and enrichment of gut-beneficial bacterial strains. Hence, a simple way to test it is through the monitoring of individual bacterial growth in a medium with the prebiotic ingredient as carbon source, leading to the design of prebiotics with a high degree of selectivity (García-Cayuela, Díez-Municio, et al., 2014). Further stages include the evaluation of the prebiotic activity through mixed cultures or co-cultures and/or by means of the simulation of gut conditions to analyze the complex interaction with microbiota organisms and its general modulation. Moreover, fecal samples can be used to study prebiotic effects simulating the human colon more accurately. This evaluation can be achieved by different strategies using both *in vitro* and *in vivo* models (Fig. 3).

In vitro tests mainly consist of the simulation of the digestion and fermentation processes under controlled conditions and at levels that cannot be reached by an *in vivo* setup. The *in vitro* models overcome the issues associated with ethical concerns and provide a cost-effective tool for research. A range of systems have been developed to model colon fermentation, from simple anaerobic batch culture systems in flasks to sophisticated multistage continuous flow models (La Fata et al., 2017). For the digestion process, using a variety of enzymes like amylase, pepsin, pancreatin, and protease, among others, is common. In contrast, the absorption process is frequently assessed using differentiated cell monolayers obtained from the human intestinal epithelium (Caco-2 cell cultures). Most enzymes utilized for the studies are extracted from pigs, rats, or human volunteers. Enzymes are specifically for starch, protein, or lipid digestion and are usually added sequentially to simulate the different steps of the digestive process. This process can be stimulated by mixing additional components within the digestive fluids to operate efficiently.

Regarding colon fermentation, simple models consist of batch incubations using anaerobic conditions and dense fecal microbiota. They provide a first assessment of the

types of microbial metabolites formed and the biodiversity due to the substrate effects. However, they are limited by substrate depletion and the accumulation of the end products of microbial metabolism. Complex models involve one or multiple-connected, pH-controlled chemostats inoculated with fecal microbiota, and represent different parts of the human colon. Some models reproduce the peristaltic mixing of proximal colonic luminal content as well as the absorption of water and fermentation products in multistages; for instance, the SHIME model (Simulator of the Human Intestinal Microbial Ecosystem), which has been developed to simulate accurately the human intestinal tract. The mucosal SHIME (M-SHIME) model incorporates a mucosal environment by adding a mucin-covered microcosm, which is replaced daily to simulate the renewal of the mucus layer allowing the simulation of surface-attached gut microbes (Pham & Mohajeri, 2018).

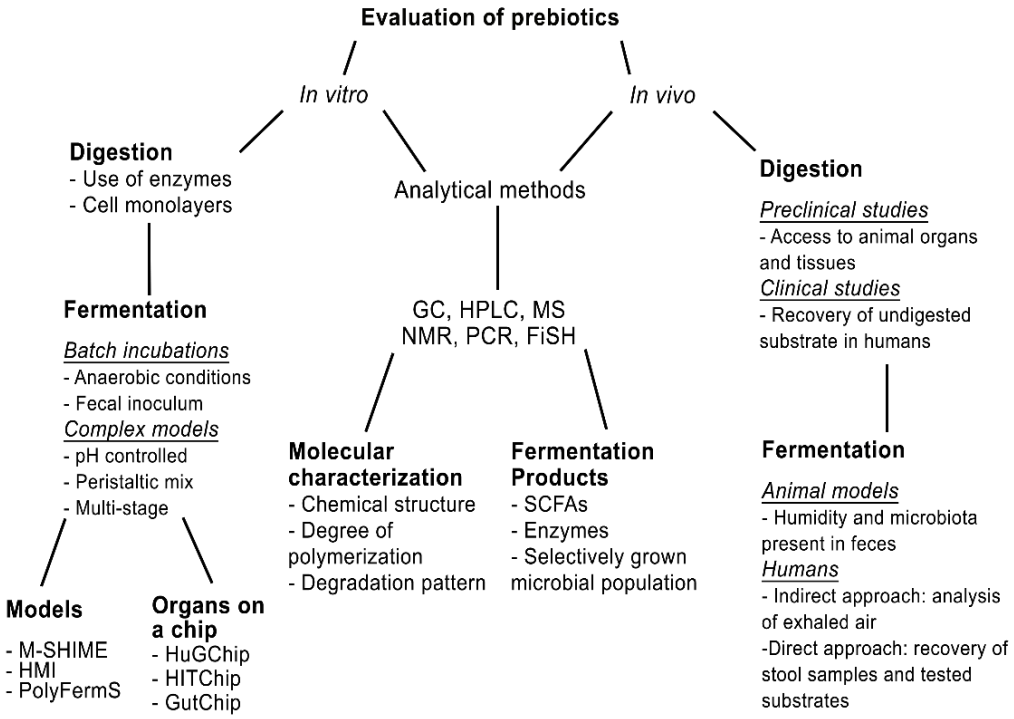


Figure 3. General validation methods for the evaluation of prebiotic properties of food ingredients. M-SHIME, Mucosal Simulator of the Human Intestinal Microbial Ecosystem; HMI, Host-Microbiota Interaction; PolyFermS, Polyfermentor Intestinal Model; HuGChip, Human Gut Chip; HITChip, Human Intestinal Tract Chip; GC, gas chromatography; HPLC, high performance liquid chromatography; MS, mass spectrometry; NMR, nuclear magnetic resonance; PCR, polymerase chain reaction; FISH, fluorescent *in situ* hybridization; SCFAs, short chain fatty acids. From Hurtado-Romero et al. (2020).

Another example is the Host-Microbiota Interaction (HMI) module, which consists of two compartments separated by an upper luminal region with a mucus layer and a lower semipermeable membrane. This double layer allows the establishment of different oxygen pressures on both sides of the membrane to establish both aerobic and anaerobic microorganisms in the luminal compartment. Cells can grow in the basal compartment allowing secretion of metabolites from the artificial mucus layer. Furthermore, the model can be combined with the SHIME system and allows the analysis of mixed bacterial communities, microbial metabolism, and the biotransformation of compounds (Williams et al., 2015).

Additionally, the Polyfermentor Intestinal Model design (PolyFermS) was developed to reduce problems of reproducibility and biological replication, while allowing stable and reproducible cultivation to test in parallel the effects of different treatments on the same complex gut microbiota. The first stage is a continuous inoculum reactor that contains immobilized fecal microbiota and mimics the upper proximal colon, continuously feeding a set of second-stage control and test reactors operating in parallel with conditions of the proximal colon (Zihler Berner et al., 2013). Recently, some *in vitro* fermentations have been performed to analyze the prebiotic potential of fibres. For example, an *in vitro* digestibility of a range of dietary carbohydrates was evaluated using rat small intestinal extract under physiological conditions of temperature and pH, showing that FOS and lactulose were the most resistant to digestion (Ferreira-Lazarte, Olano, Villamiel, & Moreno, 2017).

Also, (Ferreira-Lazarte, Gallego-Lobillo, Moreno, Villamiel, & Hernandez-Hernandez, 2019) used small intestinal brush border membrane vesicles from pig to digest GOS from lactose and lactulose, and evaluated hydrolysis rates, degree of polymerization and resistance to intestinal digestive enzymes. Results determined that the resistance to small intestinal digestive enzymes highly depends on the structure and composition of prebiotics. Furthermore, a recent study has evaluated *in vitro* the prebiotic potential of artichoke and sunflower by-products, considered promising sources of pectic compounds, while measuring bacteria populations, and SCFAs.

Results considered these by-products as good prebiotic candidates because they promoted the growth of beneficial bacteria such as *Bifidobacterium*, *Lactobacillus,* and *Bacteroides/Prevotella* (Ferreira-Lazarte, Kachrimanidou, Villamiel, Rastall, & Moreno,

2018). A recent study showed the effects of a prebiotic fibre supplementation (β-glucan, α-galactooligosaccharide, and xylooligosaccharide) on two distinct fecal microbial consortia maintained *in vitro* in the PolyFermS model. It was inoculated with immobilized adult fecal microbiota, obtaining different metabolic and compositional responses, among them a variable SCFA profile. For instance, *Bacteroidaceae-Ruminococcaceae*-dominated microbiota produced more butyrate, while the *Prevotellaceae-Ruminococcaceae*-dominated microbiota produced more propionate (Poeker et al., 2018).

These data suggest that the PolyFerms model is a suitable method with which to analyze the digestion and fermentation of prebiotic ingredients. On the other hand, the methodologies for digestion and fermentation *in vivo* are tested in animal models such as swines, zebrafish, and rodents (rats and mice), mainly because they allow direct access to intestinal contents as well as to organs and tissues. Animals may be used to analyze direct effects in microbiota after bacterial fermentation of prebiotics (SCFA production, pH variations). Other variables, such as gas production, weight changes, feces humidity, and microbiota present in feces are also evaluated. Studies evaluating the prebiotic potential have determined effects on intestinal peristalsis, bowel function improvement, and modulation of the activities of fecal bacterial enzymes, among others (Aquino et al., 2017).

Human clinical studies are the best approach to test prebiotic functionality because gut microbiota can be studied directly from the human volunteer, regardless of whether intestinal contents are more accessible in an animal study and the strict ethical codes of practice that need to be approved. The indirect approach to analyzing microbial fermentation can be conducted by using exhaled air to measure the concentration of hydrogen or methane gas, SCFAs in plasma or feces, or by directly measuring the recovery of the test carbohydrate from the collected stool sample. A direct approach is performed by collecting the stool from the volunteers after oral feeding to measure the recovery of the test substrates.

In this regard, the prebiotic activity of a maize-derived whole-grain cereal was tested in a double-blind, placebo-controlled human feeding study for 21 days, where healthy men and women consumed 48 g/d of whole-grain cereal or placebo cereal. Fecal samples were analyzed using fluorescence *in situ* hybridization with 16 S rRNA oligonucleotide probes specific for *Bifidobacterium* spp. and other bacteria strains, showing a significant increase

in fecal bifidobacteria compared with the control (Carvalho-Wells et al., 2010). The efficacy and safety of an infant formula containing bovine milk-derived oligosaccharides (BMOS) and *B. lactis* (CNCM I-3446) was evaluated recently in a multicenter, double-blind trial. Full-term infants received test or control formula (without BMOS and *B. lactis*) and fecal pH and gut microbiome were analyzed. Results indicated that BMOS promotes a beneficial microbiota composition, increasing bifidobacteria and lactobacilli and decreasing clostridia/eubacteria counts compared to the control group (Radke et al., 2017).

In addition to *in vivo* and *in vitro* models, products of the digestion and fermentation of prebiotics, as well as their structure and properties, need to be molecularly characterized. This can be achieved using diverse techniques. For example, the degree of polymerization of the fibres can be detected and quantified with gas chromatography (GC) or high-performance liquid chromatography (HPLC). These techniques can be coupled with spectroscopic instruments to obtain structural information. In this sense, nuclear magnetic resonance (NMR) and mass spectrometry (MS) are directly used for structural analysis. Some applications are for studying the degradation patterns of oligosaccharides during fermentation assays to evaluate their prebiotic effect. Also, the direct analysis of oligosaccharides can be achieved with a previous derivatization procedure and methylation analysis for their structural determination. Moreover, SCFAs derived from fermentation can be analyzed with these chromatography and spectrometry techniques (Niccolai et al., 2019).

As previously mentioned, multiple studies have shown that the fermentation of prebiotic substances in the colon promotes the growth of some specific gut strains. Some strategies to characterize microorganisms of gut microbiota are DNA–DNA hybridization or DNA sequencing encoding 16 S rRNA and strain identification by polymerase chain reaction (PCR) and fluorescent *in situ* hybridization (FISH), among others. A conventional PCR is sufficiently sensitive for the detection of microbiota organisms such as *Lactobacillus.* However, it can only be used for semiquantitative assessment due to the endpoint analysis limitations. Quantitative real-time PCR (qPCR) allows the monitoring of the complete amplification while overcoming the limitations correlated with endpoint analyses of the PCR process.

Moreover, a recent study suggests integrating qPCR-based quantitative microbiome profiling with standard next-generation sequencing (NGS)-based microbiome analysis in

order to obtain an absolute quantification and a comprehensive understanding of the dynamics and interactions of the microbiome (Jian, Luukkonen, Yki-Järvinen, Salonen, & Korpela, 2020). Novel approaches have been developed to analyze the composition of gut microbiota and the prebiotic interaction with the host, using microarray technology, a high throughput platform used to study numerous samples and to detect thousands of nucleic acid sequences simultaneously. For example, the human gut chip (HuGChip) allows 16 different samples per run to be analyzed with specificity, reducing costs, and limiting inter-microarray bias. It is also a well-adapted format to monitor the gut bacterial environment over time and gives an alternative determination of the bacterial richness and abundance of a sample (Tottey et al., 2013).

The human intestinal tract chip (HITChip) is a phylogenetic microarray also used for studying human intestinal microbiota, and consists of oligonucleotide probes targeting 16 S rRNA gene sequences. Its design allows the easy addition of probes that target newly discovered members of an ecosystem and partial 16 S rRNA sequences (Rajilić-Stojanović et al., 2009). Finally, the gut chip is a novel microfluidic 2-channel that enables human intestinal epithelium, capillary endothelium, immune cells, and commensal microbial cells to grow, coexist, and interact. It has continuous fluid flow, villi formation, and mucus production and peristalsis-like mechanical deformations *in vitro*, thus emulating the dynamic human intestinal microenvironment more faithfully than previously described *in vitro* intestine models (Bein et al., 2018).

The intake of prebiotics plays an important role in the modulating of the intestinal microbiota, favoring selectively the activity of bacteria that provide the host with certain health benefits. For this reason, the interest in using innovative technologies is not only to extract ingredients with prebiotic potential, but also to modify these ingredients in terms of their prebiotic properties. Therefore, it is important to consider some criteria before establishing the best technology to obtain prebiotic ingredients with an impact on specific host microorganisms or probiotics: the conditions applied; the matrix of food sources; the specific compound of interest; and the final delivery in the food product. For example, the high contents of DF and phytochemicals found in extracted and modified ingredients by innovative technologies from plant by-products could potentially have a prebiotic functionality modulating the activity of bifidobacteria and lactobacilli (Garcia-Amezquita, Tejada-Ortigoza, Torres, & Welti-Chanes, 2020).

2.8 By-products as functional food sources

Currently, some food industries re-process their waste and use it as functional food ingredients, making sustainable and stable economic growth. Food waste or by-products most commonly refers to edible food products that have been discarded, presenting a promising source of functional compounds because of their favorable nutritional and rheological properties. Fruit and vegetables, marine, meat and dairy by-products can be used as raw materials to obtain value added ingredients. Thus, technical and scientific growth can promote the utilization of by-products to make sustainable functional food ingredients and products (Bharat Helkar & Sahoo, 2016).

Approximately 1.4 billion tons of foods that are lost or wasted around the world come from plant sources; cereals and vegetables represent 71%, followed by fruits (25%) and in less proportion oilseed crops and legumes (4%). By-products produced from fruits and vegetables such as peels, seeds, and bagasse are of particular interest due to the bioactive compounds and nutritional content of them, being dietary fibre the most abundant compound associated to by-products. For instance, pulps contain ascorbic acid, as well as hydrophobic compounds with antioxidant activities and seeds are rich in phenolic compounds and tocopherols (Garcia-Amezquita, Tejada-Ortigoza, Serna-Saldivar, & Welti-Chanes, 2018).

Recently, the antioxidant dietary fibre concept has been proposed, referring to foods that contain the beneficial effects of both dietary fibres and antioxidants. By-products from the processing of plant-originated food materials are good sources of antioxidant dietary fibre, some examples are acaí, grapes, cabagge, apple and mango (Eskicioglu, Kamiloglu, & Nilufer-Erdil, 2016).

2.8.1 Berry by-products as a potential functional ingredient source

Global demand for berries has been driven mainly by consumer interest in foods that provide natural health benefits. Berry fruits are characterized by a high content and wide diversity of bioactive compounds such as phenolic compounds, organic acids, tannins, anthocyanins, and flavonoids. These compounds have demonstrated ant oxidative and antimicrobial activity. Utilization of antimicrobial activity of berry bioactive compounds as

natural antimicrobial agents offers many opportunities for their use in food industry and medicine (Jimenez-Garcia et al., 2013).

Berries are also valued for their sensory attributes, such as aroma, taste, size, appearance, and consistency and for being direct consumption products. Mexico's berry production has an annual average increase of 13.9%, where strawberries and blackberries are produced in greater quantities and raspberries and blueberries have a faster growth. In this context, Mexico has positioned itself as one of the major berry-producing countries, exporting between 60% and 80% of its berry production, being Jalisco the second highest producing state. In fact, berry exportation in 2017 exceeded at $55 million USD the profits reported by the export of tequila (González-Ramírez, Santoyo-Cortés, Arana-Coronado, & Muñoz-Rodríguez, 2020).

Raspberries, strawberries and blueberries are greatly used in food manufacturing for purees, juices, wines etc. Solid waste produced such as seeds and extrudates are an important by-product in the production process, but usually be discarded and thus underexploited. Significant content of antioxidant, phenolic acids, flavonoids, polyphenols and fibres, as well as the high amount of waste released during industry manufacturing (Ispiryan & Viškelis, 2019; Luchese, Uranga, Spada, Tessaro, & de la Caba, 2018; Vázquez-González et al., 2020).

During the post-harvest and transformation stages, the agro industrial by-products generated in Jalisco, including berries, leads to losses of food products that can represent up to 40% of the processed volume. These wastes represent an available and renewable source of billions of tons of biomass per year, which can be used as a source of high added value compounds (Casas Godoy & Barrera Ramírez, 2021). In this context, these by-products could be recovered and used successfully for different industry purposes, such as production of functional ingredients and purified bioactive compounds.

CHAPTER 3. SCREENING OF AUTOCHTHONOUS LACTIC ACID BACTERIA AND YEAST ISOLATES FROM HOMEMADE KEFIR: PROBIOTIC AND PREBIOTIC PROPERTIES

3.1 Introduction

Functional beverages are the most active functional foods category providing nutrition and exerting physiological effects on the body. Functional dairy products account over 40% of functional foods market. Vast majority of functional dairy products are fermented products such as fermented milks, yogurts, cheese and yogurt-type products including low-lactose or lactosefree products. These foods are supplemented with functional ingredients such as minerals, vitamins, conjugated linoleic acid (CLA) and sterols/stanols. Morevoer, dairy fermented products have long been used as probiotics and more recently, prebiotics carriers in dairy foods (Turkmen, Akal, & Özer, 2019). Kefir is a fermented dairy product with an increasing popularity due to its nutritional and reported antimicrobial, immunological, anti-tumour and hypocholesterolaemic effects. It is made of kefir grains, which contain lactic acid bacteria (LAB) and various yeasts combined with casein and complex sugars in a polysaccharide matrix. The major polysaccharide is kefiran, comprising equal amounts of glucose and galactose (Arslan, 2015).

The growing popularity of kefir and kefir grains has prompted to the use of kefir starters in dairy production like cheese; alternative substrates such as fruits and molasses have also contributed to enhanced distinct sensory characteristics for adaptation of kefir product development and marketing strategies. Kefir may be identified depending on the type of substrate used for fermentation, which can be dairy or non-dairy kefir. A wide variety of studies emphasize the advantages of kefir consumption using milk substrates for fermentation compared with their non-dairy counterpart. Dairy and non-dairy kefir grains are similar in structure, related microorganisms and metabolic products during the fermentation. However, the constitution and prevalence of microbial diversity of kefir grains and metabolic fermentation products may differ depending on the carbon and energy sources available for grain fermentation (Azizi et al., 2021).

Milk kefir grains (dry matter) are composed of approximately 58% polysaccharide, 30% protein, 7% fat, and 5% ash while the average compositional analysis for water kefir grains is unknown. Moreover, low LAB content has been reported when milk kefir grains

were used to ferment fruit juice implying low probiotic value and supporting the knowledge that milk kefir grains require a particular dairy-based growth medium. Moreover, milk kefir grains require milk or whey-based medium and sometimes can be grown in plant-based “milk” (Guzel-Seydim, Gökırmaklı, & Greene, 2021).

As a way of standardizing kefir production, the use of defined cultures has been proposed, being an interesting approach that may eliminate the problems associated with the use of kefir grains. Moreover, the use of the specific microbiota isolated from kefir grains as a starter culture can produce a fermented food whose properties are close to those of traditional kefir assuring a quality product (Chen, Wang, Chen, Liu, & Chen, 2009). Different methodologies have been employed for the discovery of new probiotic strains, being traditional *in vitro* and *in vivo* assays along with novel omics the most used approaches. For instance, during screening for novel probiotic strains, safety, antimicrobial, and survival assays are employed (Papadimitriou et al., 2015).

In adittion, several quantitative approaches are employed to determine the functional activity of prebiotics or non-digestible food ingredients during *in vitro*, regarding the stimulation of probiotcs growth or the activity of bacteria present in the colon. These methods are based on measurement of microbial populations, growth rates, substrate assimilation, and/or short-chain fatty acid production. However, fermentation of prebiotics is dependent on the bacterial strain (Huebner, Wehling, & Hutkins, 2007).

In recent years, numerous scientific investigations have been published regarding the isolation and characterization of microorganisms from kefir grains in countries such as Taiwan, China, Argentina and Russia, some of them with probiotic properties. Additionally, kefir grains have been applied in the production of dairy and non-dairy probiotic beverages (Plessas et al., 2016). Hence, the isolation of microorganisms from an artisanal milk kefir from Mexico and the probiotic characterization of its isolates is interesting, for the generation of starter cultures that are involved in the production of dairy fermented products in the food industry with attractive nutritional properties.

3.2 Materials and Methods

3.2.1 Chemicals, reagents, enzymes and bacterial strains

All chemicals and reagents used were from analytical grade. Difco MRS (Man-Rogosa-Sharpe) media and agar, M17 media and agar, Nutrient Broth and Potato Dextrose Agar (PDA) were used to isolate and grow microorganisms. Commercial probiotic strains *Lactobacillus acidophilus* La3, *Lacticaseibacillus rhamnosus* GG, *Lactiplantibacillus plantarum* 299v and pathogenic strains *Escherichia coli* ATCC-25922, *Staphylococcus aureus* ATCC-BAA-42 and *Salmonella typhi* BIOTEC019 were used as well.

Pepsin from porcine gastric mucosa (P7000), α-amylase from porcine pancreas (A3176), bile salts (B3883) and pancreatin from porcine pancreas (P1750) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Gram-positive antibiotic disks Multibac I.D. (Mexico) were used to evaluate antibiotic resistance. For the evaluation of growth of microorganisms with commercial prebiotics, lactulose was purchased from Merck, commercial inulin (enature) was acquired from a local store; dextrose and citric pectin of analytical grade were used as well.

3.2.2 Kefir grains

Kefir grains were obtained from two different homemade milk kefir beverages in two different locations at the city of Guadalajara, state of Jalisco, México. The grains were grown routinely in cow's milk at room temperature, every 24 hours, then they were propagated in the laboratory in ultra-pasteurized skim milk at the same conditions. The two sources of kefir grains were designated as KHA and KHI.

3.2.3 Isolation of bacteria and yeast from kefir grains

Ten grams of each source of kefir grains were suspended in 50 mL of sterile saline solution (0.85% NaCl) and homogenized (10-20 min, 5000 rpm) in IKA Ultra-Turrax T25 homogenizer (Zanirati et al., 2015). Dilutions were made of each resulting sample and plated in three different agars: MRS, M17 and PDA for 48 h at 30°C or 37°C and in both aerobic and anaerobic conditions. Different colonies were selected, inoculated in the respective media broth (MRS, M17 or Nutrient Broth) at the same conditions and plated again to isolate

a colony from a uniform sample. Moreover, the selected colonies were subjected to gram staining and the catalase test to select presumptive LAB (rods or cocci gram-positive, and catalase-negative). Approximately 23 morphologically distinct colonies were isolated and cultured in the respective agar plates at the same conditions for later identification by Matrix Assisted Laser Desorption/Ionization Mass Spectrometry (MALDI-TOF). The final selected isolates were stored at -80°C.

3.2.4 MALDI-TOF MS microorganism's identification

For bacteria and yeast colonies, the biomass of an isolated colony was transferred to a stainless steel plate following the "Extended Direct Transfer Method" protocol (Bruker Daltonics GmbH) and mass spectra were generated with the "MBT_FC.par" method in a Microflex LT equipment. (Bruker Daltonics GmbH). Using the MALDI BIOTYPER 3.1 software, the spectra obtained were compared with reference spectra from the BDAL database (Bruker Daltonics GmbH).

In the case of filamentous fungi, a protein extract was generated from a mycelium pellet of each microorganism following the "Formic Acid Extraction Method" protocol (Bruker Daltonics GmbH). Briefly, 1 µL of this extract was transferred to a stainless steel plate, allowed to dry and covered with 1 µL of matrix (10mg / mL of HCCA dissolved in ACN: H_2O: TFA 50: 47.5: 2.5) and mass spectra were generated with the method "MBT_FC.par" on a Microflex LT equipment (Bruker Daltonics GmbH). Using the MALDI BIOTYPER 3.1 software, the spectra obtained were compared with reference spectra from the FILAMENTOUS FUNGI and BDAL databases (Bruker Daltonics GmbH).

The software estimates a score value between 0 and 3 to determine the similarity between the sample and reference spectrum. Hence, scores between 2.300 and 3.000 represented a high identification reliability at species level; scores between 2.000 and 2.299 provided a high reliability of identification at the genus level and a probable species identification; scores between 1.700 and 1.999 represented a probable genera identification and scores of 1.699 and below represented an unreliable identification. From the identified microbial species, ten isolates were used in this study (two yeasts and eight LAB).

3.2.5 Aggregation experiments

The aggregation abilities were observed both in the identified LAB, yeast and in the commercial probiotic strains, used as controls in all the experiments. The first approach was a screening by a visual assay. Hence, microorganisms were grown in 2 mL of MRS at 30 °C for 24 h under aerobic conditions, then cultures were vortexed for 15 seconds and aggregation phenotype was observed after 3 minutes under resting conditions (formation of precipitate, clear observation of supernatant). A further analysis was developed spectrophotometrically as described by (García-Cayuela, Korany, et al., 2014). Briefly, bacterial cells (10^8 cfu/mL) were cultured overnight and harvested by centrifugation (3000×g, 20 min, 4°C), washed twice with phosphate-buffered saline PBS pH 7.1±0.2 and re-suspended in the same buffer. Buffer was prepared according to this composition: 10 mM Na_2HPO_4, 1 mM KH_2PO_4, 140 mM NaCl, 3 mM KCl, pH adjusted with NaOH 0.1 M and HCL 1 M. The mixture was vortexed, and incubated at 30 °C for 24 h without agitation, following absorbance values (OD 600) at 0, 2, 6, 20 and 24 h. Aggregation percentage was expressed as follows: $[1 - (\frac{ATime}{A0}) * 100)$ where A0 represents absorbance time t=0 h and ATime represents the absorbance of the mixture at different times.

Next, co-aggregation assays were done with an overnight culture of isolates and pathogens following the methodology described above. Then, equal volumes (500 µL) of cells of the isolated microorganisms, control stains and pathogen strains tested (*Escherichia coli* ATCC-25922, *Staphylococcus aureus* ATCC-BAA-42 and *Salmonella typhi* BIOTEC019) were mixed in pairs adjusting absorbance (between 0.8 and 1.0) and incubated at 30°C without agitation, following absorbance values (OD600) at the same time points stated above. Percentage of co-aggregation was calculated according to the absorbance values of the mix suspension at different monitored times (2, 6, 20, 24 h).

3.2.6 Antibiotic susceptibility

The bacterial isolates were cultured overnight at 2% in MRS broth or M17 broth depending on the microorganism. Bacterial isolates were vortexed and 100 µL of the overnight cultures were distributed uniformly on Nutrient agar by using sterile L-shaped cell spreaders, and susceptibilities to antibiotics were determined by standard disk-diffusion assays (MultiBac I.D., Mexico DF). Zones of inhibition were observed after 18 h of incubation at 30°C, and values were compared with interpretative standards classified as High

Resistance (+++), Intermediate Resistance (++), Moderate resistance/Moderate sensitive (+), No resistance/Sensitive (-) (MultiBac I.D., Mexico DF), depending of the grade of inhibition (diameter observed around each disk).

3.2.7 Antimicrobial activity

Screening for antimicrobial activity was performed using the agar diffusion assay. Cell cultures of all the isolates were grown overnight in MRS broth or M17 depending on the microorganism and centrifuged (10,000 g x 10 min). Then, 50 µL of the supernatant was neutralized with NaOH (1M), previously filtered through a 0.45 um pore size and placed in triplicate into wells made in nutritive agar plates. No neutralized supernatants were also placed in wells for triplicate following the same methodology. *E. coli* ATCC-25922, *S. aureus* ATCC-BAA-42 and *S. typhi* BIOTEC019 were used to inoculate 5 mL of soft-overlay (0.75% agar) nutrient medium, which was seeded onto the respective agar plates. Zones of growth inhibition were measured after an overnight incubation and inhibition halos (in millimeters) were reported.

3.2.8 *In vitro* digestion assay

The methodology followed was the improved digestion method (INFOGEST 2.0) based on the standardized protocol presented on an international consensus developed by the COST INFOGEST network (Brodkorb et al., 2019) with some modifications. The composition and preparation of simulated digestion fluids is presented in Figure 4. The digestion procedure involved the exposure of the food or in this case the culture of individual isolates to three successive digestive phases: oral, gastric and intestinal.

Overnight cell cultures (5 mL) were centrifuged (2000 g x 15 min, 4°C), washed twice and re-suspended in sterile saline solution (0.85% NaCl). After taking an initial sample (1 mL), oral phase involved dilution of the culture 1:1 (v/v) with simulated salivary fluid (SSF) containing amylase at pH 7, and incubated 2 min, 100 rmp, 37°C; these incubation conditions were constant during the assay. The oral bolus was then diluted 1:1 (v/v) with simulated gastric fluid (SGF) and gastric enzymes (pepsin in this case) and incubated at pH 3.0 for 2 h. The gastric chyme was then diluted 1:1 (v/v) with simulated intestinal fluid (SIF), bile salts and pancreatic enzymes and incubated at pH 7 for a further 2 h. Sampling process consisted in taking 1 mL of each culture after each phase, then realize serial dilutions and

plate in MRS agar in order to count viable colonies. Plates were incubated at 30°C during 48 h and viable colonies were reported in LOG (CFU/mL).

Simulated Saliva Fluid			Simulated Gastric Fluid			Simulated Duodenal Fluid		
SSF			SGF			SDF		
Volume mL	Compound	Stock g/L	Volume mL	Compound	Stock g/L	Volume mL	Compound	Stock g/L
10	KCl	46.7	28	KCl	46.7	10.8	KCl	46.7
20	KH_2PO_4	68.0	0.9	KH_2PO_4	68.0	1.6	KH_2PO_4	68.0
4	$NaHCO_3$	84.0	13	$NaHCO_3$	84.0	85.0	$NaHCO_3$	84.0
1	NaCl	120.0	10	NaCl	120.0	16.0	NaCl	120.0
1	$MgCl_2(H_2O)_6$	30.0	2	$MgCl_2(H_2O)_6$	30.0	2.2	$MgCl_2(H_2O)_6$	30.0
pH adjustment			pH adjustment			pH adjustment		
mL	Compound	mol/L	Compound	%	%	mL	Compound	mol/L
4	HCl	1	3	HCl	37	1.0	NaOH	1
1	NaOH	1				0.6	HCl	1
Final pH (inorganic): 6.8 ± 0.2 Final volume: 500 mL (distillated H_2O) Frozen at -20°C in portions			Final pH (inorganic): 1.3 ± 0.2 Final volume: 500 mL (distillated H_2O) Frozen at -20°C in portions			Final pH (inorganic): 8.2 ± 0.2 Final volume: 500 mL (distillated H_2O) Frozen at -20°C in portions		
Final volume: 5 mL/sample			Final volume: 10 mL/sample			Final volume: 20 mL/sample		
mg/mL µL/mL	Compound	Stock g/L	mg/mL µL/mL	Compound	Stock g/L	mg/mL, g/mL µL/mL	Compound	Stock g/L
0.21037 mg	α-amylase	-	0.3927 mg	Pepsin	-	0.0167 g	Bile	-
0.5 µL	$CaCl_2(H_20)_2$	588 g/L	0.13.5µL	$CaCl_2(H_20)_2$	588.0	0.15 µL	$CaCl_2(H_20)_2$	588.0
						0.011 g	Pancreatin	-
Final pH in the sample: 7.0 ± 0.2			Final pH in the sample: 3.0 ± 0.2			Final pH in the sample: 7.0 ± 0.2		

Figure 4. Composition of stock solutions of simulated digestion fluids and final composition with enzymes.

3.2.9 Evaluation of bacterial growth on commercial prebiotics

The bacterial isolates were cultured overnight at 2% in MRS broth, harvested by centrifugation (3000 g for 10 min at 4°C), washed twice and re-suspended in sterile saline solution (0.85% NaCl). MRS medium was prepared substituting dextrose with different carbon sources: lactulose, agave inulin and citric pectin (2%), using dextrose as the control. Next, bacterial growth was monitored in triplicate in 300 µL wells of sterile 96-well microplates with lid (Corning). All cultures were grown in aerobic conditions at 30°C for 48 h. The optical densities at 600 nm of the cultures growing aerobically were recorded at 60 min intervals with an automated microplate reader (Varioskan Lux, Thermo Fisher Scientific). Maximum growth rates and lag parameter (lag) of microorganisms were calculated by fitting the curves to a sigmoid model using the Microsoft Excel add-in DMfit v.2.1 (available at http://www.ifr.ac.uk/ safety/DMfit/default.html).

3.2.10 Statystical analysis

Standard deviations and mean values were calculated. Simultaneously, Minitab Software was used to carry out analysis of variance (ANOVA). In addition, Tukey test was used for means comparison using a 0.05 significance level (p-value).

3.3 Results and discussion

3.3.1 MALDI-TOF MS microorganism's identification

Eleven species-level cultures were identified as shown in Table 1, of which ten were selected. Bacteria scores ranged from 1.8 to 2.4 (genus level, probable species), being a reliable identification. Differences in score can be associated with variation in the database. Most of the lactic bacteria belong to the *Lactobacillus, Lactoccocus* and *Leuconostoc* genera, while the yeasts found belong to the *Kluyveromyces* genus. MALDI-TOF MS spectra were generated for each isolate, some examples are shown in Figure 5.

MALDI-TOF MS is a novel high-throughput identification method relying on the analysis of whole cell proteins. For microorganisms, a large majority of bacterial proteins and fragments detected by this approach are of ribosomal origin. This can be attributed both to the high abundance of ribosomal proteins in the bacteria and the extraction protocol and the detection of these basic proteins and fragments (Doan et al., 2012). Recent studies have reported the identification of LAB by MALDI-TOF MS analysis, isolated from fermented foods such as cheese and kimchi. For instance, bacteria from the genus *Lactobacillus*, *Lactoccocus* and *Leuconostoc* have been identified from MALDI-TOF MS profile of artisan cheeses; this study successfully identified more than eighty varieties of LAB species. Therefore, a good reference database should contain multiple strains per species to cover phenotypic diversity within species; misidentifications or non-identifications are likely due to insufficient number of strains in the MALDI-TOF MS reference databases (Gantzias et al., 2020).

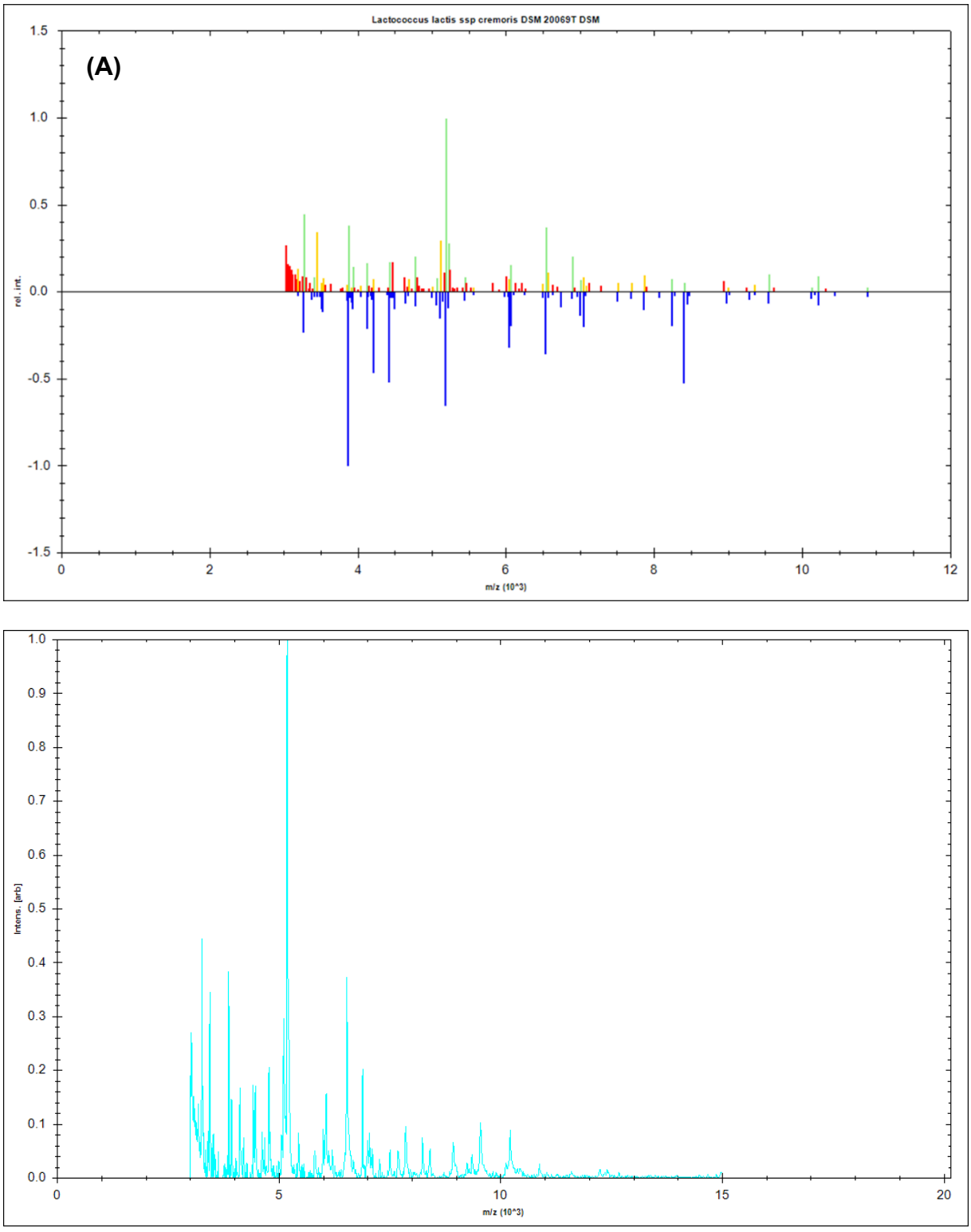


Figure 5 (Part I). MALDI-TOF mass spectrum analysis of (A) *Lactococcus lactis*, (B) *Kluyveromyces lactis*, (C) *Lactobacillus parakefiri* and (D) *Leuconostoc pseudomesenteroides*

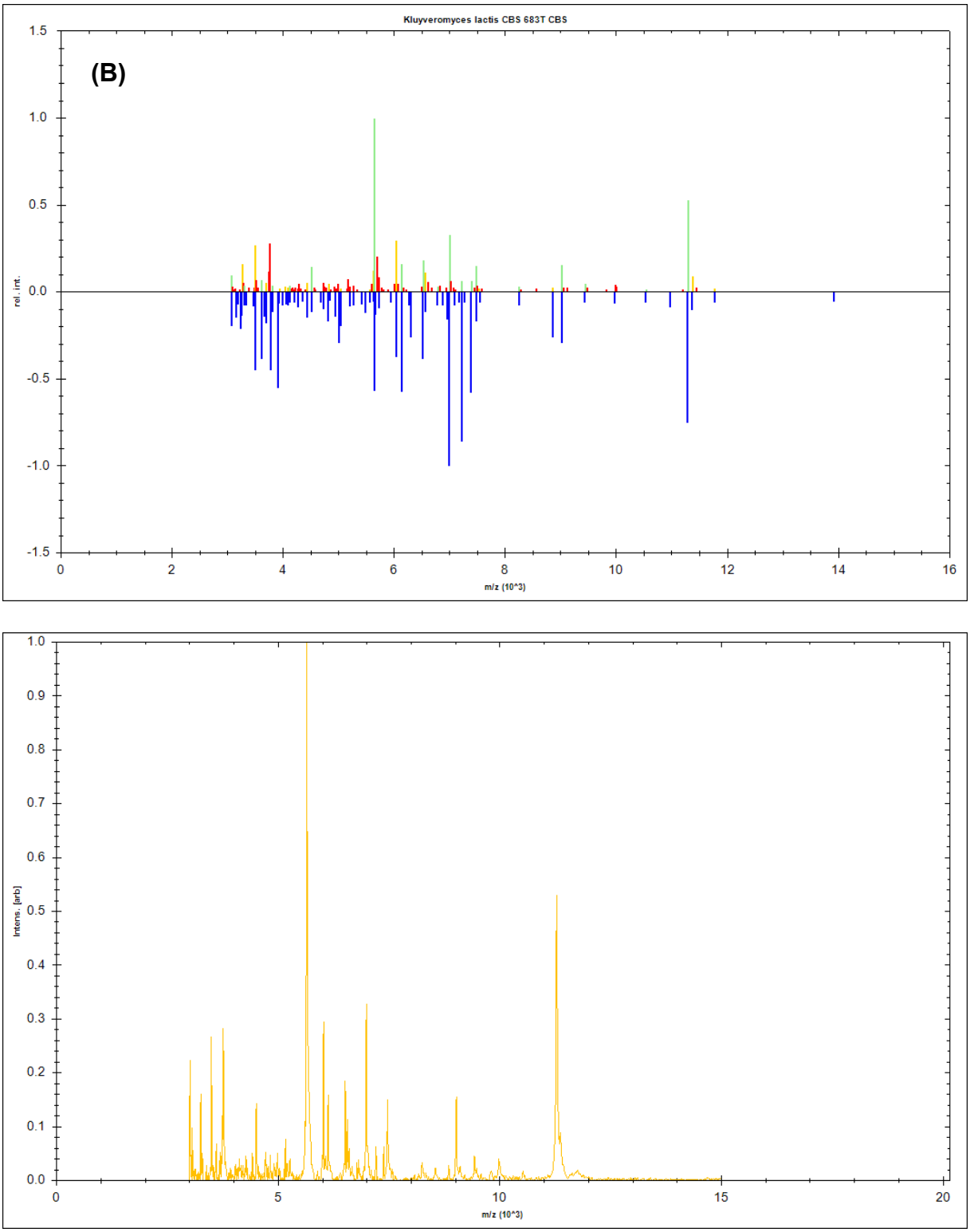


Figure 5 (Part II). MALDI-TOF mass spectrum analysis of (A) *Lactococcus lactis*, (B) *Kluyveromyces lactis*, (C) *Lactobacillus parakefiri* and (D) *Leuconostoc pseudomesenteroides*

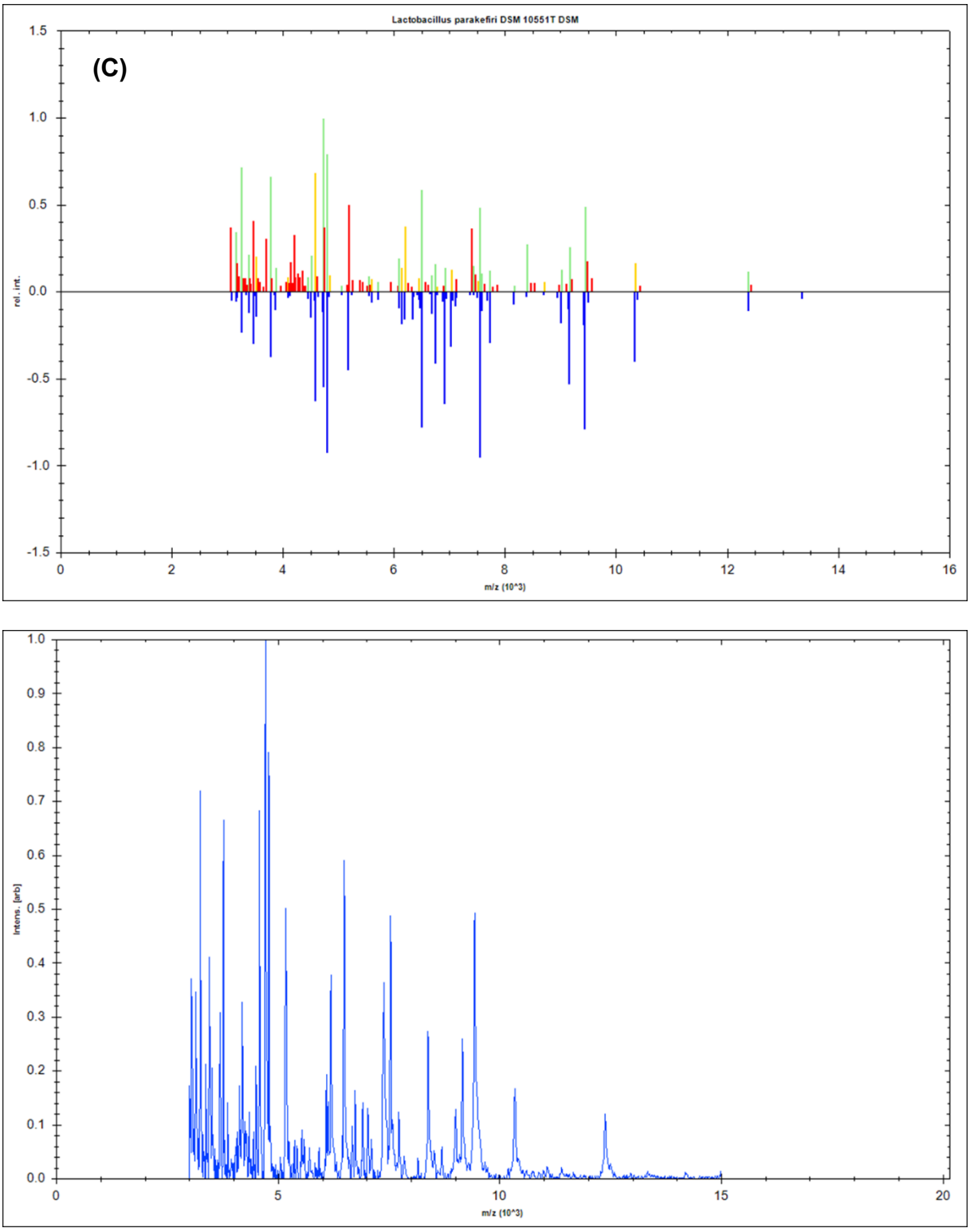


Figure 5 (Part III). MALDI-TOF mass spectrum analysis of (A) *Lactococcus lactis*, (B) *Kluyveromyces lactis*, (C) *Lactobacillus parakefiri* and (D) *Leuconostoc pseudomesenteroides*

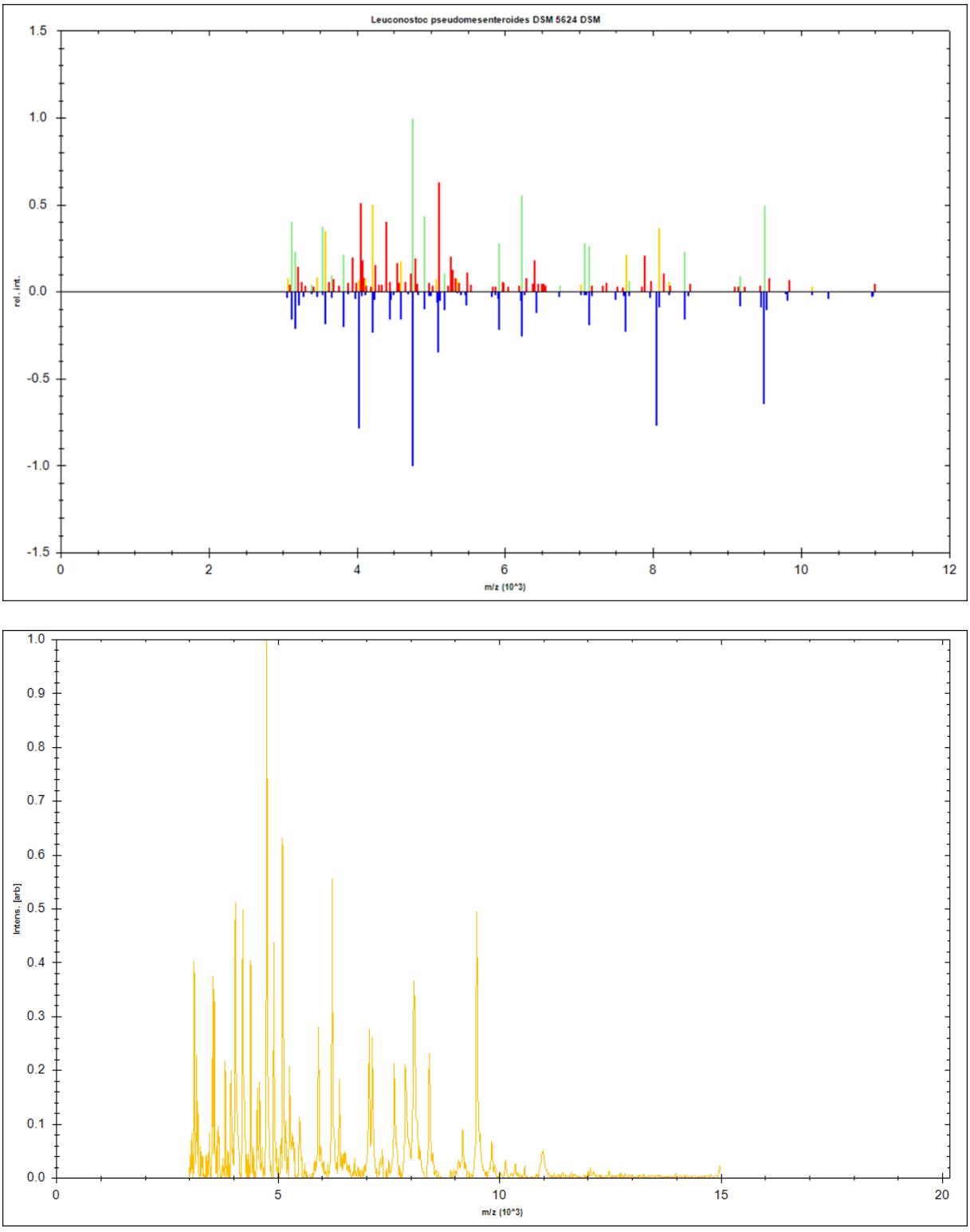


Figure 5 (Part IV). MALDI-TOF mass spectrum analysis of (A) *Lactococcus lactis*, (B) *Kluyveromyces lactis*, (C) *Lactobacillus parakefiri* and (D) *Leuconostoc pseudomesenteroides*

Table 1. MALDITOF-MS bacteria, yeast and fungi identification with code and software identification scores[a]

MALDI-TOF result	Assigned code	MALDI-TOF score	Reliability
Lactococcus lactis	BIOTEC006	2.143	Genus-level, probable species
Lactococcus lactis	BIOTEC007	2.446	Genus-level, species level
Lactococcus lactis	BIOTEC008	2.400	Genus-level, species level
Kluyveromyces lactis	BIOTEC009	2.102	Genus-level, probable species
Kluyveromyces lactis	BIOTEC010	1.937	Probable genus
Leuconostoc pseudomesenteroides	BIOTEC011	1.821	Probable genus
Leuconostoc pseudomesenteroides	BIOTEC012	1.885	Probable genus
Lactobacillus kefiri	BIOTEC013	2.006	Genus-level, probable species
Lactobacillus kefiri	BIOTEC014	2.226	Genus-level, probable species
Lactobacillus parakefiri	BIOTEC015	2.151	Genus-level, probable species
Lactococcus lactis	BIOTEC016	1.861	Probable genus
Penicillium commune	BIOTEC017	2.366	Genus-level, species level

[a] Reliability score: 2.300 to 3.000 correspond to high reliability at the species level, 2.000 to 2.299 high reliability at the genus level and probable species identification, 1.700 to 1.999 probable identification at the genus level and < 1.699 unreliable identification.

Moreover, MALDI-TOF MS analysis identified isolates at genus and the species level of kimchi, a traditional Korean fermented foods. The identified species based on MALDI-TOF MS and high-throughput sequencing indicated that similar results were obtained from both methods. While the high-throughput sequencing, MALDI-TOF MS enabled accurate identification of microorganisms at the species level as well as analysis of the viable cell communities by only identifying the live microorganisms. Therefore, the reliability of MALDI-TOF MS identification hass been reported as a good alternative to DNA sequencing identification procedures (Kim, Cho, Yang, Kim, & Kim, 2021). The use of MALDI-TOF MS profiling method has been reported also for the LAB in french cheese, with high match values, strains belonging to different genera and discrimination of phylogenetically close species into the same genus. This study indicates several advantages for this method including robustness, recognizing-based method, ultrafast tendency test, ease of use to low cost per test. Further, MALDI-TOF MS profiling provides identification in the first step of

characterization of the culturable microbial community in dairy products and have demonstrated success rates of species level assignment using MALDI-TOF MS approach than PCR (Nacef, Chevalier, Chollet, Drider, & Flahaut, 2017). Therein, the MALDI-TOF-MS method presented as affordable, sustainable, robust method could warrant a better management of microorganism identification.

3.3.2 Aggregation experiments

The aggregation assay considered the ten isolates of kefir as well as the commercial strains as controls (*L. acidophilus* La3, *L. rhamnosus* GG and *L. plantarum* 299v), the results of the visual screening and spectrophotometric test are shown in Table 2. An aggregation phenotype was observed in six isolates, rapidly forming aggregates in a stationary phase culture after few minutes (Figure 6). Besides, in the spectrophotometric aggression assay, in general a considerable percentage of auto aggregation was observed in all the isolates (>30%), being the highest percentages of aggregation at 24 hours in an incubation at 30°C. The microorganisms that showed a higher aggregation percentage (>50%) were *L. lactis* BIOTEC006 and BIOTEC007, *L. acidophilus* La3, *L. rhamnosus* GG, *L. kefiri* BIOTEC014 and *L. parakefiri* BIOTEC015. On the other hand, the isolates that showed least autoaggregated values were the bacteria of the genus *Leuconostoc* and *L. kefiri* BIOTEC013, although a precipitate was visualized, in the measurements an auto aggregation percentage of less than 35% was observed. In addition, a general linear model ANOVA was performed to compare the aggregation percentage of all the microorganisms at the different times measured.

In order to achieve the desired benefit of probiotic bacteria, isolates tested need to form a sufficiently large biomass through aggregation. Capability of bacteria to form cellular aggregates via auto aggregation (same microorganism) or via co-aggregation (genetically different microorganisms) can also contribute to persistence in the intestine (Krausova, Hyrslova, & Hynstova, 2019). The mechanism of cellular aggregation involves a complex interaction of surface and/or secreted components of the cell. Further, autoaggregation ability of cells plays a crucial role in adhesion to intestinal cells and prevention of pathogen colonisation. The highest percentage of auto-aggregation was observed after 24 h, agreeing with results reported by Krausova et. al, (2019), observing a time-dependent increase. Further, *Lactobacillus* species (*L. plantarum* SAU96 and *L. fermentum* CH58) have reported

a high capacity for self-aggregation (61.9 and 55.1%, respectively) (Ramos, Thorsen, Schwan, & Jespersen, 2013). These values are comparable with the self-aggregation percentages obtained by the *Lactobacilli* belonging to this study. According to these results, kefir isolates posses good auto-aggregation properties, usually related to adhesion capacity.

Figure 6. Aggregation phenotypes observation. From left to right: Control, Negative phenotype, Positive phenotype

Results of the co-aggregations assays between kefir isolates, control strains and pathogens *E. coli* ATCC-25922, *S. typhi* BIOTEC019 and *S. aureus* ATCC-BAA-42 are shown in Tables 3, 4 and 5, respectively. All microrganisms were shown to co-aggregate with pathogens tested with the highest percentage values at 24 h (>55%).

Co-aggregation is also one of the desired properties for probiotics and it may play an important role gastrointestinal tract by preventing adherence of pathogens to the host tissue. Thus, this ability with potential pathogens may be used for preliminary screening of potentially probiotic bacteria. Results presented here can be comparable to results reporting co-aggregation with pathogens, in a strain-pathogen combination-dependent manner, where *L. reuteri* VB4 showed a high percentage of co-agregation (>50%) against pathogen *E. faecalis* ATCC 29212 (Dlamini, Langa, Aiyegoro, & Okoh, 2019).

Table 2. Autoaggregation abilities of the kefir isolates and controls[a]

Microorganisms	Autoaggregation (%)			
	2h	6h	20h	24h
L. acidophilus La3$^{(-)}$	4.36 ± 0.29^{cd}	10.29 ± 0.10^{de}	56.54 ± 0.12^{c}	72.21 ± 0.53^{c}
L. rhamnosus GG$^{(-)}$	3.26 ± 0.29^{f}	8.17 ± 0.19^{ef}	35.17 ± 1.76^{e}	53.05 ± 0.62^{ef}
L. plantarum 299V$^{(-)}$	3.54 ± 0.35^{def}	8.00 ± 0.21^{f}	35.86 ± 3.51^{e}	46.98 ± 3.12^{fg}
L. lactis BIOTEC006$^{(-)}$	2.97 ± 0.03^{f}	12.48 ± 0.44^{c}	30.85 ± 1.06^{ef}	38.87 ± 2.52^{gh}
L. lactis BIOTEC007$^{(-)}$	10.64 ± 0.15^{a}	31.78 ± 1.08^{b}	77.00 ± 1.94^{b}	84.31 ± 1.28^{b}
L. lactis BIOTEC008$^{(-)}$	10.09 ± 0.00^{a}	32.24 ± 0.95^{b}	85.93 ± 0.85^{a}	94.33 ± 0.52^{a}
K. lactis BIOTEC009$^{(+)}$	4.14 ± 0.30^{de}	9.50 ± 0.54^{def}	28.46 ± 0.41^{f}	46.01 ± 0.38^{h}
K. lactis BIOTEC010$^{(+)}$	5.12 ± 0.19^{bc}	10.80 ± 0.38^{cd}	28.82 ± 0.53^{f}	39.00 ± 3.77^{gh}
L. pseudomesenteroides BIOTEC011$^{(+)}$	3.43 ± 0.29^{ef}	7.48 ± 0.46^{f}	26.00 ± 0.20^{f}	33.99 ± 1.42^{h}
L. pseudomesenteroides BIOTEC012$^{(+)}$	3.50 ± 0.12^{ef}	9.27 ± 0.13^{def}	26.96 ± 0.28^{f}	33.92 ± 0.29^{h}
L. kefiri BIOTEC013 $^{+)}$	5.33 ± 0.17^{b}	9.05 ± 0.43^{def}	27.67 ± 0.29^{f}	32.60 ± 3.12^{h}
L. kefiri BIOTEC014$^{(+)}$	10.53 ± 0.07^{a}	34.73 ± 0.42^{a}	57.81 ± 0.04^{c}	59.87 ± 0.03^{de}
L. parakefiri BIOTEC015$^{(-)}$	2.80 ± 0.01^{f}	10.44 ± 0.68^{cd}	50.14 ± 2.73^{d}	66.10 ± 0.65^{cd}

[a] Data are expressed as % of auto-aggregation measured after 2, 6, 20 and 24 h of incubation. Aggregation phenotype is indicated as positive (+) or negative (-). The values are means of duplicate measurements ± standard deviation. Different letters in the same column denotes significant differences among all the microorganisms studied.

Table 3. Co-aggregation abilities of isolates and controls with *E. coli* ATCC-25922[a]

Microorganisms	**Co-aggregation with *E.coli* (%)**			
	2h	**6h**	**20h**	**24h**
L. acidophilus La3	5.94 ± 0.04^{ef}	21.16 ± 0.03^{e}	61.66 ± 0.06^{f}	68.90 ± 1.15^{c}
L. rhamnosus GG	8.36 ± 0.71^{de}	14.13 ± 0.93^{e}	50.54 ± 1.20^{h}	58.05 ± 0.38^{de}
L. plantarum 299V	4.99 ± 0.77^{f}	21.11 ± 2.66^{e}	57.99 ± 0.41^{fg}	62.26 ± 0.86^{cde}
L. lactis BIOTEC006	14.44 ± 0.05^{c}	25.27 ± 0.70^{d}	74.83 ± 0.55^{e}	83.99 ± 1.77^{b}
L. lactis BIOTEC007	8.40 ± 0.36^{de}	19.73 ± 0.62^{e}	81.12 ± 1.36^{c}	85.57 ± 0.81^{b}
L. lactis BIOTEC008	13.47 ± 0.91^{c}	29.57 ± 1.46^{c}	76.90 ± 0.44^{de}	85.24 ± 0.74^{b}
K. lactis BIOTEC009	35.75 ± 0.61^{a}	45.63 ± 0.12^{b}	79.19 ± 1.00^{cd}	83.64 ± 0.46^{b}
K. lactis BIOTEC010	15.10 ± 0.30^{bc}	19.53 ± 0.20^{e}	54.41 ± 0.61^{gh}	62.94 ± 1.30^{cde}
L. pseudomesenteroides BIOTEC011	15.63 ± 0.26^{bc}	23.09 ± 0.00^{de}	54.14 ± 1.24^{gh}	68.58 ± 1.73^{cd}
L. pseudomesenteroides BIOTEC012	17.77 ± 0.21^{b}	19.47 ± 0.48^{e}	50.68 ± 0.00^{h}	63.22 ± 0.48e
L. kefiri BIOTEC013	9.59 ± 0.83^{d}	13.47 ± 0.89^{e}	56.49 ± 0.26^{g}	57.01 ± 0.57^{e}
L. kefiri BIOTEC014	35.45 ± 1.82^{a}	54.45 ± 0.24^{a}	97.71 ± 0.41^{a}	98.13 ± 0.29^{a}
L. parakefiri BIOTEC015	10.56 ± 0.05^{d}	43.54 ± 0.33^{b}	92.27 ± 2.62^{b}	98.76 ± 0.22^{a}

[a]Data are expressed as % of co-aggregation measured after 2, 6, 20 and 24 h of incubation. The values are means of duplicate measurements ± standard deviation. Different letters in the same column denotes significant differences among all the microorganisms studied.

Table 4. Co-aggregation abilities of isolates and controls with *S. typhi* BIOTEC019[a]

Microorganisms	**Co-aggregation with *S. typhi* (%)**			
	2h	**6h**	**20h**	**24h**
L. acidophilus La3	6.09 ± 0.01^{c}	19.78 ± 0.62^{de}	57.03 ± 0.39^{fg}	63.82 ± 0.07^{e}
L. rhamnosus GG	2.45 ± 0.00^{d}	17.13 ± 0.74^{fg}	54.75 ± 0.13^{g}	63.48 ± 1.13^{ef}
L. plantarum 299V	12.58 ± 0.42^{a}	20.02 ± 0.35^{de}	44.05 ± 1.06^{h}	53.76 ± 0.22^{g}
L. lactis BIOTEC006	10.19 ± 0.12^{ab}	27.09 ± 0.20^{ab}	78.42 ± 0.21^{a}	86.41 ± 1.09^{a}
L. lactis BIOTEC007	9.30 ± 0.60^{b}	20.50 ± 0.77^{de}	67.16 ± 0.24^{c}	77.74 ± 0.62^{bc}
L. lactis BIOTEC008	12.76 ± 0.16^{a}	28.42 ± 0.51^{a}	75.22 ± 0.37^{ab}	82.55 ± 1.92^{ab}
K. lactis BIOTEC009	11.81 ± 0.39^{ab}	27.13 ± 0.15^{ab}	61.20 ± 0.97^{dei}	63.57 ± 0.11^{ef}
K. lactis BIOTEC010	5.01 ± 0.25^{cd}	15.97 ± 0.42^{g}	45.82 ± 0.24^{h}	58.60 ± 0.15^{fg}
L. pseudomesenteroides BIOTEC011	5.65 ± 0.26^{c}	21.25 ± 0.15^{d}	55.42 ± 0.72^{g}	62.58 ± 0.19^{ef}
L. pseudomesenteroides BIOTEC012	10.84 ± 1.21^{ab}	24.75 ± 0.93^{bc}	64.65 ± 2.79^{cd}	75.62 ± 0.33^{cd}
L. kefiri BIOTEC013	9.53 ± 1.43^{b}	18.67 ± 1.18^{ef}	58.15 ± 0.76^{efg}	70.64 ± 2.41^{d}
L. kefiri BIOTEC014	11.33 ± 1.19^{ab}	16.19 ± 0.41^{fg}	60.86 ± 1.09^{def}	73.12 ± 2.92^{cd}
L. parakefiri BIOTEC015	9.76 ± 0.68^{b}	24.09 ± 0.76^{c}	72.50 ± 0.70^{b}	84.70 ± 1.03^{a}

[a] Data are expressed as % of co-aggregation measured after 2, 6, 20 and 24 h of incubation. The values are means of duplicate measurements ± standard deviation. Different letters in the same column denotes significant differences among all the microorganisms studied.

Among the results that stand out, it is observed that after 24 h, *Lactoccocus* species demonstrate a high coaggregation (>80%) with *E. coli* ATCC-25922 while higher percentages (>70%) are also reported for *Lactobaillus* BIOTEC013, BIOTEC014 and BIOTEC015 with *S. thyphi* BIOTEC019. Moreover, co-agregation with *S. aureus* ATCC-BAA-42 is also high in in *Lactoccocus* (>80%), *Leuconostoc* and *Lactobacillus* species (>70%). Comercial probiotics (*L. acidophilus* La3, *L. rhamnosus* GG and *L. plantarum* 299v) demonstrated aggregation values between 58-76% with the pathogens tested.

In the same way, *Lactobacillus* isolates were able to exhibit co-aggregation with both pathogens *Salmonella enterica* subsp. *enterica serovar Typhimurium* and S. *Typhi* reporting maximum co-aggregation potential with *L. fermentum* (69.00 ± 4.62%) *against S. Typhimurium* and *against S. Typhi* (68.55 ± 1.26%). Morever, *isolates L. plantarum* A5*, L.*

fermentum A8*, L. casei* LbS2 and *L. casei* LbS6 showed moderate co-aggregation potential (30–50%) after 3 hours of incubation (Mallappa et al., 2019). These results suggest the ability of kefir isolates to co-aggregate with pathogens and to compete for adhesion to the epithelial cell surface is a strain-dependent manner.

Table 5. Co-aggregation abilities of isolates and controls with *S. aureus* ATCC-BAA-42[a]

Microorganisms	**Co-aggregation with *S. aureus* (%)**			
	2h	**6h**	**20h**	**24h**
L. acidophilus La3	6.51 ± 0.90^{e}	22.63 ± 0.28^{ef}	64.94 ± 0.44^{efg}	73.84 ± 1.35^{efg}
L. rhamnosus GG	7.71 ± 0.12^{e}	21.76 ± 0.50^{f}	63.86 ± 0.44^{fg}	70.83 ± 0.02^{fg}
L. plantarum 299V	7.81 ± 0.09^{e}	23.24 ± 0.16^{ef}	67.86 ± 0.76^{def}	76.55 ± 0.72^{def}
L. lactis BIOTEC006	10.30 ± 0.08^{cd}	33.71 ± 0.23^{c}	70.67 ± 0.66^{cd}	84.35 ± 0.15^{cd}
L. lactis BIOTEC007	11.67 ± 0.14^{bc}	33.75 ± 0.26^{c}	69.70 ± 0.50^{de}	86.01 ± 0.67^{de}
L. lactis BIOTEC008	12.65 ± 0.99^{bc}	33.39 ± 1.59^{c}	72.89 ± 0.21^{bcd}	83.49 ± 0.50^{bcd}
K. lactis BIOTEC009	18.29 ± 0.01^{a}	43.33 ± 0.12^{a}	75.52 ± 0.23^{abc}	83.18 ± 0.94^{abc}
K. lactis BIOTEC010	7.00 ± 0.62^{e}	17.66 ± 0.20^{g}	48.94 ± 1.61^{h}	57.63 ± 0.42^{h}
L. pseudomesenteroides BIOTEC011	8.64 ± 0.02^{de}	25.09 ± 0.78^{e}	64.84 ± 0.19^{efg}	71.27 ± 0.01^{efg}
L. pseudomesenteroides BIOTEC012	8.06 ± 0.62^{de}	30.96 ± 1.06^{c}	60.98 ± 0.10^{g}	72.45 ± 0.53^{g}
L. kefiri BIOTEC013	13.07 ± 0.33^{b}	31.03 ± 0.12^{c}	63.87 ± 0.30^{lm}	72.87 ± 0.57^{fg}
L. kefiri BIOTEC014	6.53 ± 1.32^{e}	27.96 ± 0.66^{d}	79.56 ± 4.48^{a}	88.38 ± 0.61^{a}
L. parakefiri BIOTEC015	16.52 ± 0.10^{a}	38.69 ± 1.10^{b}	76.36 ± 0.23^{a}	79.79 ± 0.19^{ab}

[a] Data are expressed as % of co-aggregation measured after 2, 6, 20 and 24 h of incubation. The values are means of duplicate measurements ± standard deviation. Different letters in the same column denotes significant differences among all the microorganisms studied.

3.3.3 Antibiotic susceptibility

Results of susceptibility testing for the twelve tested antibiotics on the bacterial isolates and controls are shown in Table 6. From the table, it is observed that most of the isolates shown certain level or resistance to each antibiotic. However, *L. lactis* species showed no resistance to the majority of the antibiotics with an exception to gentamicin and erythromycin (BIOTEC007), penicillin and clyndamicin (BIOTEC008). Control strains and *L. parakefiri* BIOTEC015 showed high resistance to almost all antibiotics with the exception of

erythromycin; while *Leuconostoc* species showed high resistance to antibiotics like clyndamicin, sulfamethoxazole and vancomycin.

Table 6. Antibiotic resistance of the identified kefir isolates and commercial probiotics

Microorganisms	**Antibiotics**											
	AM	**CF**	**CFX**	**DC**	**CPF**	**GE**	**CLM**	**E**	**STX**	**PE**	**VA**	**TE**
L. acidophilus La3	+	+ +	+ + +	+ + +	+ + +	+ + +	-	-	+	+ + +	+ + +	+
L. rhamnosus GG	+ + +	+ + +	+ + +	+ + +	+ +	+ + +	+	-	+ + +	+ +	+ + +	+ + +
L. plantarum 299V	+ +	+ + +	+ + +	+ + +	+ +	+ + +	+	-	+ + +	+ +	+ + +	+ +
L. lactis BIOTEC006	-	-	-	-	-	-	-	-	-	-	-	-
L. lactis BIOTEC007	-	-	-	-	-	+ + +	-	+ + +	-	-	-	-
L. lactis BIOTEC008	-	-	-	+ +	+ +	+ +	+ + +	-	-	+ + +	+	-
K. lactis BIOTEC009	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +
K. lactis BIOTEC010	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +
L. pseudomesenteroides BIOTEC011	+ +	-	-	+ +	-	-	+ + +	-	+ +	-	+ + +	-
L. pseudomesenteroides BIOTEC012	+ +	-	-	+ +	-	-	+ + +	-	+ + +	-	+ +	-
L. kefiri BIOTEC013	+	-	+	+ + +	-	-	+	-	+ + +	+	-	-
L. kefiri BIOTEC014	-	-	-	-	+ + +	-	-	-	-	+	+ +	+ + +
L. parakefiri BIOTEC015	+ + +	+ + +	+ +	+ + +	+ +	+ + +	+	-	+ + +	+ + +	+ + +	+

+ + +, High resistance; + + Intermediate resistance; + Low resistance; -, No resistance; AM, Ampicilin; CF, Cephalothin; CFX, Cefotaxime; DC, Dicloxacilin; CPF, Ciprofloxacin; GE, Gentamicin; CLM, Clindamycin; E, Erythromycin; STX, Sulfamethoxazole; PE, Penicilin; VA, Vancomycin; TE, Tetracycline.

Gad et. al (2014) reported high susceptibility of LAB isolates to ampicillin and amoxicillin and more resistance to cephalosporins. Also, high vancomycin resistance rate was observed. This coincides with the results reported in this assay, since the LAB of the genus *Lactoccocus* did not demonstrate resistance to ampicillin, while most of the lactobacilli isolated from kefir showed a high resistance to vancomycin (Gad, Abdel-Hamid, & Farag, 2014). Moreover, it has been reported a substantial level of antibiotic resistance toward not only vancomycin, but also streptomycin, aztreonam, gentamicin, and/or ciprofloxacin antibiotics in dietary supplements (Wong, Ngu, Dan, Ooi, & Lim, 2015).

Antibiotic resistance of probiotics can be divided into “intrinsic” or “acquired”. Intrinsic or endogenus resistance is inherent to a bacterial species, which may be a desirable characteristic to help restore the host gut microflora during or after a course of antibiotics with usage of probiotics. Intrinsic resistance genes exist in the chromosomes of certain probiotics (mostly LAB). On the other hand, acquired resistance occurs when a bacterium that has been sensitive to antibiotics develops resistance by gene mutation of its own DNA or horizontal gene transfer, lateral exchange of genes between organisms. In this sense, LAB are considered carriers of resistance genes that could propagate their genes within the food chain between food and humans, as well as to the environment through these different mechanisms (M. Li et al., 2020).

Moreover, horizontal gene transfer among the probiotic strains have been reported for *L. rhamnosus*, *L. gasseri*, *L. paracasei*, *L. reuteri*, *L. plantarum*, and some other probiotics. These gene transfer processes in the gut may affect the host–microbe crosstalk and compromise the host health, due to the widespread dissemination of antibiotic resistance genes among many bacteria . Further, intestinal microbiota is a potential source of antibiotic resistant pathogens which may cause infections via the fecal–oral route or through nosocomial infections such as bacteremia, endocarditis, urinary tract infections, and contamination of surgical sites. Besides the antibiotic resistance genes, other factors of concern could be toxins and virulence factors (Huddleston, 2014; Lerner, Matthias, & Aminov, 2017).

However, according to the FAO and WHO, it is important to determine whether starter or probiotic cultures intended for human or animal consumption have mobile resistance genes that could be transferred to other microorganisms (M. Álvarez-Cisneros & Ponce-Alquicira, 2019). A further analysis is necesarry to detect resistant genes, with specific techniques such as PCR and novel DNA sequencing technologies.

3.3.4 Antimicrobial activity

Results of antimicrobial activity are shown in Table 7. Neutralized and non-neutralized supernatant cultures were used for the test, however, no halos were observed in the neutralized cultures. Therefore, only the halos observed in the non-neutralized cultures were measured (in milimeters) and reported (Figure 7). Halos measured were associated with the production of organic acids from the isolates. No halos were formed in

Lactoccocus species, therefore, no data is reported for *L. lactis* BIOTEC006, BIOTEC007 and BIOTEC008 in the Table 7.

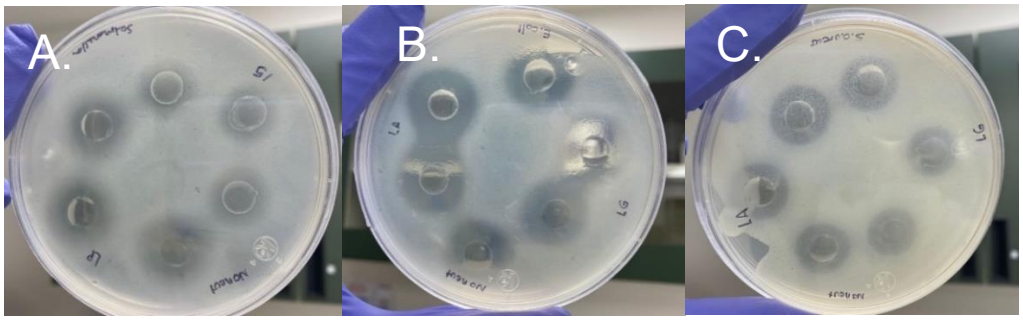


Figure 7. Halos observed in different cultures with a soft gel agar overlay of different pathogens. (A) Growing inhibition of *S. typhi* BIOTEC019 by non neutralized supernatants of *L. plantarum* 299v and *L. parakefiri* BIOTEC015; (B) Growing inhibition of *E. coli* ATCC-25922 by non neutralized supernatans of *L. rhamnosus* GG and *L. acidophilus* La3; (C) Growing inhibition of *S. aureus* ATCC-BAA-42 by non neutralized supernatants of *L. rhamnosus* GG and *L. acidophilus* La3.

Table 7. Antimicrobial activity measured in non neutralized cultures[a]

Microorganisms	**Media growth inhibition halos (in mm)**		
	Escherichia coli	***Salmonella typhi***	***Staphylococcus aureus***
L. acidophilus La3	18.33 ± 1.53^{a}	16.67 ± 0.58^{ab}	14.67 ± 0.58^{b}
L. rhamnosus GG	18.67 ± 1.53^{a}	17.67 ± 1.53^{a}	14.67 ± 0.58^{a}
L. plantarum 299V	19.00 ± 1.00^{a}	18.33 ± 0.58^{a}	15.33 ± 0.58^{b}
K. lactis BIOTEC009	14.67 ± 2.08^{a}	12.00 ± 1.00^{a}	15.33 ± 0.58^{a}
K. lactis BIOTEC010	14.33 ± 1.15^{a}	12.00 ± 0.00^{b}	15.33 ± 0.58^{a}
L. pseudomesenteroides BIOTEC011	13.00± 1.00^{b}	12.00 ± 0.00^{b}	15.33 ± 0.58^{a}
L. pseudomesenteroides BIOTEC012	15.00 ± 1.00^{a}	13.00 ± 0.00^{b}	16.33 ± 0.58^{a}
L. kefiri BIOTEC013	17.00 ± 1.15^{a}	14.67 ± 0.58^{b}	16.00 ± 1.00^{ab}
L. kefiri BIOTEC014	16.33 ± 1.15^{a}	14.67 ± 0.58^{a}	16.33 ± 0.58^{a}
L. parakefiri BIOTEC015	16.00 ± 0.58^{ab}	14.33 ± 0.58^{b}	15.33 ± 0.58^{a}

[a] The values measured are means of triplicate measurements ± standard deviation. Different letters in each row denote significant differences among production of organic acid against pathogens.

Results showed antimicrobial activity against *E. coli* ATCC-25922, *S. thyphi* BIOTEC019 and *S. aureus* ATCC-BAA-42 due to the production of organic acids and reduction of pH that inhibited pathogenic growth. Traditional fermented products can serve as vehicles for pathogenic bacteria; therefore, antimicrobial activity is an important technological aspect when selecting strains for the controlled production of fermented dairy products. The reduction in pH observed in fermented milk products is associated with the production of lactic acid and other types of organic acids by fermenting LAB (Obioha et al., 2021). It has been reported that microorganisms from fermented foods, such as *L. plantarum* isolated from Xinjiang traditional dairy product, showed strong antimicrobial activities against *Escherichia coli* and *Salmonella spp.*, being organic acids a key role in antimicrobial substances in fermentation broths (Hu, Ren, Zhou, & Ye, 2019).

Similar results were observed by Arena et. al (2016) when evaluating antimicrobial effect of *L. plantarum* strains against the pathogenic bacteria *Listeria monocytogenes, Salmonella Enteritidis, Escherichia coli* O157:H7 and *Staphylococcus aureus,* depending mostly of a pH-lowering effect of supernatants and/or on the presence of organic acids (Arena et al., 2016), similarly to the results reported in these study. This could be a considerable feature to be sought in the choice of starter or probiotic microorganisms. Indeed, live microorganisms carry out antimicrobial and preservative activity in the food when used as starters.

3.3.5 *In vitro* digestion assay

For the *in vitro* test of survival to gastrointestinal digestion samples were taken at each stage of digestion to have a number of colony-forming units and estimate the survival of each microorganism. These results are shown in Table 8. During the initial phase, all the isolates were at a high concentration levels (10^8-10^9) and after oral digestion some significant differences are observed in the concentration of *L. kefiri* BIOTEC014 bacteria, while the rest of the isolates remain at high concentrations. After the gastric phase, significant differences are observed in cell concentrations as well as a reduction of 2-3 log in *Lactococcus* species and *L. parakefiri* BIOTEC015. Finally, after the intestinal phase, significant differences are observed between isolates, with reductions of 1-4 log in *Lactoccocus* species, 2 log in *K. lactis* and *L. pseudomensenteroides* isolates. For *Lactobacillus* isolates, a reduction from 1.5 - 2.5 was observed. However, the concentration of commercial strains showed a reduction of less than 1 log.

Similar results have been reported in the survival of *Lactobacillus spp*. in fermented milk, which retained a high cell number throughout the digestion and decreased by only 1 log. As a matter of fact, gastric stage of digestion decreased cell viability, however, after the subsequent duodenal phase when the pH is re-adjusted to 7 for the intestinal phase, the cell concentration increased again 1 or 2 log, as noted in this study as well (Faye, Tamburello, Vegarud, & Skeie, 2012).

Table 8. Individual isolates survival and percentage of resistance to *in vitro* digestion[a]

Microorganisms	**LOG (CFU/mL)**			
	Initial phase	**Oral phase**	**Gastric phase**	**Intestinal phase**
L. acidophilus La3	9.41±0.01^{b}	9.62±0.01^{a}	8.88±0.02^{c}	9.15±0.01^{d}
L. rhamnosus GG	9.59±0.16^{a}	9.54±0.09^{a}	8.56±0.03^{b}	8.77±0.08^{b}
L. plantarum 299V	9.55±0.07^{b}	9.79±0.02^{a}	8.60±0.02^{d}	8.85±0.02^{c}
L. lactis BIOTEC006	8.39±0.12^{a}	8.38±0.12^{a}	4.84±0.08^{b}	4.75±0.21^{b}
L. lactis BIOTEC007	8.00±0.00^{b}	8.30±0.00^{a}	6.95±0.07^{c}	6.86±0.05^{c}
L. lactis BIOTEC008	9.35±0.49^{a}	8.65±0.07^{a}	5.45±0.21^{b}	5.22±0.21^{b}
K. lactis BIOTEC009	8.74±0.06^{a}	8.94±0.14^{a}	6.75±0.21^{b}	6.60±0.00^{b}
K. lactis BIOTEC010	8.96±0.05^{a}	9.09±0.02^{a}	6.99±0.12^{b}	6.75±0.21^{b}
L. pseudomesenteroides BIOTEC011	9.24±0.09^{a}	9.05±0.08^{a}	6.78±0.17^{b}	6.95±0.04^{b}
L. pseudomesenteroides BIOTEC012	8.80±0.02^{a}	9.02±0.03^{a}	6.90±0.00^{b}	6.99±0.12^{b}
L. kefiri BIOTEC013	9.09±0.12^{a}	9.15±0.21^{a}	6.58±0.00^{b}	6.56±0.17^{b}
L. kefiri BIOTEC014	8.09±0.04^{a}	7.84±0.00^{b}	6.46±0.06^{c}	6.50±0.00^{c}
L. parakefiri BIOTEC015	8.09±0.05^{a}	7.54±0.01^{a}	5.86±0.07^{b}	5.60±0.01^{c}

[a] The values measured are means of triplicate measurements ± standard deviation . Different letters in each column denote significant differences among each individual strain and the phases of the digestion

The results presented in the *in vitro* digestion are also consistent to the ones reporting a high survival rate of *Lactobacillus spp.* isolated from Malaysia kefir to low pH (96-98%) (Talib et al., 2019). Similar to the approach of this study, Escobar-Ramírez et al. (2020)

reported *an in vitro* digestion-resistant microorganism isolated from tepache (Mexican fermented pineapple drink), *L. plantarum* ABHEAU-05, which survived acidic conditions and the action of bile salts and pepsin. Ability of a microorganism to survive acidic conditions depends directly on the concentration of hydronium ions that accumulate inside the cell; survival under acidic conditions is positively affected by adaptation to low pH, a behavior known as the acid-tolerance response. Moreover, these study demonstrates the potential source of probiotics in fermented beverages in Mexico (Escobar-Ramírez et al., 2020).

3.3.6 Evaluation of bacterial growth on commercial prebiotics

Different commercial prebiotics (agave inulin, lactulose and citric pectin) were used as a source of fermentable carbon for the isolates of this study. Figure 8 shows the growth curves of the kefir isolates and control strains with the different substrates, using dextrose as a control. Table 9 shows maximum growth rates and lag parameter of microorganisms. As expected, the majority of the isolates grew well on dextrose or glucose, reaching maximum optical density values at 600 nm (OD_{max}) between 1.00 – 1.86. However, *Lactoccocus* species grew optimally on lactulose; moreover, the LAB and yeast reached maximum values in lactulose media, similar to the ones reported for glucose (0.77-1.85). On the other hand, citric pectin promoted the growth of only six isolates, among them *K. lactis* BIOTEC010, *L. pseudomesenteroides* BIOTEC011, *L. kefiri* BIOTEC013 and commercial probiotic strains *Lactobacillus acidophilus* La3, *Lacticaseibacillus rhamnosus* GG, *Lactiplantibacillus plantarum* 299v. In general, the maximum growth rate is constant at high optical density values and most strains have a similar growth rate, demonstrating that the strains can grow in commercial prebiotics. However, the lag time varies between 1 - 33 hours, highlighting that the longest lag times are observed for the pectin substrate, while the lag times are shorter for the other three substrates. These results can be compared to the ones reported by Chatterjee (2016), where the effect of pectin on the growth of LAB was assessed by the addition of pectin (0.4%) from different fruit waste to the MRS broth. It was found that pectin samples increased the growth of *L. casei* , showing a maximum growth of 2.4 OD at 660 nm with pectin from *S. lycopersicum* (Chatterjee & GA Manuel, 2016).

It has been reported that lactulose increases the population of *L. delbrueckii ssp. bulgaricus* (Delgado-Fernández, Corzo, Olano, Hernández-Hernández, & Moreno, 2019); yogurts or fermented milks supplemented with lactulose (4%) have shown to enhance the

acidification rate of these products and promote the growth of co-cultures of *L. acidophilus, L. rhamnosus* and *B. lactis* in combination with *S. thermophilus,* associated with lactulose metabolization (De Souza Oliveira, Rodrigues Florence, Perego, De Oliveira, & Converti, 2011). Similarly, the favorable growth of *L. acidophilus* strains has been observed in medium supplemented with lactulose (1%) in order to select potential strains to formulate synbiotics (Kneifel, 2000). Figueroa-González (2019) indicates lactulose generates good growth of several strains of lactobacilli (*L. rhamnosus* and *L. casei*). However, better indicative parameters of the stimulation of probiotics by prebiotic carbohydrates may be obtained using quantitative prebiotic parameters such as prebiotic index and prebiotic activity score. Moreover, it is also important to consider the metabolic system of each strain, due to the variations found in the utilization of a carbohydrate as a carbon source (Figueroa-GonzáLez, Rodríguez-Serrano, Gómez-Ruiz, García-Garibay, & Cruz-Guerrero, 2019). Lactulose is formed by a β-1,4- glycosidic bond, such as the prebiotic GOS. Therefore, the use of genes related to GOS metabolism have been used to predict potential lactulose-metabolizing bacteria. Although lactulose is a disaccharide composed of fructose and galactose, while GOS is a complex with different degrees of galactose polymerization, they may possess similar genes transporters or glycosidases, allowing the fermentation of this substrate by LAB (Mao et al., 2014).

Agave inulin promoted the growth of ten isolates (*Leuconostoc, Lactobacillus* and *Kluyveromyces* species), with maximum values between 0.57 – 1.22. Agave inulin has been reported to favor the growth of probiotic bacteria such as *Ligilactobacillus salivarius* and *Enterococcus faecium*. The growth was related to the molecular structure of the polymer, composed of linear fructose chains. In addition, the degree of polymerization of the molecule affects the degradation of inulin, promoting greater solubility, which favors its degradation and use (Ayala Monter et al., 2018). Another *Kluyveromyces* specie has been reported to ferment a fructan similar to inulin (agavin); *Kluyveromyces marxianus,* isolated from residua of tequila industry, produces a dimeric β-D-fructan fructohydrolase, with exo-inulinase activity on agavin and inulin (Trapala, Bustos-Jaimes, Manzanares, Bárzana, & Montiel, 2020).

Similarly, Garcia-Gamboa et. al, (2018) reported that probiotics *L. casei* and *L. paracasei* are able to metabolize agave fructans obtained from several species (*A. salmiana spp. crassipina, A. salmiana var. liso, A. atrovirens, A. tequilana spp*). This is also dependent of the polymerization degree of the fructan and the agave specie. Moreover, extracellular

and intracellular enzyme activity was observed by these probiotic bacteria when dextrose or fructans were used as carbon source (García Gamboa et al., 2018). Fructans from *A. salmiana* have also been reported as useful prebiotic due to its structural heterogeneity and to because of the maintenance of probiotic strains such as *Lacticaseibacillus casei*, *Lactobacillus acidophilus*, *Bifidobacterium longum subsp. infantis, Bifidobacterium longum subsp. longum*; since their effect showed a high growth rate, high production of SCFA and decrease of the pH value (Martinez-Gutierrez et al., 2017).

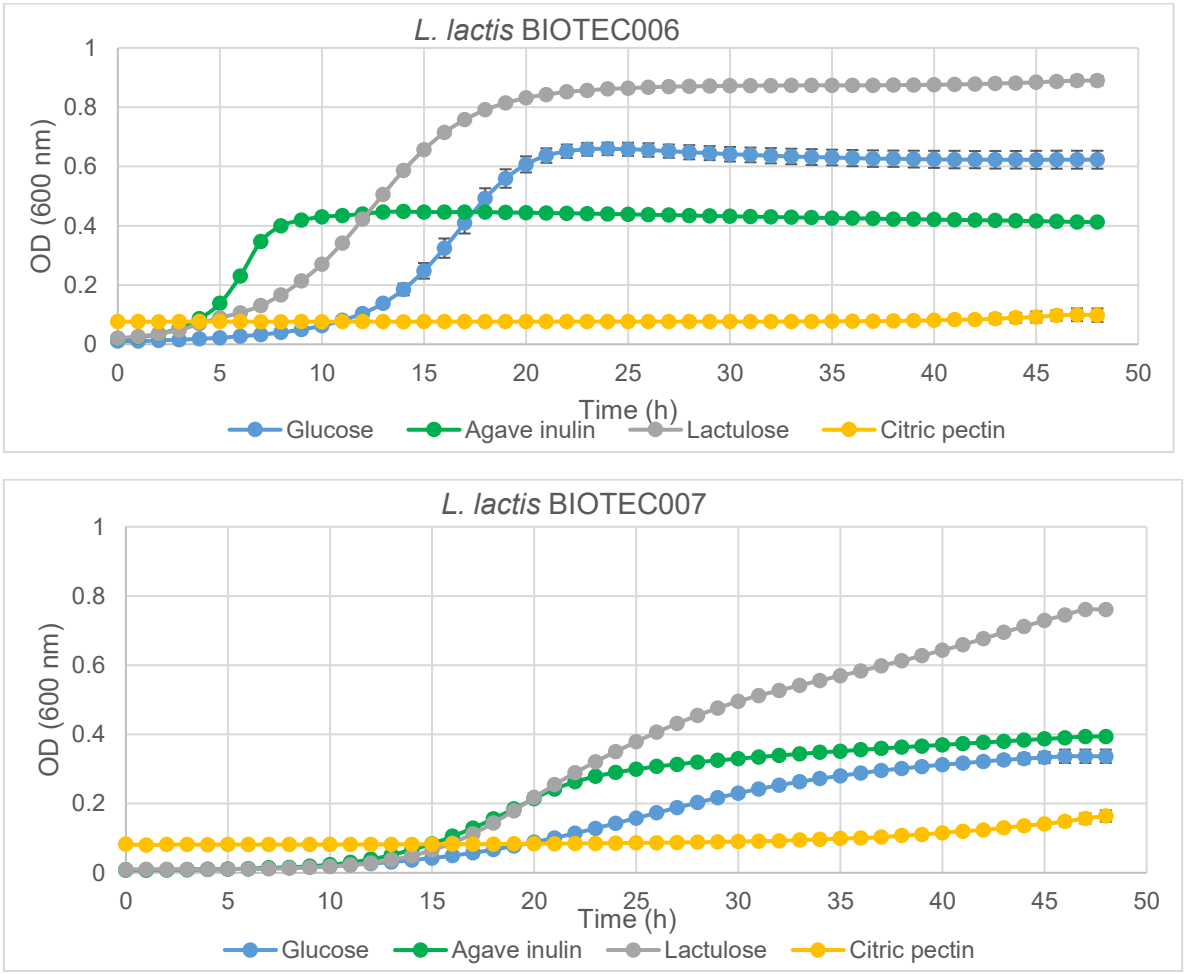


Figure 8 (part I). Growth curves of isolated LAB (*Lactoccocus, Lactobacillus, Leuconostoc*) and yeast (*Kluyveromyces*) on dextrose, agave inulin, lactulose and citric pectin (at 1%). Standard deviation was calculated and curves were done in triplicate.

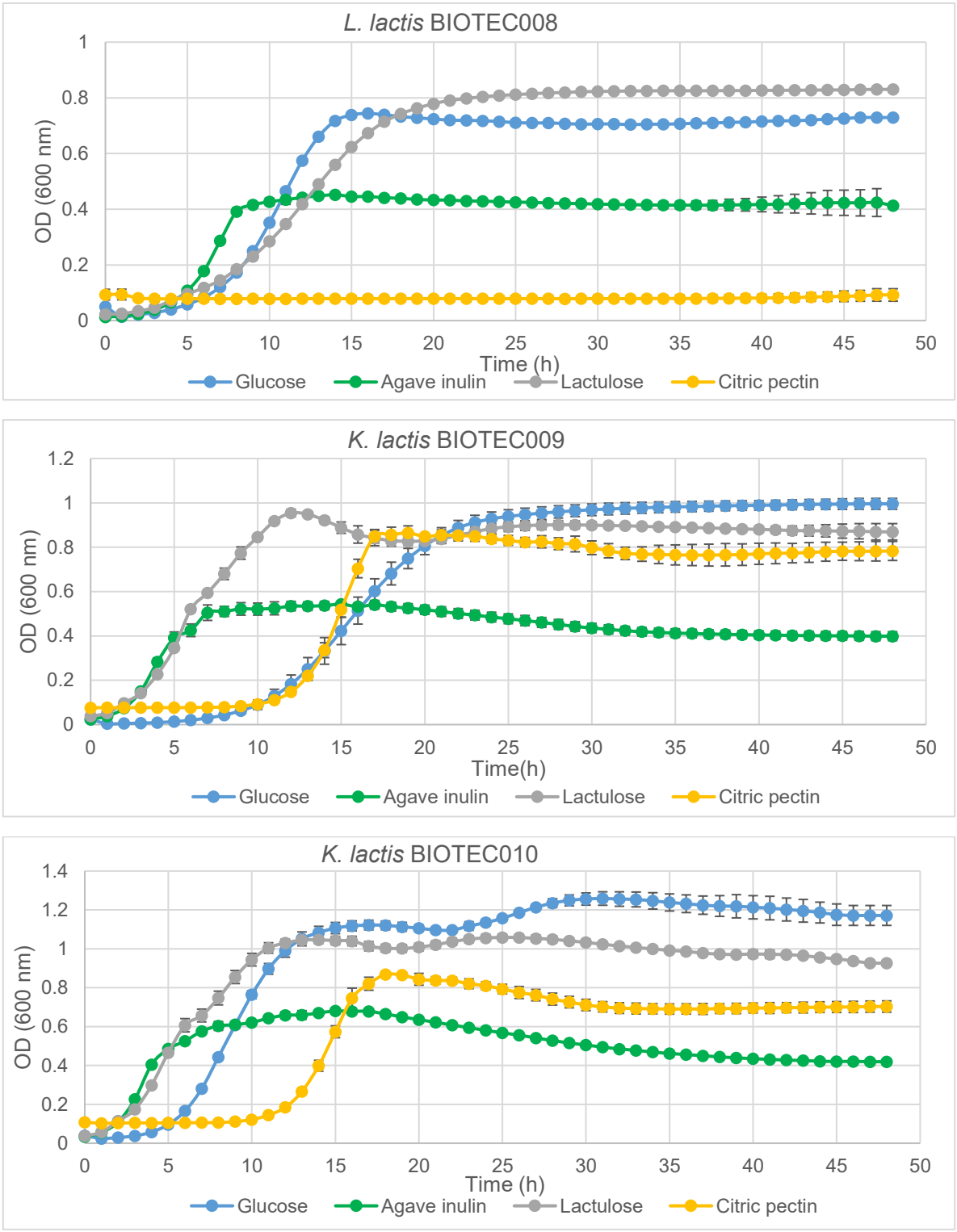


Figure 8 (part II). Growth curves of isolated LAB (*Lactoccocus, Lactobacillus, Leuconostoc*) and yeast (*Kluyveromyces*) on dextrose, agave inulin, lactulose and citric pectin (at 1%). Standard deviation was calculated and curves were done in triplicate.

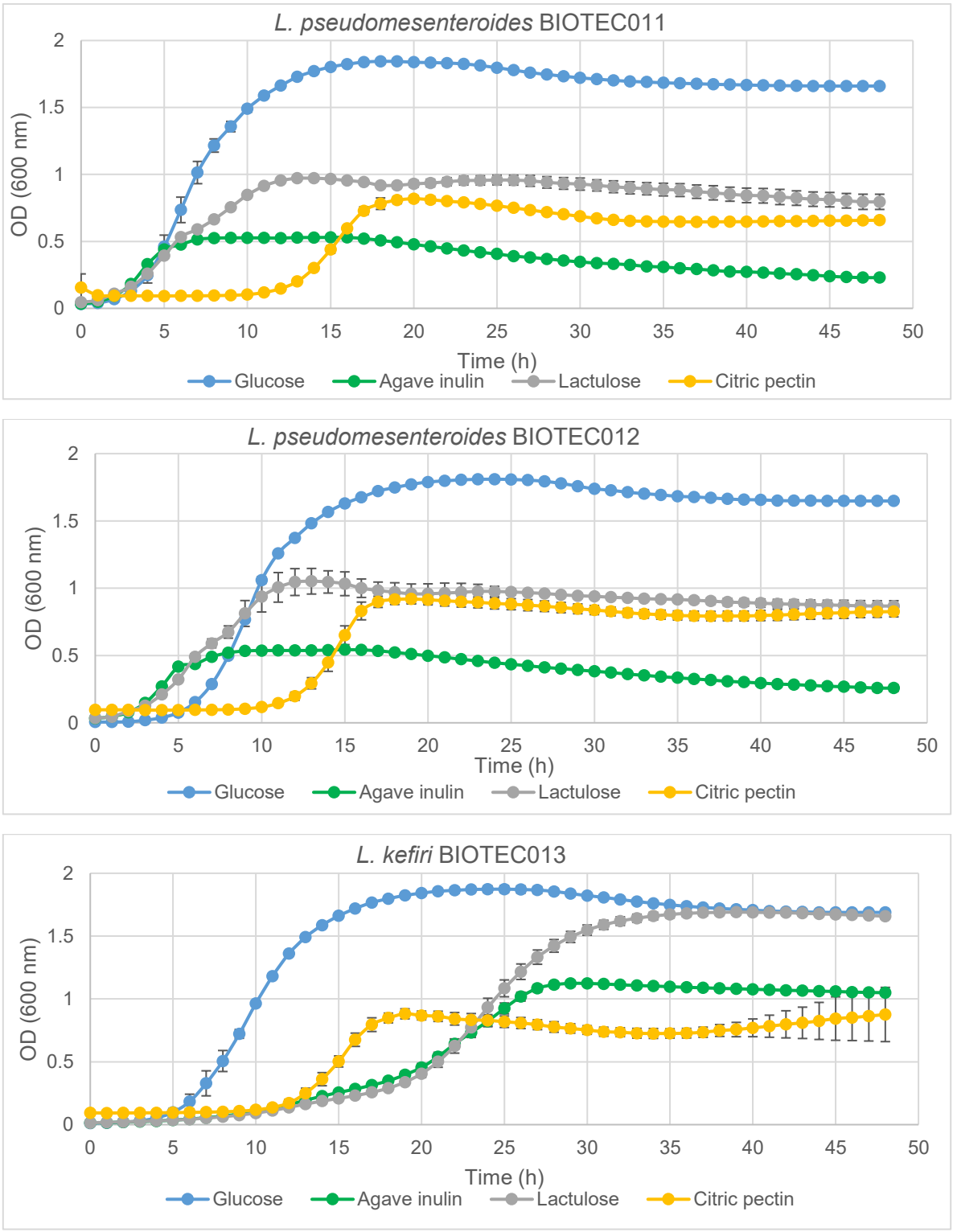


Figure 8 (part III). Growth curves of isolated LAB (*Lactoccocus, Lactobacillus, Leuconostoc*) and yeast (*Kluyveromyces*) on dextrose, agave inulin, lactulose and citric pectin (at 1%). Standard deviation was calculated and curves were done in triplicate.

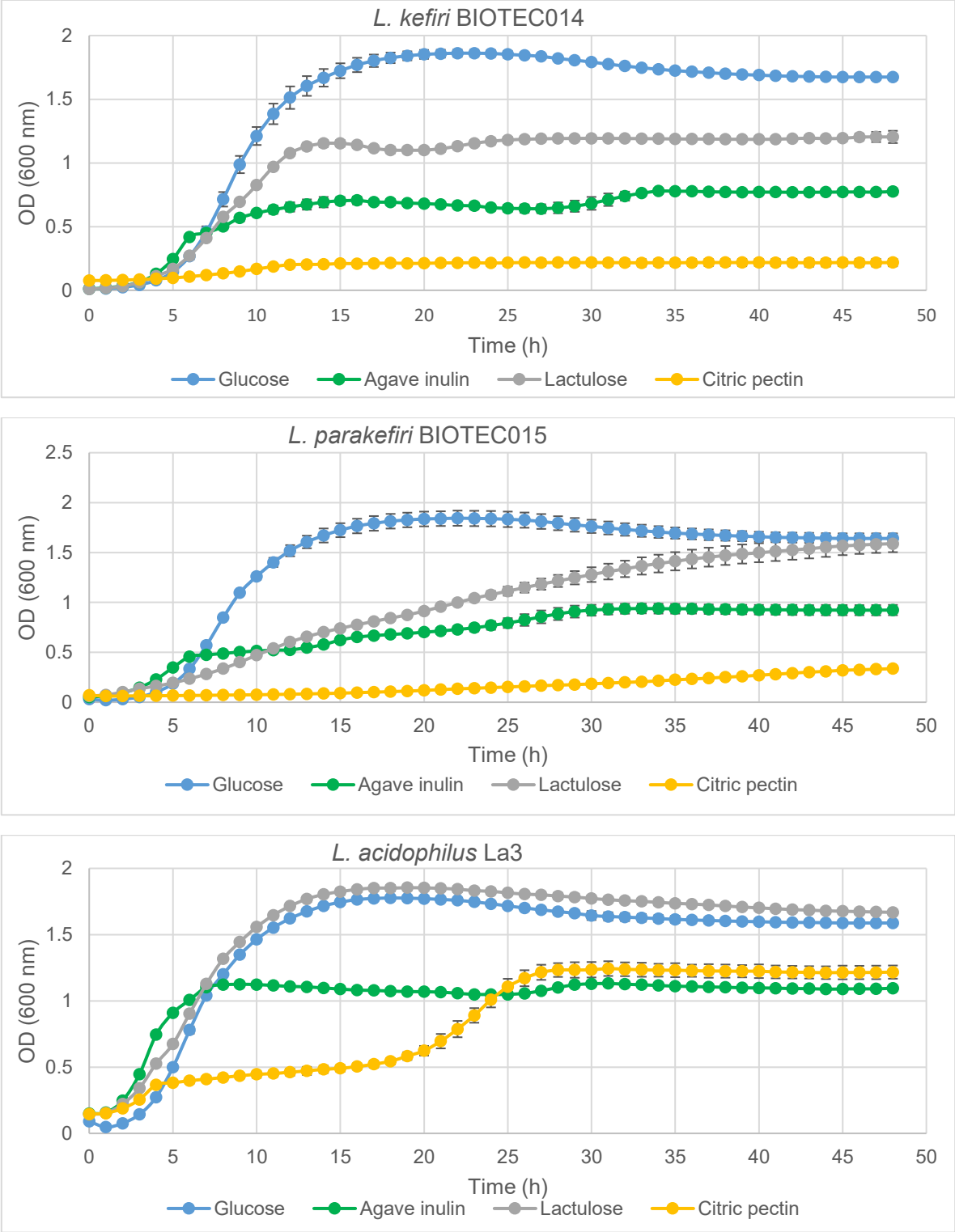


Figure 8 (part IV). Growth curves of isolated LAB (*Lactoccocus, Lactobacillus, Leuconostoc*) and yeast (*Kluyveromyces*) on dextrose, agave inulin, lactulose and citric pectin (at 1%). Standard deviation was calculated and curves were done in triplicate.

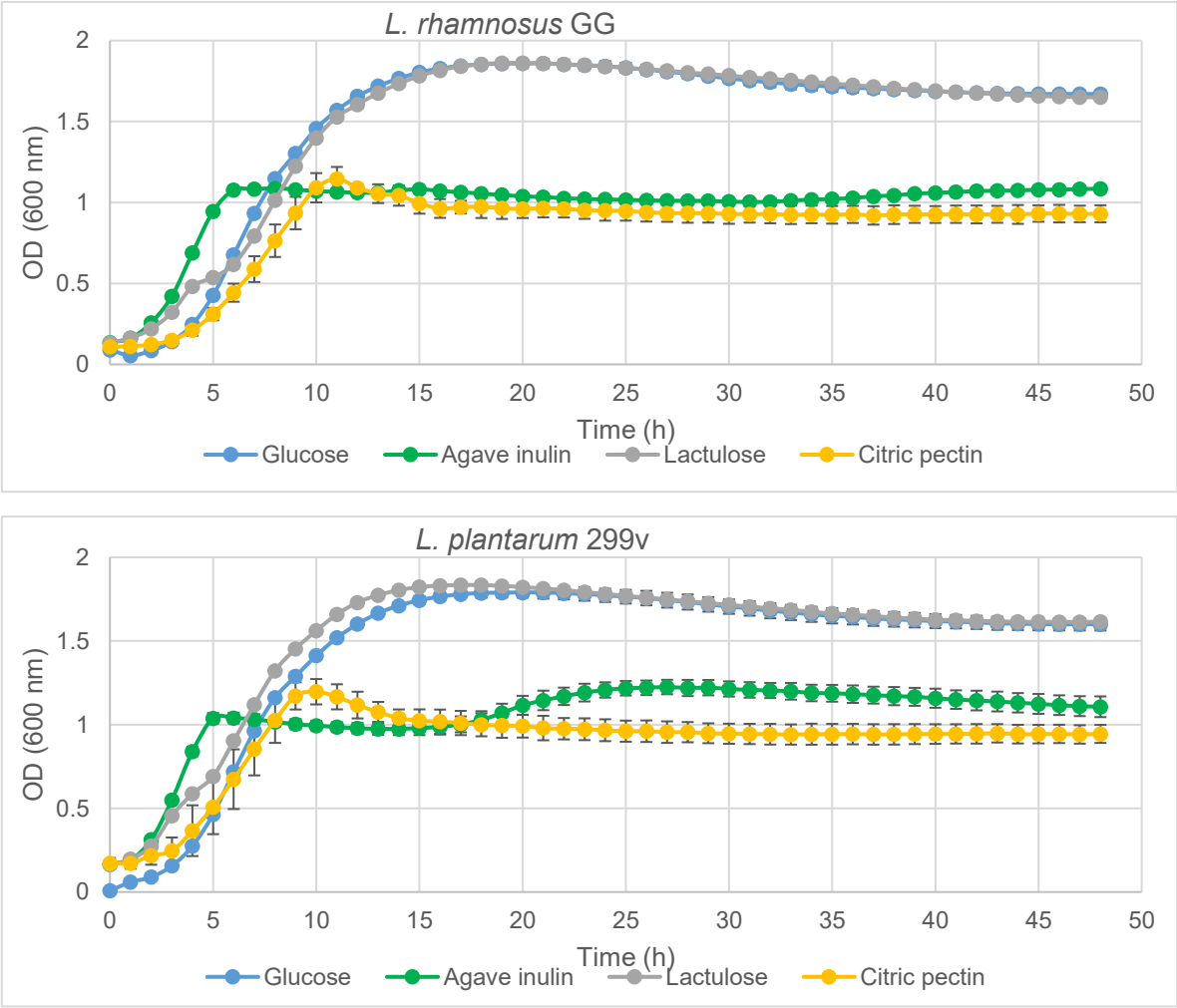


Figure 8 (part V). Growth curves of isolated LAB (*Lactoccocus, Lactobacillus, Leuconostoc*) and yeast (*Kluyveromyces*) on dextrose, agave inulin, lactulose and citric pectin (at 1%). Standard deviation was calculated and curves were done in triplicate.

Table 9. Maximum optical density at 600 nm (ODmax), maximum growth rate (μmax, h-1) and lag (h) parameters of bacteria growing under aerobic conditions on glucose, lactulose,agave inulin and citric pectin as carbon sources

Microorganisms		Glucose	Lactulose	Agave inulin	Citric pectin
L. acidophilus La3	OD_{max}	1.78 ± 0.01	1.85 ± 0.02	1.13 ± 0.02	1.24 ± 0.04
	μ_{max}	0.21 ± 0.01	0.19 ± 0.02	0.22 ± 0.02	0.09 ± 0.04
	lag	1.72 ± 0.01	1.95 ± 0.02	1.02 ± 0.02	16.80 ± 0.04
L. rhamnosus GG	OD_{max}	1.85 ± 0.02	1.86 ± 0.01	1.09 ± 0.02	1.15 ± 0.05
	μ_{max}	0.21 ± 0.02	0.18 ± 0.01	0.25 ± 0.02	0.16 ± 0.05
	lag	3.04 ± 0.02	3.21 ± 0.01	1.30 ± 0.02	3.04 ± 0.05
L. plantarum 299v	OD_{max}	1.79 ± 0.03	1.83 ± 0.02	1.22 ± 0.04	1.20 ± 0.07
	μ_{max}	0.19 ± 0.03	0.19 ± 0.02	0.27 ± 0.04	0.16 ± 0.07
	lag	1.32 ± 0.03	2.02 ± 0.02	1.56 ± 0.04	2.00 ± 0.07
L. lactis BIOTEC006	OD_{max}	0.66 ± 0.03	0.89 ± 0.01	0.45 ± 0.00	0.09 ± 0.02
	μ_{max}	0.08 ± 0.03	0.19 ± 0.01	0.27 ± 0.00	0.16 ± 0.02
	lag	12.02 ± 0.03	2.02 ± 0.01	1.56 ± 0.00	2.00 ± 0.02
L. lactis BIOTEC007	OD_{max}	0.34 ± 0.02	0.76 ± 0.00	0.40 ± 0.01	0.16 ± 0.02
	μ_{max}	0.01 ± 0.02	0.02 ± 0.00	0.02 ± 0.01	0.005 ± 0.02
	lag	14.01 ± 0.02	11.00 ± 0.00	10.74 ± 0.01	33.21 ± 0.02
L. lactis BIOTEC008	OD_{max}	0.74 ± 0.00	0.83 ± 0.00	0.45 ± 0.05	0.09 ± 0.02
	μ_{max}	0.11 ± 0.00	0.06 ± 0.00	0.10 ± 0.05	0.00 ± 0.02
	lag	7.03 ± 0.00	5.76 ± 0.00	4.40 ± 0.05	1.66 ± 0.02
K. lactis BIOTEC009	OD_{max}	0.99 ± 0.06	0.95 ± 0.04	0.54 ± 0.03	0.86 ± 0.05
	μ_{max}	0.08 ± 0.06	0.11 ± 0.04	0.11 ± 0.03	0.18 ± 0.05
	lag	9.89 ± 0.06	1.25 ± 0.04	1.14 ± 0.03	12.54 ± 0.05
K. lactis BIOTEC010	OD_{max}	1.26 ± 0.06	1.06 ± 0.03	0.68 ± 0.02	0.87 ± 0.05
	μ_{max}	0.14 ± 0.06	0.12 ± 0.03	0.10 ± 0.02	0.18 ± 0.05
	lag	5.02 ± 0.06	1.53 ± 0.03	0.00 ± 0.02	12.26 ± 0.05
L. pseudomesenteroides BIOTEC011	OD_{max}	1.84 ± 0.02	0.97 ± 0.05	0.53 ± 0.01	0.82 ± 0.02
	μ_{max}	0.21 ± 0.02	0.11 ± 0.05	0.13 ± 0.01	0.16 ± 0.02
	lag	1.78 ± 0.02	1.84 ± 0.05	1.27 ± 0.01	12.77 ± 0.02
L. pseudomesenteroides BIOTEC012	OD_{max}	1.81 ± 0.00	1.05 ± 0.04	0.54 ± 0.01	0.92 ± 0.03
	μ_{max}	0.22 ± 0.00	0.13 ± 0.04	0.12 ± 0.01	0.19 ± 0.03
	lag	5.60 ± 0.00	1.65 ± 0.04	1.47 ± 0.01	12.08 ± 0.03
L. kefiri BIOTEC013	OD_{max}	1.87 ± 0.02	1.69 ± 0.02	1.12 ± 0.02	0.89 ± 0.05
	μ_{max}	0.21 ± 0.02	0.13 ± 0.02	0.08 ± 0.02	0.16 ± 0.05
	lag	5.52 ± 0.02	17.35 ± 0.02	14.65 ± 0.02	12.28 ± 0.05
L. kefiri BIOTEC014	OD_{max}	1.86 ± 0.03	1.20 ± 0.02	0.78 ± 0.02	0.22 ± 0.02
	μ_{max}	0.23 ± 0.03	0.14 ± 0.02	0.07 ± 0.02	0.02 ± 0.02
	lag	4.90 ± 0.03	4.31 ± 0.02	0.00 ± 0.02	4.39 ± 0.02
L. parakefiri BIOTEC015	OD_{max}	1.84 ± 0.05	1.59 ± 0.04	0.94 ± 0.03	0.33 ± 0.02

μ_{max}	0.22 ± 0.05	0.04 ± 0.04	0.03 ± 0.03	0.01 ± 0.02
lag	4.33 ± 0.05	0.00 ± 0.04	0.00 ± 0.03	15.24 ± 0.02

The values measured are means of triplicate measurements ± standard deviation .

3.4 Conclusions

The results of this study showed the reliable identification of LAB and yeasts isolated from kefir grains through MALDI-TOF MS analysis, identifying species mostly belonging to *Lactoccocus*, *Leuconostoc*, *Kluyveromyces* and *Lactobacillus* genus. Overall, the isolates revealed the following desirable probiotic-related properties: autoaggregation, co-aggregation with patogens, antimicrobial activity related to the production of organic acids, resistance to diffrerent antibiotics and good growth in both laboratory media and media supplemented with commercial prebiotics such as lactulose and agave inulin. Moreover, isolated LAB and yeasts demonstrated to resist *in vitro* gastrointestinal digestion conditions. Thus, these data suggested that isolates such *as L. lactis* BIOTEC007, *K. lactis* BIOTEC009, *L. kefiri* BIOTEC014 and *L. pseudomesenteroides* BIOTEC012 are good candidates for further studies including fermentation of potential prebiotics from by-products, as well as growth and survival in dairy matrices.

CHAPTER 4. CHARACTERIZATION OF THE PHYSICOCHEMICAL PROPERTIES, BIOACTIVE COMPOUNDS, NUTRITIONAL CONTENT AND PREBIOTIC POTENTIAL OF BERRY WHOLE FRUITS AND BY-PRODUCTS

4.1 Introduction

Berry fruits are popularly consumed in fresh, frozen and processed forms, including yogurts, beverages and jams. Berries such as blackberry (some *Rubus* species), black raspberry (*Rubus occidentalis*), blueberry (*Vaccinium corymbosum*), red raspberry (*Rubus idaeus*), and strawberry (*Fragaria ananassa*) are popularly used in the human diet and also as part of the trend of functional food products and suplements. Berries contain high levels of a diverse range of phytochemicals, most of which are phenolic molecules. Additionally, they contain essential minerals, vitamins, fatty acids, and dietary fibers (Nile & Park, 2014).

Fruit and vegetable by-products such as bagasse, peels, trimmings, stems, shells, bran, and seeds account for more than 50% of fresh fruits and sometimes contain significant amounts of phytochemicals and essential nutrients in higher concentrations than the final product, displaying in addition, an impact on environmental, economic, and social sectors. Food development applying food by-products from different agroindustries is a great alternative to use secondary food products and revalorize them due to their low price, high existing amounts, and because they are sources of biomolecules and precursors of bioactive components (Ruiz Rodríguez et al., 2021). Moreover, the by-products of various fruits like mango, banana, watermelon, grape, pomegranate, papaya and apple are considered a good source of carbohydrates and antioxidants, wich could be potential prebiotics ingredients, generating changes in the composition or activity of the gastrointestinal microbiota and enhancing health benefits (Akter & M.S., 2020).

Multiple fruit by-products have been reported as possible prebiotic ingredients, such as seeds, skins and pomace of fruits like grape, goji, blackcurrant or berry combinations (Campanella et al., 2017; Fratianni et al., 2014; Skenderidis, Mitsagga, Lampakis, Petrotos, & Giavasis, 2019). However, an interesting approach is to explore specific regional varieties of berry by-products. Hence, the objective of this study was to obtain and characterize the bagasse of different Jalisco berries (raspberry, strawberry, blackberry and blueberry), determining bioactive compounds, antimicrobial activity and fiber and carbohydrate content;

and evaluate its potential use as a fermentable prebiotic ingredient by kefir isolates. In adittion, the substrate consumption during fermentation by the isolates was monitored.

4.2 Materials and methods

4.2.1 Chemicals, reagents and fruit material

All chemicals and reagents used were from analytical grade. Frozen fruits of raspberries (*Rubus idaeus*), strawberries (*Fragaria ananassa*), blueberries (*Vaccinium corymbosum*) and blackberries (*Rubus fruticosus*) were obtained from a local store in Zapopan, Jalisco, México (Global Premier frozen berries, México). Folin & Ciocalteu's phenol reagent, gallic acid standard and other reagents used for the assays were purchased from Sigma-Aldrich (St. Louis, MO, USA).

4.2.2 Physicochemical properties of whole fruit

A batch of each fruit was selected to evaluate the physicochemical characteristics. Apical caliber (cm), equatorial caliber (cm), weight (g) and moisture content (%) were measured for triplicate. Berries juices were obtained using a blender (Oster) and a kitchen strainer, and soluble solids, titratable acidity and pH were evaluated for triplicate. Soluble solids (°Brix at 25°C) were measured using a digital refractometer (Hanna instruments Hi 96801); titratable acidity (mL of citric acid/ mL of juice) was determined by neutralization of juice with NaOH 0.1 N until a pH of 8.3. Finally, pH was measured with a pHmeter (HORIBA LAQUAact-PH110-K).

4.2.3 Recovery of berries bagasse

After characterization, a part of the whole fruit was lyophilized at -83°C, 0.35 mPa (LABCONCO Triad 11044030774F) for subsequent tests. Another part of the fruit was processed to obtain a bagasse similar to the by-product generated in the agro-industry (Figure 9). Briefly, berries varieties were blended and filtered with cheese cloth to separate the pulp and seeds from the juice. Subsequently, pulp and seeds were centrifuged 4000 rpm x 15 min (GYFROZEN 1580R) in 50 mL tubes to maximize juice extraction and pulp separation. The juice was collected and stored at -20°C while the bagasse obtained was lyophilized under the same conditions as the whole fruit. The lyophilized powder was

subsequently grounded (IKA A10), reducing the particle size and stored at -20°C for later tests.

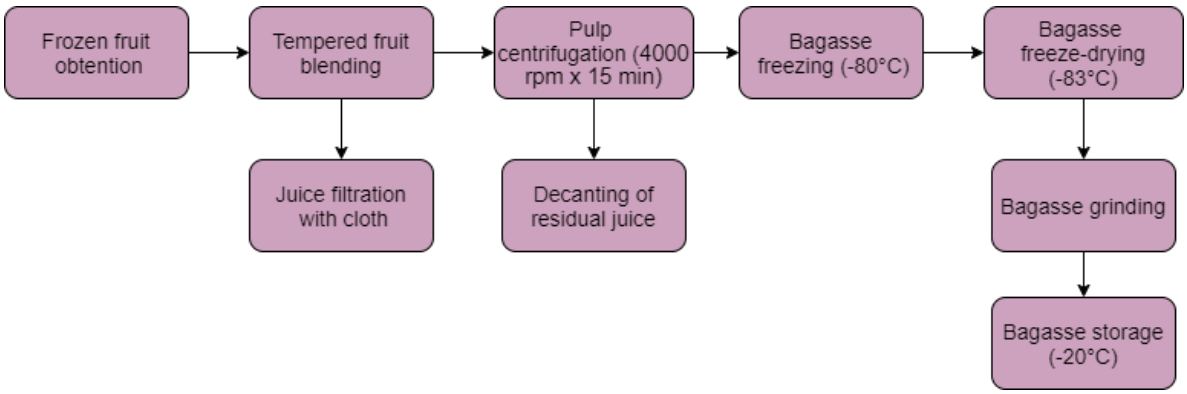


Figure 9. Bagasse obtention diagram

4.2.4 Nutritional content of berry bagasse

To know the composition of berry bagasses, a proximal analysis assay was carried out. First, total humidity content of bagasse was calculated according to the method 920.151 of the AOAC, where a sample was dried in a convection oven (BINDER) at 60 ºC until constant weight was achieved. Then, lyophilized berry bagasse powders were used for the other assays of the proximal analysis. Its humidity content was calculated using the same method in order to know the correction factor of the next assays. Ashes content was determined according to AOAC's method 940.26 where the sample was heated in a muffle (Thermolyne FB1215M) at 525 ºC for 5 hours. Protein content was calculated using AOAC 920.152 method, consisting in carrying out Kjeldahl's method of digesting, distilling and measuring the amount of nitrogen in the sample. In parallel, the lipid content was obtained using Goldfisch's modified protocol according to AOAC 960.39, where petroleum ether is used to extract the fat in the sample. At last, the dietary fiber content was calculated using AOAC's method 2011.25, which consists on a series of enzymatic digestions, filtrations and precipitations of the sample. All assays were carried out at least in duplicates.

4.2.5 Characterization of bioactive compounds in berry whole fruit and bagasse

a) Quantification of total phenolic content

The total phenolic content was determined by using Folin-Ciocaltue assay adapted to 96-well microplate (Sánchez-Rangel et al., 2013). For the obtention of methanolic

extracts, lyophilized berry powders were weighted (1 g) and suspended in methanol (4 mL). Additionally, other variables were evaluated to check if the extraction performance was increased, such as the use of solvent mixtures or the extraction assisted by ultrasonic bath. Briefly, different combinations of solvents with or without ultrasonic treatment were evaluated: methanol, methanol and water, acidified methanol, acidified methanol and water; in order to select the combination capable of extracting more bioactive compounds. However, the best extraction was observed using methanol without ultrasonic bath due to no significant differences were observed between treatments; thus, all the extracts were obtaining using methanol. The mixture was vortexed and centrifuged (4000 rpm for 5 min, 4°C). The supernant was collected in a separate tube and the operation was repeated washing the sample with 3 mL of methanol twice, until the obtainment of 10 mL of methanolic extract. Thereafter, the final extract (60 µL) was mixed with distillated water (960 µL) and Folin-Ciocalteu reagent (0.25 N, 60 µL) and after 3 min Na_2CO_3 (1 N, 120 µL) was added. The mix was incubated in a 2 mL microtube for 30 min at room temperature under dark conditions, then placed into wells of a 96-well microplate. Absorbance was measured at 765 nm using a microplate reader (Varioskan LUX multimode microplate reader, Thermo Scientific). Gallic acid was used as standard and the total phenolic content was expressed as mg of gallic acid equivalents per 100 g of fresh weight using a standard curve.

b) Measurement of monomeric anthocyanin content

Total anthocyanin content was determined according to the pH-differential method by UV-visible spectroscopy (Wrolstad & Acree, 2005). This methodology was carried out with the methanolic extract described above in this section. Two dilutions were prepared: 50 µL of each extract were diluted in 950 µL of 0.025 M potassium chloride buffer (pH 1.0±0.2) and another 50 µL of each extract were diluted in 950 µL of 0.4 M sodium acetate buffer (pH 4.5±0.2). Dilutions were equilibrated for 15 min at room temperature. After that, the absorbance of each dilution was measured at 510 nm (cyanidin-3-glucoside maximum absorbance value) and at 700 nm (to correct for haze), using distilled water as the blank. The final absorbance value of the diluted sample was calculated using the following formula: $A = (A510 - A700)\, pH1 - (A510 - A700)\ pH\ 4.5$, where A510 is the maximum absorbance value of the sample at 510 nm and A700 the absorbance correction value of the sample at 700 nm. The monomeric anthocyanin pigment (MAP) concentration in the original sample is calculated using the following equation: $MAP\ (mg/liter) = (A * MW * DF *$

$1000)/(E*1)$ where MW is the molecular weight (449.2), DF is the dilution factor (in this case 20), and E is the molar absorptivity (26900). Values used in this formula correspond to the predominant anthocyanin in the sample, cyanidin-3-glucoside.

4.2.6 Antimicrobial activity of berry bagasse extracts

The methanolic berry extracts described in the above section were evaporated to dryness at 40°C in rotary evaporator (BUCHI R-100). The dry material was redissolved in a 10% methanol-water solution and stored at -20°C for further tests. Antimicrobial assay was performed according to an adapted method (Krisch et al., 2008) of broth microdilution developed by the Clinical and Laboratory Standards Institute (Ferraro & Clinical and Laboratory Standards Institute, 2009). Inocula from pathogen bacterium *E. coli* ATCC-25922, *S. aureus* ATCC-BAA-42 and *S. typhi* BIOTEC019 (10^5- 10^7 cells/mL) were prepared in nutrient broth and 100 µL of fivefold diluted extract was mixed with 100 µL cell suspension in triplicates, incubated at 30°C for 36 h. Growth curves were developed recording optical density (600 nm) each hour.

4.2.7 Formulation of berry bagasse media as fermentative substrate

A culture medium was formulated based on the methodology of Jadhav et al. (2018) with some modifications. MRS culture medium was used as a base (proteose peptone 10 g/L, beef extract 10 g/L, yeast extract 5 g/L, dextrose 20 g/L, polysorbate 80 1 g/L, ammonium citrate 2 g/L, sodium acetate 5 g/L, magnesium sulfate 0.1 g/L, manganese sulfate 0.05 g/L, dipotassium phosphate 2 g/L), substituting dextrose for the lyophilized powder of the bagasse of the raspberry, blueberry, blackberry and strawberryp. Hence, 1 g of powder was homogenized in 100 mL of MRS medium without carbon source at 50°C with stirring for 2-3 hours. Subsequently, it was filtered (Whatman) and the pH was adjusted to 6.5±0.2 and autoclaved for 15 min at 121°C.

4.2.8 Evaluation of bacterial growth on berry bagasse media

The microorganisms were cultured overnight at 2% in MRS broth or M17 depending on the strain, harvested by centrifugation (3000 g for 10 min at 4°C), washed twice and re-suspended in sterile saline solution (0.85% NaCl). The different variations of MRS media prepared using dextrose (control) or berry bagasse powders as carbon sources were inoculated with microorganisms cultures (2%). Next, bacterial growth was monitored in triplicate in 300 µL wells of sterile 96-well microplates with lid (Corning). All cultures were grown in aerobic conditions at 30°C for 48 h. The optical densities at 600 nm of the isolates growing aerobically were recorded at 60 min intervals with an automated microplate reader (Varioskan Lux, Thermo Fisher Scientific). Maximum growth rates and lag parameter (lag) of microorganisms were calculated by fitting the curves to a sigmoid model using the Microsoft Excel add-in DMfit v.2.1 (available at http://www.ifr.ac.uk/ safety/DMfit/default.html), following the methodology for commercial prebiotics described in the section 3.2.9.

4.2.9 Analysis of substrate consumption

In order to elucidate the specific substrates used by the microorganisms and to quantify the growth of viable cells generated by the decomposition and use of the substrates, growth kinetics and 3,5-Dinitrosalicylic acid (DNS) method of reducing sugars were carried out. Briefly, overnight cultures were centrifuged (3000 g for 10 min at 4°C), washed twice, re-suspended in sterile saline solution (0.85% NaCl) and inoculated (2%) for triplicate in the four different media described in section 5.2.2 of this document. Sampling was carried out at 0, 5, 10, 20, 24, 30, 36, 46 and 48 h; 100 µL of each culture were taken and diluted to plate in MRS agar in order to count viable colonies. Meanwhile, 15 µL of supernatant were used for the DNS assay, following the methodology of reducing sugars adapted to a 96-well microplate (Wood et al., 2012). Briefly, 96-well microplate with lid (Corning) were used, each well contained 15 µL of sample and 285 µL of DNS solution (1:20, sample: DNS reagent). The resulting solutions were heated in a thermocycler (Biometra Tone 96G) typically at 100°C for 5 minutes. After cooling for 2 minutes, the plate was analysed using an automated microplate reader (Varioskan Lux, Thermo Fisher Scientific). Readings were made at 540 nm, and values were compared to a DNS standard curve.

4.2.10 Statystical analysis

Standard deviations and mean values were calculated for all the assays. In addition, Minitab Software was used to carry out analysis of variance (ANOVA), to determine significant differences in the analysis of physicochemical characteristics, bioactive compounds, growth of microorganisms with berry bagasse media, consumption of substate and *in vitro* digestion results of the differtent microorganisms tested. Furthermore, Tukey test was used for means comparison using a 0.05 significance level (p-value).

4.3 Results and discussion

4.3.1 Physicochemical properties of whole fruit

The physical appearance and physicochemical characteristics of berry species are presented in Table 10. Among varieties, there were significant differences observed in terms of apical caliber (cm), equatorial caliber and weight (g). Also between pH, soluble solids (° Brix at 25°C) and titratable acidity (g citric acid/ 100 g of fresh weight), which is interesting to to its potential different effects as fermentation substrate. However, there were no significant differences among moisture content. Characterization values of pH, soluble solids and titratable acidity are comparable to those reported for strawberry and blueberry, with pH values between 3.4 - 3.55 and soluble solids between 9 – 11 (Casati, Baeza, & Sánchez, 2019; Espinoza et al., 2017).

Table 10. Physicochemical characteristics of local berries*

Characteristics	Berry species			
	Strawberry	**Raspberry**	**Blueberry**	**Blackberry**
Apical caliber (cm)	2.84 ± 0.32[a]	1.26 ± 0.26b[c]	1.06 ± 0.09[c]	2.04 ± 0.15[d]
Equatorial caliber (cm)	2.34 ± 0.24[a]	1.50 ± 0.10[b]	0.82 ± 0.04[c]	1.54 ± 0.23[b]
Weight (g)	11.75 ± 2.90[a]	3.84 ± 0.47[bc]	1.54 ± 0.20[c]	6.25 ± 0.77[bc]
Soluble solids (° Brix at 25°C)	10.25 ± 0.25[ab]	9.08 ± 0.38[ab]	10.67 ± 1.53[a]	8.33 ± 0.58[b]
pH	3.56 ± 0.05[b]	3.15 ± 0.02[c]	4.25 ± 0.02[c]	3.15 ± 0.01[a]
Titratable acidity (g citric acid/ 100 g of fresh weight)	0.51 ± 0.03[a]	0.69 ± 0.01[b]	0.20 ± 0.01[a]	0.71 ± 0.02[c]
Moisture content (%)	82.20 ± 0.87[a]	80.85 ± 1.78[a]	82.09 ± 1.60[a]	82.83 ± 0.52[a]

*The values measured are means of triplicate measurements ± standard deviation. Different letters in each row denote significant differences among the characteristics measured in berry species

4.3.2 Nutritional content of whole fruit and proximal analysis of berry bagasse

Berry bagasses proximate composition for 100 g of fresh weight are presented in Table 10. Nutritional content of bagasses was compared to the nutritional content of frozen berries reported in the U.S. Department of Agricultute National Nutrient Database for Standard Reference, since the nutritional content of the frozen fruit was provided by the supplier and is comparable to that of the database. In general, the fiber content in the bagasse was higher than the reported in frozen or fresh berries, with blueberry containing the highest fiber content. Protein levels and fat content presented are also higher than in frozen or fresh fruits. This is mainly due to the processing of the fruit to obtain the bagasse, since results in accordance with this study have been reported for the proximal analysis of blueberry pomace, with a content of 26 g of fiber, 6 g of protein and 4 g of fat (in dry weight). The differences observed with fresh fruit are mainly attributed to the skins and seeds with a considerable content of fiber (mostly lignin and hemicellulose) that constitute blueberry pomace and its high content of seeds rich in fatty acids, resulting in an increase of the amount of fat. Moreover, carbohydrates content is lower because non-structural carbohydrates remain in the juice, which in this study was removed to obtain the bagasse (Tagliani, Perez, Curutchet, Arcia, & Cozzano, 2019).

Mainly, it has been reported that high contents of insoluble dietary fiber are found in the skins of berry pomaces, this is why the content is higher than the documented for fresh fruits. Other observed differences can beattributed to berry composition, the effectiveness of juice extraction, but also to low molecular mass fibre and trace amounts of starch (Reißner et al., 2019). High amount of fatty acids, carbohydrates and proteins are also documented for raspberry pomaces, due to the grinding of seeds and other factors besides the processing method of the fruit. For instance, concentration of these compounds in raspberries is strongly influenced by extrinsic factors, such as variations in plant type, growth stage, climate, season, temperature, and degree of ripeness. As a matter of fact, approximately 80% of the raspberry pulp consists of seeds, which have 23% content of oil (Fotschki et al., 2019).

Table 11. Proximate analysis of berry bagasses

	Berry bagasses proximal analysis (100 g of fresh weight)			
	Raspberry	**Strawberry**	**Blackberry**	**Blueberry**
Energetic content (Cal)	55.05 ± 0.16	69.93 ± 0.01	64.12 ± 5.83	116 ± 5.95
Total carbohydrates* (g)	6.42 ± 0.11	14.29 ± 0.18	8.02 ± 1.48	21.22 ± 0.52
Total dietary fiber (g)	17.90 ± 0.00	7.94 ± 0.00	18.94 ± 1.67	22.39 ± 1.20
Insoluble fiber (g)	17.28 ± 0.00	7.31 ± 0.00	17.67 ± 1.27	20.13 ± 1.68
Soluble fiber (g)	0.62 ± 0.00	0.63 ± 0.00	1.27 ± 0.06	2.26 ± 0.47
Protein (g)	1.83 ± 0.03	2.28 ± 0.12	3.04 ± 0.02	1.93 ± 0.17
Fat (g)	2.45 ± 0.07	0.40 ± 0.03	2.20 ± 0.00	2.66 ± 0.35
Ashes (g)	0.70 ± 0.05	0.81 ± 0.05	0.64 ± 0.22	1.23 ± 0.16

*Carbohydrates were obtained by difference. The values measured are means of duplicate measurements ± standard deviation

4.3.3 Characterization of bioactive compounds in whole fruit and berry bagasse

Total phenolic acids content (TPC) and total anthocyanin content (TAC) were measured in both fruit and bagasse, indicated in Table 12 and 13, respectively. Significant differences are observed between the TPC of each fruit, as well as the TPC of the bagasses.

In general, the content of bioactive compounds is higher in the whole fruit than in bagasse, this might be due to the process to obtain the baggase and juice is removed to generate the by-product; it has been reported different amounts of bioactive compounds in fruit, juice and berry puree (Oszmiański & Wojdyło, 2009) . Both TPC and TAC highest content in whole fruit and bagasse is found in blueberry. Regarding the anthocyanin content, differences are observed both in fruit and in bagasse and notably, TAC of strawberry and raspberry is significantly different from the TAC of blackberry and blueberry.

The phenolic content found in berries is comparable to that reported by Zia (2014). It is mentioned also that fruits exhibit different antioxidant capacity due to variations in vitamin C and E contents, phenolic, flavonoid and anthocyanin contents, solvents used for extraction and method used to assess antioxidant activity (Zia-Ul-Haq, Riaz, De Feo, Jaafar, & Moga, 2014)

Table 12. Characterization of bioactive compounds in whole fruit

Berry	Total phenolic acids content (mg of GAE/ 100 g of fresh weight)	Total anthocyanin content (mg of Cyd-3-glu/ 100 g of fresh weight)
Strawberry	147.16±1.00^{d}	97.86±0.18^{d}
Raspberry	205.25±0.95^{c}	148.62±0.03^{c}
Blueberry	299.03±0.90^{a}	468.47±0.18^{b}
Blackberry	210.67±1.04^{b}	1293.50±1.04^{a}

*GAE, Gallic Acid Equivalents; Cyd-3-glu, Cyanidin-3-glucoside. The values measured are means of triplicate measurements ± standard deviation. Different letters in each column denote significant differences among berries.

Table 13. Characterization of bioactive compounds in berry bagasse

Berry	Total phenolic acids content (mg of GAE/ 100 g of fresh weight)	Total anthocyanin content (mg of Cyd-3-glu/ 100 g of fresh weight)
Strawberry	52.52 ± 1.00^{d}	53.05 ± 0.17^{c}
Rasbberry	66.56 ± 0.95^{c}	41.26 ± 0.02^{c}
Blueberry	297.90 ± 0.91^{a}	1288.62 ± 1.04^{a}
Blackberry	213.61 ± 1.05^{b}	232.45 ± 0.17^{b}

*GAE, Gallic Acid Equivalents; Cyd-3-glu, Cyanidin-3-glucoside. The values measured are means of triplicate measurements ± standard deviation. Different letters in each column denote significant differences among berries.

4.3.4 Antimicrobial activity of berry bagasse extracts

The effects of the methanolic berry extracts on the pathogen growth are observed in Figure 10. All the berry methanolic extracts inhibited the growth of *E. coli* ATCC-25922, with a maximum absorbance value of 0.28 (OD 600 nm). Similar results are observed for *S. typhi* BIOTEC019 and *S. aureus* ATCC-BAA-42 growth, indicating maximum absorbance values of 0.17 and 0.35 respectively. Hence, berry methanolic extracts had a less inhibiting effect on *S. aureus* ATCC-BAA-42 growth, compared to the other pathogens tested. Blackberry extract presented the highest pathogen inhibition while strawberry extract showed the lowest antimicrobial activity.

These results can be compared to the ones reported by Krisch et al. (2008). In that study, the *in vitro* antibacterial activity of fruit juices and pomace extracts were evaluated by microdilution plate assays, using a methanolic extract redissolved in a 10% methanol-water solution, as indicated in this study as well. Black currant, cornelian cherry and European rowan had the highest growth inhibition capacity, reporting that *E. coli* was the most sensitive strain to the juices and methanolic extracts. Moreover, raspberry juice totally inhibited the growth of *E. coli* while the extracts had a moderate inhibitory effect of *B.subtilis*. The low pH of fruit juices caused by weak organic and phenolic acids, generates undissociated compounds that are able to interact with cell membranes and penetrate into the cells causing acidification of the cytoplasm. Moreover, antimicrobial potential of anthocyanic extracts of strawberry against *S. aureus* has been reported. Ethanolic extracts showed significant antibacterial activity ($p \leq 0.05$) on the growth of *S. aureus* ATCC 27543, since all the assayed amounts inhibited its growth. In addition, inhibition percentages observed oscillated between 2.4% and 53.6%, which increased in proportion to the applied amount. Inhibitory potential present of strawberry extracts was attributed to the synergy between the anthocyanins and other phenolic compounds present in the extracts, added to the acidic conditions of these (Cárdenas-Valdovinos et al., 2018).

Antimicrobial activity of methanolic and ethanolic extracts of different fruits may be due to dissolving fruit components in the solvent. Further, activity of extracts at even low concentrations could change the membrane permeability of Gram negative and positive bacteria, and fungi. It is also reported that a synergistic effect of the phenolic compounds in fruit and vegetables extracts, particularly anthocynins mixture, may explain the potent

antibacterial action and significant antifungal activity reported in the literature (Hafidh, 2011; Nirmala et al., 2018).

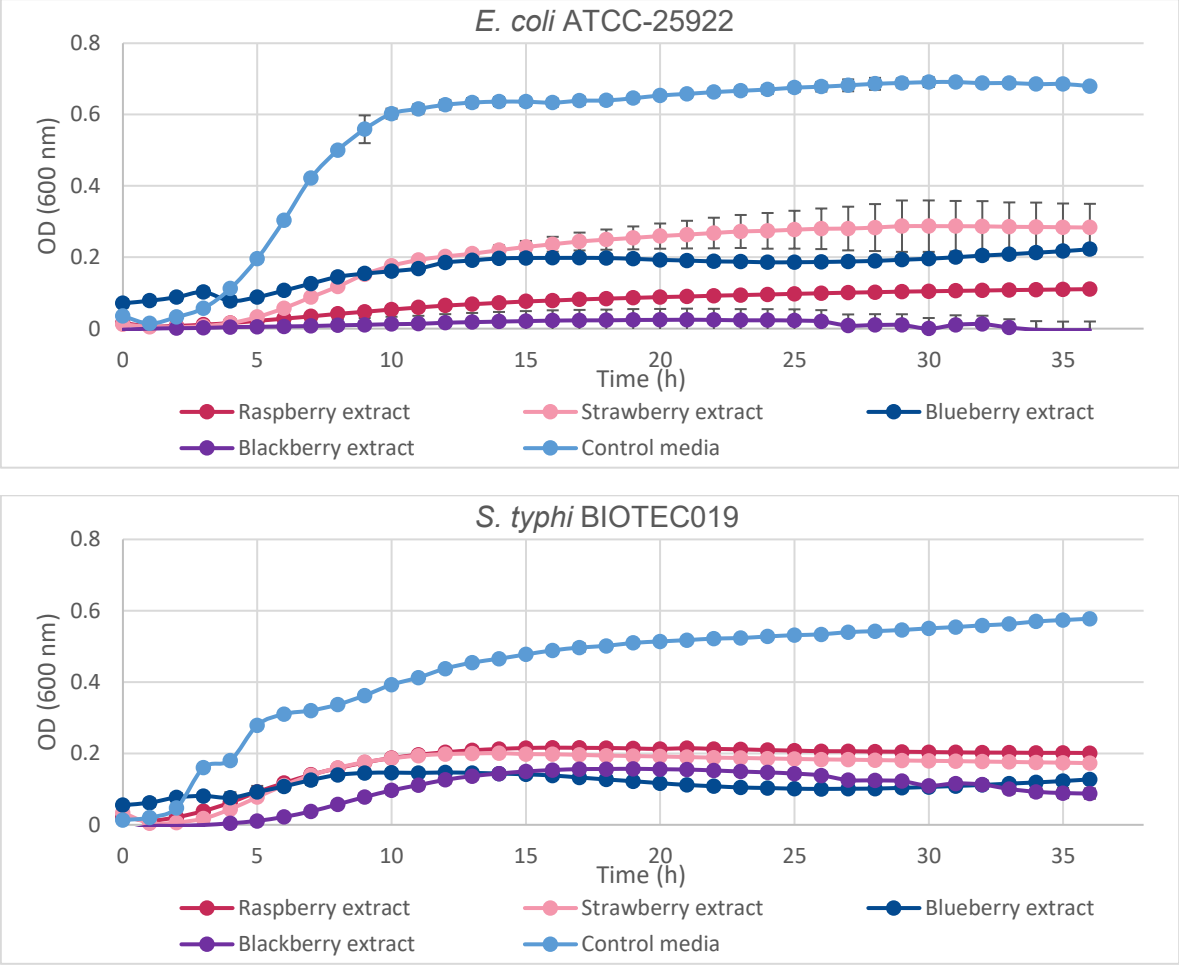


Figure 10 (Part I). Effect of raspberry, strawberry, blueberry and blackberry methanolic extracts on the growth of pathogenic strains, compared to the control (nutrient broth). Standard deviation was calculated and curves were done in triplicate.

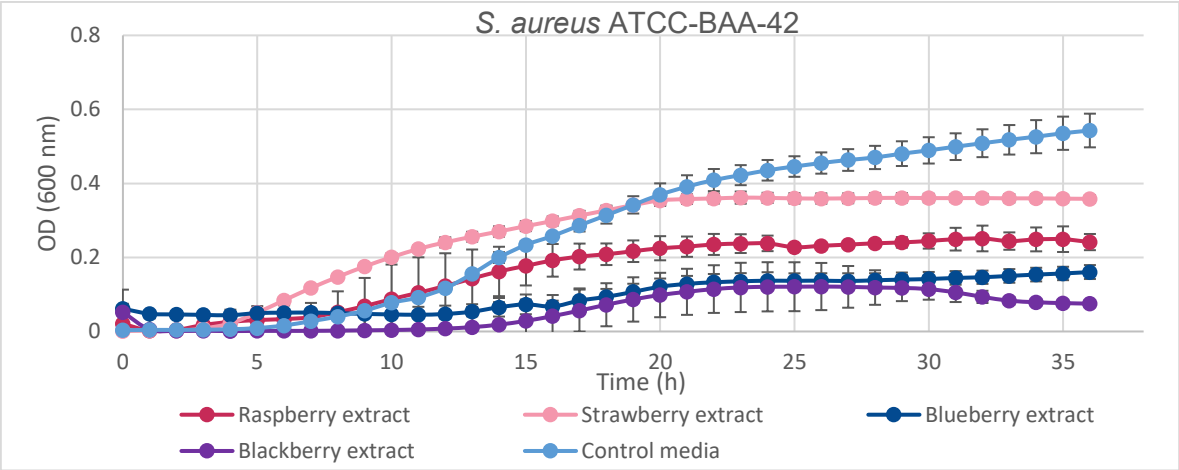


Figure 10 (Part II). Effect of raspberry, strawberry, blueberry and blackberry methanolic extracts on the growth of pathogenic strains, compared to the control (nutrient broth). Standard deviation was calculated and curves were done in triplicate.

Recently, antimicrobial activity of different fruits was investigated. Lyophilised blackcurrant and apple by-products inhibited several pathogenics strains (*E. coli* ATCC 25922, *E. coli* ATCC 35218, *S. aureus* ATCC 25923, *S. aureus* ATCC 29213*, P. aeruginosa* ATCC 27853, *E. faecalis* ATCC 29212 and *L. monocytogenes*). Furthermore, accorging to this study, lyophilisation is an optimal tecqnique for the dehydration of by-products since a broader spectrum of the antimicrobial properties was reported by their lyophilised samples, in comparison with vacuum dried fruits (Bartkiene et al., 2019).

4.3.5 Evaluation of bacterial growth on berry bagasse media

Growth of the kefir isolates of the genus *Lactoccocus, Lactobacillus*, *Leuconostoc* and *Kluyveromyces*, as well as the commercial probiotic strains were evaluated on different media supplemented with lyophilized berry bagasses. Growth curves are presented in Figure 11 and Table 14 shows maximum growth rates and lag parameter of microorganisms in each media. It is notable that the maximum growth rate coincides with the high optical densities observed in the blueberry and strawberry media and in general all lag times are short (1-5 hours). The results indicate that most of the isolates can grow on berry sutrates in a short period of time and with growth rates that are comparable to the control. Blackberry and raspberry media showed lower growth rates as well as lower optical densities. In general, microorganisms reached maximum growth values (OD 600nm) in media supplemented with strawberry and blueberry bagasse. Microorganisms showed optical

density values between 0.51 and 1.22 in strawberry bagasse media, while in blueberry bagasse media values vere among 0.54 and 1.19. Lower growth values were showed in raspberry and blackberry media (0.41-0.98). The high growth of the isolates in medium supplemented with strawberry or blueberry may be due to the carbohydrate content in the strawberry bagasse, or the fiber content of the blueberry bagasse, reported in the previous section of this chapter. The microorganisms that showed the highest growth were *L. rhamnosus* GG (among the commercial strains), *L. lactis* BIOTEC007, *L. lactis* BIOTEC008 and *L. kefiri* BIOTEC014.

Potential prebiotic activity of agro-industrial co-products derived of fruit processing have been previously reported. For instance, apple bagasse flour presented a similar performance as compared to glucose to promote the growth of lactic acid bacteria. Maximum specific growth rates were observed with disaccharides and oligosaccharides as compared to monosaccharide. The ability to utilize a prebiotic is strain-dependent, conditioned on the ability to depolymerizing capacity and chemical structure and polymerization degree of the carbon sources (Hernández-Alcántara, Totosaus, & Pérez-Chabela, 2016). Apple and banana total dietary fiber have been also reported to increase the probiotic viability when incorporated to skim milk yoghurts fermented by four different probiotics strains: *Lactobacillus acidophilus* L10 and *Bifidobacterium animalis* subsp. *lactis* BL04, HN019 and B94, helping to preserve its viability until the fourth week of cold storage (do Espírito Santo et al., 2012).

Addtionally, it has been documented that guava and acerola by-products could be novel prebiotic ingredients because they can stimulate the growth and metabolism of probiotics and induce overall beneficial changes in human colonic microbiota. These by-products contain insoluble and soluble fibers and fructans, also small amounts of monosaccharides such as glucose and fructose, enhancing the use of these materials as sources of direct fermentable substrates by *Bifidobacterium*, *Lactobacillus* and *Enteroccocus* (Menezes et al., 2021).

Regarding berries or similar fruits, the potential prebiotic action of Goji berry powder on selected probiotic bacteria has been investigated showing that the addition of the Goji extracts promoted the proliferation of *Bifidobacterium* and *Lactobacillus* species (Skenderidis et al., 2019). Moreover, the commercial grape seed extracts added to growth media have presented specific phenolic profiles and hase allowed a maximal growth *of L. plantarum*, *L. casei*, and *L. bulgaricus* strains in the presence of phenolic extracts in the

growth medium. However, The transformation of the different polyphenols in the gut depends on microbial esterase and glucosidase, as well as on demethylation, dehydroxylation and decarboxylation activities. Further, unabsorbed dietary polyphenols and their metabolites can behave as activators or inhibitors of bacterial growth depending on their chemical structure (Tabasco et al., 2011).

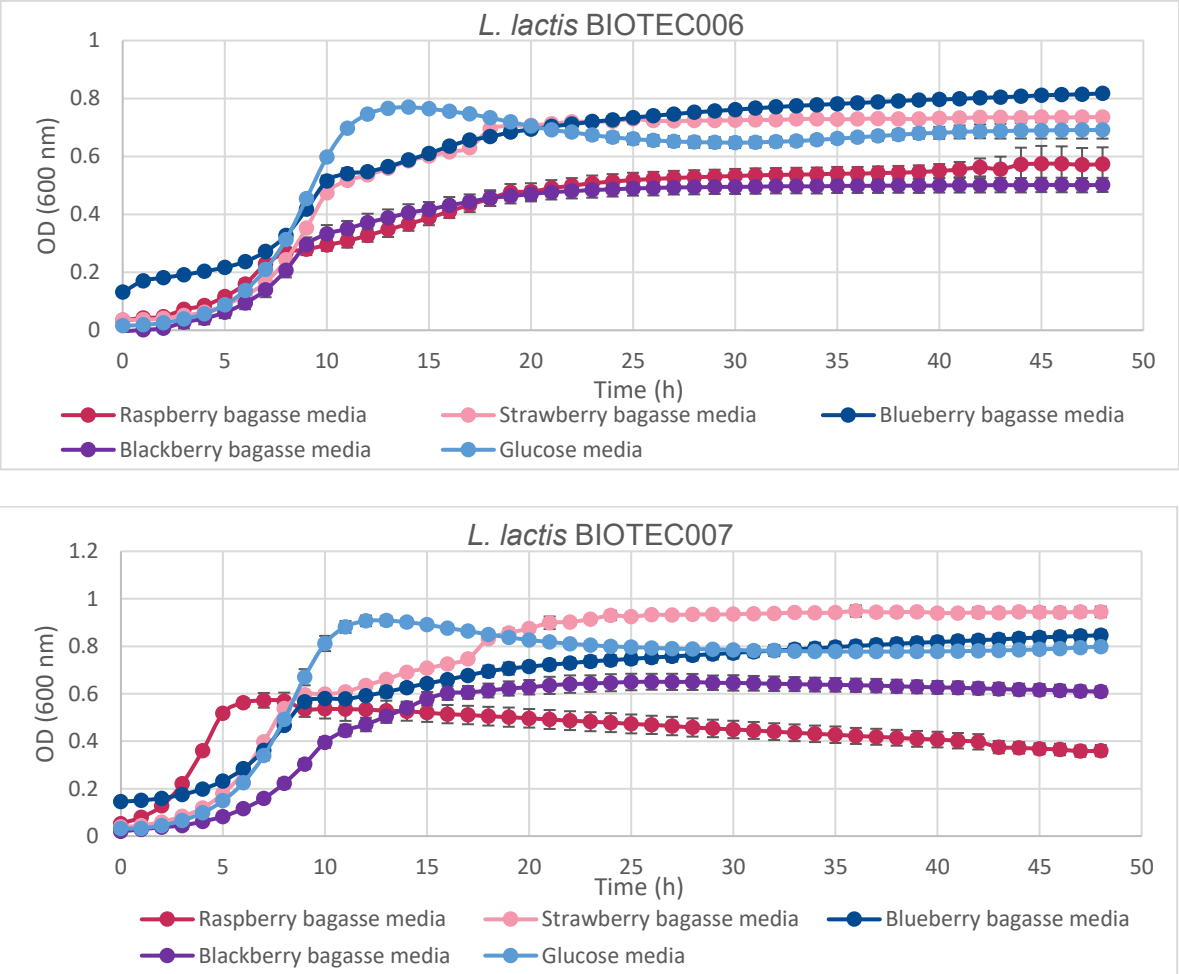


Figure 11 (Part I). Growth curves of isolated LAB (*Lactoccocus, Lactobacillus, Leuconostoc*) and yeast (*Kluyveromyces*) on dextrose (control), raspberry, strawberry, blackberry and blueberry bagasse (1%) media. Standard deviation was calculated and curves were done in triplicate.

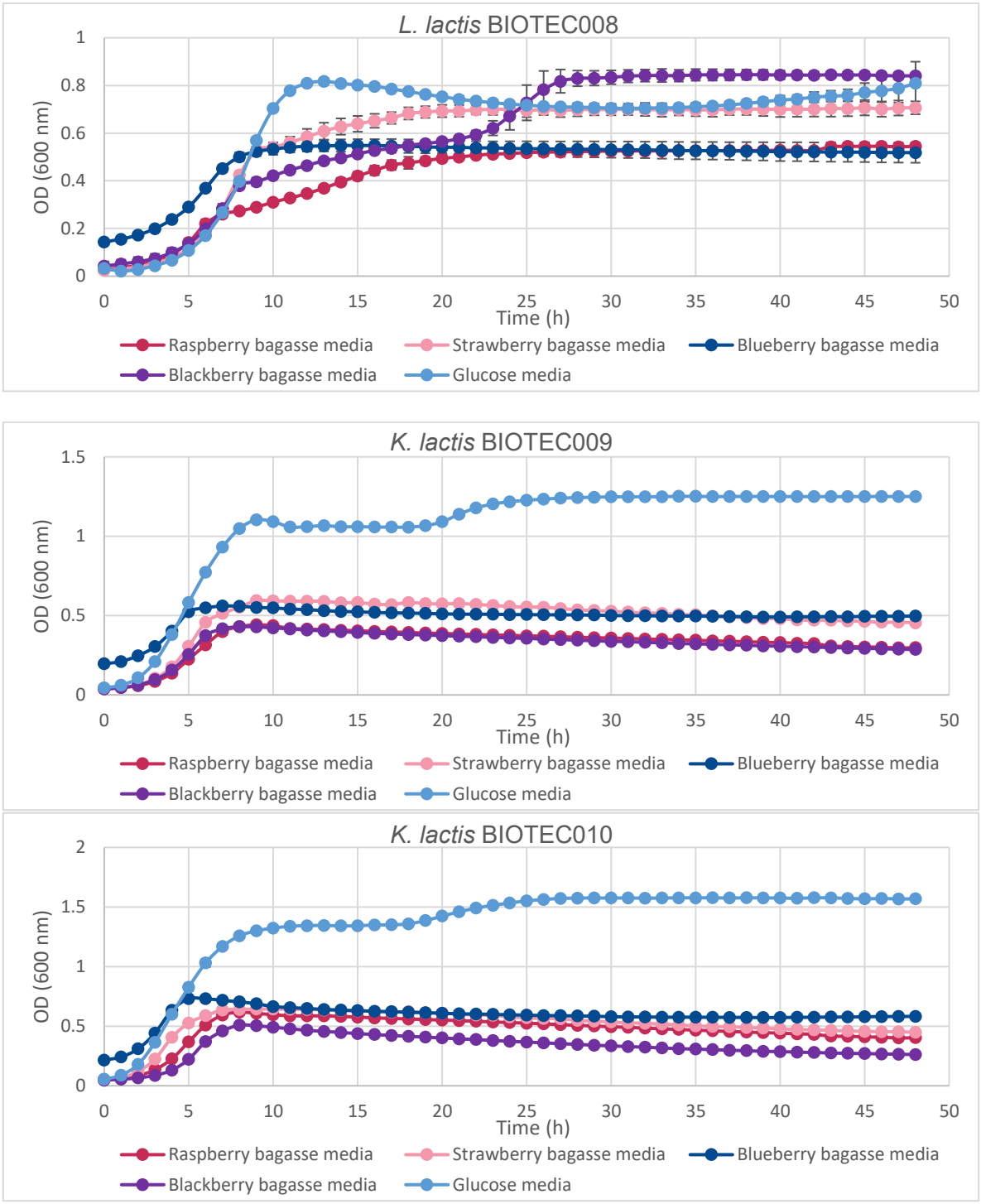
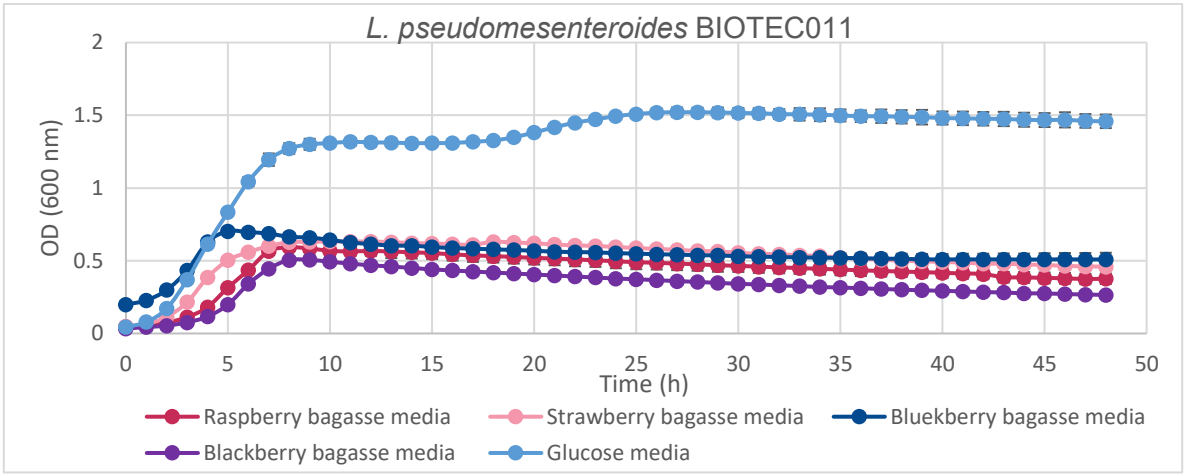
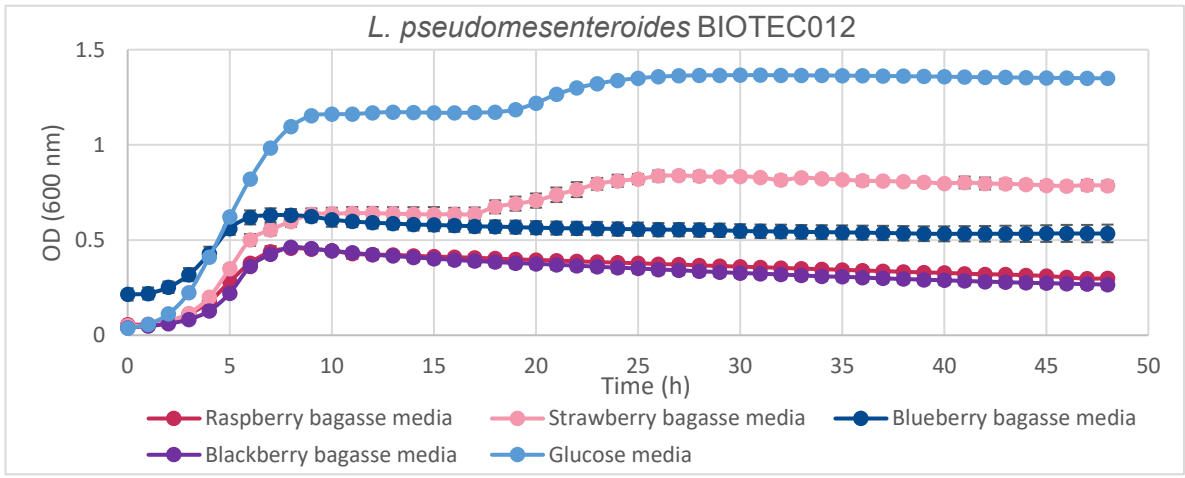


Figure 11 (Part II). Growth curves of isolated LAB (*Lactoccocus, Lactobacillus, Leuconostoc*) and yeast (*Kluyveromyces*) on dextrose (control), raspberry, strawberry, blackberry and blueberry bagasse (1%) media. Standard deviation was calculated and curves were done in triplicate.





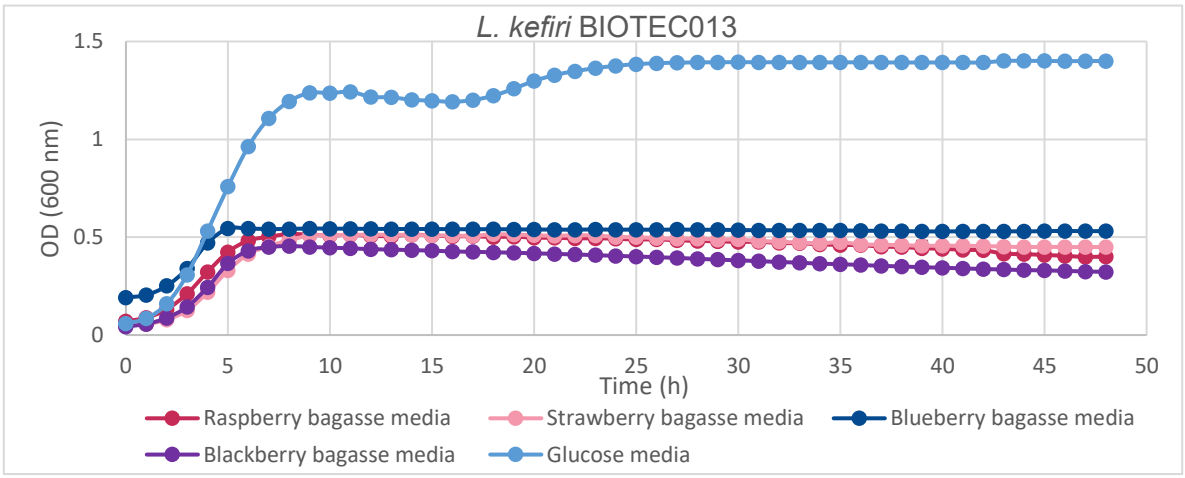


Figure 11 (Part III). Growth curves of isolated LAB (*Lactoccocus, Lactobacillus, Leuconostoc*) and yeast (*Kluyveromyces*) on dextrose (control), raspberry, strawberry, blackberry and blueberry bagasse (1%) media. Standard deviation was calculated and curves were done in triplicate.

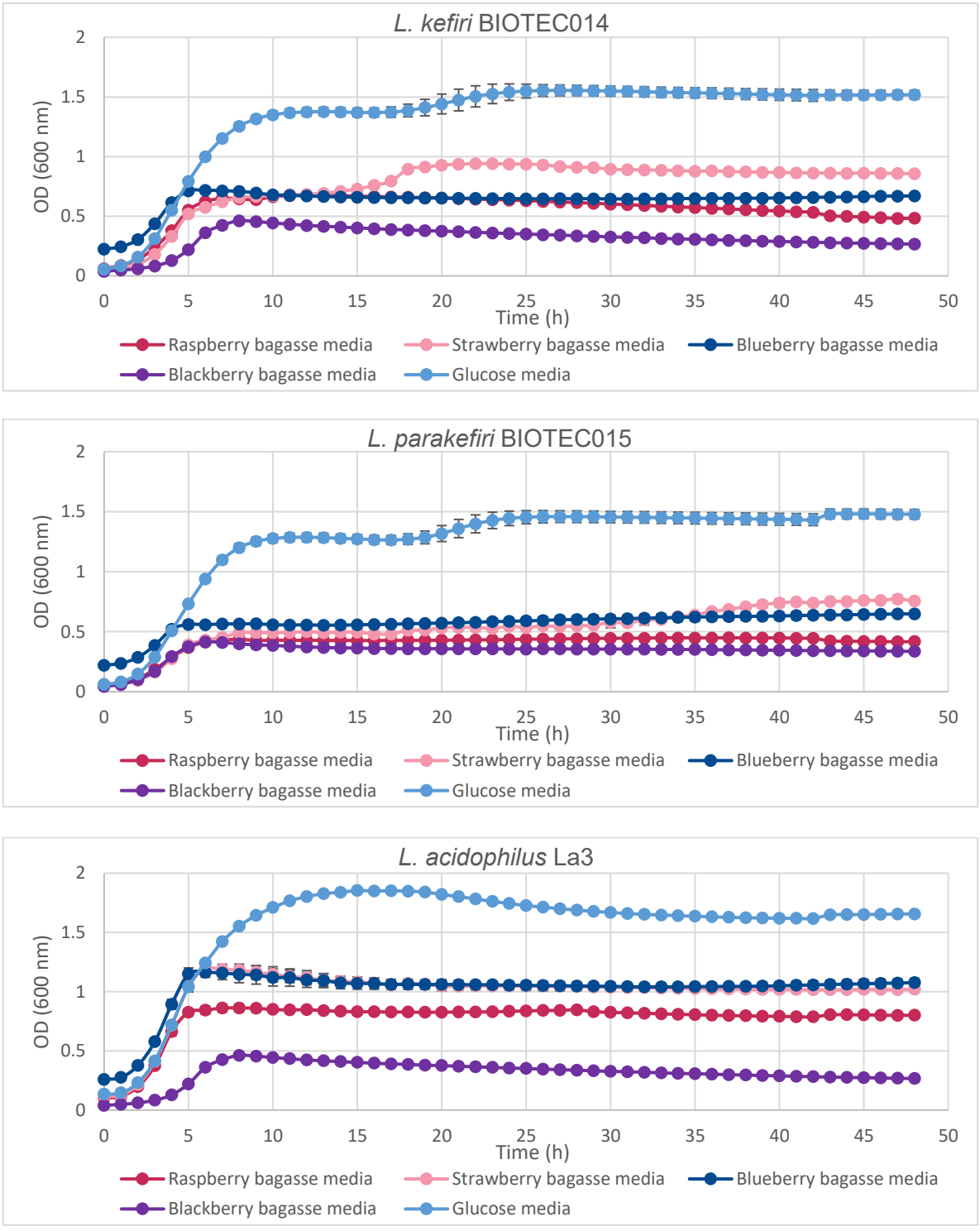
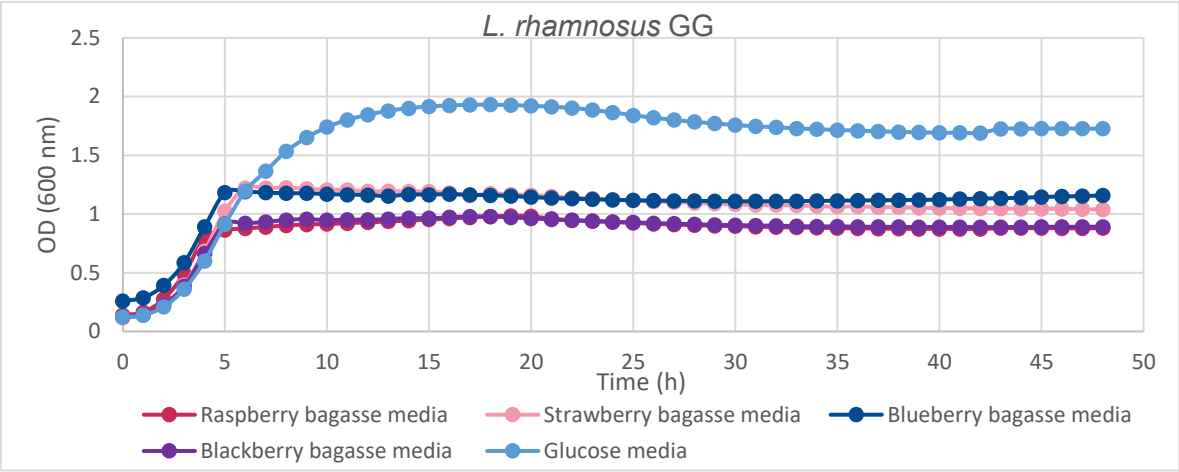


Figure 11 (Part IV). Growth curves of isolated LAB (*Lactoccocus, Lactobacillus, Leuconostoc*) and yeast (*Kluyveromyces*) on dextrose (control), raspberry, strawberry, blackberry and blueberry bagasse (1%) media. Standard deviation was calculated and curves were done in triplicate



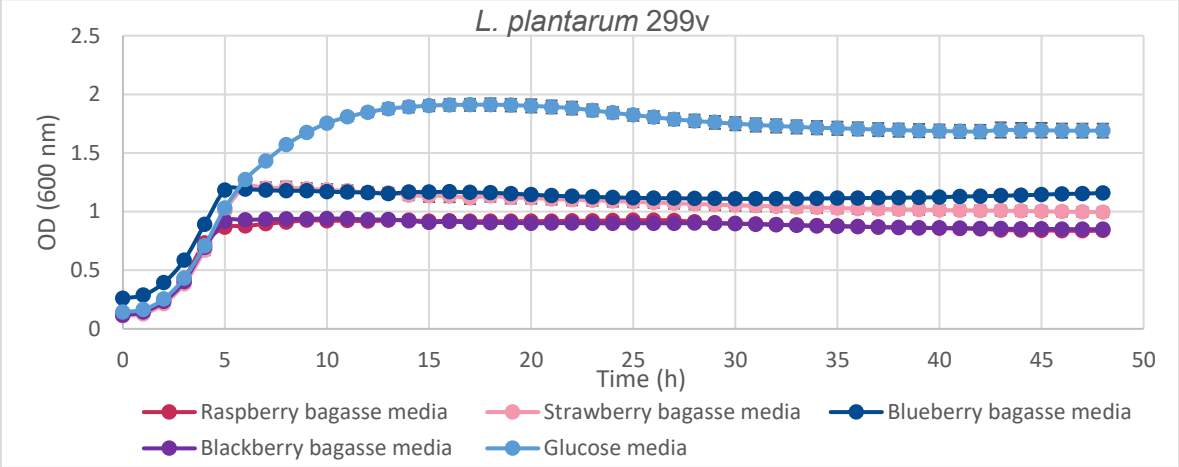


Figure 11 (Part V). Growth curves of isolated LAB (*Lactoccocus, Lactobacillus, Leuconostoc*) and yeast (*Kluyveromyces*) on dextrose (control), raspberry, strawberry, blackberry and blueberry bagasse (1%) media. Standard deviation was calculated and curves were done in triplicate.

Table 14. Maximum optical density at 600 nm (ODmax), maximum growth rate (μmax, h-1) and lag (h) parameters of bacteria growing under aerobic conditions on raspberry, strawberry, blueberry and blackberry bagasse as carbon sources

Microorganisms		Glucose media	Raspberry media	Strawberry media	Blueberry media	Blackberry media
L. acidophilus La3	OD_{max}	1.85 ± 0.00	0.86 ± 0.02	1.19 ± 0.03	1.16 ± 0.04	0.46 ± 0.02
	μ_{max}	0.25 ± 0.00	0.25 ± 0.02	0.31 ± 0.03	0.30 ± 0.04	0.10 ± 0.02
	lag	0.86 ± 0.00	1.43 ± 0.02	1.57 ± 0.03	1.44 ± 0.04	2.51 ± 0.02
L. rhamnosus GG	OD_{max}	1.93 ± 0.01	0.98 ± 0.01	1.22 ± 0.02	1.19 ± 0.02	0.97 ± 0.00
	μ_{max}	0.24 ± 0.01	0.25 ± 0.01	0.30 ± 0.02	0.29 ± 0.02	0.26 ± 0.00
	lag	1.02 ± 0.01	1.09 ± 0.01	1.55 ± 0.02	1.37 ± 0.02	1.47 ± 0.00
L. plantarum 299v	OD_{max}	1.91 ± 0.04	0.93 ± 0.02	1.21 ± 0.04	1.19 ± 0.02	0.94 ± 0.01
	μ_{max}	0.25 ± 0.04	0.24 ± 0.03	0.31 ± 0.04	0.29 ± 0.02	0.25 ± 0.01
	lag	1.50 ± 0.04	1.16 ± 0.03	1.60 ± 0.04	1.37 ± 0.02	1.37 ± 0.01
L. lactis BIOTEC006	OD_{max}	0.76 ± 0.01	0.57 ± 0.02	0.73 ± 0.00	0.82 ± 0.01	0.50 ± 0.02
	μ_{max}	0.14 ± 0.01	0.02 ± 0.02	0.06 ± 0.00	0.04 ± 0.01	0.03 ± 0.02
	lag	5.87 ± 0.01	0.00 ± 0.00	3.31 ± 0.00	1.68 ± 0.01	0.00 ± 0.00
L. lactis BIOTEC007	OD_{max}	0.91 ± 0.02	0.57 ± 0.03	0.95 ± 0.01	0.84 ± 0.02	0.65 ± 0.03
	μ_{max}	0.14 ± 0.02	0.14 ± 0.03	0.05 ± 0.01	0.04 ± 0.02	0.06 ± 0.03
	lag	3.76 ± 0.02	1.22 ± 0.03	0.00 ± 0.00	0.00 ± 0.02	4.30 ± 0.03
L. lactis BIOTEC008	OD_{max}	0.82 ± 0.02	0.54 ± 0.00	0.71 ± 0.02	0.55 ± 0.03	0.85 ± 0.02
	μ_{max}	0.15 ± 0.02	0.02 ± 0.00	0.07 ± 0.02	0.07 ± 0.03	0.03 ± 0.02
	lag	5.55 ± 0.02	0.00 ± 0.00	3.09 ± 0.02	1.73 ± 0.03	0.00 ± 0.00
K. lactis BIOTEC009	OD_{max}	1.25 ± 0.00	0.44 ± 0.02	0.60 ± 0.00	0.56 ± 0.00	0.43 ± 0.00
	μ_{max}	0.17 ± 0.00	0.08 ± 0.02	0.12 ± 0.00	0.10 ± 0.00	0.09 ± 0.00
	lag	1.89 ± 0.00	1.97 ± 0.02	2.20 ± 0.00	1.30 ± 0.00	1.79 ± 0.00
K. lactis BIOTEC010	OD_{max}	1.58 ± 0.00	0.62 ± 0.00	0.64 ± 0.01	0.73 ± 0.01	0.51 ± 0.00
	μ_{max}	0.16 ± 0.00	0.12 ± 0.00	0.15 ± 0.01	0.11 ± 0.01	0.05 ± 0.00
	lag	0.00 ± 0.00	1.74 ± 0.00	1.29 ± 0.01	0.00 ± 0.00	0.00 ± 0.00
L. pseudomesenteroides BIOTEC011	OD_{max}	1.52 ± 0.03	0.60 ± 0.03	0.63 ± 0.00	0.70 ± 0.02	0.50 ± 0.00
	μ_{max}	0.17 ± 0.03	0.11 ± 0.03	0.14 ± 0.00	0.19 ± 0.02	0.05 ± 0.00
	lag	0.00 ± 0.00	1.93 ± 0.03	1.16 ± 0.00	1.76 ± 0.02	0.00 ± 0.00
L. pseudomesenteroides BIOTEC012	OD_{max}	1.37 ± 0.00	0.46 ± 0.01	0.84 ± 0.02	0.63 ± 0.03	0.46 ± 0.02
	μ_{max}	0.14 ± 0.00	0.08 ± 0.01	0.07 ± 0.02	0.12 ± 0.03	0.10 ± 0.02
	lag	0.00 ± 0.00	1.84 ± 0.01	0.00 ± 0.00	1.63 ± 0.03	2.51 ± 0.02
L. kefiri BIOTEC013	OD_{max}	1.40 ± 0.00	0.51 ± 0.01	0.51 ± 0.01	0.54 ± 0.01	0.45 ± 0.01
	μ_{max}	0.19 ± 0.00	0.10 ± 0.01	0.09 ± 0.01	0.12 ± 0.01	0.11 ± 0.01
	lag	1.31 ± 0.00	1.07 ± 0.01	1.53 ± 0.01	1.22 ± 0.01	1.47 ± 0.01
L. kefiri BIOTEC014	OD_{max}	1.55 ± 0.04	0.67 ± 0.01	0.94 ± 0.01	0.72 ± 0.01	0.46 ± 0.02
	μ_{max}	0.16 ± 0.04	0.15 ± 0.01	0.06 ± 0.01	0.16 ± 0.01	0.09 ± 0.02
	lag	0.00 ± 0.00	1.20 ± 0.01	0.00 ± 0.00	1.23 ± 0.01	2.13 ± 0.02

L. parakefiri BIOTEC015	OD_{max}	1.48 ± 0.01	0.45 ± 0.01	0.78 ± 0.01	0.65 ± 0.03	0.42 ± 0.01
	μ_{max}	0.18 ± 0.01	0.09 ± 0.01	0.07 ± 0.01	0.11 ± 0.03	0.11 ± 0.01
	lag	1.11 ± 0.01	1.57 ± 0.01	0.00 ± 0.00	1.51 ± 0.03	1.24 ± 0.01

4.3.6 Analysis of substrate consumption

In addition to the bacterial growth on berry bagasse media, a substrate consumption analysis was developed to determine the substrates in the media that LAB might utilize to grow during 48 hours. In order to facilitate high-throughput screening of biomass, implementation of the DNS colorimetric assay was developed. This method is recomended to analyse hydrolysates containing 0-100 g/L reducing sugars and can be used in complex substrates (Wood et al., 2012). Figure 12 shows the graph of the controls for each medium, indicating the initial sugar content in the medium, with different concentrations probably due to the carbohydrate content of each bagasse, reported above. Following this methodology, it was observed an initial reduction of sugars during the first hours of fermentation (Figure 13). However, it was noted that after 5 and 24 hours, sugars in the media increased. This observation is more emphasized in the fermentations on blueberry and strawberry media, which coincide to the substrates that generated a high microorganism growth. During a fermentation process, the DNS analysis can be performed to test the reduction of sugars in the media and to indirectly evaluate the enzymatic activity of microorganisms (Whiny Hardiyati Erliana, Widjaja, Ali Altway, & Lily Pudjiastuti, 2020).

Recent studies have found that the bacteria use complexes of proteins that span the bacterial outer membrane and allow them to capture and break down complex polysaccharides into their monosaccharide components; sets of genes known as polysaccharide utilization loci (Inman, 2011). In addition to fiber degradation, fermentation of bagasse by microorganisms can biotransform phenolic compounds in the culture medium. It has been reported that LAB-fermented pomegranate juices can increase the bioaccessibility of phenolic compounds, ensuring the survival of bacteria even after digestion. Moreover, fermentation modifies the amount and type of polyphenols with respect to those that are not fermented, and metabolites excreted by the LAB could produce health benefits through bioaccessibility or bioactivity even though they are not absorbed in the gut (Valero-Cases, Nuncio-Jáuregui, & Frutos, 2017). Therefore, the results obtained suggest that it is possible that LAB ferment the fiber increasing the available polyphenols increase. Similar results were reported by Mashitoa et al. (2021), hypothesizing that LAB fermentation

increases total phenol content, different phenolic metabolites, and antioxidant capacity in fermented papaya puree after fermentation (Mashitoa et al., 2021).

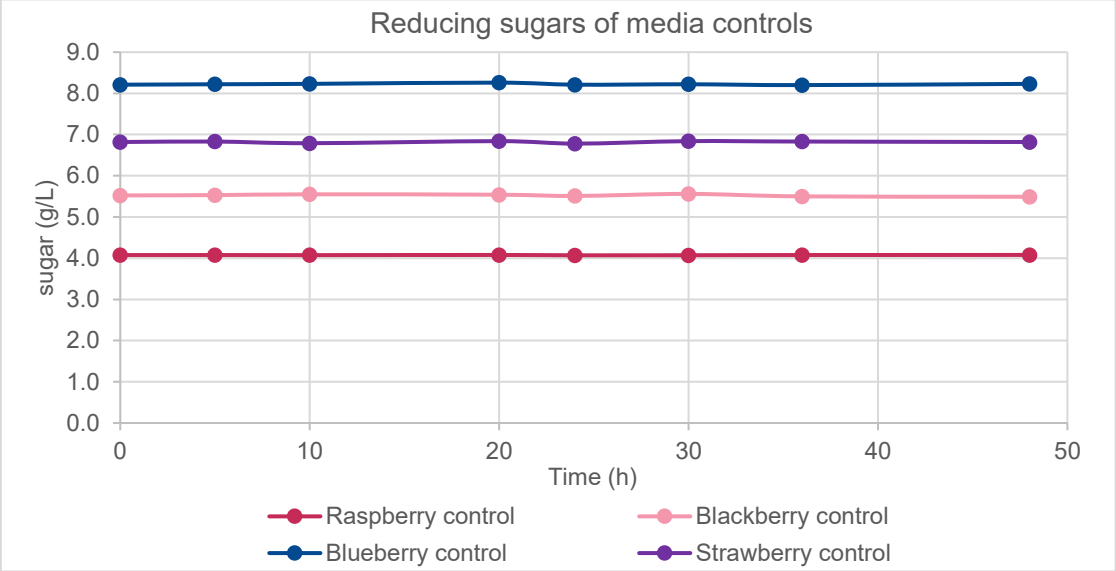
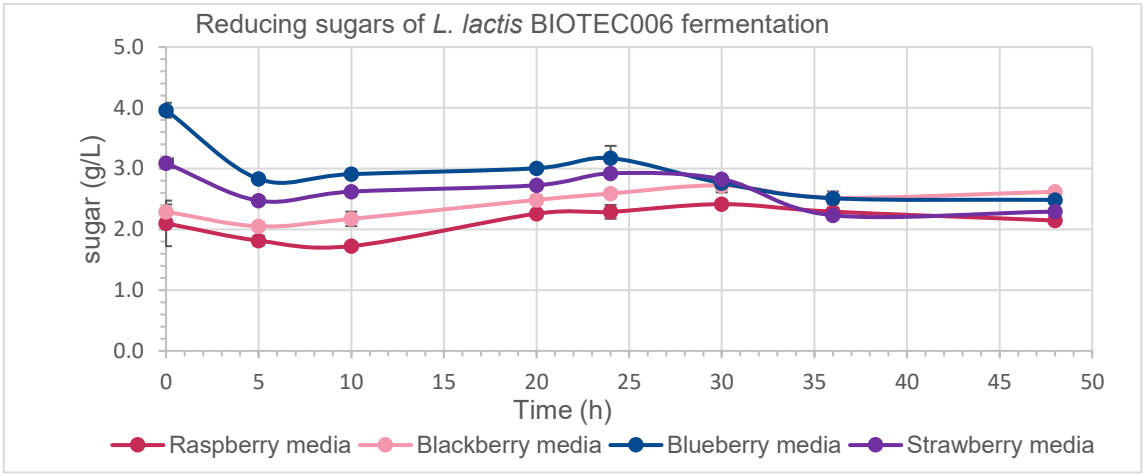
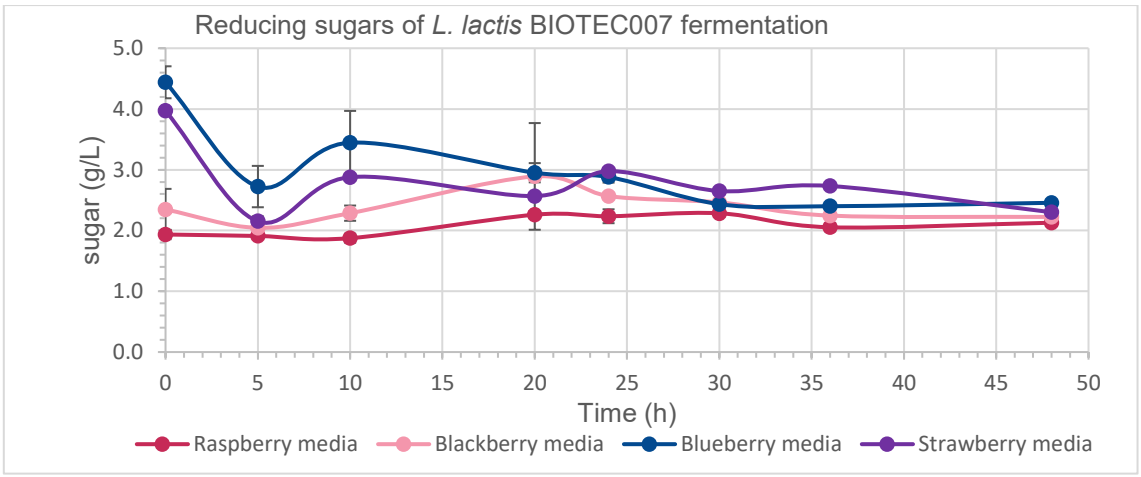


Figure 12. Reducing sugars measured in raspberry, strawberry, blackberry and blueberry bagasse controls media during the fermentation.





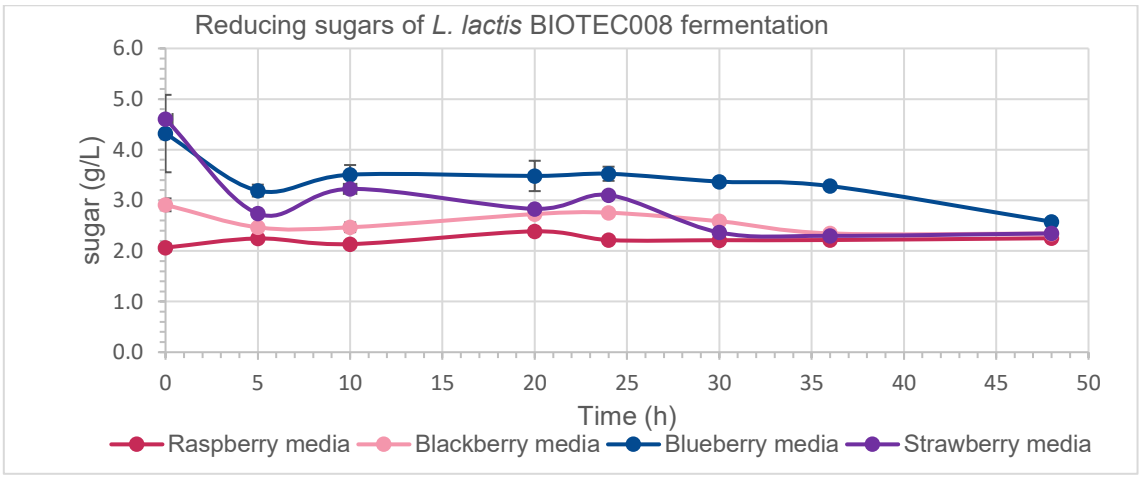
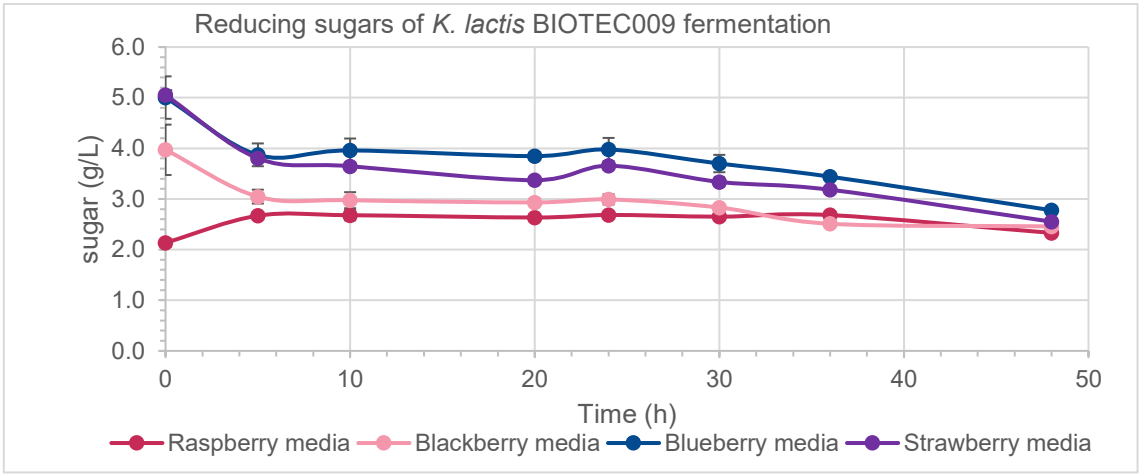
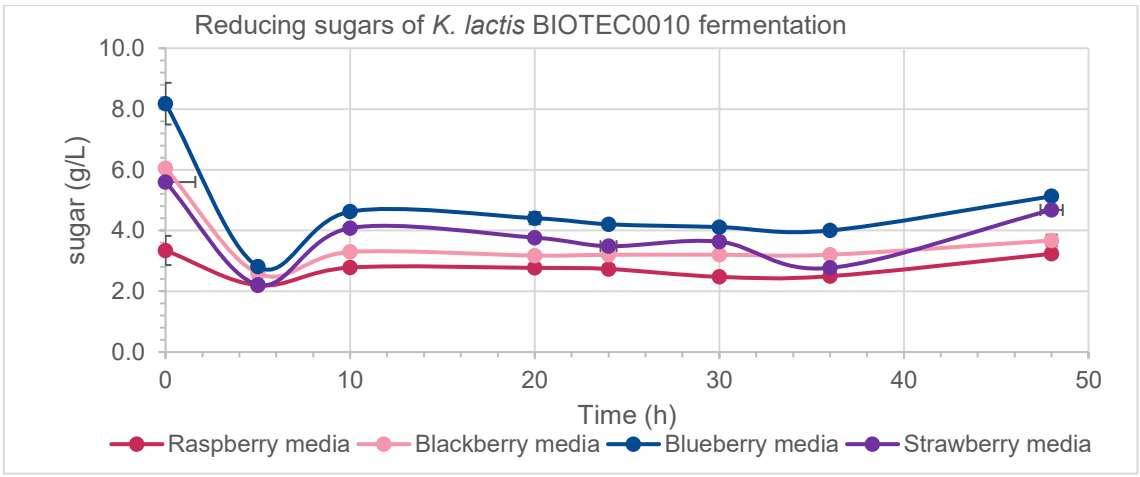


Figure 13 (Part I). Reducing sugars measured in raspberry, strawberry, blackberry and blueberry bagasse media during the fermentation with cultures of isolated LAB (*Lactoccocus, Lactobacillus, Leuconostoc*) and yeast (*Kluyveromyces*). Standard deviation was calculated and curves were done in triplicate.





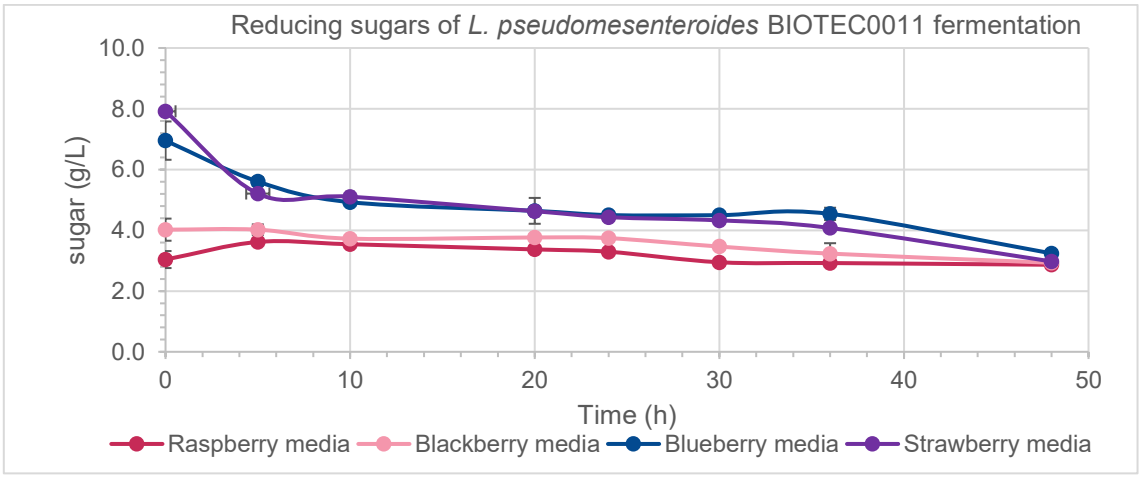
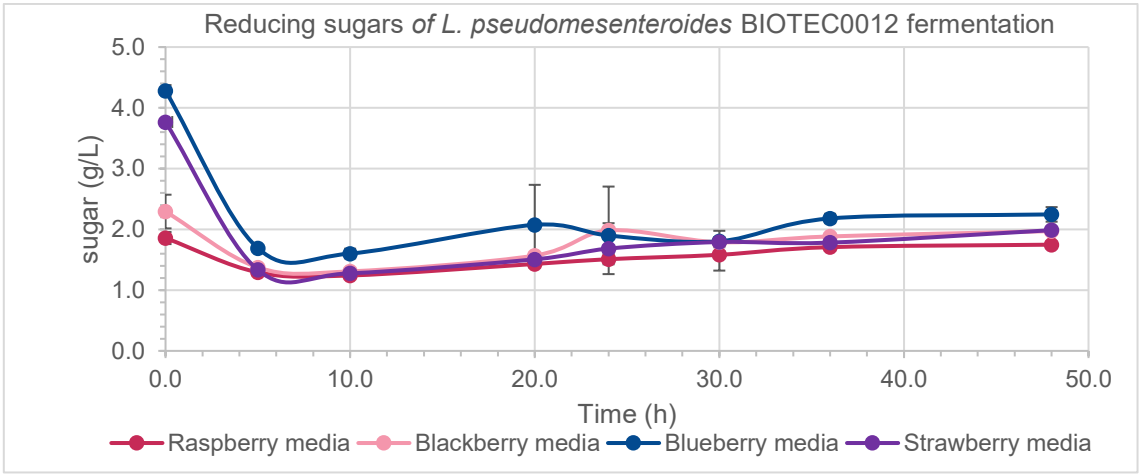
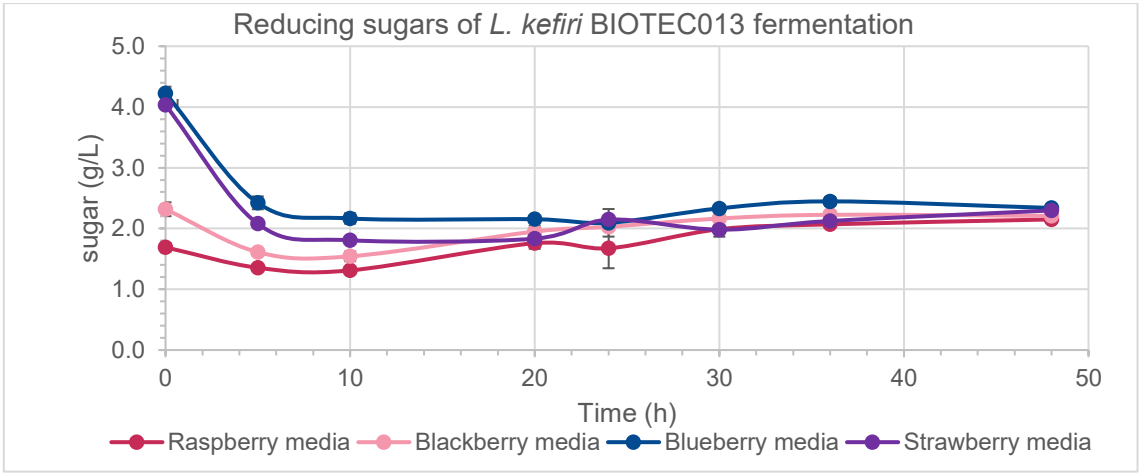


Figure 13 (Part II). Reducing sugars measured in raspberry, strawberry, blackberry and blueberry bagasse media during the fermentation with cultures of isolated LAB (*Lactoccocus, Lactobacillus, Leuconostoc*) and yeast (*Kluyveromyces*). Standard deviation was calculated and curves were done in triplicate.





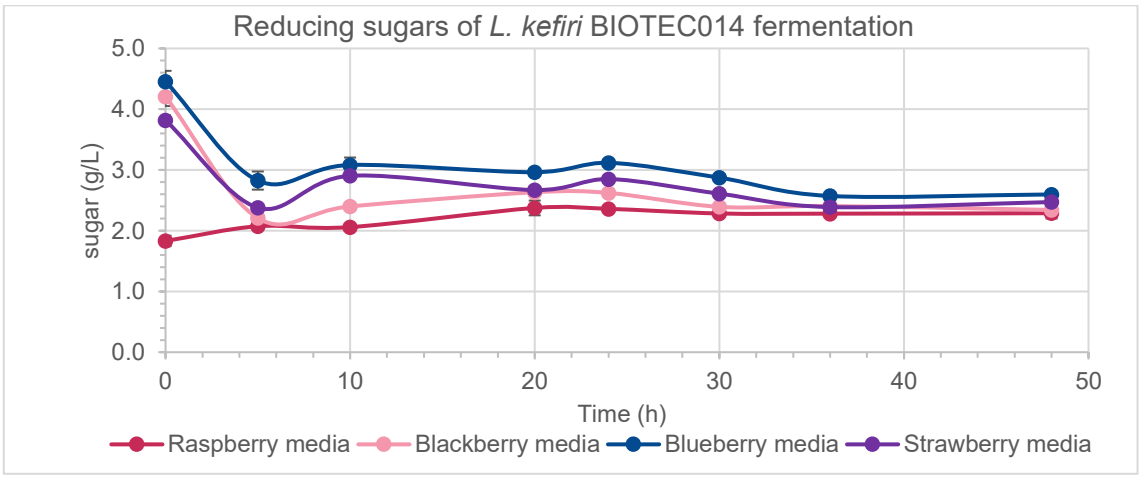
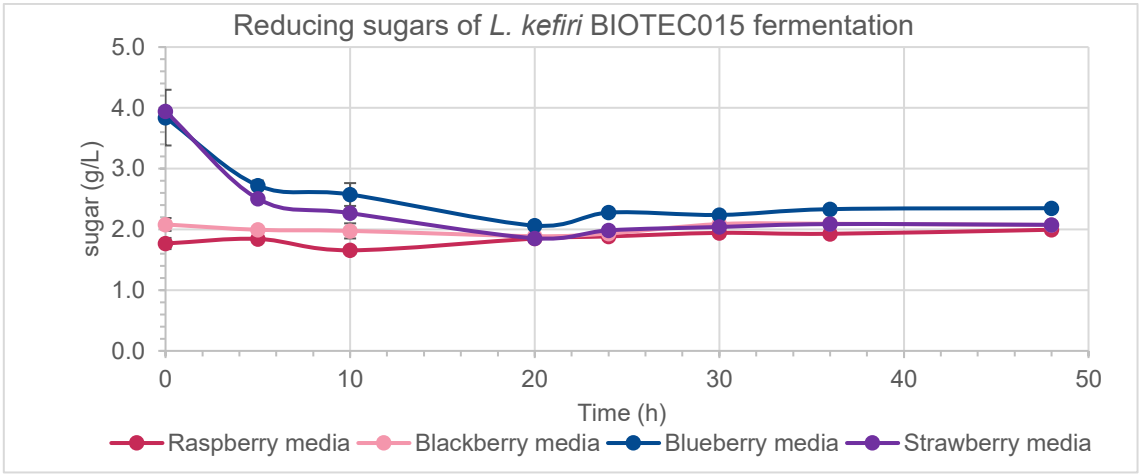
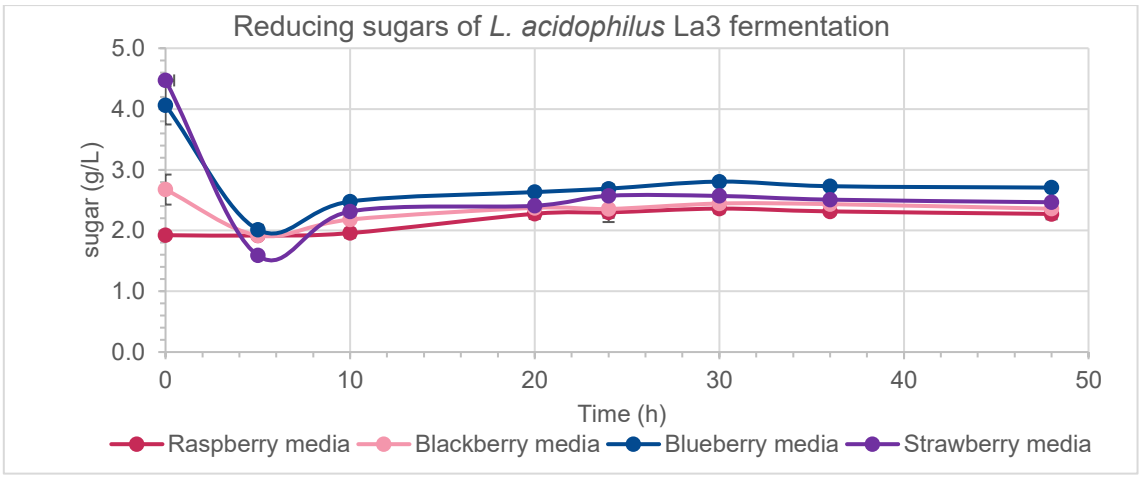


Figure 13 (Part III). Reducing sugars measured in raspberry, strawberry, blackberry and blueberry bagasse media during the fermentation with cultures of isolated LAB (*Lactoccocus, Lactobacillus, Leuconostoc*) and yeast (*Kluyveromyces*). Standard deviation was calculated and curves were done in triplicate.





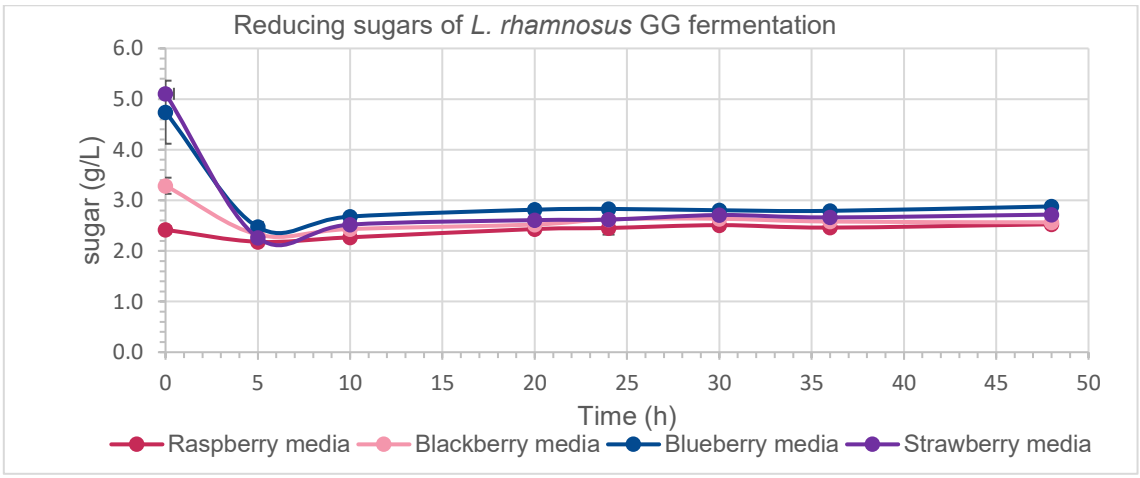


Figure 13 (Part IV). Reducing sugars measured in raspberry, strawberry, blackberry and blueberry bagasse media during the fermentation with cultures of isolated LAB (*Lactoccocus, Lactobacillus, Leuconostoc*) and yeast (*Kluyveromyces*). Standard deviation was calculated and curves were done in triplicate.

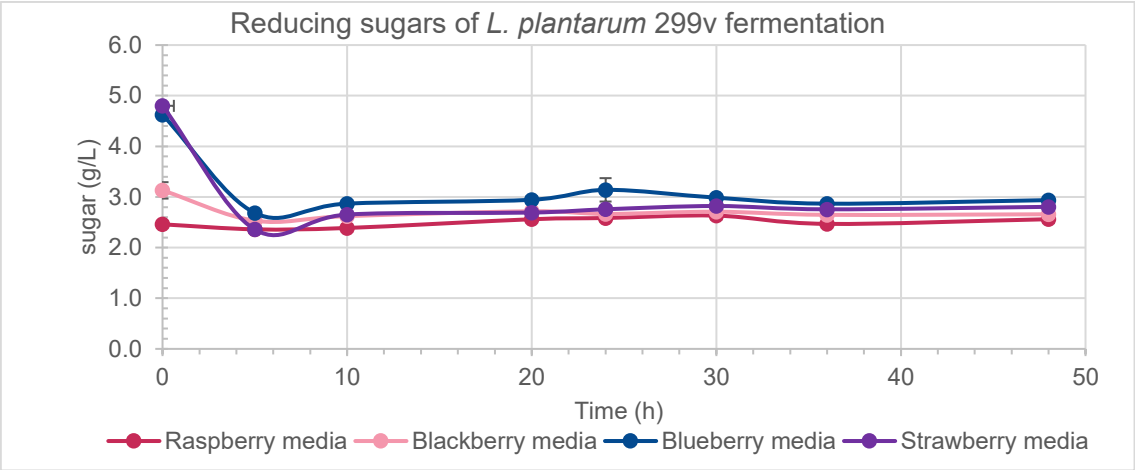


Figure 13 (part V). Reducing sugars measured in raspberry, strawberry, blackberry and blueberry bagasse (1%) media, during its fermentation with cultures of isolated LAB (*Lactoccocus, Lactobacillus, Leuconostoc*) and yeast (*Kluyveromyces*). Standard deviation was calculated and curves were done in triplicate

4.4 Conclusions

Different bagasses of berries were obtained and characterized by determining the physicochemical properties of the fruits from which they were obtained, the contents of total phenolics and anthocyanins. Also, methanolic extracts were obtained, showing antimicrobial activity against pathogens such as *E. coli, S. typhi* and *S. aureus*. Regarding the nutritional content, high fiber and carbohydrate contents were found in the bagasse, being potential substrates for microbial fermentation. In this respect, fermentation of kefir isolates in different media supplemented with berry bagasses was evaluated, showing maximum growth values in the case of *L. lactis* BIOTEC007, *L. kefiri* BIOTEC014 and *L. rhamnosus* GG in the blueberry and strawberry bagasse media. The substrate consumption measurements indicated that the sugars present in the media are consumed during the first hours but a slight increase is observed after 5-10 hours of fermentation, depending on the strain and the medium. Therefore, it is possible that LAB metabolize the fiber and polyphenols, releasing linked sugar molecules.

CHAPTER 5. FORMULATION OF A POTENTIAL SYNBIOTIC PRODUCT WITH BACTERIA ISOLATED FROM KEFIR AND BLUEBERRY BAGASSE

5.1 Introduction

Synbiotics are combinations of probiotics and prebiotics designed to improve the survival of the ingested microorganisms and their colonization of the intestinal tract. Current synbiotic approaches have focused on characterised probiotics and fermentable prebiotic substrates. Well characterised probiotics such as *Bifidobacterium* and *Lactobacillus* genera ferment human indigestible sugars, producing organic acids (SCFAs and lactic acid) which have shown positive health effects (Gurry, 2017).

In the development of functional fermented foods, the selection of a suitable food system to deliver probiotics and retain viability and sensory characteristics is a key factor. Technological conditions while producing the probiotic foods can significantly reduce the viability of probiotic cells due to heat, mechanical damage or osmotic stress. However, in dairy based probiotic foods, the physicochemical composition of milk, which is rich in protein and lipids (fats), acts as a protective matrix for the probiotics and these factors help the survival of probiotics from adverse conditions of the stomach and small intestine. Further processed fruits and vegetables which have good matrices, have been considered as ideal substrates for probiotics due to the presence of minerals, vitamins, antioxidants and fibers (Vijaya Kumar, Vijayendra, & Reddy, 2015).

Recently, the formulation of functional foods has been directed towards the use of fruit processing by-products as they are rich in bioactive compounds and dietary fibers, being also a practical and economic sources of antioxidants. Moreover, antioxidant activity in yogurt may be favourable in terms of reducing lipid oxidation process responsible for unwanted chemical compounds and the formation of undesired flavour. For instance, yogurts formulated with pomegranate peel extracts or powders, pinnaple powder or grape skin have shown to increase viable cells as well as a higher antioxidant activity and phenolic content (Fazilah, Ariff, Khayat, Rios-Solis, & Halim, 2018).

Therefore, the objective of this study was to evaluate the effect of the addition of lyophilized blueberry bagasse on the overall perception, physicochemical characteristics and viability of potential probiotic bacteria isolated from kefir in a model dairy fermented milk.

5.2 Materials and methods

5.2.1 Chemicals and reagents

Ingredients to formulate the fermented milk were purchased in a local store (Guadalajara, Jalisco, México).

5.2.2 Formulation of a synbiotic product

After evaluating the growth of kefir isolates in media supplemented with berry bagasse as carbon source (chapter 4), the blueberry bagasse and three LAB (*Lacticaseibacillus rhamnosus* GG, *Lactobacillus kefiri* BIOTEC014 and *Lactococcus lactis* BIOTEC007) were selected to formulate a synbiotic fermented milk. Four blueberry fermented milks were formulated (Table 15): a fermented milk fermented with all the microorganisms and three varieties using each strain to ferment. Formulations contained part-skim ultrapasteurized milk (Alpura), low-fat milk powder (Nestlé), lyophilized blueberry bagasse powder, gelatin, cornstarch, sweetener and microbial inoculum (4%). A Control milk was prepared with the formulation ingredients but without the addition of the inoculum, only the blueberry bagasse for the physicochemical, phenolics and anthocyanin determination assays. Other control fermented milks were also prepared for the *in vitro* digestion assay using the same ingredients proportions indicated and its respective inoculum, with no addition of blueberry bagasse powder. The fermented milk was prepared as follows: gelatin was hydrated and the cornstarch was dispersed with cold milk. Subsequently, the milk was heated and the cornstarch was added when the milk was at 30°C, then the skim milk powder was incorporated when the mixture was at 40°C. Next, the milk was heated to 60°C, in this step the lyophilized powder of blueberry bagasse was added together with the gelatin and then temperature was raised to 80°C. Finally, it was allowed to cool to 35°C and inoculated according to Table 15. The fermented milk was homogenized and refrigerated after 24 hours of incubation at 30°C. Controls were prepared following the same methodology with the exception of the addition of blueberry bagasse powder. Fermented milks are presented in Figure 14.

Table 15. Fermented milk formulations

Fermented milk	A	B	C	D
Inoculum	*L. rhamnosus* GG, *L. kefiri* BIOTEC014, *L. lactis* BIOTEC007 (4%)	*L. lactis* BIOTEC007 (4%)	*L. kefiri* BIOTEC014 (4%)	*L. rhamnosus* GG (4%)
Ingredients	Part-skim ultrapasteurized milk (89.85%) Low-fat milk powder (2.6%) Lyophilized blueberry bagasse powder (2.5%) Gelatin (0.15%) Cornstarch (0.4%) Sweetener (0.5%)			

Figure 14. Blueberry bagasse fermented milks after 24 h of incubation

5.2.3 Physicochemical characterization of product

Different characteristics such as density, water holding capacity, viscosity, soluble solids and titratable acidity were determined. First, density was calculated trifold using 10 mL volumetric flax that were previously placed in an oven to achieve constant weight, which were filled with the blueberry fermented milk and then weighed. Viscosity was measured in triplicates at room temperature using a Scientific VE-8S viscosimeter according to the

manufacturer's instructions and maintaining the torque percentage between 30 and 35%. Next, water holding capacity was calculated by triplicate according to (Bancalari et al., 2020), where 20 grams of the sample were centrifuged (GYFROZEN 1580R) at 2,600 rpm for 15 min and the supernatants were collected and weighed. Soluble solids (°Brix at 25°C) were measured using a digital refractometer (Hanna instruments Hi 96801); titratable acidity (mL of citric acid/ mL of juice) was determined by neutralization of juice with NaOH 0.1 N until a pH of 8.3. Finally, pH was measured with a pHmeter (HORIBA LAQUAact-PH110-K), according to the Mexican Official Standard 155-SCFI-2012.

5.2.4 Characterization of bioactive compounds in the product

Methanolic extracts, total phenolic compounds and total anthocyanin content were obtained as described in the section 4.2.4 of this document. For the obtention of methanolic extracts, 1 g of fermented milk was weighted and suspended in methanol, as described previously. Moreover, total phenolic content of the fermented milk was determined by using Folin-Ciocaltue assay adapted to 96-well microplate and measurement of monomeric anthocyanin content in fermented milk was carried out using the same methanolic extract with the spectrophotometric protocol described also in section 4.2.4 of this document.

5.2.5 *In vitro* gastrointestinal survival analysis of bacteria in product

The assay was carried out as stated in the section 3.2.8 of this documents with some modifications. An aliquot of each fermented milk was taken as initial sample (1 mL), then 5 mL of the product were mixed with the simulated salivary fluid (SSF) containing amylase at pH 7, and incubated 2 min, 100 rmp, 37°C; these incubation conditions were constant during the assay. The following steps for the digestions are the ones described in the section 3.2.8 of this document. Sampling process for each phase consisted in taking 1 mL of the fermented milks, realize serial dilutions and plate in MRS agar in order to count viable colonies. Plates were incubated at 30°C during 48 h and viable colonies were reported in LOG (CFU/mL).

5.2.6 Sensorial analysis of product

In order the evaluate the sensory attributes of the different blueberry fermented milk formulations, a preliminar sensorial assay with semi-trained panel of consumers was

performed. Five participants from 24 to 26 years old were asked to evaluate on a hedonic scale from 1 to 9, the fermented milks in different attributes: texture, smell, flavor, color, sweetness, acidity, and over all perception. The first filter besides food allergies that the participants had to pass was whether they consumed fermented dairy products regularly. If they did, they were asked to evaluate the fermented milks.

5.2.7 Statystical analysis

Standard deviations and mean values were calculated for all the assays. In addition, Minitab Software was used to carry out analysis of variance (ANOVA) to determine significant differences in the analysis of physicochemical characteristics, bioactive compounds and *in vitro* digestion results of the differtent fermented milks tested. Besides, Tukey test was used for means comparison using a significance level (p- value) of 0.05.

5.3 Results and discussion

5.3.1 Physicochemical characterization of fermented milks

Chemical parameters like density, water holding capacity, viscosity, soluble solids and titratable acidity were assessed and indicated in Table 16. Parameters were measured in both ferermented milk formulations and in a control without inoculum . The lowest pH was found in fermented milk D and fermented milk B, indicating a significant decrease during the period of fermentation. Moreover, Fermented milk D had the greatest acidity value, followed by fermented milk B and A, whose values were statistically different to the acidity of fermented milk C. Fermented milks B and D were also the samples with the higher viscosity and soluble solids. Fermented milk C indicated the highest water holding capacity, while B and D had the lowest values. These results can be compared to the ones reported by Islam et. al (2021), where acidity values ranges from 1.26 – 1.55 and soluble solids from 8.17 – 16-57 in a fermented probiotic beverage using whey production and pineapple juice (Islam et al., 2021). Regarding the results observed for viscosity might indicate that this characteristic is due to the fermentation than to the addition of lyophilized bagasse, since significant differences are obserd among all the fermentations. It has been observed thatLAB speciesposses the ability to increase the apparent viscosity of fermented milk, due

the production of exopolysaccharides (W. Li, Mutuvulla, Chen, Jiang, & Dong, 2012). It should be noted that bacteria isolated from kefir have previously been reported capable of producing exopolysaccharides, which is determinative for the kefiran polymer, among them, *Lactobacillus delbrueckii* subsp*. bulgaric*us HP1 is one example (Frengova, Simova, Beshkova, & Simov, 2002). Hence, high viscosity observed here might be produced by the fermentation of LAB isolated from milk kefir and its inner activity and potential production of exopolysaccharides.

Table 16. Physicochemical characterization of blueberry fermented milks[a]

Characteristics	**Fermented milk**				
	A	**B**	**C**	**D**	**Control**
pH	4.39 ± 0.00[b]	4.33 ± 0.02[c]	5.02 ± 0.00[a]	4.15 ± 0.02[d]	5.47 ± 0.05[a]
Soluble solids (° Brix at 25°C)	10.97 ± 0.05[c]	13.67 ± 0.12[a]	11.37 ± 0.23[b]	13.33 ± 0.15[a]	11.66 ± 0.15[b]
Viscosity (mPa x s)	804.73 ± 8.00[b]	2778.96 ± 97.07[a]	155.27 ± 4.32[d]	2886.00 ± 150.41[a]	771.63 ± 6.85[c]
Density	1.05 ± 0.01[a]	1.07 ± 0.00[a]	1.04 ± 0.01[a]	1.05 ± 0.00[a]	1.03 ± 0.00[a]
Water holding capacity (%)	16.23 ± 0.17[b]	14.02 ± 0.40[c]	29.09 ± 0.85[a]	13.10 ± 0.36[c]	24.76 ± 0.33[a]
Titratable Acidity (g of lactic acid/g of sample)	1.97 ± 0.04[b]	2.07 ± 0.13[a]	1.31 ± 0.08[c]	2.17 ± 0.04[b]	1.30 ± 0.17[a]

[a] Fermented milk A is fermented with *L. rhamnosus* GG, *L. kefiri* BIOTEC014, *L. lactis* BIOTEC007; B is fermented with *L. lactis* BIOTEC007; C is fermented with *L. kefiri* BIOTEC014 and D is fermented with *L. rhamnosus* GG. Control is the milk without any strain fermentation. The values measured are means of triplicate measurements ± standard deviation . Different letters in each row denote significant differences among the parameter measured in fermented milks.

5.3.2 Characterization of bioactive compounds in the product

Total phenolic acids content (TPC) and total anthocyanin content (TAC) were measured for each blueberry fermented milk variety and indicated in Table 17. Fermented milk D had the highest values of both phenolics (65.14 mg of GAE/100 g of fresh weight) and anthocyanins (19.46 mg of Cyd-3-glu/100 g of fresh weight), being significantly different from the rest of the fermented milks. Fermented milk C had the lowest TPC and fermented milk A the lowest TAC.

Table 17. Bioactive compounds measured in fermented milk varieties.

Fermented milk	Bioactive compounds*	
	Total phenolic acids content (mg of GAE/ 100 g of fresh weight)	**Total anthocyanin content (mg of Cyd-3-glu/ 100 g of fresh weight)**
A	50.05 ± 0.85^{c}	11.16 ± 0.15^{d}
B	56.04 ± 0.72^{b}	15.96 ± 0.23^{b}
C	49.72 ± 1.76^{c}	14.34 ± 0.45^{c}
D	65.14 ± 1.50^{a}	19.46 ± 0.32^{a}
Control	63.60 ± 1.50^{a}	16.22 ± 0.30^{b}

*GAE, Gallic Acid Equivalents; Cyd-3-glu, Cyanidin-3-glucoside. The values measured are means of triplicate measurements ± standard deviation . Different letters in each column denote significant differences among bioactive compounds measured

The results observed in the phenolic content are comparable to those measured by Dos Santos et. al (2017), where phenolic concentrations ranged from 25 - 47 mg of GAE for 100 g of fresh weight in a fermented goat milk with grape pomace extract added and fermented by *Lactobacillus acidophilus* LA-5 or *Lacticaseibacillus rhamnosus* HN001 (dos Santos et al., 2017). Moreover, TPC and TAC values reported in a fermented milk beverage containing blueberry juice were also similar to the ones reported in this study. . Nevertheless, the total phenolic content in the fermented milk is dependent on the fruits and bacteria cultures utilized, and even reducing molecules present in milk that might have potentially reacted with Folin and Ciocalteu's reagent, as reported in the study by Silva et. al, (2017) . Additionally, a recent study documented the formulation and evaluation of aronia kefir, a fermented milk with a berry native from eastern North America, looking for protect the berry juice polyphenols from the degradation in the small intestine. Fermentation of aronia kefir was found to produce metabolites with higher antioxidant capacity and to enhance the bioavailability of dietary polyphenols, being a promising alternative when consuming foods rich in polyphenols (Du & Myracle, 2018; Silva et al., 2017).

Regarding the anthocyanin content, a similar study of fermented milk with sweet cherry puree reported TAC values likewise the ones documented in this study, with values of approximately 2 mg of anthocyanins per 100 g of cherry puree (later incorporated into different varieties of milk fermented with *L casei*, *L. paracasei* or *L. helveticus*) (Sánchez-Bravo, Zapata, Martínez-Esplá, Carbonell-Barrachina, & Sendra, 2018). The results reported here for fermented milk with blueberry bagasse are slightly higher (11-19 mg of

Cyd-3-glu /100 g of fresh weight), but this may be due to the fact that the processing for obtainin bagasse and puree are different, also the moisture content of the final substrate and the anthocyanin content of the fresh fruits that varies considerably. For the blueberry, the reported content ranges between 60 - 400 mg of Cyd-3-glu /100 g of fresh weight (Peña-Sanhueza, Inostroza-Blancheteau, Ribera-Fonseca, & Reyes-Díaz, 2017), while the sweet cherry presents a content of between 10 - 100 mg of Cyd-3-glu /100 g of fresh weight (Ferretti, Bacchetti, Belleggia, & Neri, 2010).

5.3.3 *In vitro* gastrointestinal survival analysis of bacteria in product

For the *in vitro* assay of survival to gastrointestinal digestion, samples were taken at each stage of digestion to count viable cells and also to estimate the percentage of resistance. Fermented milks with blueberry bagasse (2.5%) were compared to the control fermented milks, whose are fermented with the same isolate(s) but with no blueberry bagasse powder. These results are shown in Table 18. During the initial phase, all the milks were fermented at a high concentration levels (10^7-10^9), demonstrating microorganisms are able to grow in a matrix with bagasse. The results indicate that both control fermented milks and those containing blueberry bagasse have a high resistance to gastrointestinal conditions *in vitro*, due to the fact that both fermented milks and controls remained at high concentrations (10^5-10^9). Regarding survival, fermented milks presented a lower reduction in cell viability than the control, since the concentration of bacteria was reduced between 0.17 - 2.17 log, while in the controls the reduction was 1.7 - 3.93 log.

Formulations of synbiotic fermented products such as yogurt contain matrices with a beneficial effect on the survival of probiotic organisms and provide optimal protection to bacteria even after storage. Formulations of synbiotic fermented products such as yogurt contain matrices with a beneficial effect on the survival of probiotic organisms and provide optimal protection to bacteria even after storage. For instance, growth rate and generation time of probiotic bacteria stored in different synbiotic matrices has been reported, observing that after four weeks of refrigerated aerobic storage probiotics such as *B. breve*, *L. acidophilus* and *L. reuteri* remained at high levels in synbiotic matrices that were supplemented with fructo-oligosaccharides, inulin and pectic-oligosaccharides compared to cultures not stored as synbiotics (Chaluvadi et al., 2012). Similar results were obtained in this assay when adding blueberry bagasse to the fermented milk, ensuring the optimal

growth, and survival of the kefir isolates and even its resistance through all the digestion process.

Table 18. Fermented milk microorganisms survival to *in vitro* digestion[a]

	LOG (CFU/mL)							
	FM A	**Control A**	**FM B**	**Control B**	**FM C**	**Control C**	**FM D**	**Control D**
Initial phase	9.30 ± 0.00	9.18 ± 0.00	7.85 ± 0.06	9.30 ± 0.00	9.00 ± 0.00	8.63 ± 0.04	9.24 ± 0.08	8.57 ± 0.04
Oral phase	9.48 ± 0.00	8.98 ± 0.00	7.78 ± 0.25	9.07 ± 0.00	8.92 ± 0.11	8.84 ± 0.09	9.08 ± 0.00	8.60 ± 0.00
Gastric pase	7.60 ± 0.00	7.78 ± 0.00	6.60 ± 0.00	4.60 ± 0.00	6.30 ± 0.00	5.44 ± 0.09	8.93 ± 0.21	7.43 ±0.02
Intestinal pase	7.60 ± 0.00	7.84 ± 0.05	5.15 ± 2.05	5.37 ± 0.10	6.60 ± 0.00	5.58 ± 0.03	9.07 ± 0.00	6.80 ± 0.57

[a] Fermented milk A is fermented with *L. rhamnosus* GG*, L. kefiri* BIOTEC014, *L. lactis* BIOTEC007; B is fermented with *L. lactis* BIOTEC007; C is fermented with *L. kefiri* BIOTEC014 and D is fermented with *L. rhamnosus* GG. The values measured are means of duplicate measurements ± standard deviation. Different letters in each row denote significant differences among the parameter measured in fermented milks

In addition, these results agree with those observed when evaluating the viability and resistance to simulated gastrointestinal stress of the strain *L. rhamnosus* GG in a symbiotic ice cream of Amazonian palm berry (açai), finding that in comparison with the fresh culture, *Lb. rhamnosus* GG showed greater survival under simulated gastrointestinal conditions when it was incorporated into açai ice cream, indicating that the presence of the food matrix contributed to the survival of the microorganism and attributed it to the buffering effect of dairy foods (Costa et al., 2017). However, in the present study, both dairy matrices were evaluated, showing that the microorganisms in the blueberry bagasse fermented milk presented a greater resistance to simulated gastrointestinal conditions. Nevertheless, further studies are necessary to investigate the role of each ingredient on the strain resistance.

5.3.4 Sensorial analysis of product

The results obtained from the 9-point hedonic scale evaluation are illustrated in Figure 15. The mean scores of texture, color, aroma, sweetness, acidity, flavor and overall acceptability are reported. All types of beverages had similar color and sweetness, but

values for texture and overall perception differ among fermented milk variety. The color and sweetness are similar in all the fermented milks because the same proportions of sweetener and blueberry bagasse powder were used for the elaboration of all the fermented milks. However, the texture was affected by the fermentation of each strain, which produced different viscosities (reported in the physicochemical characterization section). These fermentations also produced different scores for flavor, acidity, and general perception, being fermented milks B and D the most preferred by consumers.

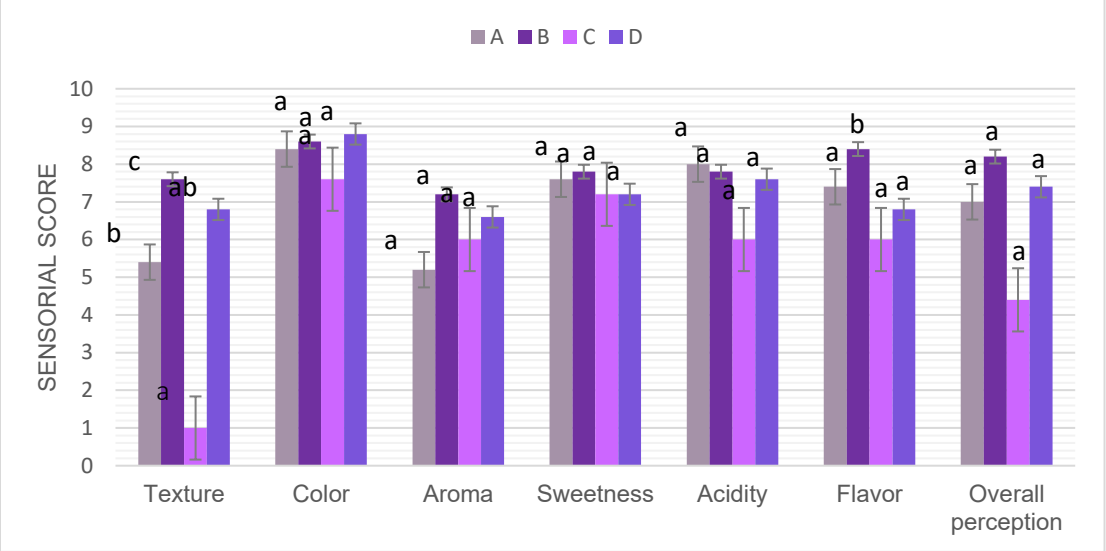


Figure 15. Sensory attributes of different types of fermented beverages using hedonic scale from 1 to 9 (1, lowest score, 9, highest score). Fermented milk A is fermented with *L. rhamnosus* GG*, L. kefiri* BIOTEC014, *L. lactis* BIOTEC007; B is fermented with *L. lactis* BIOTEC007; C is fermented with *L. kefiri* BIOTEC014 and Fermented milk D is fermented with *L. rhamnosus* GG. Different letters denote significant differences among the parameter measured in fermented milks.

Panelists considered that fermented milks with kefir isolates (as singles or co-culture) depending of the variety of milk, had a positive perception and are sensorially acceptable, with good flavor, color and acidity. However, the texture of fermented milk C affected the overall perception of the product. A similar study reported the sensory profiles of fermented milk containing isolates from kefir in co-culture. In this study, a group of trained panelists considered the fresh fermented milks obtained with the two- and three-strain starter cultures to be sensorially acceptable. However, sensory tests demonstrated that the milk fermented with the three-strain starter culture did not exhibit a desired acceptability after storage due to the high concentration of *K. marxianus* (a yeast specie) in the fermented product (Kakisu

et al., 2011). Therefore, it is important to consider in future work to develop tests after considerable storage time, where all sensory attributes can be evaluated and the general acceptability determined after days or weeks of elaboration.

It should be noted that the development of these functional products should be accompanied by specific sensory analyzes to allow for the acquisition of the best conditions and of real knowledge in the search for products well accepted on the consumer market, obtaining a greater detail concerning the addition of the probiotics and prebiotics to the product and their interaction with the consumer (Cruz et al., 2010).

5.4 Conclusions

Dairy fermented beverages by different kefir isolates and with blueberry bagasse as a functional ingredient were formulated. The beverages that had a better acceptance by the consumer panel were those with the highest viscosity values and which were fermented by *L. lactis* BIOTEC007 and *L. rhamnousus* GG. This formulation also contained considerable levels of bioactive compounds including phenolic acids and anthocyanins. Finally, it was observed that this formulation with bagasse promotes the growth of microorganisms in the beverage and exerts in some way a protective effect on the microorganisms, due to the fact that they resisted the digestion stages in a better way than the isolates that were in a milk matrix with no bagasse as a control. Taken together, these properties allowed the fermented blueberry milk to be considered a good dairy product, comparable with commercially available fermented milks. Moreover, blueberry bagasse may be considered as a potential prebiotic ingredient due to the activity observed *in vitro*. However, more studies are needed to elucidate all the mechanisms, including the evaluation and quantification of the metabolites produced by fermentation.

CHAPTER 6. CONCLUSIONS AND FUTURE WORK

1. Lactic acid bacteria and yeasts were isolated from artesanal kefir, and a reliable identification at genus and specie levels was performed through MALDI-TOF MS analysis, identifying species belonging to *Lactoccocus*, *Leuconostoc*, *Kluyveromyces* and *Lactobacillus* genus. Probiotic screening assays were developed and most of LAB and yeasts showed adequate properties of autoaggregation, co-aggregation with patogens, antimicrobial activity related to the production of organic acids, resistance to diffrerent antibiotics and the ability of ferment commercial prebiotics such as lactulose and agave inulin. In adittion, isolated LAB and yeasts demonstrated high resistance to in vitro gastrointestinal digestion conditions. Based on that, *L. lactis* BIOTEC007, *K. lactis* BIOTEC009, *L. kefiri* BIOTEC014 and *L. pseudomesenteroides* BIOTEC012 showed great probiotic potential.

2. Bagasse from different berries were obtained (raspberry, strawberry, blueberry and blackberry) and characterized by determining the physicochemical properties, the contents of total phenolics and anthocyanins, and the antimicrobial activity against *E. coli*, *S. typhi* and *S. aureus*. Regarding the nutritional content, high fiber and carbohydrate contents were found in the bagasse, being potential substrates for microbial fermentation.

3. High growth values were reported by the fermentation of LAB in different media supplemented with berry bagasse, finding the maximum growth in blueberry and strawberry bagasse media with *L. lactis* BIOTEC007, *L. kefiri* BIOTEC014 and *L. rhamnosus* GG. The substrate consumption measurements indicated that the sugars present in the media are consumed during the first hours but a slight increase is observed after 5-10 hours of fermentation, depending on the strain and the medium. Therefore, it is possible that LAB from kefir metabolize the fiber and polyphenols, releasing linked sugar molecules.

4. A synbiotic fermented milk was formulated by selected potential probiotc isolates from kefir and adding blueberry bagasse as a functional ingredient. Physicochemical properties and bioactive compounds including total phenolic content and total anthocyanin content were determined. Sensorial quality results showed that fermented beverages containing *L. lactis* BIOTEC007 and *L. rhamnousus* GG had a better acceptance by the consumer panel due to its sensorial atributes including texture, coinciding with high viscosity values. Results

showed that this dairy model promotes growth and survival of microorganisms in the beverage, exerting a protective effect on the isolates. Overall these characteristics allowed the fermented blueberry milk to be considered a good nutritional dairy synbiotic product

Future research to complement the results obtained in this dissertation are the following:

- To determine the enzymatic activity of the kefir isolates to elucidate the mechanisms by which the potential prebiotic ingredient (berry bagasse) is fermented.
- To monitor the production of short chain fatty acids through an *in vitro* fermentation using berry bagasse as a substrate and microorganisms characterized as potential probiotics, for their quantification and analysis.
- To characterize at a structural level the fiber content found in berry bagasse. Thus, knowing its degree of polymerization, chemical bonds, among other characteristics, the mechanism by which it is reduced to simple carbon sources can be determined.
- To evaluate the bioavailability of the phenolic compounds present in the bagasse during the fermentation of the substrate by the microorganisms, to analyze if its absorption in the organism can be potentiated.
- To difference the potential prebiotic substrates used by the microorganisms to grow in medium supplemented with bagasse (simple sugars, fiber, fruit polyphenols).

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VITA

Alejandra Hurtado Romero earned the Biotechnology Engineering degree from the Instituto Tecnológico y de Estudios Superiores de Monterrey, Guadalajara Campus in May 2019. She was accepted in the Biotechnology Master program in August 2019, working under the supervision of Dr. Tomás García Cayuela. This thesis represents the termination of her Master studies.

She has made a professional stance in the area of Plant Biotechnology at the Centro de Investigación y Asistencia en Tecnología y Diseño del Estado de Jalisco (CIATEJ) and has participated in projects such as the Design of Bioprocesses in the area of bioengineering of the Tecnológico de Monterrey Campus Guadalajara and in collaboration with the government of Tlajomulco for the bioremediation of Cajititlán Lake.

She participated in two congresses:

- An oral session presenting “Nonthermal technologies for the production of food ingredients with prebiotic potential” and a poster entitled “Sanitizing procedures applied before storage of fresh-cut carrots affect the wound-induced biosynthesis of chlorogenic acid” at the IFT-EFFoST International Nonthermal Processing Workshop 2019, Monterrey, Mexico.
- A poster presentation introducing the first part of her investigation thesis project titled “Screening of autochthononous lactic acid bacteria and yeast strains isolated from homemade kefir: probiotic and prebiotic properties” at the 51st Congress of Research and Development 2021, Monterrey Mexico.

Currently, she has four published scientific articles:

- **Hurtado-Romero, A.,** Del Toro-Barbosa, M., Garcia-Amezquita, L. E., & García-Cayuela, T. (2020). Innovative technologies for the production of food ingredients with prebiotic potential: Modifications, applications, and validation methods. Trends in Food Science & Technology, 104, 117-131.
- Toro-Barbosa, M., **Hurtado-Romero, A.,** Garcia-Amezquita, L. E., & García-Cayuela, T. (2020). Psychobiotics: Mechanisms of Action, Evaluation Methods and Effectiveness in Applications with Food Products. Nutrients, 12(12), 3896.

- Gastélum-Estrada, A., **Hurtado-Romero, A.,** Santacruz, A., Cisneros-Zevallos, L., & Jacobo-Velázquez, D. A. (2020). Sanitizing after fresh-cutting carrots reduces the wound-induced accumulation of phenolic antioxidants compared to sanitizing before fresh-cutting. Journal of the Science of Food and Agriculture, 100(13), 4995-4998.
- Berumen-Guerrero, L., Ortega-Hernández, E., Gastélum-Estrada, A., **Hurtado-Romero, A.,** Navarro-López, D., Benavides, J., & Jacobo-Velázquez, D. A. (2020). Chitosan enhances the production of antioxidant phenolic compounds in carrot through a synergistic effect with wounding stress. Revista Mexicana De Ingeniería Química, 19(Sup. 1), 375-384.

Additionally, she has accredited various courses in the areas of microbiology, food safety, research, industrial biotechnology, and analytical techniques such as HPLC. She is also the founder and owner of the natural biocosmetics trademark AHURA BIO.