Integrated Master in Bioengineering

Branch – Biological Engineering

Analysis of the organic acids and exopolysaccharides produced by the lactic acid microflora present in kefir and their potential applications

Dissertation for Master Thesis

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July 2012
Success is not final, failure is not fatal: it is the courage to continue that counts.

Winston Churchill
Acknowledgements

First of all, I would like to thank Doctor Isabel Afonso, for all the support, guidance, and availability always given. For always answering my calls, and try to find a way with me, when everything was going wrong.

I also would like to acknowledge Doctor Margarida Bastos for her wise counsel and availability.

I couldn’t forget to express my gratitude towards Professor Luís Melo for being one of the Professors who have chosen me for being a Bioengineering student when I transferred from Chemical Engineering; and for being one of the Professors that made me choose this branch.

I have to thank Professor Manuel Simões for always offering me help, and being always available.

My thanks also go to the researchers in Lab E007, especially to Madalena, Anabela, Renato, Luciana and Ana.

I have to express my gratitude towards Carla Ferreira, for her patience and help always given. Without you I couldn’t have done it.

Another person who always has listened to me and helped me when I need it, without me asking, was Dina Martins. Thank you!

To my “helpers” in the Lab, Paula and Sílvia I have no words to express my thankfulness. Along these years you were there for me, anytime, every day.

I would like to thank the Escola Superior Agrária – Instituto Politécnico de Viana do Castelo for letting me use the HPLC, particularly Eng. Virgílio Peixoto for his technical support,

I would like to thank my Bioengineering and “lunch” friends, Carol, Inês, Catarina, Diogo, Soraia and Carolina, for the funny moments and for always making me laugh.
I couldn’t forget Ana Luísa for always being there for me, listen and given me her opinion and help; and Francisco for making me laugh and always offering me help. You two brightened my days.

I would also like to thank Rita Fulgêncio for her friendship and help towards our shared BB-12.

Finally I am grateful to my family, specially my parents, sister and André, for being there for me unconditionally.

My special gratitude is to my mother who always tried to understand my theme and tried to help me.
Abstract

The present work comprised the evaluation of the potential production of added-value metabolites, such as organic acids and exopolysaccharides, by several microorganisms normally present in kefir. The promising application of the metabolites as food additives was also analysed.

To accomplish the aims of this work, multiple microorganisms combinations (lactic acid bacteria, yeasts and *Bifidobacterium*) were studied; and the analysis of organic acids and EPS production, was performed by HPLC and colorimetric methods.

Relatively to the organic acids, the higher concentration was obtained for succinic acid ($5.39 \times 10^{-1}$ g/L) for *C. krusei* after 6.5 hours of experiment. For possible applications, this production has to be optimized.

The identification of the monosaccharides that constitute the exopolysaccharide produced was not possible. However the reducing sugars present in the samples were quantified and the highest concentration value was obtained for *L. lactis* subsp. *lactis* (47.66 g/L, after 30 hours of growth). This microorganism also showed properties for hydrolysing the exopolysaccharides and consuming the hydrolysates.

The yeasts inhibit the microorganisms from producing the exopolysaccharides and *B. animalis* subsp. *lactis* was not able to produce exopolysaccharides.

The bifidogenic effect of *B. animalis* subsp. *lactis* was evident by the exopolysaccharide hydrolysis and consume of the resulting sugars. The prebiotic effect of the exopolysaccharides was also proved by the increased growth of *B. animalis* subsp. *lactis* for higher exopolysaccharides concentrations.

The metabolites produced could have several applications and they are of great interest to research and food industry. In one hand, they can be applied as additives in the preservation and stabilization of food products; and in the other hand they could have the potential to originate functional food.

Keywords: organic acids, exopolysaccharides, yeasts, bacteria, prebiotic, probiotic.
Resumo

O presente trabalho consistiu na avaliação do potencial de produção de metabolitos de valor acrescentado, como os ácidos orgânicos e os exopolissacarídeos, através de diversos microrganismos normalmente presentes no kefir. A aplicação promissora destes metabolitos como aditivos alimentares também foi analisada.

Para alcançar os objetivos do trabalho, foram estudadas múltiplas combinações de microrganismos (bactérias do ácido lático, leveduras e Bifidobacterium); a análise da produção de ácidos orgânicos e de exopolissacarídeos foi realizada através de HPLC e métodos colorimétricos.

Relativamente aos ácidos orgânicos, a concentração mais elevada foi atribuída ao ácido succínico (5,39x10^{-1} g/L) para a levedura C. krusei após 6,5 horas de experiência. Para possíveis aplicações, esta produção teria de ser otimizada.

A identificação dos monossacarídeos que constituem o exopolissacarídeo produzido, não foi possível. Contudo, os açúcares redutores presentes nas amostras foram quantificados e o valor de concentração mais elevado foi obtido para L. lactis subsp. lactis (47,66 g/L, após 30 horas de crescimento). Este microrganismo demonstrou também propriedades de hidrólise para com o exopolissacarídeo e o posterior consumo dos hidrolisados.

As leveduras inibiram os microrganismos de produzirem os exopolissacarídeos e o B. animalis subsp. lactis não foi capaz de produzir exopolissacarídeos.

O efeito bifidogênico do B. animalis subsp. lactis foi evidente pela hidrólise do exopolissacarídeo e o consumo dos açúcares resultantes. O efeito prebiótico do exopolissacarídeo foi também demonstrado, uma vez que concentrações mais elevadas deste polissacarídeo, promoveram o crescimento do B. animalis subsp. lactis.

Os metabolitos produzidos podem ter diversas aplicações e possuem um grande interesse para a investigação e para a indústria alimentar. Por um lado, podem ser aplicados como aditivos na conservação e estabilização de produtos alimentares; por outro lado, podem possuir o potencial para originar alimentos funcionais.
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Palavras-chave: ácidos orgânicos, exopolissacarídeos, leveduras, bactérias, prebiótico, probiótico.
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<tbody>
<tr>
<td>AAB</td>
<td>Acetic Acid Bacteria</td>
</tr>
<tr>
<td>Abs</td>
<td>Absorbance</td>
</tr>
<tr>
<td>AMRS</td>
<td>Altered Man, Rogosa and Sharpe</td>
</tr>
<tr>
<td>CBA</td>
<td>Corynebacterium Agar</td>
</tr>
<tr>
<td>CoA</td>
<td>Coenzyme A</td>
</tr>
<tr>
<td>DNS</td>
<td>3,5-Dinitrosalicylic acid</td>
</tr>
<tr>
<td>EPS</td>
<td>Exopolysaccharides</td>
</tr>
<tr>
<td>FDA</td>
<td>Food and Drug Administration</td>
</tr>
<tr>
<td>FOS</td>
<td>Fructo-oligosaccharide</td>
</tr>
<tr>
<td>GOS</td>
<td>Galacto-oligosaccharide</td>
</tr>
<tr>
<td>GRAS</td>
<td>Generally Recognized As Safe</td>
</tr>
<tr>
<td>HPLC</td>
<td>High Performance Liquid Chromatography</td>
</tr>
<tr>
<td>LAB</td>
<td>Lactic Acid Bacteria</td>
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<tr>
<td>MRS</td>
<td>Man, Rogosa and Sharpe</td>
</tr>
<tr>
<td>OD</td>
<td>Optic Density</td>
</tr>
<tr>
<td>PCA</td>
<td>Plate Count Agar</td>
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<tr>
<td>RI</td>
<td>Refractive Index</td>
</tr>
<tr>
<td>RP</td>
<td>Reversed phase</td>
</tr>
<tr>
<td>TDP</td>
<td>Thymidine diphosphate</td>
</tr>
<tr>
<td>UDP</td>
<td>Uridine Diphosphate</td>
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<table>
<thead>
<tr>
<th>Acronym</th>
<th>Description</th>
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<tbody>
<tr>
<td>UV</td>
<td>Ultraviolet</td>
</tr>
<tr>
<td>Vis</td>
<td>Visible</td>
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<tr>
<td>YMA</td>
<td>Yeast Malt Agar</td>
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1. Introduction

1.1. Thesis Presentation and Objectives

The current microbial biotechnology encompasses the use of microorganisms for the production and development of products that consumers want and need. Microorganisms have an important role in the generation of a wide variety of products, and therefore have an extensive range of applications in many areas, specially the pharmaceutical, environmental and food industries.

Modern consumers are becoming increasingly aware of the importance of healthy, natural and nutritional food. This demand leads to the importance of isolate, purify and characterise microorganisms that may be useful in the referred areas (Laws and Marshall, 2001).

Based on these concepts, it is an objective to produce food with high nutritional value and that may have health properties that increase the commercial and nutritional value of products. These products can be defined as functional food which are consumed in the regular diet, have beneficial effects and reduce the risk of disease; or as probiotics, which are microorganisms that confer a beneficial effect to the host, when ingested in adequate quantities (Farnworth, 2005).

An example of this type of food is kefir, which in addition of having the properties mentioned above has a composition that makes it interesting in the biotechnology point of view. The complex set of microorganisms which coexist in symbiosis is capable of producing metabolites of high interest (Farnworth, 2005; Irigoyen et al., 2005).

These metabolites can be thickening and stabilizers agents, such as polysaccharides (Badel et al., 2011), or preservatives such as organic acids. Since they are produced by natural means, they become favourites in the area, being possible food additives (Welman and Maddox, 2003; Theron and Lues, 2011).
These thickening agents, such as polysaccharides, have a low fat and sugar contents and are associated with health improvements, properties that consumers prefer and look for (Welman and Maddox, 2003). The organic acids are antimicrobial agents, applied mainly in the beverage industry. The consumers will prefer this preservatives, if their origin is natural and not synthetic (Theron and Lues, 2011).

Some of the carbohydrates that are produced by specific strains of microorganisms can promote the growth of *Bifidobacterium* species, which are known for their probiotic effects, beneficial for the human health (Hattner, 2009; Neri *et al.*, 2009).

Consequently, the main objectives for this thesis were to extract the organic acids and exopolysaccharides (EPS) produced by the strains (isolated and mixed in different combinations); to analyse their production, in terms of concentration and composition. These goals were attempted by different extraction techniques, by HPLC (High Performance Liquid Chromatography) analysis and by colorimetric methods.

The possible bifidogenic effect over the EPS extracted was also studied, by the verification of the EPS hydrolysis, by *Bifidobacterium animalis* subsp. *lactis*.

1.2. Thesis Organization

In Chapter 1 the thesis theme and the main objectives of the work are outlined.

The Chapter 2 is a review in kefir production, microorganisms’ diversity in this fermented milk, the health interest behind this product and the importance of the metabolites produced by the microorganisms enclosed in kefir. It also focuses on the possible and potential applications that these products of metabolism could have in several areas: food and health domains. The investigation in probiotics and prebiotics is also addressed in this chapter.

Chapter 3 contains the methodology used to accomplish the objectives of the work, in terms of organic acids extraction and quantification, as for EPS extraction, hydrolysis and quantification and for the study of the bifidogenic effect of the EPS produced.

In Chapter 4 are presented all the results and their discussion.

The conclusions and suggestions for a future work are summarized in Chapter 5.
2. Theoretical Fundamentals

2.1. Kefir

Kefir is a fermented milk beverage originated from Eastern Europe, obtained by lactic and alcoholic fermentation (Assadi et al., 2000; Yaman et al., 2006). The production is carried out from kefir grains, which can be preserved at low temperatures or freeze-dried (Witthuhn et al., 2005a).

This drink differs from other milk products since it results from the interaction of different organisms (Garrote et al., 1997). Although it shares certain organoleptic properties with yogurt, there are some significant differences. Kefir is not as creamy as yogurt, it is effervescent, due to the carbon dioxide content, and is slightly alcoholic (Latorre-García et al., 2007; Farnworth and Mainville, 2008).

It has a pH between 4 and 4.5, which is lower than milk (pH 6) and varies in fat content, in accordance to the milk used as raw material. The grains used to prepare this drink look like small cauliflowers as it can be seen in Figure 2.1 (Irigoyen et al., 2005).

Figure 2.1 – Kefir grains (each square is equal to 1 cm).
The grains have a diameter varying from 5 to 45 mm. This variation is considerable due to the growing process and the complex structure of the grains. Smaller grains are formed around the original grain, until it reaches a breaking point where the grains detach, due to the fact that the original grain cannot sustain the weight, forming multiple grains (Güzel-Seydim et al., 2000). They are gelatinous, their colour can be white or yellowish and their shape is irregular (Garrote et al., 2001; Miguel et al., 2010).

The grains composition depends on their origin, but they are mainly composed by water ($\approx 90\%$), proteins ($\approx 3\%$), lipids ($\approx 0.3\%$), carbohydrates ($\approx 6\%$) and ashes ($\approx 0.7\%$) (Garrote et al., 1997; Garrote et al., 2001). The microflora is entrapped in the proteins and polysaccharides matrix that constitute the grains (Garrote et al., 1997).

### 2.1.1. Production

Kefir production and commercialisation are more common in Central and East Europe, being quite new in North America. In Russia this product is considered an essential food alongside with bread, milk, sugar and salt. Some countries, like Germany, France and Norway already have regulation related to kefir production and composition (Farnworth and Mainville, 2008; Cogulu et al., 2010).

The traditional production consists in the simple addition of the grains to the milk for an incubation time of 24 hours. At the end of this period, the grains are separated from the milk by filtration processes and the final product is conserved at 4 °C. The same process can be repeated several times, with the same grains (Lopitz-Otsoa et al., 2006). The optimal temperature to produce kefir is 20 - 30 °C and it can be produced with any type of milk (buffalo, cow, goat) (Irigoyen et al., 2005; Siritat and Jelena, 2010). This process is not aseptic (Marshall et al., 1984; Irigoyen et al., 2005).

At an industrial level, the production is not with the grains, but with the strains typically present in kefir. Firstly, the milk is homogenised and undergoes a heat treatment at high temperatures (e.g. pasteurization). Afterwards the product is cooled down until it reaches 20 °C and at this point the inoculation with specific strains is done. The incubation time is the same as for the traditional process, as well as the conservation conditions (Assadi et al., 2000; Otles and Cagindi, 2003).
2.1.2. Microorganisms’ Diversity

The microbial population present in the grains coexists in symbiosis (Goršek and Tramšek, 2008; Balabanova and Panayotov, 2011) and is mostly constitute by lactic acid bacteria (LAB), acetic acid bacteria (AAB), yeasts, and it can also contain mycelial fungi. The grains have certain specific microbial species that are always present, but there may be present other species, depending on the origin of the grains (Marshall et al., 1984; Witthuhn et al., 2005b). In Figure 2.2, the coexistence of different microorganisms in a kefir grain can be observed.

![Image](image.png)

Figure 2.2 – Optical microscope photographs of a kefir grain (1000x with immersion lens). A – Evidence of the presence of Gram-positive and Gram-negative bacilli; B – Evidence of the presence of yeasts and bacilli.

Some referred LAB are *Lactobacillus acidophilus*, *Lactobacillus brevis*, *Lactobacillus casei*, *Lactobacillus fermentum*, *Lactobacillus helveticus*, *Lactobacillus kefir*, *Lactobacillus parakefiri*, *Lactococcus lactis*, *Leuconostoc mesenteroides* (Witthuhn et al., 2005b) and *Lactobacillus delbrueckii* subsp. *bulgaricus* (Simova et al., 2002).

The LAB are Generally Recognized As Safe (GRAS) and had been known for their EPS production (Duboc and Mollet, 2001; Welman and Maddox, 2003) and complex carbohydrates utilization for their own growth (Gobbetti, 1998). Due to their GRAS status and their specific characteristics and products of metabolism, these microorganisms are of great interest for industry (Cogan et al., 1997).

These microorganisms are also referred as having immunological properties, the ability of maintaining the gastrointestinal system healthy and producing different metabolites with a positive effect in the human health (Turpin et al., 2010).
Several yeasts had also been referred: *Kluyveromyces marxianus*, *Torula kefir*, *Saccharomyces exiguis*, *Candida lambica* (Witthuhn et al., 2005b), *Candida kefir*, *Saccharomyces cerevisiae* (Abraham and de Antoni, 1999), *Candida krusei* and *Candida famata* (Witthuhn et al., 2005b; Adriana et al., 2009).

These yeasts are fungi that play a key role in food industry (Fleet, 2007) and particularly in the flavour and aroma of kefir (Simova et al., 2002). In addition, they provide essential nutrients for bacteria growth (e.g. vitamins and amino acids) stimulating them (Farnworth, 2005; Irigoyen et al., 2005; Álvarez-Martín et al., 2008), and they also remove toxic products from metabolism. Furthermore they inhibit the growth of microorganisms, by decreasing the pH of the medium and producing ethanol, carbon dioxide and organic acids (Viljoen, 2001).

There are two types of yeasts: the ones that can ferment lactose, and the ones that cannot ferment lactose and need the assistance of LAB to degrade it (Adriana and Socaciu, 2008).

The microorganisms’ distribution in the grains had been referred as specific and the yeasts are predominantly located in the periphery of the grains (Farnworth, 2005).

### 2.1.3. Health Interest

Kefir has been described to possess several health benefits: antitumor (Irigoyen et al., 2005; Urdaneta et al., 2007; Chen et al., 2008), antibacterial and anti-inflammatory properties (Rodrigues et al., 2005). It stimulates the immune system and has hypocholesterolemic effects (Irigoyen et al., 2005; Urdaneta et al., 2007; Chen et al., 2008). It was also reported that it has beneficial effects for the gastrointestinal tract (Lopitz-Otsoa et al., 2006), facilitating the digestion of lactose (Urdaneta et al., 2007).

These antimicrobial properties are related to the microorganisms that are present in the microflora and to the products of their metabolism, such as organic acids, EPS, carbon dioxide, acetaldehyde, diacetyl (Adriana and Socaciu, 2008; Siritat and Jelena, 2010).

It is considered a complex probiotic due to the microorganisms that constitute it (Urdaneta et al., 2007; Adriana and Socaciu, 2008). A probiotic can be defined as living
microorganisms that have a positive effect on the health of the host (Lopitz-Otsoa et al., 2006).

There are several criteria that must be followed to be considered a probiotic strain: the microorganism cannot harm the host; it has to subsist in the gastrointestinal tract and be in a sufficient number to have any benefit to the host, being able to survive and be resistant in that environment (Lopitz-Otsoa et al., 2006; Xie et al., 2012).

The probiotics mostly applied and known for their properties are the LAB and Bifidobacterium (more applied in the industry). Lactobacilli are the LAB that have more attention from the researchers, due to their common presence in fermented food (Turpin et al., 2010).

2.2. Metabolic Products with Industrial Applications

The microorganisms listed in subsection 2.1.2 normally present in kefir have the characteristic of being able to produce different compounds: organic acids, EPS, carbon dioxide, ethanol, acetaldehyde, acetoain, diacetyl, among many others (Adriana and Socaciu, 2008; Siritat and Jelena, 2010). From what was stated above, these products of metabolism can have several applications in the industry, consequently their study is an important field of investigation.

2.2.1. Organic Acids

The characteristic flavour of kefir is the result of the presence of various compounds, such as organic acids (lactic, propionic, citric, acetic, orotic, butyric, hippuric, uric, pyruvic and succinic acids). These compounds can also be used in food industry as flavouring and preservative agents (Mullin and Emmons, 1997; Güzel-Seydim et al., 2000; Zeppa et al., 2001) or even in the production of biodegradable plastics (Theron and Lues, 2011). These acids may be present due to the fatty acids (from the milk) hydrolysis, or as a result of the metabolic processes (Güzel-Seydim et al., 2000).
The microorganisms present in kefir, metabolise the substrate (glucose) to produce energy through glycolysis (or other degradative pathway). The final product formed is pyruvate (pyruvic acid) (Güzel-Seydim et al., 2000; Madigan et al., 2009).

If there is oxygen available and the microorganisms are aerobic or, at least are oxygen tolerant, the pyruvic acid is integrated in the Krebs cycle (Figure 2.3), in which take place several oxidations and decarboxylations. Citric and succinic acids are two organic acids produced by this means. In the absence of oxygen, the pyruvic acid is fermented, and other products are formed (Madigan et al., 2009).

![Krebs cycle](image)

Figure 2.3 – Krebs cycle (adapted from Madigan et al., 2009).

In kefir, the fermentation process could be heterolactic or homolactic (Güzel-Seydim et al., 2000). The first process involves the partial oxidation of carbohydrates, resulting in the release of energy, lactic acid, ethanol and carbon dioxide, while in the
second process the only products are lactic acid and energy (Madigan et al., 2009). A representative scheme of these processes can be seen in Figure 2.4.

![Diagram of heterolactic fermentation]

The glucose could also be metabolised by the microorganisms through mixed acid fermentation, being produced ethanol, hydrogen, carbon dioxide, acetate (acetic acid), lactate (lactic acid) and succinate (succinic acid) (Figure 2.5) (Madigan et al., 2009).

The organic acids produced can be used in food industry. The preservation of food has always been performed by man, through various methods, often without the microbiological knowledge that is inherent. The organic acids are compounds used as food preservatives due to the fact that they are naturally present in food, as ingredients. Consequently, it is common to add organic acids to food in order to control microbial contamination (Theron and Lues, 2011).

Many organic acids are FDA (Food and Drug Administration) and EC (European Commission) approved and are used as preservatives, antioxidants, flavouring agents, acidulants and pH regulators. They have the GRAS status and are applied in the pharmaceutical industry (Theron and Lues, 2011).
Analysis of the organic acids and exopolysaccharides produced by the lactic acid microflora present in kefir and their potential applications

Figure 2.5 – Mixed acid fermentation (adapted from MetaCyc, 2010).

The acids usually present in kefir and other fermented products are typically identified by chromatographic techniques (Mullin and Emmons, 1997; Güzel-Seydim et al., 2000; Zeppa et al., 2001). The acids investigated in this study are shown in Figure 2.6.

Figure 2.6 – Chemical structure of succinic, acetic, citric, pyruvic, lactic and propionic acid.
Succinic acid is used as a buffer agent in food industry, or as a chemical intermediate in medicine and in perfumes esters manufacture. Acetic acid is a weak acid used as a preservative and a food additive and has GRAS status. It is soluble in lipids and therefore it is able to diffuse through the plasmatic membrane, affecting the pH of the cells, causing their death (Nielsen, 2004; Theron and Lues, 2011).

The citric acid is the preservative and flavouring agent generally applied in food and pharmaceutical industries. This acid acts as a chelate agent of the metallic ions present in the medium, preventing microbial proliferation (Theron and Lues, 2011). Pyruvic acid is also applied as a flavouring agent and as a preservative, inhibiting the microorganisms’ growth (Nielsen, 2004; DCCC, 2010; Theron and Lues, 2011).

Lactic acid has GRAS status and is a preservative, an acidulant, a flavouring agent and a pH regulating agent (Theron and Lues, 2011). Propionic acid is used as a preservative and as a flavouring agent (Nielsen, 2004). Furthermore it can be utilized to produce polymers (Theron and Lues, 2011).

2.2.2. Exopolysaccharides

The matrix that entraps the microorganisms in kefir is composed of EPS, produced by some LAB (especially microorganisms from the genera *Lactobacillus* and *Lactococcus*) (Welman and Maddox, 2003; Irigoyen et al., 2005; Miguel et al., 2010).

A schematic representation of the metabolic production of EPS from lactose is present in Figure 2.7. The molecule of glucose-6-phosphate can be involved in EPS production (anabolic pathways) or in the catabolic pathways (subsection 2.2.1) (Boels et al., 2001). In this pathway, this molecule is used to produce sugar nucleotides (UDP-glucose and TDP-glucose). The final sugars formed are linked together to constitute the EPS molecules. This mechanism has not been full studied yet (De Vuyst and Degeest, 1999; Welman and Maddox, 2003).

The EPS production is influenced by several factors, such as microorganisms, pH, temperature, agitation, incubation time, oxygen, carbon source and nitrogen sources (Aslim et al., 2005).
There are two types of EPS that can be produced: the homopolysaccharides and heteropolysaccharides. The homopolysaccharides are composed of only one type of monosaccharide (repeated units); and the heteropolysaccharides are formed by two or more units of different monosaccharides (e.g. glucose, galactose, rhamnose), which are repeated along the polysaccharide chain (De Vuyst and Degeest, 1999; Welman and Maddox, 2003; Mozzi et al., 2006).

The EPS are important to the rheology and consequently to the texture of milk products. It acts like a thickener, emulsifying and stabilizing agent (Laws and Marshall, 2001; Frengova et al., 2002). The EPS increases the viscosity, which leads to a superior contact time in the mouth for the products. This allows a better perception of the taste to the consumer (Duboc and Mollet, 2001). The mode of action is explained by the capability of the EPS to interact with the proteins of the milk (caseins) increasing the viscosity of the whey (Duboc and Mollet, 2001).
Normally, these properties are reached by the addition of sugars, lipids and other stabilizers (alginate, gelatine or, starch), but since the actual consumers are becoming more aware of the health issues, the demand is for products with low sugar and fat content but, at the same time, with an identical texture. This can be achieved with these natural additives avoiding the artificial ones (Duboc and Mollet, 2001; Welman and Maddox, 2003).

Furthermore it has been published that EPS have favourable effects in health, specially related to gastrointestinal diseases (Habibi et al., 2011) and possible antitumor effects (Duboc and Mollet, 2001; Welman and Maddox, 2003).

The EPS relation with health issues leads to another important definition, which is prebiotics. These are food ingredients that are not digestible for man but can stimulate the growth of certain species of microorganisms, like probiotics. In other words, prebiotics stimulate the presence of probiotics in the gut, leading to the beneficial effects (Barreteau et al., 2006; Sarkar, 2011).

The EPS are polysaccharides that can be hydrolysed to oligosaccharides which some of them have been described as prebiotics. The two main oligosaccharides of interest are galacto-oligosaccharide (GOS) and fructo-oligosaccharide (FOS). The microorganisms’ species that can metabolise these oligosaccharides have to possess enzymes that can break the glycosidic bonds. These enzymes are hydrolases called β-galactosidase (3.2.1.23) and β-fructosidase (3.2.1.80) (Barreteau et al., 2006; Splechtna et al., 2006). The resulting molecules are fermented by the intestinal microflora in lactate, propionate and acetate, providing means for the proliferation of the probiotic species (Kouya et al., 2007) and the decreased growth for the pathogenic ones (Fanaro et al., 2005).

Due to the presence of those enzymes, kefir improves the lactose maldigestion, since the degradation of lactose requires the presence of certain enzymes, which some individuals do not possess. In the presence of kefir, some probiotic strains persist and stay in the microflora of the gut being stimulated by carbohydrates, like lactose, which they degrade to more simple molecules, reducing the maldigestion (Hertzler and Clancy, 2003).

One of the EPS produced from microorganisms present in kefir is called kefiran and it is a heteropolysaccharide with distinct properties, like others EPS (Farnworth, 2005; Adriana and Socaciu, 2008). It is soluble in water and has a branched structure.
composed by D-glucose and D-galactose in equal amounts (Figure 2.8) (Micheli et al., 1999; Rimada and Abraham, 2001; Badel et al., 2011).

The kefiran application is still very limited due to its low viscosity, therefore it has to be combined with other hydrocolloids agents, like λ-carrageenan (Frengova et al., 2002; Farnworth, 2005).

![Figure 2.8 – Kefiran structure (adapted from Micheli et al., 1999).](image)

The EPS can be identified and quantify by different techniques, but the most commonly applied are: molish test (Yang et al., 1999), anthrone method (Rimada and Abraham, 2001; Piermaria et al., 2008), Dubois method (Frengova et al., 2002; Ghasemlou et al., 2012), gas chromatography (Yang et al., 1999; Frengova et al., 2002), HPLC (Gassem et al., 1997; Piermaria et al., 2008), thin layer chromatography (La Rivière and Kooiman, 1967; Rimada and Abraham, 2001; Piermaria et al., 2008) and gel filtration (Yang et al., 1999).

These carbohydrates are less sweet than the usual sugars used in the industry and can enhance the probiotic activities (Sako et al., 1999), whereby they are of great interest to investigation and food industry.

Despite all the advantages referred, considerable research involving its specific properties has to be made to apply this biological EPS in the industry. Only xanthan, gellan and curdlan had been deeply investigated and applied as additives industrially (Badel et al., 2011).
3. Materials and Methods

3.1. Microorganisms

The complete study involved seven different strains: *Lactobacillus delbrueckii* subsp. *bulgaricus*, *Lactobacillus kefir*, *Lactococcus lactis* subsp. *lactis* (the three were acquired from DSMZ - German Collection of Microorganisms and Cell Cultures), *Candida famata*, *Candida krusei* (both isolated from kefir), *Bifidobacterium animalis* subsp. *lactis* (BB-12 CHR-HANSEN, acquired from Promolac) and *Escherichia coli* (gently given by UTAD – Universidade de Trás-os-Montes-e-Alto-Douro).

The characteristics, the mediums and the cultivation conditions for all the strains are listed in the Table 3.1. The composition of all the mediums used is described in the Annex A.1.

At the end of the experiments, the microorganisms were preserved at -80 °C (except *E. coli*) with freezing medium specific for each strain. This freezing medium consists basically in the mediums referred in the Table 3.1 for each strain, but doubly concentrated and with 30% of glycerol.

All the growth studies in skim milk were evaluated by the dry weight method and by filtration of a specific volume of sample. The first method was accomplished by placing 1 mL of the sample in an eppendorf, left to dry in a heater (Selecta) at 60 °C, until constant weight. In the second method, a specific volume of sample was filtered (5 mL) and the retained biomass was washed with 15 mL of distilled water. After heating at 105 °C for 8 hours, the weight of the sample was determined (g weighted/mL filtered).

---

1 The temperature chosen was 60 °C due to the medium applied (skim milk).
Table 3.1 – Microorganisms applied in the study, their characterisation, origins and cultivations conditions

<table>
<thead>
<tr>
<th>Microorganisms</th>
<th>Photography (1000x with immersion lens)</th>
<th>Characteristics</th>
<th>Isolated from</th>
<th>Medium</th>
<th>Cultivation Conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>L. delbrueckii</em> subsp. <em>bulgaricus</em> (DSM 20081)</td>
<td><img src="lactic-acid-microflora.jpg" alt="Image" /></td>
<td>Microaerophilic Gram - positive</td>
<td>Bulgarian yogurt</td>
<td>MRS</td>
<td>37 °C, 2 days anaerobic jar</td>
</tr>
<tr>
<td><em>L. kefir</em> (DSM 20587)</td>
<td><img src="lactic-acid-microflora.jpg" alt="Image" /></td>
<td>Aerobic Gram - positive</td>
<td>Kefir grains</td>
<td>MRS</td>
<td>30 °C, 2 days</td>
</tr>
<tr>
<td><em>L. lactis</em> subsp. <em>lactis</em> (DSM 20481)</td>
<td><img src="lactic-acid-microflora.jpg" alt="Image" /></td>
<td>Aerobic Gram - positive</td>
<td>Not specified</td>
<td>CBA</td>
<td>30 °C, 3 days</td>
</tr>
</tbody>
</table>

2 With the exception of *L. delbrueckii* subsp. *bulgaricus*, in which the magnification, of the present picture, is 400x.
3 Information given by the producer.
Table 3.1 – Microorganisms applied in the study, their characterisation, origins and cultivations conditions (cont.)

<table>
<thead>
<tr>
<th>Microorganisms</th>
<th>Photography (1000x with immersion lens)</th>
<th>Characteristics</th>
<th>Isolated from</th>
<th>Medium</th>
<th>Cultivation Conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>C. famata</em></td>
<td></td>
<td>Anaerobic facultative</td>
<td>Kefir grains</td>
<td>YMA</td>
<td>30 °C, 2 days</td>
</tr>
<tr>
<td><em>C. krusei</em></td>
<td></td>
<td>Anaerobic facultative</td>
<td>Kefir</td>
<td>YMA</td>
<td>30 °C, 3 days</td>
</tr>
<tr>
<td><em>B. animalis subsp. lactis</em> (BB-12 CHR-HANSEN)</td>
<td></td>
<td>Anaerobic (slightly oxygen tolerant) Gram - positive</td>
<td>Not specified</td>
<td>MRS with cysteine 0.05%</td>
<td>37 °C, 2 days anaerobic jar</td>
</tr>
<tr>
<td><em>E. coli</em> (UTAD private collection)</td>
<td></td>
<td>Anaerobic facultative Gram – negative</td>
<td>Dog faeces</td>
<td>PCA</td>
<td>30 °C, 2 days</td>
</tr>
</tbody>
</table>
3.2. Organic Acids

The organic acids production was performed using the single microorganisms (referred as isolated microorganisms) and in pulled mixtures with different combinations of microorganisms (Table 3.2).

Table 3.2 – List of the different combinations applied in the study for organic acids production

<table>
<thead>
<tr>
<th>Isolated microorganisms</th>
<th>Combinations of Microorganisms</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>L. delbrueckii</em> subsp. bulgaricus</td>
<td><em>L. delbrueckii</em> subsp. bulgaricus + <em>L. kefir</em></td>
</tr>
<tr>
<td><em>L. kefir</em></td>
<td><em>L. kefir</em> + <em>L. lactis</em> subsp. lactis</td>
</tr>
<tr>
<td><em>L. lactis</em> subsp. lactis</td>
<td><em>L. lactis</em> subsp. lactis + <em>L. delbrueckii</em> subsp. bulgaricus</td>
</tr>
<tr>
<td><em>C. famata</em></td>
<td><em>L. delbrueckii</em> subsp. bulgaricus + All the yeasts (C. famata + C. krusei)</td>
</tr>
<tr>
<td><em>C. krusei</em></td>
<td><em>L. kefir</em> + All the yeasts (C. famata + C. krusei)</td>
</tr>
<tr>
<td></td>
<td><em>L. lactis</em> subsp. lactis + All the yeasts (C. famata + C. krusei)</td>
</tr>
<tr>
<td></td>
<td>All the bacteria (<em>L. delbrueckii</em> subsp. bulgaricus + <em>L. kefir</em> + <em>L. lactis</em> subsp. lactis)</td>
</tr>
<tr>
<td></td>
<td>All the bacteria (<em>L. delbrueckii</em> subsp. bulgaricus + <em>L. kefir</em> + <em>L. lactis</em> subsp. lactis) + All the yeasts (C. famata + C. krusei)</td>
</tr>
<tr>
<td></td>
<td><em>C. famata</em> + C. krusei</td>
</tr>
</tbody>
</table>

The microorganisms were inoculated into 100 mL Erlenmeyer’s (with 100 mL of skim milk \(^4\)) in 24-hour tests, in an orbital incubator (Aralab) at 120 rpm (rotation per minute) and 30 °C (Figure 3.1).

The samples removal was performed under sterile conditions. Seven samples were collected for each condition, for the time zero, after 2.5, 4.0, 6.5, 8.5, 10.0 and 24 hours of growth.

\(^4\) These Erlenmeyer’s used had the volume of skim milk, and not a higher volume, due to the fact that one strain is microaerophilic. In this way the volume of air is reduced.
3.2.1. Extraction

For the organic acids extraction it was applied a liquid-liquid extraction, which consists in a separation process that relies on the miscibility of liquids.

Firstly, 25 mL of 0.4% ortho-phosphoric acid (H₃PO₄ 85% (Carlo Erre)), at pH 2.10 (pH meter: Hanna HI 8424) were added to a 4 mL sample. The solutions were stirred and homogenised with a vortex for 5 min (Güzel-Seydim et al., 2000). At this point the samples were centrifuged (5810 R Eppendorf) (Zeppa et al., 2001) for 40 min at 4000 g (relative centrifugal force) at a temperature of 4 °C. The resulting supernatant was filtered using a paper filter with a porosity of 11 µm (Whatman). Samples were preserved in the freezer at -15 °C until HPLC analysis (Güzel-Seydim et al., 2000).

3.2.2. Identification and Quantification by HPLC-UV/Vis

To identify which acids were present and quantify them, it was applied HPLC-UV/Vis (Mullin and Emmons, 1997; Güzel-Seydim et al., 2000; Zeppa et al., 2001; Baptista, 2004).

The mobile phase (0.4% H₃PO₄) has the same composition as the solution used in the extraction. This solution was filtered in vacuum through a membrane filter of cellulose nitrate with a 0.45 µm pore (Pall Corporation) and afterwards it was degasified (Elma Transsonic 420) (Baptista, 2004).
Mixtures of organic acids standards were prepared in order to obtain composed standards (pyruvic (Merck), acetic (VWR), lactic (Sigma-Aldrich), citric (Panreac), propionic (Sigma-Aldrich) and succinic acids (Merck)) were prepared with 0.4% H$_3$PO$_4$ pH 2.10. From one 0.1 g/L stock composed standard solution different concentrations were prepared: $1 \times 10^{-3}$, $5 \times 10^{-3}$, $8 \times 10^{-3}$, $1 \times 10^{-2}$, $2 \times 10^{-2}$, $5 \times 10^{-2}$, $8 \times 10^{-2}$ and $1 \times 10^{-1}$ g/L.\(^5\)

For succinic acid, higher concentration solutions were also prepared: $5 \times 10^{-2}$, $8 \times 10^{-2}$, $1 \times 10^{-1}$, $2 \times 10^{-1}$, $3 \times 10^{-1}$, $5 \times 10^{-1}$ and $7 \times 10^{-1}$ g/L. The calibration curves are present in Annex A.2.1.

The standards and samples were filtered with a nylon filter with a 0.45 µm pore (VWR) for 2 mL vials (VWR). Each standard solution/sample was injected twice (20 µL each injection) with a running time of 30 min.

Between the samples it was carried out a column cleaning process of 17 min (to make sure that the next sample didn’t have traces of the previous one), using a gradient of mobile phase and methanol (Figure 3.2). Firstly, for 5 min it was passed mobile phase in the column, then, for 1 minute it was performed the passage of mobile phase to methanol. Subsequently, the methanol was left running for 5 min, then it was made a new passage from methanol to mobile phase for 1 minute, and finally it was stabilized with mobile phase for 5 min.

![Figure 3.2](image)

Figure 3.2 – Schematic representation of the column cleaning process. The mobile phase (0.4% H$_3$PO$_4$) is represented in green and the methanol is represented in orange.

---

\(^5\) Several acids from an organic acids kit (Sigma-Aldrich) were also tested, but the peaks weren’t able to be distinguished clearly. The time did not allow the optimization of these organic acids identification.
In parallel the samples were also analysed with the composed standards added (500 µL sample + 500 µL composed standards) to confirm that the analysed acids were present.

**Equipment:**

- **Column:** C$_{18}$ (Merck, LiChroCART® 250-4 Purospher® STAR RP-18 endcapped (5 µm))
- **Oven:** Jasco CO-2060 Plus
- **Injector:** Jasco AS-2057 Plus
- **Detector:** UV-Vis (Jasco MD-2515 Plus)
- **Pump:** Jasco PU-2080 Plus
- **Ternary Gradient Unit:** Jasco LG-2080-02
- **Degasifier:** Jasco DG-1580-54
- **Software:** Jasco ChromPass Chromatography Data System 1.8.2.1

**Conditions:**

- **Flow:** 0.7 mL/min
- **Oven temperature:** 25 °C
- **$\lambda_{\text{detection}}$:** 210 nm

### 3.3. Exopolysaccharides

The EPS production was achieved applying the isolated and combined microorganisms (Table 3.3). It has to be stated that the yeasts weren’t applied without a bacteria in the combination, due to their incapability of producing EPS.

The microorganisms were inoculated into 100 mL Erlenmeyer’s (with 100 mL of skim milk) in 48-hour tests, in an incubator (Foc 225E refrigerated incubator) at 30 °C.

It was also tested the growth with whole milk, but it was interrupted, since the extraction applied was not efficient.

The samples removal was performed under sterile conditions and in duplicated. Four samples were collected for each strain combination, for the time zero, after 24, 30 and 48 hours of growth.
Table 3.3 – List of the different combinations applied in the study of EPS production

<table>
<thead>
<tr>
<th>Isolated microorganisms</th>
<th>Combinations of Microorganisms</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>L. delbrueckii</em> subsp. bulgaricus</td>
<td><em>L. delbrueckii</em> subsp. bulgaricus + <em>L. kefir</em></td>
</tr>
<tr>
<td><em>L. kefir</em></td>
<td><em>L. kefir</em> + <em>L. lactis</em> subsp. lactis</td>
</tr>
<tr>
<td><em>L. lactis</em> subsp. lactis</td>
<td><em>L. lactis</em> subsp. lactis + <em>L. delbrueckii</em> subsp. bulgaricus</td>
</tr>
<tr>
<td><em>B. animalis</em> subsp. lactis</td>
<td><em>L. delbrueckii</em> subsp. bulgaricus + All the yeasts</td>
</tr>
<tr>
<td></td>
<td>(<em>C. famata</em> + <em>C. krusei</em>)</td>
</tr>
<tr>
<td></td>
<td><em>L. kefir</em> + All the yeasts (<em>C. famata</em> + <em>C. krusei</em>)</td>
</tr>
<tr>
<td></td>
<td><em>L. lactis</em> subsp. lactis + All the yeasts (<em>C. famata</em> + <em>C. krusei</em>)</td>
</tr>
<tr>
<td></td>
<td>All the bacteria (<em>L. delbrueckii</em> subsp. bulgaricus + <em>L. kefir</em> + <em>L. lactis</em> subsp. lactis)</td>
</tr>
<tr>
<td></td>
<td>All the bacteria (<em>L. delbrueckii</em> subsp. bulgaricus + <em>L. kefir</em> + <em>L. lactis</em> subsp. lactis) + All the yeasts (<em>C. famata</em> + <em>C. krusei</em>)</td>
</tr>
</tbody>
</table>

### 3.3.1. Extraction

Initially, two different extraction methods were tested: an ethanolic extraction (Rimada and Abraham, 2001; Taniguchi *et al*., 2001; Yuksekdag and Aslim, 2008; Ghasemlou *et al*., 2012) and another one with a Dowex resin (with Na⁺-form, strongly acidic (Sigma Aldrich)) (Simões *et al*., 2011) (Annex A.3). The extraction chosen was the first one, due to the matrix used (skim milk).

The ethanolic extraction performed had several steps. First, and immediately after removing the samples, they have to be heated in a bath (Grant GD 120) at 90 °C for 20 min. This was performed in order to inactivate the enzymes that could hydrolyse the polymer and to detach it from the cells (Kimmel *et al*., 1998; Rimada and Abraham, 2001).

At this point the samples were centrifuged (5810 R Eppendorf) at 4000 g, for 30 min at 20 °C. Subsequently the supernatants were precipitated with ethanol 96% (1:1) and left 4 °C overnight (Rimada and Abraham, 2001; Yuksekdag and Aslim, 2008; Ghasemlou *et al*., 2012).
Afterwards a second centrifugation step was performed for 30 min, at 4000 g and at 4 °C (5810 R Eppendorf). The pellet (EPS) were resuspended with 1 mL of distilled water (Rimada and Abraham, 2001; Yuksekdag and Aslim, 2008; Ghasemlou et al., 2012). The steps of this process are present in Figure 3.3.

![Figure 3.3 – Process of extraction. A – After heating; B – First step of centrifugation after heating; C – Precipitation with ethanol; D – Pellet after second centrifugation; E – Resuspending the pellet in distilled water.](image)

### 3.3.2. Hydrolysis

Several hydrolysis were tested (Table 3.4) with sulphuric acid 95% (VWR) in different concentrations, temperature and time conditions. To each 500 µL of sample were added 5 mL of H$_2$SO$_4$.  

<table>
<thead>
<tr>
<th>Concentration</th>
<th>Temperature (°C)</th>
<th>Time (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>95%</td>
<td>Room temperature</td>
<td>-</td>
</tr>
<tr>
<td>1 M</td>
<td>100</td>
<td>30</td>
</tr>
<tr>
<td>2 M</td>
<td>100</td>
<td>30</td>
</tr>
<tr>
<td>2 M</td>
<td>130</td>
<td>30</td>
</tr>
<tr>
<td>2 M</td>
<td>150</td>
<td>30</td>
</tr>
</tbody>
</table>

The procedures with high temperatures were executed in a controlled temperature digester (spectroquant TR 420, Merck).

---

6 The hydrolysis procedure was executed only for the identification and quantification by HPLC-RI and for the quantification by DNS Method.
Despite the published literature (La Rivière and Kooiman, 1967; Grobben et al., 1997; Petry et al., 2000), the hydrolysis condition chosen was H$_2$SO$_4$ 2M, at 150 °C for 30 min. Posteriorly the hydrolysis with concentrated H$_2$SO$_4$ (95%) at room temperature was also analysed.

### 3.3.3. Identification and Quantification by HPLC-RI

To identify and quantify the carbohydrates present, it was applied HPLC with a refractive index detector (RI) (Gassem et al., 1997; Piermaria et al., 2008).

The mobile phase (H$_2$SO$_4$ 0.005 M) was prepared with distilled water and sulphuric acid 95-97% (VWR).

The composed standards (glucose (Fluka), galactose (AppliChem), rhamnose (Sigma-Aldrich) and lactose (Liofilmchem)) were prepared with distilled water. From one 5.00 g/L composed standards stock solution different concentrations were prepared: 0.01, 0.05, 0.08, 0.10, 0.20, 0.40, 0.80, 1.00, 1.50, 2.00, 2.50, 3.00, 3.50, 4.00 and 4.50 g/L. The calibration curves are present in Annex A.2.2.

These standards were chosen mainly due to the fact that glucose, galactose and rhamnose are reported to be the component sugars of EPS produced by microorganisms, such as *L. delbrueckii* subsp. *bulgaricus* (Cerning, 1995), while lactose’s presence was also analysed since there was the possibility that the hydrolysis was not complete.

The standard solutions were filtered with a nylon filter of 0.45 µm (VWR) for 2 mL vials (VWR). The hydrolysed samples were not filtered because of their composition in sulphuric acid. Each standard solution/sample was injected twice (20 µL each injection) with a running time of 20 min.

**Equipment:**

- Column: Sugar SH1011 (Shodex), particle size: 6 µm
- Oven: Jasco CO-2060
- Injector: Jasco AS-2057
- Detector: Jasco RI-2031
- Pump: Jasco PU-2089
- Software: ChromNAV
Analysis of the organic acids and exopolysaccharides produced by the lactic acid microflora present in kefir and their potential applications

**Conditions:**
Flow: 0.7 mL/min
Oven temperature: 35 °C

### 3.3.4. Quantification by DNS Method

To quantify the reducing sugars present in the EPS hydrolysed samples, the DNS method was employed, using glucose as standard (Miller, 1959). From one 5.0 g/L glucose stock solution different concentrations standard solutions were prepared: 0.1, 0.2, 0.4, 0.6, 0.8, 1.0, 1.4, 2.0, 2.5 and 3.0 g/L. The calibration curve is present in Annex A.2.3.

For each 500 µL of hydrolysed sample, 500 µL of DNS (the reagent preparation is described in Annex A.3) were added. The solution was mixed and heated (GD 120 Grant) at 80 °C, for 5 min. Afterwards the samples were cooled down (until they reached room temperature) and 5 mL of distilled water were added. The mixture was homogenised and the absorbance was read with a plastic cuvette at 540 nm in the UV-Vis spectrophotometer (V-1200 VWR). In the blank, the volume of sample was replaced by distilled water with H$_2$SO$_4$ (in the same proportion as in the hydrolysis).

### 3.3.5. Quantification by Dubois Method

The total amount of sugars was measured by the Dubois method using glucose as standard (Dubois et al., 1956). From one 1.00 g/L glucose stock solution different concentrations were prepared: 0.01, 0.08, 0.10, 0.20, 0.40, 0.80 g/L. In Annex A.2.4 the calibration curve is presented.

The EPS samples used in this method were not hydrolysed. To perform the method were used glass tubes. For each 250 µL of sample (EPS extracted), 2.5 mL of H$_2$SO$_4$ 95-97% (Merck) and 500 µL of phenol 50 g/L (Merck) were added. The mixture was homogenised and allowed to stand for 15 min. The absorbance was read at 490 nm in the UV-Vis spectrophotometer (V-1200 VWR) with a glass cuvette (Nahita). In the blank the volume of sample was replaced by distilled water.
3.4. Bifidogenic Effect

The bifidogenic effect was evaluated by the EPS hydrolysis, using specific microorganisms’ strains - *B. animalis* subsp. *lactis* as the hydrolysing agent and *E. coli* as the control.

It was studied the potential bifidogenic effect in *B. animalis* subsp. *lactis* due to the fact that this microorganism is considered a probiotic and has beneficial effects in the human health (Kearney *et al.*, 2008). As *E. coli* is present in the intestinal area and cannot hydrolyse exopolysaccharides, it was used as the control (Sako *et al.*, 1999).

To perform this experiment it was used MRS as the medium but altered (AMRS), in order to substitute all the carbon sources by the EPS extracted from kefir (employing the same procedure described in the section 3.3.1). Additionally, the EPS was added to the AMRS at three different concentrations (C<sub>1</sub> - half of the sugars concentration present in the original MRS; C<sub>2</sub> – the same concentration of sugars present in the original MRS; C<sub>3</sub> – 10 times higher than C<sub>1</sub>). The composition of the AMRS is in Annex A.1.

It should be noted that the EPS is only added to the solution after autoclaving, since at 121 °C it was formed a precipitate. To sterilize the EPS, it was used UV light in a laminar flux camera (SG 403A-HE-INT, Baker) for 30 min.

The microorganisms were inoculated into 100 mL Erlenmeyer’s (with 100 mL of AMRS) during 48 hours, in an incubator (Foc 225E refrigerated incubator) at 37 °C.

The samples collection was performed in duplicate under sterile conditions. In total, samples at five different times (for the time zero, after 6, 24, 30 and 48 hours of growth) were collected, for each strain, to evaluate the hydrolysis. The growth study was measured by the optic density (OD) with an UV-Vis spectrophotometer (VWR) at 610 nm.

### 3.4.1. Quantification by DNS Method

To verify the EPS hydrolysis it was used the DNS method, already described in the subsection 3.3.4. In the blank, the volume of sample was replaced by a sample of the medium without the EPS.
4. Results and Discussion

4.1. Organic Acids

In this subsection the production of organic acids for the combination of strains referred in the section 3.2 will be evaluated.

Before further analysis, it has to be explained that the growth study of the strains in skim milk was not able to be demonstrated. This can be justified by the fact that this medium does not allow an OD determination, because it has to be diluted 10 times, which implicates an enormous error. To solve this problem two different solutions were applied: filtration of a volume of a sample and determination of the dry weight residue; and the dry weight technique. The two possible solutions were unsuccessful. The first one was not possible due to filter clogging. The second one was not representative of the cells, as a small volume (1 mL) was used which involves errors. As a consequence of these errors the results were not conclusive and they are not presented.

From the retention times obtained for the standards (Table 4.1), the organic acids presence in the samples was identified. The chromatograms for the standards and samples are presented in Annex A.4.

Table 4.1 – Retention times for the organic acids standards

<table>
<thead>
<tr>
<th>Standards</th>
<th>Retention Time (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pyruvic Acid</td>
<td>4.63</td>
</tr>
<tr>
<td>Citric Acid</td>
<td>5.46</td>
</tr>
<tr>
<td>Lactic Acid</td>
<td>6.68</td>
</tr>
<tr>
<td>Acetic Acid</td>
<td>7.33</td>
</tr>
<tr>
<td>Propionic Acid</td>
<td>9.90</td>
</tr>
<tr>
<td>Succinic Acid</td>
<td>13.82</td>
</tr>
</tbody>
</table>
From the acids identified, all of them were reported as metabolic products for *C. famata* and *L. lactis* subsp. *lactis* (Álvarez-Martín et al., 2008).

The results were classified as significant or not significant through the Student’s *t*-test, whose theoretical basis is present in the Annex A.5.

The pyruvic acid production (Figure 4.1) starts, approximately, 2 hours after the beginning of the experiment, which indicates the occurrence of glycolysis (or other degradative pathways of glucose) leading to pyruvate production. Subsequently, this pyruvate is consumed; after 8/10 hours of experiment its concentration decreases until it is not detected. This indicates that at this point (8/10 hours) starts the fermentation and the other acids are produced (Güzel-Seydim et al., 2000).

As regards to Figure 4.1A, one can see that *L. lactis* subsp. *lactis* is the strain that produces a higher value of pyruvic acid concentration. The other two bacteria have a similar behaviour between them. These results were not significantly different (P>0.05).

On the other hand, when the bacteria are combined with each other (Figure 4.1B), *L. lactis* subsp. *lactis* + *L. delbrueckii* subsp. *bulgaricus* is the one combination that had a higher concentration value, no significantly different (P>0.05) from isolated *L. lactis* subsp. *lactis*. In addition *L. delbrueckii* subsp. *bulgaricus* + *L. kefir* and *L. kefir* + *L. lactis* subsp. *lactis* had a similar performance between each other, not being significantly different (P>0.05).

The isolated bacteria + yeasts (Figure 4.1C), all the bacteria and all the bacteria + yeasts (Figure 4.1D) exhibit an analogous behaviour and their pyruvic acid concentration was not significantly different (P>0.05).

The highest values for pyruvic acid production were obtained for the isolated and mixed yeasts (Figure 4.1E). Once again, the difference in pyruvic acid concentration was not considered significant (P>0.05).

The highest concentration exhibited by the yeasts (despite the variations in pyruvic acid concentrations are not considered significant (P>0.05)) can be justified by the fact that the bacteria are using the substrate (lactose) to produce the EPS, while the yeasts cannot produce EPS and use the substrate to survive. Therefore the pyruvic acid concentrations are neither higher nor detected, as it is being used.

The results obtained (Figure 4.1E) can also demonstrate that the yeasts are able to degrade lactose, not needing the LAB to hydrolyse the disaccharide (van den Tempel and Jakobsen, 1998; Viljoen, 2001).
Figure 4.1 – Pyruvic acid production along growth time, for all the combination of strains. A – Isolated bacteria (L. lactis subsp. lactis, L. delbrueckii subsp. bulgaricus and L. kefir); B – Mixed bacteria (L. lactis subsp. lactis + L. delbrueckii subsp. bulgaricus, L. delbrueckii subsp. bulgaricus + L. kefir and L. kefir + L. lactis subsp. lactis); C – Isolated bacteria mixed with yeasts (L. lactis subsp. lactis + yeasts, L. delbrueckii subsp. bulgaricus + yeasts, L. kefir + yeasts); D – All the bacteria mixed (L. lactis subsp. lactis + L. delbrueckii subsp. bulgaricus + L. kefir) and all the bacteria mixed with yeasts (L. lactis subsp. lactis + L. delbrueckii subsp. bulgaricus + L. kefir + C. krusei + C. famata); E – isolated yeasts and mixed yeasts (C. krusei, C. famata and C. krusei + C. famata).
The pyruvic acid concentration observed was only for microorganisms’ survival: production and consumption. The maximum concentration achieved was $1.23 \times 10^{-2}$ g/L for *C. krusei*, after 8 hours of study. The value obtained in literature for *L. lactis* subsp. *lactis* is higher, but in the same order of magnitude ($6.00 \times 10^{-2}$ g/L) and for *C. famata* is also higher but very similar ($2.00 \times 10^{-2}$ g/L) (Álvarez-Martín *et al*., 2008). These literature results have the opposite tendency of the results obtained in the present work. This difference is explained by the different conditions applied: microorganisms with different origins and the medium was milk (not skim milk).

Concerning the citric acid production, the results obtained are presented in Figure 4.2.

![Figure 4.2 – Citric acid production along growth time. A – Isolated bacteria (*L. lactis* subsp. *lactis* and *L. kefir*); B – Mixed bacteria (*L. lactis* subsp. *lactis* + *L. delbrueckii* subsp. *bulgaricus* and *L. kefir* + *L. lactis* subsp. *lactis*); C – Isolated bacteria mixed with yeasts (*L. lactis* subsp. *lactis* + yeasts, *L. delbrueckii* subsp. *bulgaricus* + yeasts, *L. kefir* + yeasts); D – All the bacteria mixed (*L. lactis* subsp. *lactis* + *L. delbrueckii* subsp. *bulgaricus* + *L. kefir* and *C. krusei* + *C. famata*).](attachment:image.png)
The citric acid was not detected for isolated *L. delbrueckii* subsp. *bulgaricus*, *L. delbrueckii* subsp. *bulgaricus* + *L. kefir*, *C. famata*, *C. krusei* and *C. krusei* + *C. famata*. This does not mean that these microorganisms did not produce this acid, but under these growth conditions it was not detected.

The variation in concentration between the different strains combinations and along the time of the study, was not considered significant (P>0.05) and the same tendency is exhibit as shown in Figure 4.2.

After 5 hours of fermentation, the citric acid production started, which is in agreement with the published literature (Güzel-Seydim *et al.*, 2000). In some cases (*L. lactis* subsp. *lactis* + *L. delbrueckii* subsp. *bulgaricus* (Figure 4.2B) and for all the bacteria (Figure 4.2D)) a slight decrease (P>0.05) in citric acid concentration can be seen, which indicates that the microorganisms are consuming the citric acid in their metabolism in order to survive (Güzel-Seydim *et al.*, 2000).

The maximum concentration reached was 1.85\times10^{-1} \text{g/L} for *L. lactis* subsp. *lactis*, after 24 hours of growth. The value obtained in literature for *L. lactis* subsp. *lactis* is higher (2.35 \text{g/L}) (Álvarez-Martín *et al.*, 2008). Once more, it has to be said that the microorganisms have different origins and the milk used was not skim milk.

For lactic acid production, the evolution in concentration can be observed in Figure 4.3.

![Figure 4.3 – Lactic acid production along the growth time, for *C. krusei* and *C. krusei* + *C. famata*.](image-url)
The lactic acid production was only detected for *C. krusei* and *C. krusei* + *C. famata*. This was not expected, since the bacteria applied in the study are LAB (Simova *et al.*, 2002; Witthuhn *et al.*, 2005b). Nevertheless, it can be concluded that the lactose present in the medium is being applied by the bacteria to produce the EPS.

Isolated *C. famata* did not produce this acid, probably due to the fact that this strain perform another type of fermentation (mixed acid fermentation), in addition to lactic fermentation.

The production of lactic acid started after 8 hours of the beginning of the growth. This increase in concentration is consistent with the consumption of pyruvic acid after 8/10 hours of experiment; i.e. after total consumption of the substrate (lactose), microorganisms are using pyruvate as a carbon source producing lactic acid, through the fermentation process (Güzel-Seydim *et al.*, 2000). During this growth period the variation in concentration was not considered significant (P>0.05).

It can be inferred that up until 5 hours of growth the microorganisms use the respiration to survive. From 5 to 10 hours the process of fermentation, simultaneously with respiration, appears to begin. After 10 hours of experiment, when there is no substrate available, the fermentation process prevails (Güzel-Seydim *et al.*, 2000).

The decrease in concentration that occurs at the end of the experiment, in the mixture of yeasts, is not significant (P>0.05) and is due to the assimilation of lactic acid by microorganisms. The results obtained can be explained by the fact that the mixture of yeasts conditioned the strains to extreme conditions of survival, since they have to compete for the carbon and energy source, also consuming the produced lactate. Similarly the consumption of lactic acid by yeasts was reported by Viljoen (2001).

The highest concentration of lactic acid obtained was 1.34x10^{-1} g/L for *C. krusei* after 24 hours. This value is very low when compared to 70 g/L - concentration value obtained for *Saccharomyces cerevisiae*, with glucose as a substrate (Sauer *et al.*, 2008). However, this production has been optimized and uses another yeast and glucose as carbon source. Whereas in the present work skim milk is used, in which *C. krusei* spends energy splitting the lactose into smaller molecules.

As regards to acetic acid, the results with the different conditions of microorganisms can be observed in Figure 4.4.
For *L. lactis* subsp. *lactis*, *L. delbrueckii* subsp. *bulgaricus*, *L. kefir*, *L. delbrueckii* subsp. *bulgaricus* + *L. kefir* and *L. kefir* + *L. lactis* subsp. *lactis* the acetic acid production was not detected. The acetic acid production verified was not considered significant (P>0.05) for any of the combinations of microorganisms.

*L. lactis* subsp. *lactis* + *L. delbrueckii* subsp. *bulgaricus* (Figure 4.4A) exhibited several peaks of acetic acid production, that were always followed by an acetic acid consumption (not significantly different (P>0.05)). To the isolated bacteria with yeasts, all the bacteria and all the bacteria combined with yeasts, the acetic acid production is very similar, as can be seen from Figure 4.4B and Figure 4.4C.

There is a peak of acetic acid production at 5 hours of fermentation and afterwards there is a consumption, which indicates that the microorganisms incorporate this acid in...
their metabolism, when there is no more substrate left (Güzel-Seydim et al., 2000). The increase in concentration at 5 hours of experimentation is consistent with the consumption of pyruvic acid. Acetic acid production has been identified in previous studies with kefir (Rea et al., 1996).

However, in the yeasts (Figure 4.4D), the behaviour is different: they produce fewer quantities than in the other combinations of microorganisms; however this difference was not considered significant (P>0.05).

The highest value of acetic acid produced was \(3.80 \times 10^{-2}\) g/L for all the bacteria mixed with both yeasts after 4 hours of growth.

Álvarez-Martín et al. (2008) did not detect acetic acid production for isolated \(L.\ lactis\) subsp. \(lactis\) as well, so this is consistent with the published work. For \(C.\ famata\) they detected \(5.00 \times 10^{-1}\) g/L, which is much higher than the values obtained for \(C.\ famata\) in this study. However Álvarez-Martín and his co-workers (2008) did not detect any acetic acid production for \(L.\ lactis\) subsp. \(lactis\) + \(C.\ famata\), and in this work acetic acid was produced by \(L.\ lactis\) subsp. \(lactis\) and the yeasts. The conditions applied are distinct (the microorganisms applied have different origins and the medium used was milk (and not skim milk)), therefore the results differ.

The propionic acid production was another acid studied in this work (Figure 4.5) and as it was verified for the others acids, the production was not considered significant (P>0.05) for any of the combinations of microorganisms.

The production of propionic acid was not detected for all the isolated bacteria, for \(L.\ lactis\) subsp. \(lactis\) + \(L.\ delbrueckii\) subsp. \(bulgaricus\), for \(L.\ kefir\) + yeasts and for the isolated and mixed yeasts.

\(L.\ delbrueckii\) subsp. \(bulgaricus\) + \(L.\ kefir\) (Figure 4.5A) only start to produce propionic acid after 8 hours of fermentation in a non-significant quantity (P>0.05) which is also being consumed in their metabolism.

For the other combinations (Figure 4.5B and Figure 4.5C) and for \(L.\ kefir\) + \(L.\ lactis\) subsp. \(lactis\) (Figure 4.5A) the behaviour is different. Apparently the microorganisms began the production of propionic acid after 2 hours of fermentation and it is consumed and produced again during the process of fermentation, with the exception of \(L.\ delbrueckii\) subsp. \(bulgaricus\) + yeasts that consume what was initially produced. This behaviour is explained by their altered metabolisms when they coexist together with each other and compete for the same substrate.
Figure 4.5 – Propionic acid production along the growth time. A – Mixed bacteria (L. delbrueckii subsp. bulgaricus + L. kefir and L. kefir + L. lactis subsp. lactis); B – Isolated bacteria mixed with yeasts (L. lactis subsp. lactis + yeasts and L. delbrueckii subsp. bulgaricus + yeasts); C – All the bacteria mixed (L. lactis subsp. lactis + L. delbrueckii subsp. bulgaricus + L. kefir) and all the bacteria mixed with yeasts (L. lactis subsp. lactis + L. delbrueckii subsp. bulgaricus + L. kefir + C. krusei + C. famata).

The combination of L. kefir + L. lactis subsp. lactis are responsible for the highest value obtained (3.97x10\(^{-1}\) g/L) after 2.5 hours of growth, which is a low value when compared to the value obtained for L. lactis subsp. lactis in Álvarez-Martín et al. (2008) (1.28 g/L). For isolated C. famata they obtained 3.90x10\(^{-1}\) g/L, which is more similar to the maximum value obtained in the present study. Once again, it cannot be forgotten that these values were obtained for different growth conditions (microorganisms with different origins and milk as the growth medium (instead of skim milk)).

Concerning the succinic acid, Figure 4.6 shows the evolution in its concentration during the experiments.
Succinic acid production was detected for other strains conditions, however the concentration detected was lower than the one detected for the skim milk (time zero), therefore the results are not presented. The microorganisms did not produce this acid but they consumed the one that was present in the medium. These results were not considered significantly different (P>0.05).

After 5 hours of experiment there is an increase in the succinic acid concentration (not significant (P>0.05)). The decrease in concentration that occurs at the end of the test is not significant (P > 0.05) and is due to the assimilation of succinic acid by the microorganisms, thus they can survive (Viljoen, 2001).

In this particular case, the production of succinic acid in a higher concentration was observed for C. krusei after 6.5 hours of experiment. Of all the acids, this was the one that was produced at a higher concentration (5.39x10^{-1} g/L). Comparing this value to the values obtained by Álvarez-Martín and his group (2008) (2.20x10^{-1} g/L for L. lactis subsp. lactis and 6.00x10^{-1} g/L for C. famata), it can be said that the results are very similar, despite the differences in the growth conditions (microorganisms with different origins and milk as the growth medium (instead of skim milk)).

However, when comparing with other published studies (83 g/L for Anaerobiospirillum succiniciproducens with glucose as the carbon source (Sauer et al., 2008)), the concentration obtained in the present study is much lower. Nevertheless the production described by Sauer et al. (2008) has been optimized: uses another microorganism and glucose as carbon source. In the present study, it was used skim
milk as carbon source, in which *C. krusei* has to split the lactose into smaller molecules, which implies energy that is being deviated for this purpose.

As it was observed by previous studies done in this area (Meireles *et al.*, 2012) and by published literature (Cock and de Stouvenel, 2006), the enhancement of the organic acids production could be reached through the controlled of the pH in a basic environment. This can be explained by the natural metabolism of the microorganisms. When they grow at uncontrolled conditions, the pH levels decrease during time. This means that, when an alkaline (or neutral) pH is imposed, they struggle to maintain the pH values in a low range. To decrease the pH, they automatically produce more acids (Meireles *et al.*, 2012). Instead of using the nutrients to grow and reproduce, the cells have to deviate their sustainability to survive producing the organic acids.

The skim milk could also be influencing the results. Possibly if it was applied whole milk the microorganisms’ growth would be improved, since the whole milk contains vitamins that are not present in the skim milk (vitamin A and D). However, the organic acids yield is not directly proportional with microorganisms’ enhanced growth. This was verified from previous studies, since at pH 8 the microorganisms had an enhanced organic acids production and their growth was diminished, when compared to uncontrolled pH growth conditions (Meireles *et al.*, 2012).

The organic acids production could also be influenced by the oxygen availability for the microorganisms, since the volume of oxygen in the beginning of the experiment was reduced. This condition was performed due to microaerophilic *L. delbrueckii* subsp. *bulgaricus*, thus the conditions had to be the same for all the microorganisms.

The microorganisms applied have different incubation times and it is possible that the bacteria would exhibit different results if the experiment time was longer. Though, the incubation time was chosen due to the fact that yeasts have a more rapid growth, as well as the incubation time applied in kefir production is normally 24 hours.

Furthermore, agitation used in the incubation conditions could also have a certain influence in the results. The agitation leads to some kind of aeration (not recommended for microaerophilic species) and the mechanical stress is not well tolerated for all the species of microorganisms (Madigan *et al.*, 2009).

Despite the addition of the standards to the samples, the accuracy could not be determined. The organic acids concentration determined were lower than expected, and
the standard concentrations added to the samples was higher, comparatively to the acids concentrations in the samples, therefore the accuracy was not possible to be determined.

4.2. Exopolysaccharides

The results obtained for the EPS extraction, identification and quantification will be discussed in this subsection.

4.2.1. Extraction

In the process of extracting the EPS it was chosen the ethanolic extraction, due to the matrix used (skim milk). The difference between the samples that had EPS present could be seen from the ones that had not. This could be noted from the first step of extraction – heating and centrifuging (Figure 4.7A).

When the EPS was not being produced by the cells, the separation was not complete, because the supernatant was not a polysaccharide. The difference is easily seen in Figure 4.7B, where the separation is efficient and the supernatant is visible.

![Figure 4.7 – First step of centrifugation after heating. A – In the absence of EPS. B – In the presence of EPS.](image)

Despite the poor separation of the samples where there was no EPS present, they were extracted (as the ones where there was EPS present), thus it could be seen the difference in the extracts in the posteriorly analysis executed. In the last step of centrifugation, after precipitation of ethanol the difference between the final pellets can be observed (Figure 4.8A and B), fact evidenced by the consistency and colour of the EPS extracted samples.
Due to these previous conclusions drawn, the presence and absence of EPS production in the combination of strains applied for EPS production was registered (Table 4.2).

Table 4.2 – Observed results after the process of extraction (nd – EPS not detected; d – EPS detected)

<table>
<thead>
<tr>
<th>Combinations of Microorganisms</th>
<th>Time (hours)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td><em>L. delbrueckii</em> subsp. <em>bulgaricus</em></td>
<td></td>
</tr>
<tr>
<td><em>L. kefir</em></td>
<td></td>
</tr>
<tr>
<td><em>L. lactis</em> subsp. <em>lactis</em></td>
<td></td>
</tr>
<tr>
<td><em>B. animalis</em> subsp. <em>lactis</em></td>
<td></td>
</tr>
<tr>
<td><em>L. delbrueckii</em> subsp. <em>bulgaricus</em> + <em>L. kefir</em></td>
<td></td>
</tr>
<tr>
<td><em>L. kefir</em> + <em>L. lactis</em> subsp. <em>lactis</em></td>
<td></td>
</tr>
<tr>
<td><em>L. lactis</em> subsp. <em>lactis</em> + <em>L. delbrueckii</em> subsp. <em>bulgaricus</em></td>
<td></td>
</tr>
<tr>
<td><em>L. delbrueckii</em> subsp. <em>bulgaricus</em> + All the yeasts (C. famata + C. krusei)</td>
<td></td>
</tr>
<tr>
<td><em>L. kefir</em> + All the yeasts (C. famata + C. krusei)</td>
<td></td>
</tr>
<tr>
<td><em>L. lactis</em> subsp. <em>lactis</em> + All the yeasts (C. famata + C. krusei)</td>
<td></td>
</tr>
<tr>
<td>All the bacteria (<em>L. delbrueckii</em> subsp. <em>bulgaricus</em> + <em>L. kefir</em> + <em>L. lactis</em> subsp. <em>lactis</em>)</td>
<td></td>
</tr>
<tr>
<td>All the bacteria (<em>L. delbrueckii</em> subsp. <em>bulgaricus</em> + <em>L. kefir</em> + <em>L. lactis</em> subsp. <em>lactis</em>) + All the yeasts (C. famata + C. krusei)</td>
<td></td>
</tr>
</tbody>
</table>
From these results it is notorious that *L. lactis* subsp. *lactis* is the microorganism that had a faster metabolism for the EPS production, while *L. kefir* and *L. delbrueckii* subsp. *bulgaricus* have a slower metabolism, producing only the EPS after 48 hours. Whenever *L. lactis* subsp. *lactis* was present in the combination, the EPS was produced.

Other observation that has to be stated is that *B. animalis* subsp. *lactis* was not able to produce EPS in the time of the study. It should be noted that *Bifidobacterium* are microorganisms that have difficulty in growing in milk (Kearney *et al.*, 2008). Moreover, there is no published literature that shows EPS production by *B. animalis* subsp. *lactis*.

### 4.2.2. Identification and Quantification by HPLC-RI

The retention times obtained for the standards and the chromatograms for the standards and samples are present in Table 4.3 and in Annex A.4, respectively.

<table>
<thead>
<tr>
<th>Standards</th>
<th>Retention Time (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lactose</td>
<td>9.73</td>
</tr>
<tr>
<td>Glucose</td>
<td>10.99</td>
</tr>
<tr>
<td>Galactose</td>
<td>11.43</td>
</tr>
<tr>
<td>Rhamnose</td>
<td>11.96</td>
</tr>
</tbody>
</table>

The identification and quantification of the sugars present in EPS structure by HPLC-RI was not possible. This could have many possible reasons. The main reason is the hydrolysis process applied to accomplish this purposes.

In one hand, the temperature (150 °C) and time applied (30 min) could have been not enough to hydrolyse the complex polysaccharide, since it was noticed that the extracted EPS could not be frozen and the hydrolysed samples couldn’t either, which means that EPS was still present in the hydrolysed samples.

On the other hand, the temperature applied could possibly be too high, leading to polysaccharide damage. This can be explained by the caramelisation of the samples, or/and by the Maillard reaction (non-enzymatic browning).
The caramelisation is reached with high temperatures in the presence of acidic and/or alkaline catalysts, originating brown pigments – furosines (Belitz et al., 2009). These compounds can be identified by RP-HPLC, or by an anion-exchange chromatography with pulsed amperometric detection (Beloque et al., 2009). This explanation can be accurate since the samples acquired a brown colour after adding the sulphuric acid.

The Maillard reaction is typical in heat processing milk and involves the presence of amino acids and reducing sugars. The carbonyl group of the sugars react with the nucleophilic group of the amino acids (mainly lysine), resulting in molecules called melanoidins (Huppertz and Kelly, 2009). Therefore, lactose can react with free amino acids (that could be present in the samples), leading to the formation of melanoidins (brown pigment) (Walstra et al., 2006).

Furthermore, the products formed by the Maillard reaction can also react, when acid hydrolysis is applied, forming furosines (Beloque et al., 2009).

The acidic environment associated with high temperatures, could also be responsible for the sugars dehydration and subsequently formation of furan derivatives. These compounds can condensate and form a dark solution (Brummer and Cui, 2005).

The hydrolysis with concentrated H$_2$SO$_4$ lead to unconclusive results as well, but in this case the concentration of acid is suspected to be inadequate to this kind of meticulous analysis.

The results of the samples are present in Figure 4.9 for both the hydrolysis tested. Observing the first peak (6 min for Figure 4.9A and 9 minutes for Figure 4.9B) is the solvent one. The second peak could be some compound present in the sample (possibly furosines or melanoidins), although it was not identified.

With the possibility that it did not occurred hydrolysis, the EPS is a very complex molecule that could be retained in the column, leading to these results. The other possibilities (caramelisation, Maillard reaction and sugar dehydration) lead to the reaction of the sugars, therefore they are not possible to be identified. The molecules formed could have the retention time of 15 min (the only peak observed), or could have been retained in the column, or could not be detected by the RI-detector.
Analysis of the organic acids and exopolysaccharides produced by the lactic acid microflora present in kefir and their potential applications

Figure 4.9 – Chromatograms obtained for the growth with all the bacteria after 48 hours of study. A - Hydrolysis with $\text{H}_2\text{SO}_4$ 2 M at 150 °C for 30 min. B - Hydrolysis with concentrated $\text{H}_2\text{SO}_4$ at room temperature.

In Figure 4.10 is shown a superposition for the chromatograms of the samples and for the standards. One can noticed that none peak of the standards is present in the samples.

Figure 4.10 – Superposition of the chromatograms obtained for some samples and a chromatogram for a standard.
It has to be stated that the standards were prepared in water and the matrix of the sample was not water. In one case it was H₂SO₄ 2 M and in the other was concentrated H₂SO₄. Therefore, in future works the standards would have to be prepared like the samples. This was not done, because the concentration of H₂SO₄ used was so high (in both conditions) that the molecule of lactose could be hydrolysed into glucose and galactose, not being able to detect the lactose’s peak.

It has been published that the polysaccharides are difficult to extract from milk (Cerning, 1995; Habibi et al., 2011), because of the complexity of the composition of the medium. The hydrolysis is also a difficult process due to the connection between the monosaccharides (Badel et al., 2011).

4.2.3. Quantification by DNS Method

Since the DNS method permits to quantify reducing sugars, and the previous results (subsection 4.2.2) shown that there was a possibility that the hydrolysis did not occurred completely, it was done this method to verify the occurrence of EPS hydrolysis. Unfortunately the addition of DNS to the samples and to the blank (prepared in the same conditions as the samples, with H₂SO₄) did not allow the sugars quantification.

By adding DNS to the blank, it was observed that the yellow colour, characteristic from DNS reagent, disappeared (Figure 4.11A). A blank without H₂SO₄ have the yellow colour of Figure 4.11C.

![Figure 4.11 – DNS method performed. A – Blank prepared like the sample (water and H₂SO₄). B – Sample. C – Blank with only distilled water.](image-url)
Additionally, adding the DNS to the samples originated a precipitate and the solution did not have the colour expected (Figure 4.11B). This could be explained by the fact that the dinitrosalicylic acid (present in DNS reagent) may be able to precipitate proteins (Sumner, 1921). Therefore, the proteins that could be present in the samples would precipitate. These proteins (and the amino acids that compose them) could be responsible for this precipitation reaction as well as the Maillard reaction mentioned in subsection 4.2.2.

The acidic conditions could also affect the capacity of the DNS reagent to develop the yellow colour, in the presence of reducing sugars. According to Miller (1959) La Rochelle salt is crucial to colour stability, which means that the tartarate present in this salt must be responsible for the stabilization of 3-amino-5-nitrosalicylic acid (formed in the presence of reducing sugars and responsible for the colour).

### 4.2.4. Quantification by Dubois Method

To be able to quantify the sugars present in the samples the Dubois Method was executed. Therefore the EPS concentration is expressed in terms of glucose concentration (not total sugars since the standard used was glucose).

It must be noticed that this method was not performed for the samples where there was no EPS production. This decision was taken due to the fact that the time-zero samples and no EPS production samples, exhibit a higher value of absorbance. This situation originated misleading results showing that the concentration in sugars was higher for no EPS production samples, which is a negative aspect of the colorimetric methods.

As it was stated before (subsection 4.2.1), it was observed that the samples where no EPS production was noticed had a different colour unable to identify what was extracted. Therefore it is not known what the Dubois method was quantifying in these samples. In the samples with no EPS production the extracted material was possibly constituted by lactose. This fact would explain the higher values for the Dubois Method, because the lactose present in the medium would be in a higher concentration than the EPS.
Consequently, it was admitted that those samples had a zero value, which is recognised not to be true, but it was the only option for the analysis of the other results.

As it was stated before, *B. animalis* subsp. *lactis* was not able to produce EPS for the conditions used in this study; therefore the Dubois Method was also not performed for the samples from the growth with this microorganism.

The results obtained for the strains that exhibit EPS production are present in Figure 4.12. From the isolated bacteria (Figure 4.12A), *L. lactis* subsp. *lactis* is the one microorganism with higher glucose content. The difference between these concentrations is considered significant (P<0.05). After 30 hours of study, a significant decrease in glucose’s concentration (P<0.05) was observed. This can be explained by the fact that *L. lactis* subsp. *lactis* has enzymes able to degrade the EPS and consume the hydrolysed glucose obtained.

The combination of bacteria (Figure 4.12B) resulted in significantly higher (P<0.05) glucose’s concentrations (when comparing to isolated *L. delbrueckii* subsp. *bulgaricus* and *L. kefir* (Figure 4.12A)), except for *L. lactis* subsp. *lactis* + *L. kefir*, which also exhibit the lower glucose concentration (comparing to the results where occurred production). A glucose significant consumption (P<0.05) is also present, but only for *L. lactis* subsp. *lactis* + *L. delbrueckii* subsp. *bulgaricus*, which did not permit to conclude if *L. delbrueckii* subsp. *bulgaricus* can also produce enzymes that degrade the EPS.

A similar result was observed for the bacteria combined with the yeasts (Figure 4.12C) and the isolated bacteria. However, the glucose concentration for the bacteria combined with the yeasts had significantly lower values (P<0.05).

Despite the fact that the yeasts produce components that improve bacteria growth (Farnworth, 2005; Irigoyen *et al*., 2005; Álvarez-Martín *et al*., 2008) and remove toxic products from metabolism (Viljoen, 2001), they could also inhibit the growth of microorganisms, as they produce ethanol and carbon dioxide that may be prejudicial (Viljoen, 2001).

In this case it can be concluded that the yeasts are producing components that inhibit these particular bacteria from producing the EPS. Therefore, the glucose’s concentration is minor, when compared to the glucose concentration for the isolated bacteria.
Analysis of the organic acids and exopolysaccharides produced by the lactic acid microflora present in kefir and their potential applications

Figure 4.12 – Glucose’s concentration along the study, to the strains that exhibit production of EPS. A – Isolated bacteria (L. lactis subsp. lactis, L. delbrueckii subsp. bulgaricus and L. kefir); B – Mixed bacteria (L. lactis subsp. lactis + L. delbrueckii subsp. bulgaricus, L. delbrueckii subsp. bulgaricus + L. kefir and L. kefir + L. lactis subsp. lactis); C – Isolated bacteria mixed with yeasts (L. lactis subsp. lactis + yeasts, L. delbrueckii subsp. bulgaricus + yeasts, L. kefir + yeasts); D – All the bacteria mixed (L. lactis subsp. lactis + L. delbrueckii subsp. bulgaricus + L. kefir) and all the bacteria mixed with yeasts (L. lactis subsp. lactis + L. delbrueckii subsp. bulgaricus + L. kefir + C. krusei + C. famata).

The significant (P<0.05) consumption of glucose is once again verified (Figure 4.12C) but only for L. lactis subsp. lactis + yeasts leading to the conclusion that L. lactis subsp. lactis has the enzymes necessary to break the connections between the monosaccharides.

When observing the results for all the bacteria and for the bacteria + yeasts (Figure 4.12D), it becomes obvious that the yeasts have a negative influence on EPS production, leading to significantly lower (P<0.05) contents of glucose concentration.

This result was not the expected since the yeasts improve bacteria growth in kefir and the microorganisms coexist in symbiosis (Farnworth, 2005). It can only be stated
that at the implied conditions (48 hours) the yeasts, which have a shorter incubation time, deviate the nutrients in the medium to their particular growth producing prejudicial compounds, that have a negative effect in the EPS production.

The glucose’s concentration for all the bacteria (Figure 4.12D) exhibit a significant decrease (P<0.05), due to the fact that the microorganisms are consuming the glucose, possible originated from the EPS hydrolysis by *L. lactis* subsp. *lactis*. In this particular case a significant increase (P<0.05) in glucose concentration after 48 hours is observed, which means that the microorganisms are still producing the EPS and the rate of production is higher than the rate of hydrolysis.

The higher glucose’s concentration was obtained for *L. lactis* subsp. *lactis* after 30 hours of growth (47.66 g/L). Comparing these results with the published literature, it can be stated that it was obtained a higher glucose content in this work, than in the work done by Cheirsilp and his group (2003) (~25 g/L), for the same time (30 hours) but for two different microorganisms (*Lactobacillus kefiranofaciens* and *Saccharomyces cerevisiae*) and with pH control (value not mentioned).

In the work performed by Gassem and his group (1997), with *L. delbrueckii* subsp. *bulgaricus*, at 32 °C and with no pH control, a maximum value of glucose concentration for 36 hours of growth (~10 g/L) was obtained. In the present work, the maximum value obtained for *L. delbrueckii* subsp. *bulgaricus* was 33.58 g/L after 48 hours of study.

The differences with the literature are explained by the different conditions and strains of microorganisms. It cannot be forgotten that the values obtained in the present study may be not absolutely right, due to the fact that the samples could not be analysed by this quantification method. The methods applied by these two groups of study were different: glucose analyser (Cheirsilp *et al.*, 2003) and HPLC (Gassem *et al.*, 1997).

Additionally, it has to be said that the whole milk initially tested, was not used because the extraction method applied was not appropriate for this type of milk. The works published do not use whole milk (Gassem *et al.*, 1997; Micheli *et al.*, 1999; Yang *et al.*, 1999; Cheirsilp *et al.*, 2003; Rimada and Abraham, 2003), therefore the influence in the EPS production is not known. However, it has been published that the yield of EPS is not directly proportional to the microorganisms’ growth (like in the organic acids production) (Cerning, 1995).
4.3. Bifidogenic Effect

The Bifidogenic effect was evaluated by the process of hydrolysis performed by *B. animalis* subsp. *lactis* using *E. coli* as the control (Figure 4.13). The results were classified as significant or not significant, through the Student’s *t*-test, whose theoretical basis is present in the Annex A.5.

![Figure 4.13 – Glucose concentration along the study, to B. animalis subsp. lactis (A) and E. coli (B) to the three different concentrations of EPS. The orange lines mark the value obtained for the solution of AMRS in the initial of the study (time zero). C1 - half of the sugars concentration present in the original MRS; C2 – the same concentration of sugars present in the original MRS; C3 – 10 times higher than C1.](image)

As can be verified in Figure 4.13A, *B. animalis* subsp. *lactis* is able to hydrolyse the EPS extracted from kefir. After 6 hours of study the value of glucose’s concentration was higher than in the beginning due to the hydrolysis of the EPS in small molecules of sugars. In this phase, the production of sugars (hydrolysis of EPS) was in a higher rate than the consumption the value obtained is significantly different (P<0.05) than the initial one. The results indicate that *B. animalis* subsp. *lactis* has the enzymes needed to hydrolyse the EPS.

For the remaining time of the study, the consumption of sugars is higher than the hydrolysis (the EPS must have been totally hydrolysed), leading to a significant decrease (P<0.05) in glucose’s concentration, which indicates that, not only *B. animalis* subsp. *lactis* is able to hydrolyse the EPS, as it can consume the resulting sugars to its own growth.
In what concerns the three different concentrations of EPS, *B. animalis* subsp. *lactis* has a similar behaviour. The variation between the glucose concentrations is significantly different (P<0.05), for all the EPS concentrations.

*B. animalis* subsp. *lactis* is also able to hydrolyse the EPS at higher concentrations (C₃=10xC₁) and the difference between the initial value and the value after 6 hours of growth is higher (but not significantly - P>0.05) for this concentration of EPS (C₃).

The promoted growth of *B. animalis* subsp. *lactis* by higher concentrations of EPS, shows the prebiotic effect of the EPS extracted from kefir. This is demonstrated by the growth study present in Figure 4.14A, where *B. animalis* subsp. *lactis* had a stimulated growth for the higher concentrations of EPS (the higher concentration of EPS lead to a higher growth of *B. animalis* subsp. *lactis*).

*E. coli* was not able to use this polysaccharide to their survival, as expected, since *E. coli* was used as the control, due to the fact that this microorganism is present in the gut and cannot hydrolyse exopolysaccharides (Sako et al., 1999).

The glucose’s concentration values measured (Figure 4.13B) were always lower than the values obtained for the time zero and an increased in the glucose concentration was not registered. This means that *E. coli* is unable to use this EPS as carbon source, which is shown by Figure 4.14B, where it is clear that this strain did not grow properly.
These results show that the EPS extracted from kefir can be recognized as a prebiotic, since it is hydrolysed by *B. animalis* subsp. *lactis*, improving its growth; and also because it was not used by *E. coli*.

These results cannot be compared with the literature, because there is no published work with the bifidogenic effect of *B. animalis* subsp. *lactis* and the prebiotic effect of the EPS extracted from kefir.
5. Concluding Remarks and Future Outlook

5.1. Conclusions

Currently, the application of microorganisms for the production of certain compounds used in food or pharmaceutical industry has been greatly developed. Therefore the main objectives of this work were related with the metabolites production by microorganisms usually present in kefir, in this particular case, organic acids and exopolysaccharides, and the potential of these metabolites for food applications.

In what concerns the organic acids, the maximum concentrations achieved were: 1.23x10^{-2} g/L for *C. krusei* after 8 hours of growth, for pyruvic acid; 1.85x10^{-1} g/L for *L. lactis* subsp. *lactis* after 24 hours of study, for citric acid; 1.34x10^{-1} g/L for *C. krusei* after 24 hours of experiment, for lactic acid; 3.80x10^{-2} g/L for all the bacteria mixed with both yeasts, after 4 hours of growth, for acetic acid; 3.97x10^{-1} g/L for *L. kefir + L. lactis* subsp. *lactis*, after 2.5 hours of growth, for propionic acid; 5.39x10^{-1} g/L for *C. krusei* after 6.5 hours of study, for succinic acid. Of all the acids, the succinic acid was the one produced in a higher concentration.

Relatively to the EPS, the extraction process was successful for the samples where there were EPS present. When no EPS was present the extract obtained was composed of other components of milk, which did not permit to obtain a value for these samples for the remaining tests executed.

The process of identification of the sugars, present in the EPS extracted, by HPLC-RI did not lead to any conclusions, concerning the polysaccharide composition. These results have shown that the process of hydrolysis has to be optimized.

The DNS method is not appropriate to quantify the reducing sugars hydrolysed by the sulphuric acid, since the acid reacts with the components in the DNS reagent.

From the results obtained by the Dubois method, it was possible to conclude that *L. lactis* subsp. *lactis* was the microorganism that had a faster metabolism, in what
concerns the EPS production, and the strain that originated the higher glucose content (47.66 g/L, after 30 hours of growth).

Not only did \textit{L. lactis} subsp. \textit{lactis} produce more EPS, as it was able to hydrolyse the EPS itself. From the results achieved it was observed that this microorganism had the necessary enzymes to break the connections between the monosaccharides of the EPS.

Another notorious observation made was the fact that the yeasts inhibited the production of EPS by the bacteria used in the study. \textit{B. animalis} subsp. \textit{lactis} was not able to produce EPS in the time of the study, but it was able to hydrolyse the EPS extracted from kefir and consume the resulting sugars to his own growth.

The growth of \textit{B. animalis} subsp. \textit{lactis} was promoted by high EPS concentrations and \textit{E. coli} was not able to use this polysaccharide to survive. This showed the prebiotic effect of the EPS extracted from kefir.

\subsection*{5.2. Future Outlook}

Through this thesis is possible to recognize that more work is yet to be done and multiple options need to be investigated, but unfortunately they could not be tested yet, due to time constraints.

The microorganisms’ growth is influenced by many parameters such as temperature, pH, agitation and incubation time, which may be influencing the organic acids production as well as the EPS production. It will be interesting to analyse the organic acids production, for the combination of microorganisms used, in a controlled growth at pH 6, since this can promote their production (Cock and de Stouvenel, 2006) or at pH 8 (Meireles \textit{et al.}, 2012).

The organic acids accuracy should be determined, to make sure that the acids identified are in fact present and being produced by the microorganisms.

In what concerns the EPS, hydrolysis is the one step that must be clarified and improved. It can be tested the hydrolysis with other acids – 4 N trifluoroacetic acid (110 °C for 16 hours) (Petry \textit{et al.}, 2000) – or with H\textsubscript{2}SO\textsubscript{4} but in different conditions (4 M H\textsubscript{2}SO\textsubscript{4}, for 8 hours at 105 °C) (Frengova \textit{et al.}, 2002).
It can also be investigated an enzymatic hydrolysis. Micheli et al. (1999) use porcine pancrease to perform the hydrolysis at 34 °C for 24 hours with stirring.

For the enzymatic hydrolysis another option can be verified. Since *B. animalis* subsp. *lactis* can hydrolyse the EPS from kefir, instead of acquiring an enzyme, in future works, could be purified the enzyme (with precipitation and chromatographic techniques (Scopes, 1994)) from *B. animalis* subsp. *lactis* and use it to hydrolyse the EPS. It cannot be forgotten that this type of hydrolysis is much more time consuming.

To verify the influence of certain parameters (referred above), it could be investigated if the EPS production is enhanced at 37 °C and at controlled pH 5.8 (Kimmel et al., 1998).

The EPS extraction from the growth with whole milk should be explored, to develop a deeper knowledge in the parameters that influence the EPS production.

To complete the bifidogenic study, the same test should be performed to the bacteria applied in the study (*L. delbrueckii* subsp. *bulgaricus*, *L. lactis* subsp. *lactis* and *L. kefir*), to verify these microorganisms’ potential and if they are able to produce β-galactosidase.

Furthermore, by the fact that *L. lactis* subsp. *lactis* had the ability to hydrolyse the EPS, the possible probiotic effect of this microorganism should also be studied (it has to follow several criteria to be considered a probiotic), since it is a promising characteristic.

It would be interesting to implement Metabolic Control Analysis or Metabolic Flux Analysis to enhance the microorganisms’ production of these metabolites.

The analyses of the EPS rheology, to develop more knowledge in the food applications that this EPS could have, should be an interesting feature.
Analysis of the organic acids and exopolysaccharides produced by the lactic acid microflora present in kefir and their potential applications

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Analysis of the organic acids and exopolysaccharides produced by the lactic acid microflora present in kefir and their potential applications


Analysis of the organic acids and exopolysaccharides produced by the lactic acid microflora present in kefir and their potential applications


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Annexes

A.1. Culture Media Composition

All the mediums were autoclaved for 15 min at 121 °C. The solid mediums compositions of YMA, CBA and MRS were the same of the broth mediums, with the addition of 20.0 grams of agar.

**Plate Count Agar (PCA) - Liofilchem**

*Grams per litter*

- Casein Peptone ................................................................. 5.0
- Yeast Extract ............................................................... 2.5
- Glucose ................................................................. 1.0
- Agar ............................................................... 15.0

**Yeast Malt Agar (YMA)**

*Grams per litter*

- Yeast Extract ................................................................. 3.0
- Malt Extract ................................................................. 3.0
- Glucose ................................................................. 10.0
- Bacteriologic Peptone .................................................. 5.0

**Corynebacterium Agar (CBA)**

*Grams per litter*

- Casein Peptone ................................................................. 10.0
- Yeast Extract ................................................................. 5.0
- Glucose ................................................................. 5.0
- NaCl ................................................................. 5.0
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**Man, Rogosa and Sharpe (MRS) - Liofilchem**

*Grams per litter plus 1 mL Tween 80*

- Peptospecial ................................................................. 10.0
- Beef Extract ............................................................... 10.0
- Yeast Extract ............................................................. 5.0
- Glucose ................................................................. 20.0
- Triammonium Citrate ................................................... 2.0
- Sodium Acetate .......................................................... 5.0
- Magnesium Sulphate ................................................... 0.20
- Manganese Sulphate ..................................................... 0.05
- Di-Potassium Phosphate ............................................... 2.0

**Altered Man, Rogosa and Sharpe (AMRS)**

*Grams per litter plus 1 mL Tween 80 (Liofilchem)*

- Bacteriologic Peptone ................................................... 10.0
- Casein ........................................................................ 0.05
- Yeast Extract ............................................................. 5.0
- EPS (C₁/C₂/C₃) ............................................................. 10.0/20.0/100.0
- Sodium Acetate .......................................................... 5.0
- Magnesium Sulphate ................................................... 0.20
- Manganese Sulphate ..................................................... 0.05

**Skim Milk**

- Skim Milk Powder (VETAGRI HUMANA S.A.) ....................... 100 g
- Distilled Water .......................................................... 900 mL

7 The EPS weight corresponds to its humid weight.
8 Only added to the solution after autoclaving.
A.2. Calibration Curves

A.2.1. Organic Acids Calibration Curves for HPLC-UV/VIS

In this section, the calibration curves for pyruvic acid (Figure A.1), citric acid (Figure A.2) lactic acid (Figure A.3), acetic acid (Figure A.4), propionic acid (Figure A.5) and succinic acid (smaller concentrations - Figure A.6; and higher concentrations – Figure A.7) are presented.

![Pyruvic Acid Calibration Curve](image)

Figure A.1 – Calibration curve for pyruvic acid.
Analysis of the organic acids and exopolysaccharides produced by the lactic acid microflora present in kefir and their potential applications

Figure A.2 – Calibration curve for citric acid.

Figure A.3 – Calibration curve for lactic acid.
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Figure A.4 – Calibration curve for acetic acid.

Figure A.5 – Calibration curve for propionic acid.
Analysis of the organic acids and exopolysaccharides produced by the lactic acid microflora present in kefir and their potential applications

Figure A.6 – Calibration curve for succinic acid smaller concentrations.

![Figure A.6](image)

\[ y = 6.4388x + 0.0745 \]
\[ R^2 = 0.9871 \]

Figure A.7 – Calibration curve for succinic acid higher concentrations.

![Figure A.7](image)

\[ y = 5.4739x + 0.1434 \]
\[ R^2 = 0.9998 \]
A.2.2. Carbohydrates Calibration Curves for HPLC-RI

In the Figures A.8, A.9, A.10 and A.11 the calibration curves for lactose, glucose, galactose, and rhamnose, respectively, described in the subsection 3.3.3, are presented.

Figure A.8 – Calibration curve for lactose.

Figure A.9 – Calibration curve for glucose.
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Figure A.10 – Calibration curve for galactose.

Figure A.11 – Calibration curve for rhamnose.
A.2.3. Glucose Calibration Curve for DNS Method

In the Figure A.12 the calibration curve for glucose, described in the subsection 3.3.4, is presented.

![Glucose Calibration Curve for DNS Method](image1)

Figure A.12 – Calibration curve for glucose.

A.2.4. Glucose Calibration Curve for Dubois Method

In the Figure A.13 the calibration curve for glucose, described in the subsection 3.3.5, is presented.

![Glucose Calibration Curve for Dubois Method](image2)

Figure A.13 – Calibration curve for glucose.
A.3. Additional Material and Methods

**Resin Extraction**

For each 20 mL of sample (previous heated at 80 °C for 20 min and centrifugated) 2 g of resin (Dowex with Na\(^+\)-form, strongly acidic (Sigma Aldrich)) were added. The mixture was left stirring for 2 hours (Simões et al., 2011).

**DNS Reagent Preparation**

5 g of 3,5-dinitrosalicylic acid were dissolved in 250 mL of distilled water at 80 °C. The mixture was cooled down and 100 mL of 2 M sodium hydroxide were added. To the homogenised solution 150 g of La Rochelle salt (sodium and potassium tartarate) were added. The previous mixture was homogenised and diluted to 500 mL with distilled water. The solution was stored in a dark bottle (Miller, 1959).
A.4. Chromatograms

A.4.1. Organic Acids Standards

In Figure A.14 the chromatogram obtained for all the standards, described in the subsection 3.2.2, is presented.

Figure A.14 – Chromatogram obtained for all the organic acids standards.

A.4.2. Carbohydrates Standards

In Figure A.15 the chromatogram obtained for several of the standards is presented.

Figure A.15 – Chromatogram obtained for the carbohydrates standards.
A.4.3. Organic Acids Samples

The chromatograms obtained for the samples are shown below.

Figure A.16 – Chromatogram obtained for *L. delbrueckii* subsp. *bulgaricus*, for the organic acids.

Figure A.17 – Chromatogram obtained for *L. lactis* subsp. *lactis*, for the organic acids.
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Figure A.18 – Chromatogram obtained for *L. kefir*, for the organic acids.

Figure A.19 – Chromatogram obtained for *L. delbrueckii* subsp. *bulgaricus* + *L. kefir*, for the organic acids.

Figure A.20 – Chromatogram obtained for *L. kefir* + *L. lactis* subsp. *lactis*, for the organic acids.
Analysis of the organic acids and exopolysaccharides produced by the lactic acid microflora present in kefir and their potential applications

Figure A.21 – Chromatogram obtained for *L. lactis* subsp. *lactis* + *L. delbrueckii* subsp. *bulgaricus*, for the organic acids.

Figure A.22 – Chromatogram obtained for *L. delbrueckii* subsp. *bulgaricus* + yeasts, for the organic acids.

Figure A.23 – Chromatogram obtained for *L. kefir* + yeasts, for the organic acids.
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Figure A.24 – Chromatogram obtained for L. lactis subsp. lactis + yeasts, for the organic acids.

Figure A.25 – Chromatogram obtained for all the bacteria, for the organic acids.

Figure A.26 – Chromatogram obtained for all the bacteria + yeasts, for the organic acids.
Analysis of the organic acids and exopolysaccharides produced by the lactic acid microflora present in kefir and their potential applications

Figure A.27 – Chromatogram obtained for *C. krusei*, for the organic acids.

Figure A.28 – Chromatogram obtained for *C. famata*, for the organic acids.

Figure A.29 – Chromatogram obtained for *C. krusei* + *C. famata*, for the organic acids.
A.4.4. Carbohydrates Samples

The chromatograms obtained for the samples were all similar. Therefore, it is only presented a representative example.

Figure A.30 – Chromatogram obtained for the time zero, for the carbohydrates.
A.5. Student’s *t*-test

To evaluate the obtained results and to determine if the difference between the values was significant, it was applied Student’s *t*-test. This determination was possible, assuming that the samples follow a normal distribution.

The test has the following steps (Magalhães and Vicente, 2008):

1. Formulate the null hypothesis and the alternative hypothesis and determine the *α* value;
2. Calculate the *t*-value by equation A1;

\[
|t| = \frac{\bar{X} - \mu}{EPM} \text{ with } n - 1 \text{ degrees of freedom} \tag{A.1}
\]

\(\bar{X}\) - average value that is intended to be verified;
\(\mu\) - average value, that is used as comparison with \(\bar{X}\);
\(EPM\) - standard deviation of the samples that are intended to be verified;
\(n\) - number of samples.

3. With the *t*-value and with the degrees of freedom, it can be determined the evidential value (*P*), consulting a distribution table with the *t*-values (Magalhães and Vicente, 2008);
4. Conclusions:
   a. If \(P > \alpha\), accept the null hypothesis (the samples are not significantly different);
   b. If \(P < \alpha\), reject the null hypothesis (the samples are significantly different).