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

Dissertation for Master Thesis

Ana Catarina Marcelino Pinto de Meireles

Supervisors: **Doctor Margarida Bastos**

Doctor Isabel Afonso

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

Winston Churchill

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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 ($5.39x10^{-1}$ 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 láctico, 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 ($5{,}39 \times 10^{-1}$ 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 bifidogénico 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.

Palavras-chave: ácidos orgânicos, exopolissacarídeos, leveduras, bactérias, prebiótico, probiótico.

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List of Abbreviations

AAB	Acetic Acid Bacteria
Abs	Absorbance
AMRS	Altered Man, Rogosa and Sharpe
CBA	Corynebacterium Agar
CoA	Coenzyme A
DNS	3,5-Dinitrosalicylic acid
EPS	Exopolysaccharides
FDA	Food and Drug Administration
FOS	Fructo-oligosaccharide
GOS	Galacto-oligosaccharide
GRAS	Generally Recognized As Safe
HPLC	High Performance Liquid Chromatography
LAB	Lactic Acid Bacteria
MRS	Man, Rogosa and Sharpe
OD	Optic Density
PCA	Plate Count Agar
RI	Refractive Index
RP	Reversed phase
TDP	Thymidine diphosphate
UDP	Uridine Diphosphate

UV	Ultraviolet
Vis	Visible
YMA	Yeast Malt Agar

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).

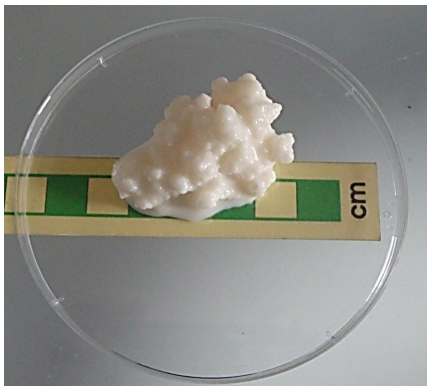


Figure 2.1 – Kefir grains (each square is equal to 1 cm).

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 (≈ 90%), proteins (≈ 3%), lipids (≈ 0.3%), carbohydrates (≈ 6%) and ashes (≈ 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.

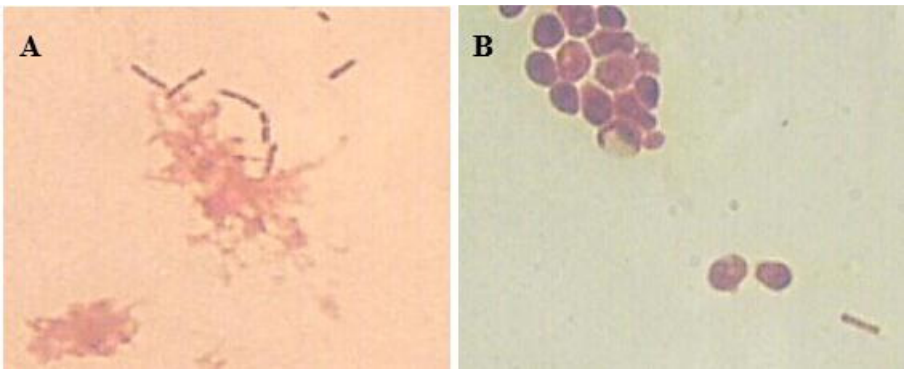


Figure 2.2 – Optical microscope photographs of a kefir grain (1000x with immersion lens). A – Evidence of the presence of Gram-positive and Gram-negative bacilli; B – Evidence of the presence of yeasts and bacilli.

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 exiguus*, *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, acetoin, 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).

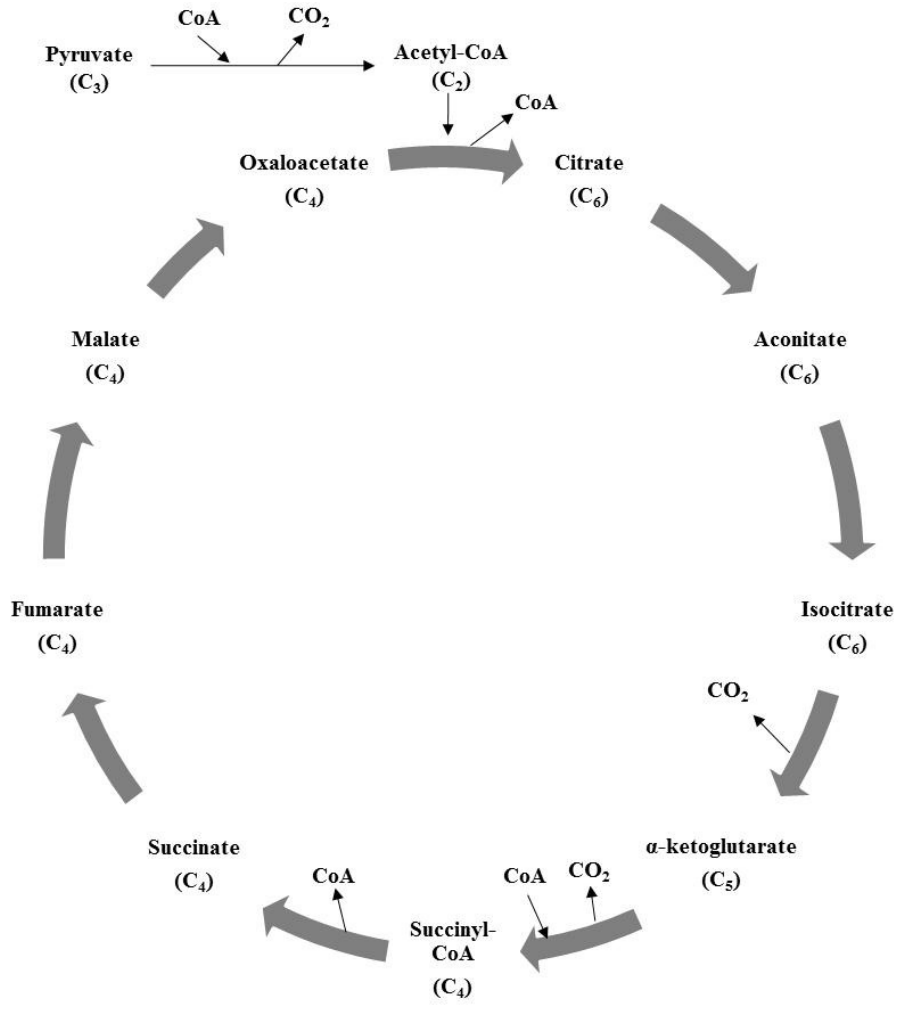


Figure 2.3 – Krebs cycle (adapted from Madigan *et al.*, 2009).

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.

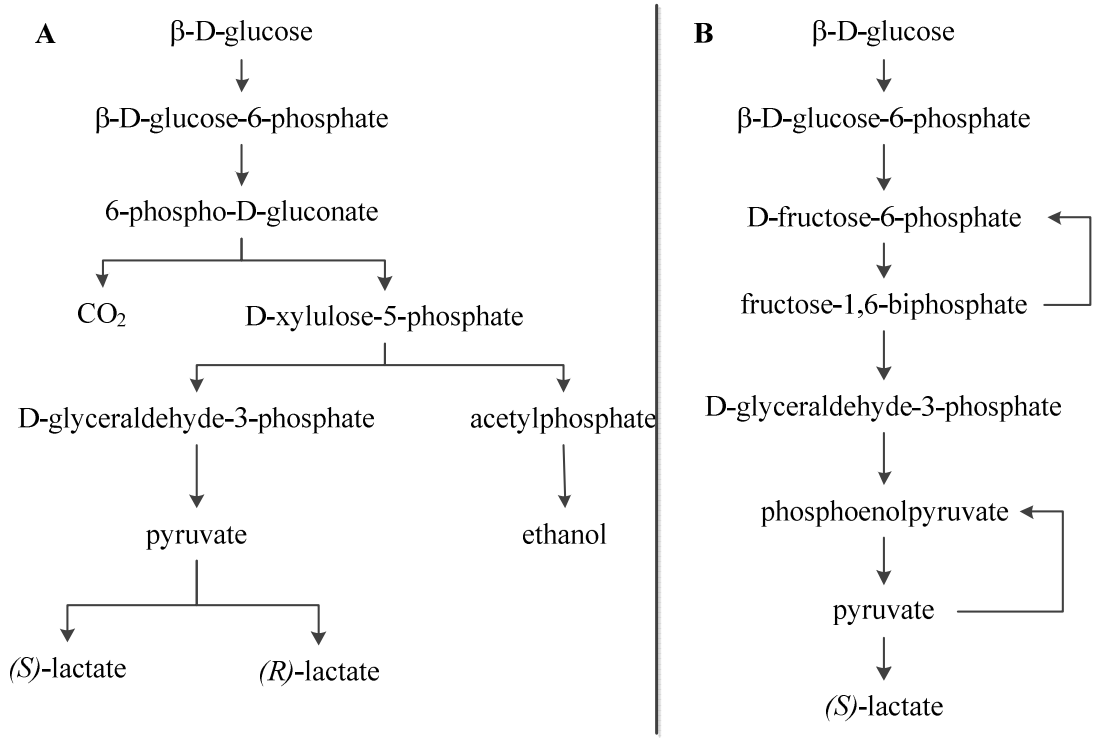


Figure 2.4 – Schematic representation of heterolactic fermentation (A) (adapted from MetaCyc, 2007a) and homolactic fermentation (B) (adapted from MetaCyc, 2007b).

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).

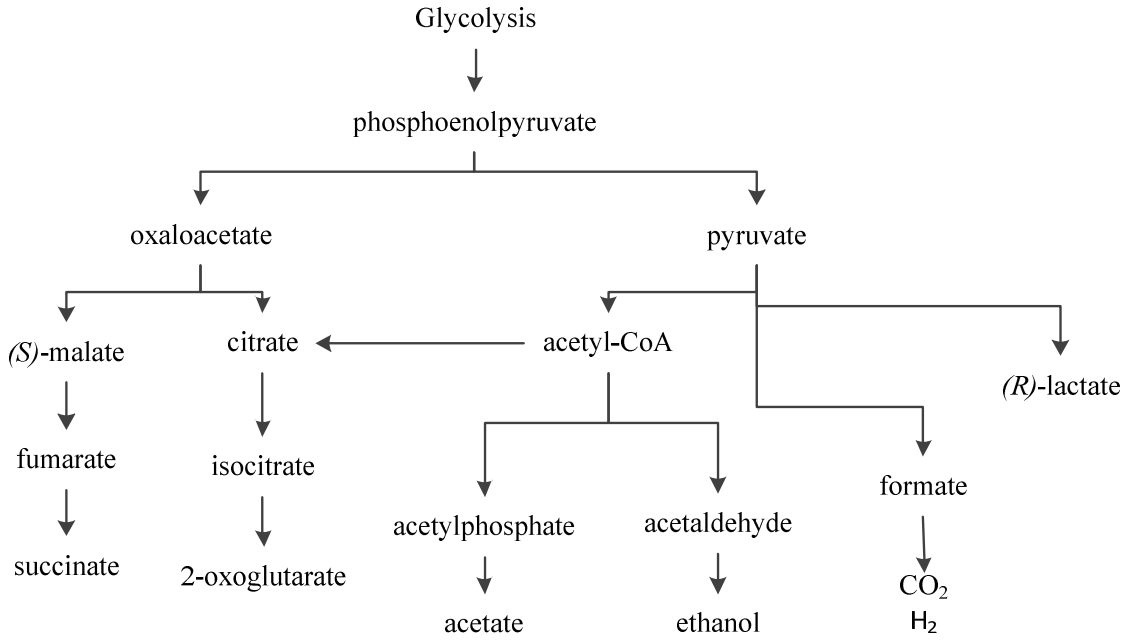


Figure 2.5 – Mixed acid fermentation (adapted from MetaCyc, 2010).

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.

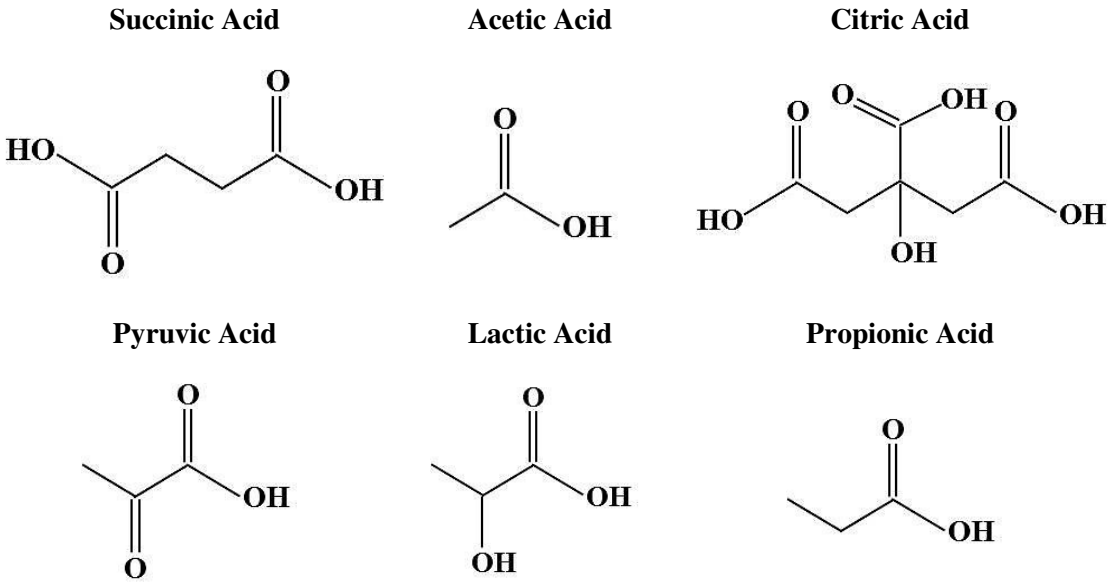


Figure 2.6 – Chemical structure of succinic, acetic, citric, pyruvic, lactic and propionic acid.

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).

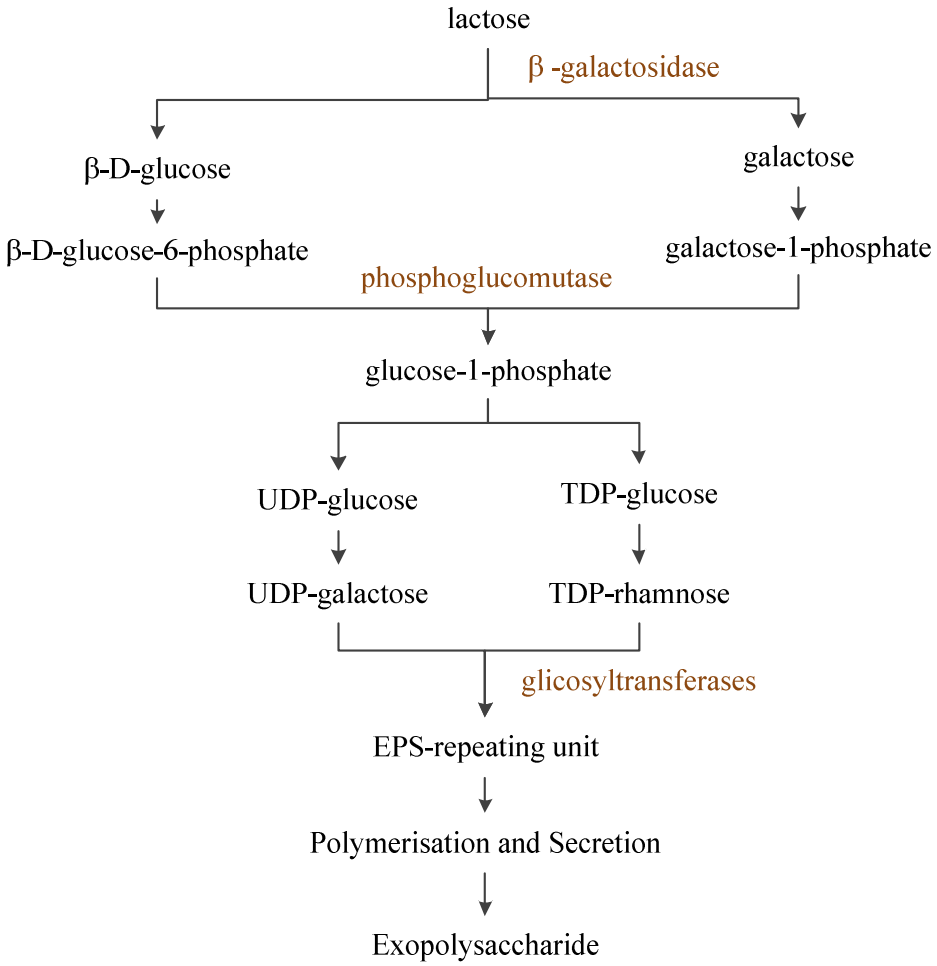


Figure 2.7 – Schematic representation of the EPS production (adapted from Welman and Maddox, 2003).

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).

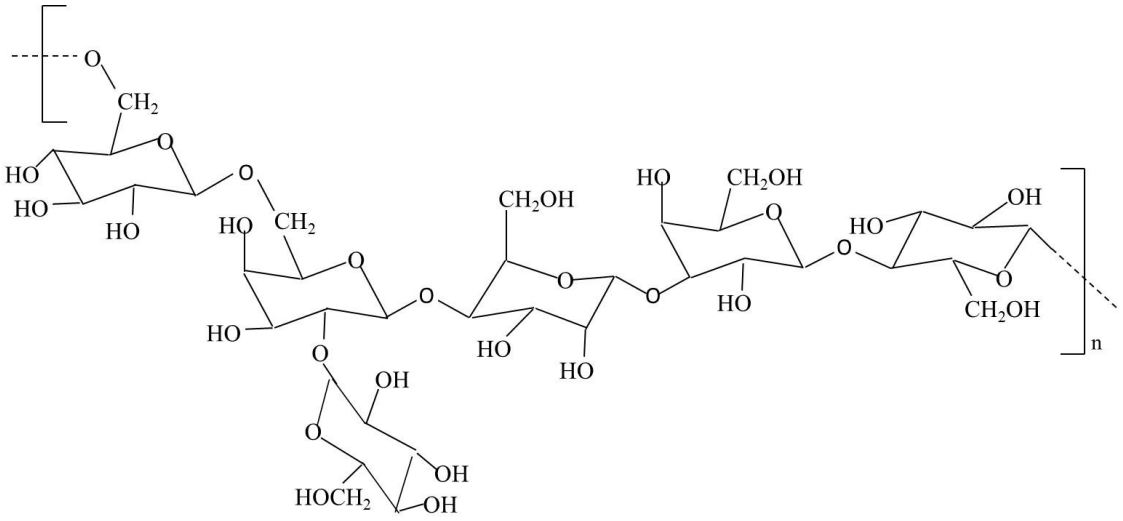


Figure 2.8 – Kefiran structure (adapted from Micheli *et al.*, 1999).

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).

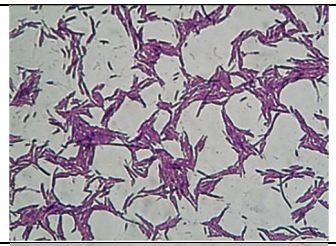
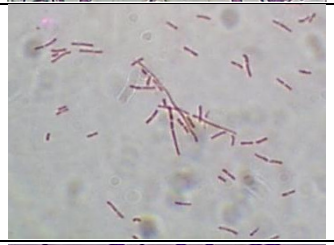
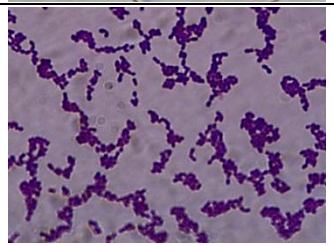
The characteristics, the mediums and the cultivation conditions for all the strains are listed in the Table 3.1. The composition of all the mediums used is described in the Annex A.1.

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 [1], 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).

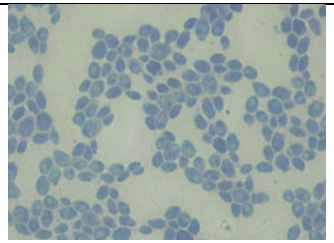
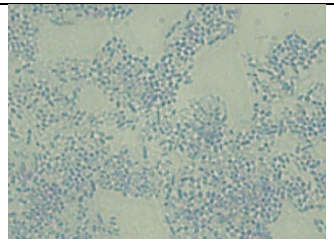
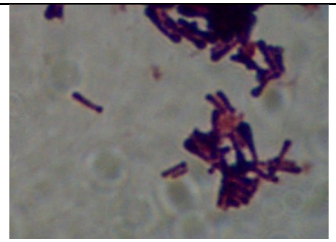
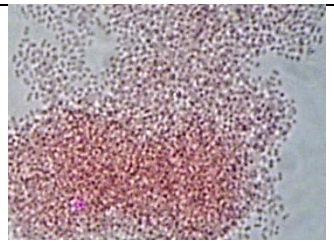
[1] The temperature chosen was 60 °C due to the medium applied (skim milk).

Table 3.1 – Microorganisms applied in the study, their characterisation, origins and cultivations conditions

Microorganisms	**Photography (1000x with immersion lens [2])**	**Characteristics**	**Isolated from**	**Medium**	**Cultivation Conditions**
L. delbrueckii subsp. *bulgaricus* (DSM 20081)		Microaerophilic Gram - positive	Bulgarian yogurt [3]	MRS	37 ºC, 2 days anaerobic jar
L. kefir (DSM 20587)		Aerobic Gram - positive	Kefir grains [3]	MRS	30 ºC, 2 days
L. lactis subsp. *lactis* (DSM 20481)		Aerobic Gram - positive	Not specified [3]	CBA	30 ºC, 3 days

[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.)

Microorganisms	Photography (1000x with immersion lens)	Characteristics	Isolated from	Medium	Cultivation Conditions
C. famata		Anaerobic facultative	Kefir grains	YMA	30 °C, 2 days
C. krusei		Anaerobic facultative	Kefir	YMA	30 °C, 3 days
B. animalis subsp. *lactis* (BB-12 CHR-HANSEN)		Anaerobic (slightly oxygen tolerant) Gram - positive	Not specified [3]	MRS with cysteine 0.05%	37 °C, 2 days anaerobic jar
E. coli (UTAD private collection)		Anaerobic facultative Gram – negative	Dog faeces	PCA	30 °C, 2 days

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

Isolated microorganisms	**Combinations of Microorganisms**
L. delbrueckii subsp. *bulgaricus*	*L. delbrueckii* subsp. *bulgaricus* + *L. kefir*
L. kefir	*L. kefir* + *L. lactis* subsp. *lactis*
L. lactis subsp. *lactis*	*L. lactis* subsp. *lactis* + *L. delbrueckii* subsp. *bulgaricus*
C. famata	*L. delbrueckii* subsp. *bulgaricus* + All the yeasts (*C. famata* + *C. krusei*)
C. krusei	*L. kefir* + All the yeasts (*C. famata* + *C. krusei*)
	L. lactis subsp. *lactis* + All the yeasts (*C. famata* + *C. krusei*)
	All the bacteria (*L. delbrueckii* subsp. *bulgaricus* + *L. kefir* + *L. lactis* subsp. *lactis*)
	All the bacteria (*L. delbrueckii subsp. bulgaricus* + *L. kefir* + *L. lactis* subsp. *lactis*) + All the yeasts (*C famata* + *C. krusei*)
	C. famata + *C. krusei*

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.

Figure 3.1 – Tests performed with skim milk.

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 (H_3PO_4 85% (Carlo Erra)), 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% H_3PO_4) 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_3PO_4 pH 2.10. From one 0.1 g/L stock composed standard solution different concentrations were prepared: $1x10^{-3}$, $5x10^{-3}$, $8x10^{-3}$, $1x10^{-2}$, $2x10^{-2}$, $5x10^{-2}$, $8x10^{-2}$ and $1x10^{-1}$ g/L. [5]

For succinic acid, higher concentration solutions were also prepared: $5x10^{-2}$, $8x10^{-2}$, $1x10^{-1}$, $2x10^{-1}$, $3x10^{-1}$, $5x10^{-1}$ and $7x10^{-1}$ 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.

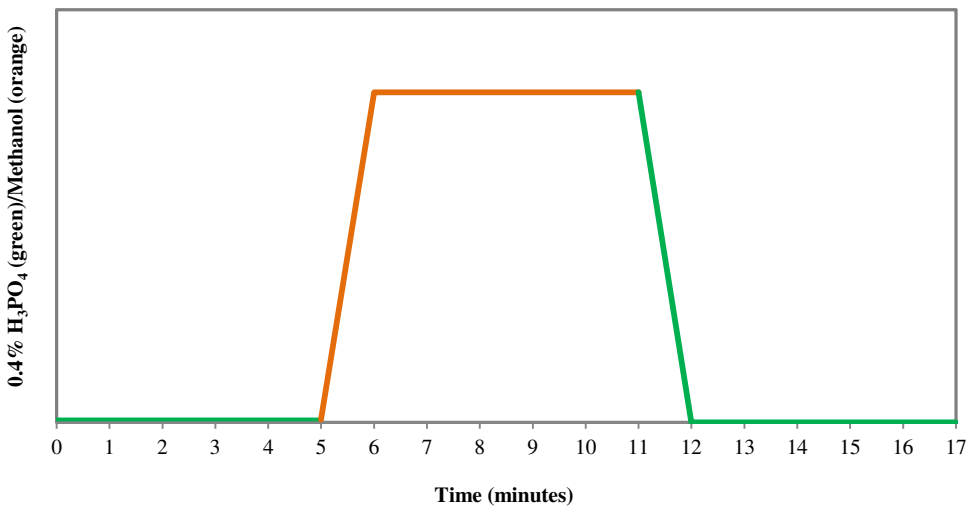


Figure 3.2 – Schematic representation of the column cleaning process. The mobile phase (0.4% H_3PO_4) is represented in green and the methanol is represented in orange.

[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

$\lambda_{detection}$: 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

Isolated microorganisms	**Combinations of Microorganisms**
L. delbrueckii subsp. *bulgaricus*	*L. delbrueckii* subsp. *bulgaricus* + *L. kefir*
L. kefir	*L. kefir* + *L. lactis* subsp. *lactis*
L. lactis subsp. *lactis*	*L. lactis* subsp. *lactis* + *L. delbrueckii* subsp. *bulgaricus*
B. animalis subsp. *lactis*	*L. delbrueckii* subsp. *bulgaricus* + All the yeasts (*C. famata* + *C. krusei*)
	L. kefir + All the yeasts (*C. famata* + *C. krusei*)
	L. lactis subsp. *lactis* + All the yeasts (*C. famata* + *C. krusei*)
	All the bacteria (*L. delbrueckii* subsp. *bulgaricus* + *L. kefir* + *L. lactis* subsp. *lactis*)
	All the bacteria (*L. delbrueckii* subsp. *bulgaricus* + *L. kefir* + *L. lactis* subsp. *lactis*) + All the yeasts (*C. famata* + *C. krusei*)

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 ressuspended 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.

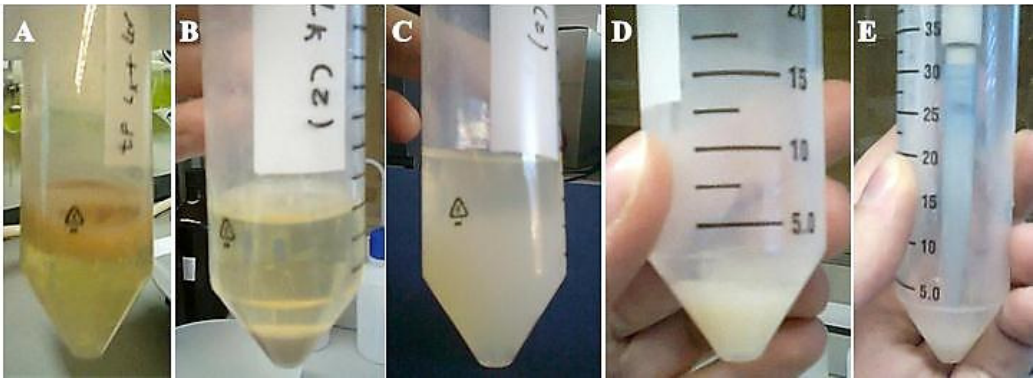


Figure 3.3 – Process of extraction. A – After heating; B – First step of centrifugation after heating; C – Precipitation with ethanol; D – Pellet after second centrifugation; E – Ressuspending the pellet in distilled water.

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_2SO_4. [6]

Table 3.4 – Conditions tested for the sulphuric acid hydrolysis

Concentration	**Temperature (°C)**	**Time (min)**
95%	Room temperature	-
1 M	100	30
2 M	100	30
2 M	130	30
2 M	150	30

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_2SO_4 2M, at 150 °C for 30 min. Posteriorly the hydrolysis with concentrated H_2SO_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_2SO_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

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_2SO_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_2SO_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_1 - half of the sugars concentration present in the original MRS; C_2 – the same concentration of sugars present in the original MRS; C_3 – 10 times higher than C_1). 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

Standards	Retention Time (min)
Pyruvic Acid	4.63
Citric Acid	5.46
Lactic Acid	6.68
Acetic Acid	7.33
Propionic Acid	9.90
Succinic Acid	13.82

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).

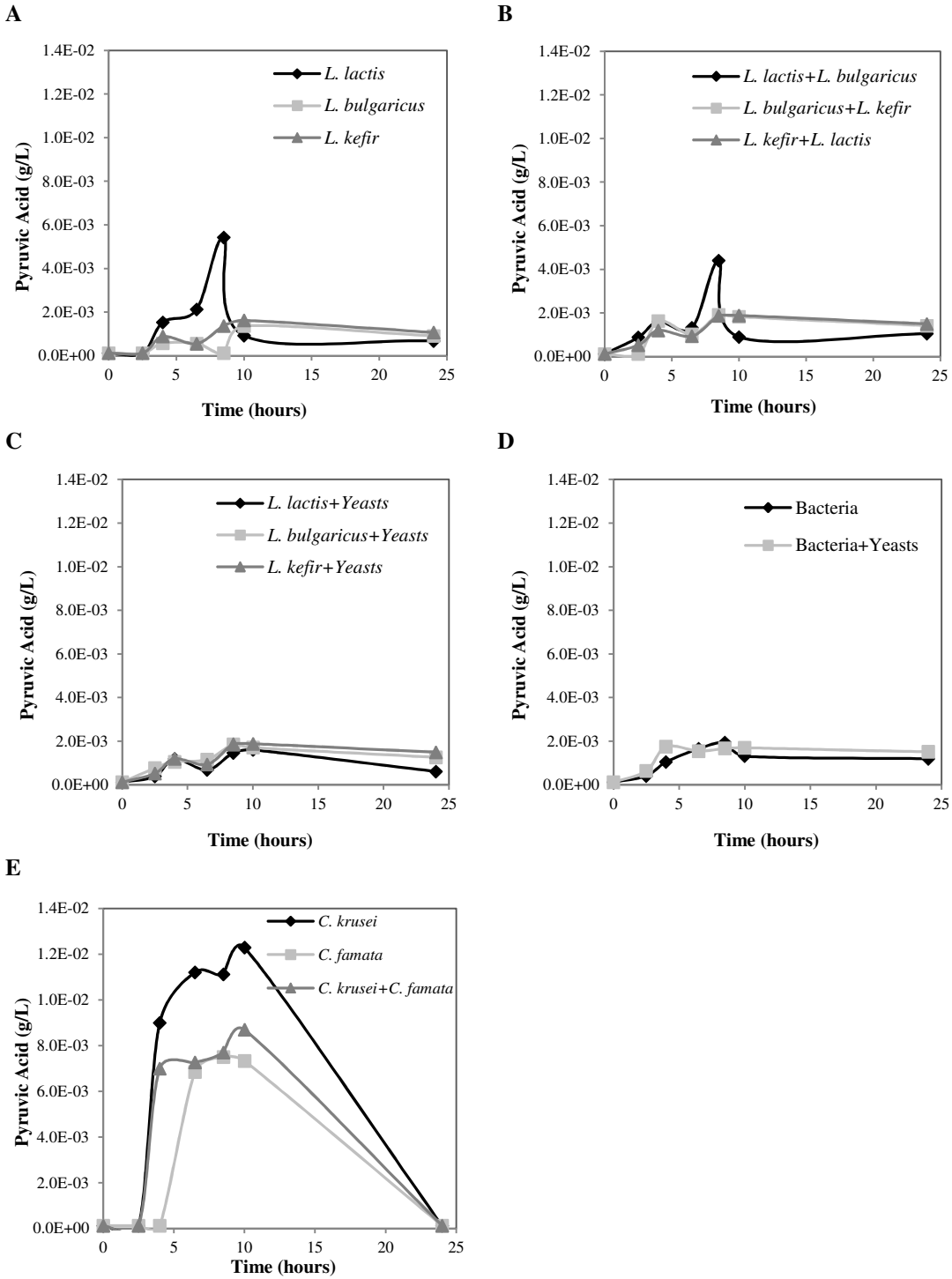


Figure 4.1 – Pyruvic acid production along growth time, for all the combination of strains. A – Isolated bacteria (*L. lactis* subsp. *lactis*, *L. delbrueckii* subsp. *bulgaricus* and *L. kefir*); B – Mixed bacteria (*L. lactis* subsp. *lactis* + *L. delbrueckii* subsp. *bulgaricus*, *L. delbrueckii* 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 $1.23x10^{-2}$ 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 ($6.00x10^{-2}$ g/L) and for *C. famata* is also higher but very similar ($2.00x10^{-2}$ 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.

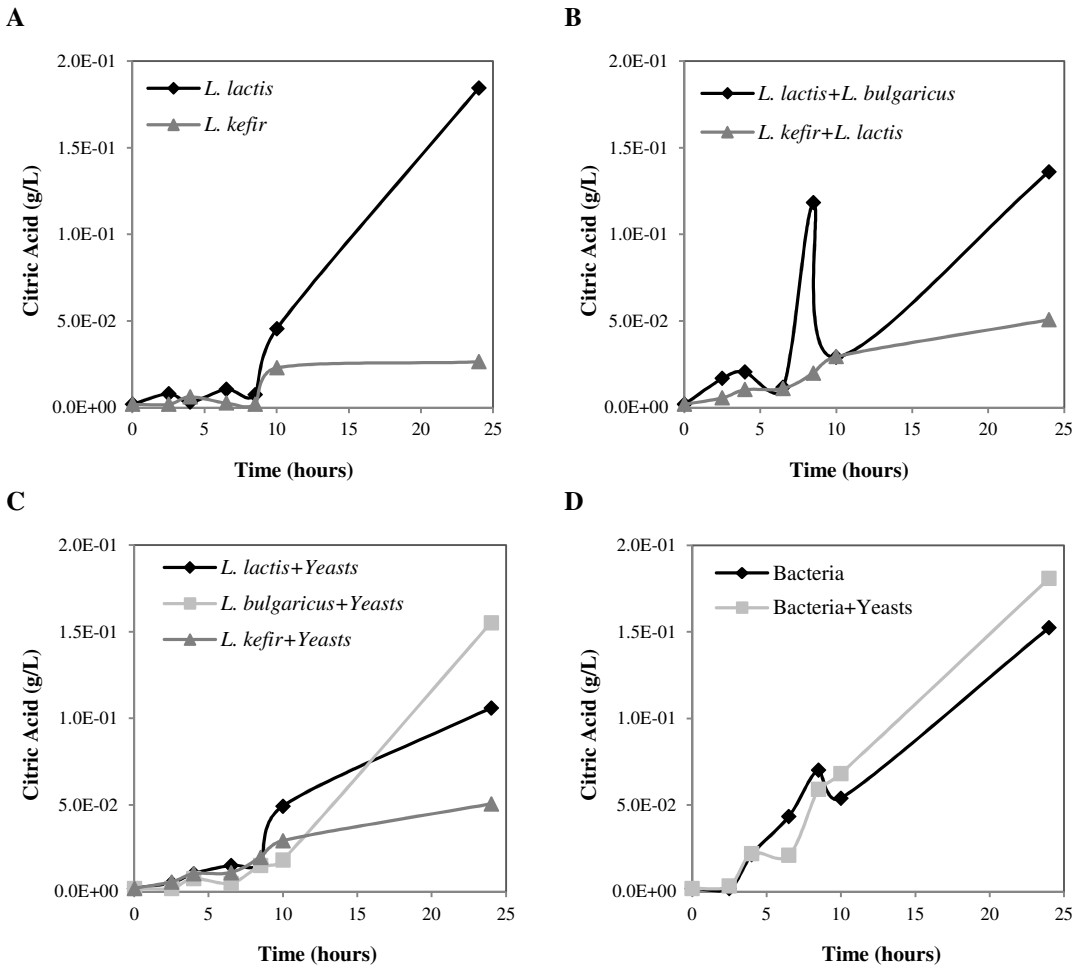


Figure 4.2 – Citric acid production along growth time. A – Isolated bacteria (*L. lactis* subsp. *lactis* and *L. kefir*); B – Mixed bacteria (*L. lactis* subsp. *lactis* + *L. delbrueckii* subsp. *bulgaricus* and *L. kefir* + *L. lactis* subsp. *lactis*); C – Isolated bacteria mixed with yeasts (*L. 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 all the bacteria mixed with yeasts (*L. lactis* subsp. *lactis* + *L. delbrueckii* subsp. *bulgaricus* + *L. kefir* + *C. krusei* + *C. famata*).

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 1.85×10^{-1} 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 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.

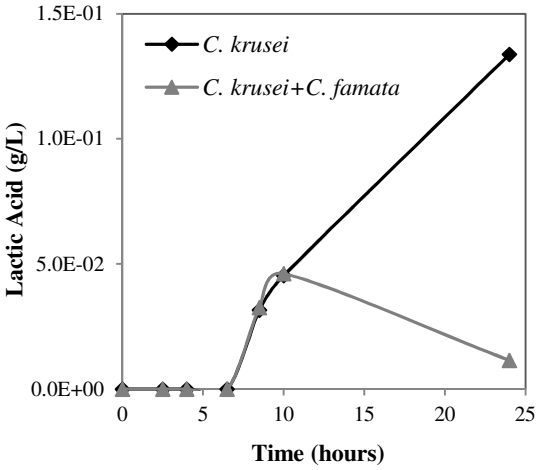


Figure 4.3 – Lactic acid production along the growth time, for *C. krusei* and *C. krusei* + *C. famata*.

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 1.34×10^{-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.

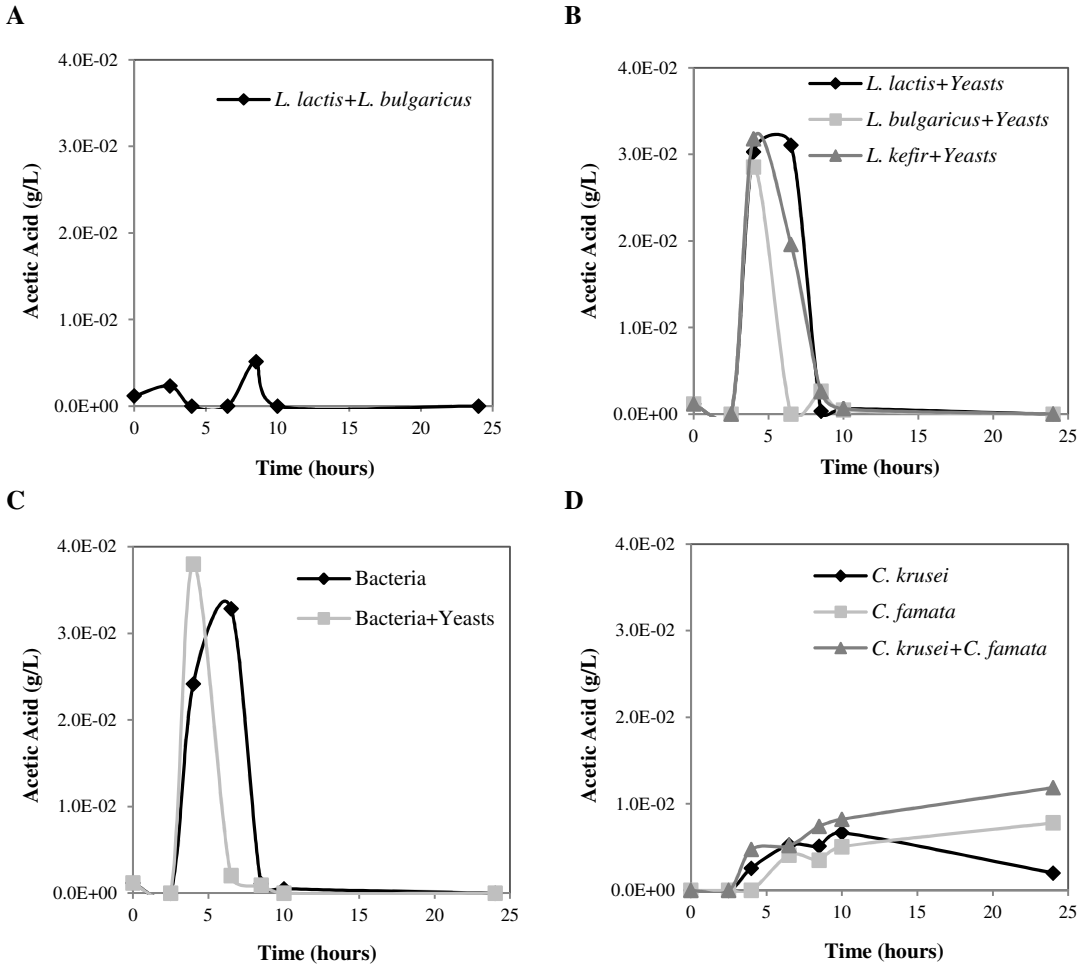


Figure 4.4 – Acetic acid production along growth time. A – Mixed bacteria (*L. lactis* subsp. *lactis* + *L. delbrueckii* subsp. *bulgaricus*); B – Isolated bacteria mixed with yeasts (*L. lactis* subsp. *lactis* + yeasts, *L. delbrueckii* subsp. *bulgaricus* + yeasts, *L. kefir* + yeasts); C – All the 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*); D – isolated yeasts and mixed yeasts (*C. krusei*, *C. famata* and *C. krusei* + *C. famata*).

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 $3.80x10^{-2}$ 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 $5.00x10^{-1}$ 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.

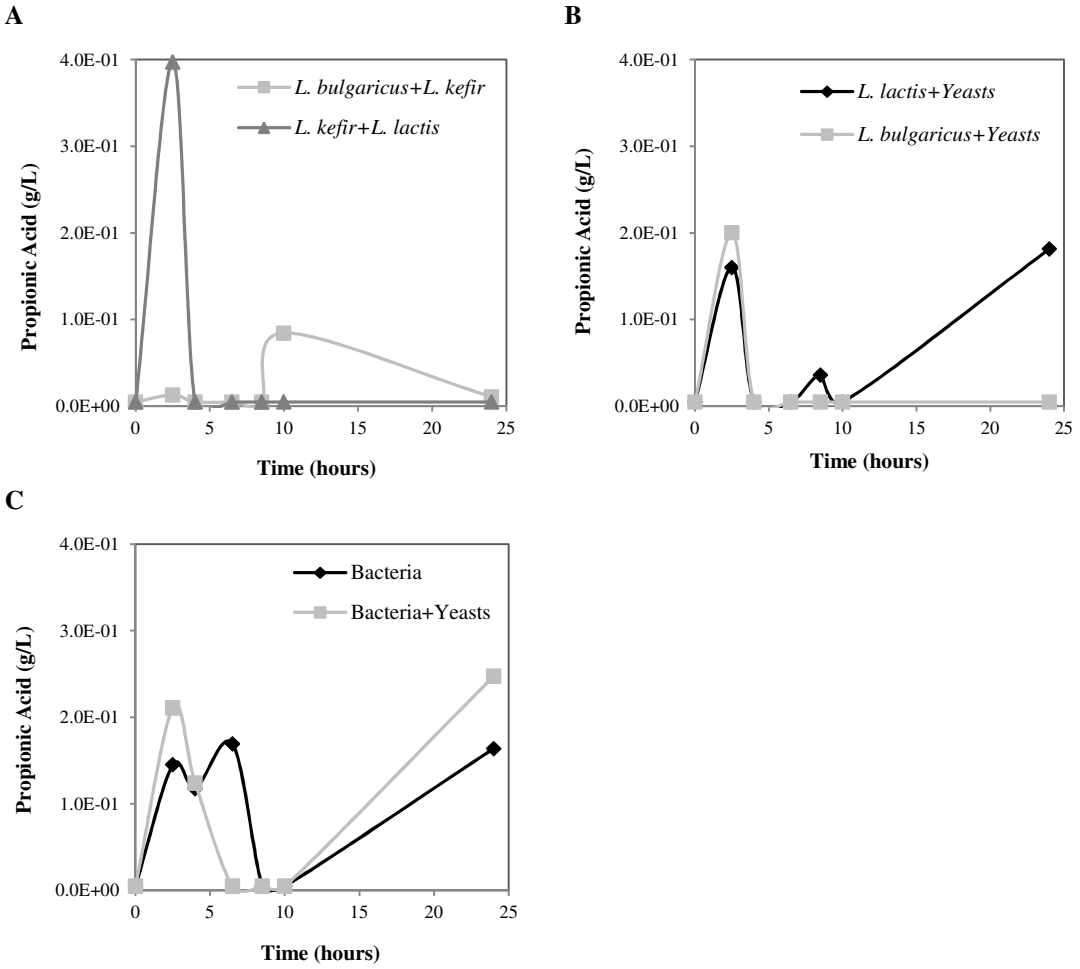


Figure 4.5 – Propionic acid production along the growth time. A – Mixed bacteria (*L. delbrueckii* subsp. *bulgaricus* + *L. kefir* and *L. kefir* + *L. lactis* subsp. *lactis*); B – Isolated bacteria mixed with yeasts (*L. lactis* subsp. *lactis* + yeasts and *L. delbrueckii* subsp. *bulgaricus* + yeasts); 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.

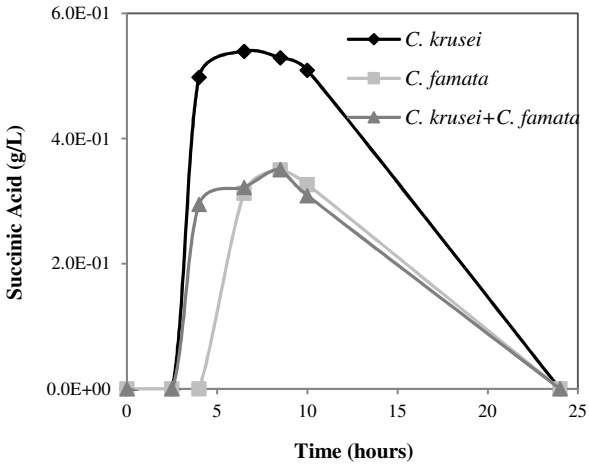


Figure 4.6 – Succinic acid production along the growth time, for the yeasts isolated and mixed.

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.

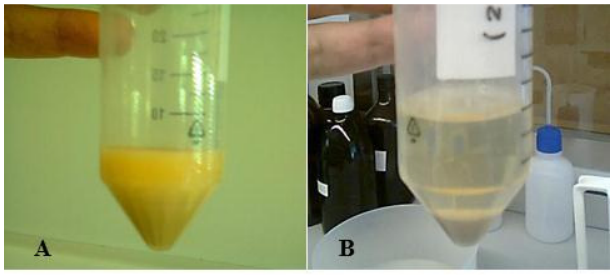


Figure 4.7 – First step of centrifugation after heating. A – In the absence of EPS. B – In the presence of EPS.

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.

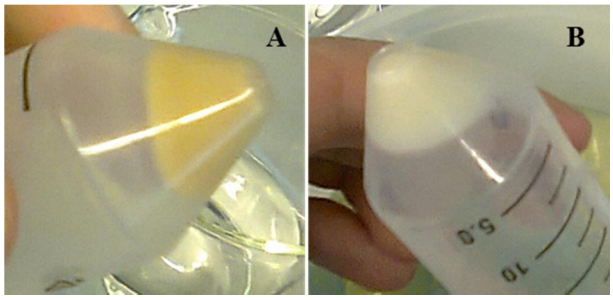


Figure 4.8 – Last step of centrifugation after precipitation with ethanol. A – In the absence of EPS. B – In the presence of EPS.

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)

	Time (hours)			
Combinations of Microorganisms	**0**	**24**	**30**	**48**
L. delbrueckii subsp. *bulgaricus*	nd	nd	nd	d
L. kefir	nd	nd	nd	d
L. lactis subsp. *lactis*	nd	d	d	d
B. animalis subsp. *lactis*	nd	nd	nd	nd
L. delbrueckii subsp. *bulgaricus* + *L. kefir*	nd	nd	nd	d
L. kefir + *L. lactis* subsp. *lactis*	nd	d	d	d
L. lactis subsp. *lactis* + *L. delbrueckii* subsp. *bulgaricus*	nd	d	d	d
L. delbrueckii subsp. *bulgaricus* + All the yeasts (*C. famata* + *C. krusei*)	nd	nd	nd	d
L. kefir + All the yeasts (*C. famata* + *C. krusei*)	nd	nd	nd	d
L. lactis subsp. *lactis* + All the yeasts (*C. famata* + *C. krusei*)	nd	d	d	d
All the bacteria (*L. delbrueckii* subsp. *bulgaricus* + *L. kefir* + *L. lactis* subsp. *lactis*)	nd	d	d	d
All the bacteria (*L. delbrueckii* subsp. *bulgaricus* + *L. kefir* + *L. lactis* subsp. *lactis*) + All the yeasts (*C. famata* + *C. krusei*)	nd	d	d	d

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 4.3 – Retention times for the sugars standards

Standards	Retention Time (min)
Lactose	9.73
Glucose	10.99
Galactose	11.43
Rhamnose	11.96

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_2S0_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.

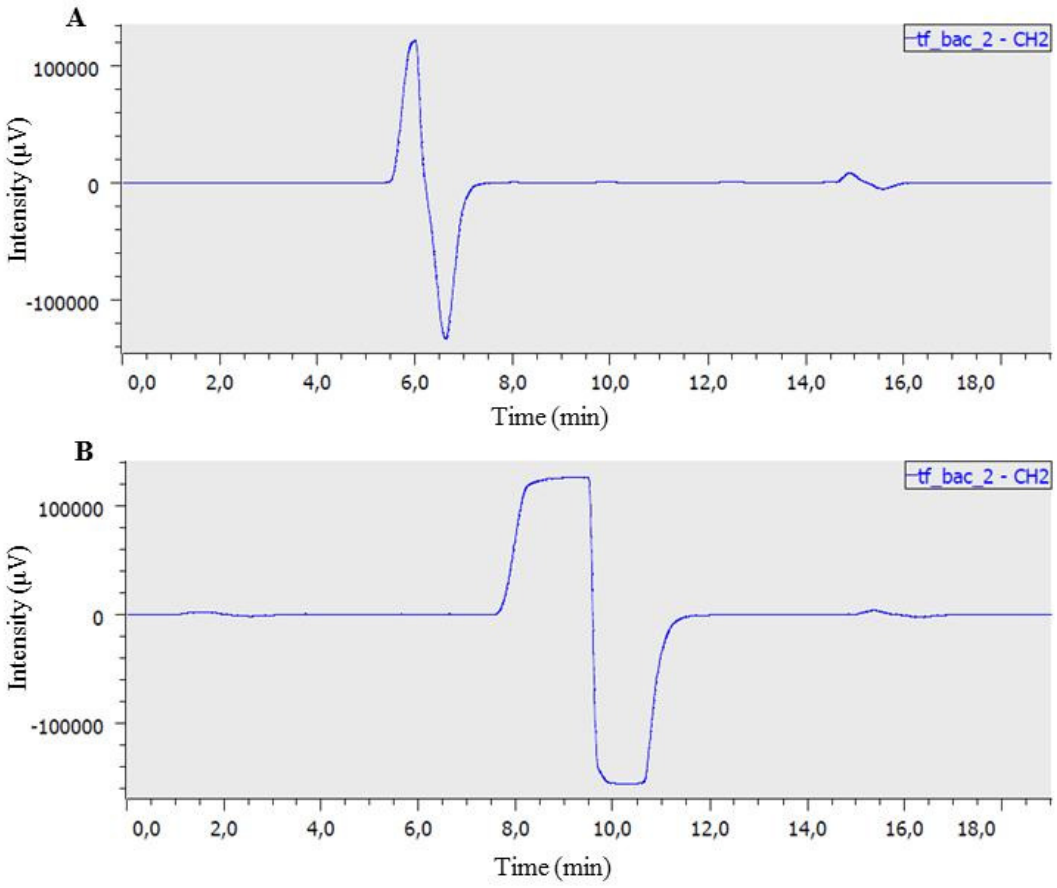


Figure 4.9 – Chromatograms obtained for the growth with all the bacteria after 48 hours of study. A - Hydrolysis with H_2SO_4 2 M at 150 °C for 30 min. B - Hydrolysis with concentrated H_2SO_4 at room temperature.

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.

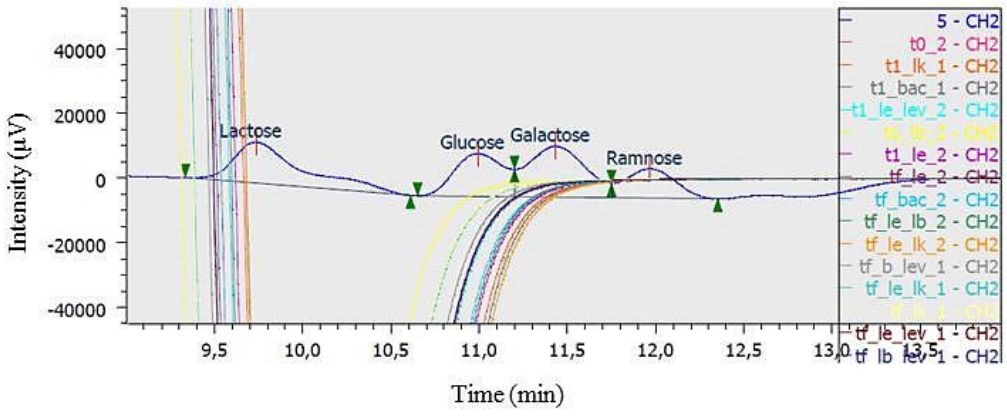


Figure 4.10 – Superposition of the chromatograms obtained for some samples and a chromatogram for a standard.

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 H_2SO_4 2 M and in the other was concentrated H_2SO_4. Therefore, in future works the standards would have to be prepared like the samples. This was not done, because the concentration of H_2SO_4 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 H_2S0_4) 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 H_2SO_4 have the yellow colour of Figure 4.11C.

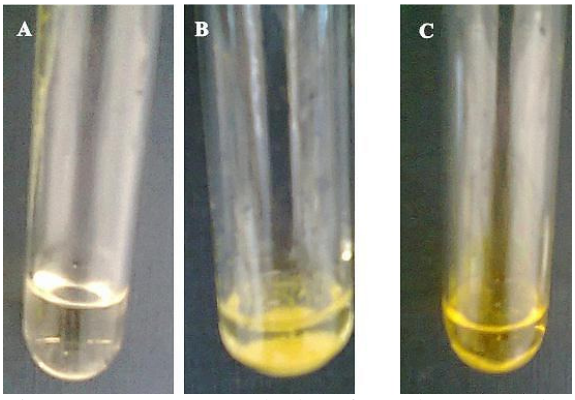


Figure 4.11 – DNS method performed. A – Blank prepared like the sample (water and H_2SO_4). B – Sample. C – Blank with only distilled water.

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.

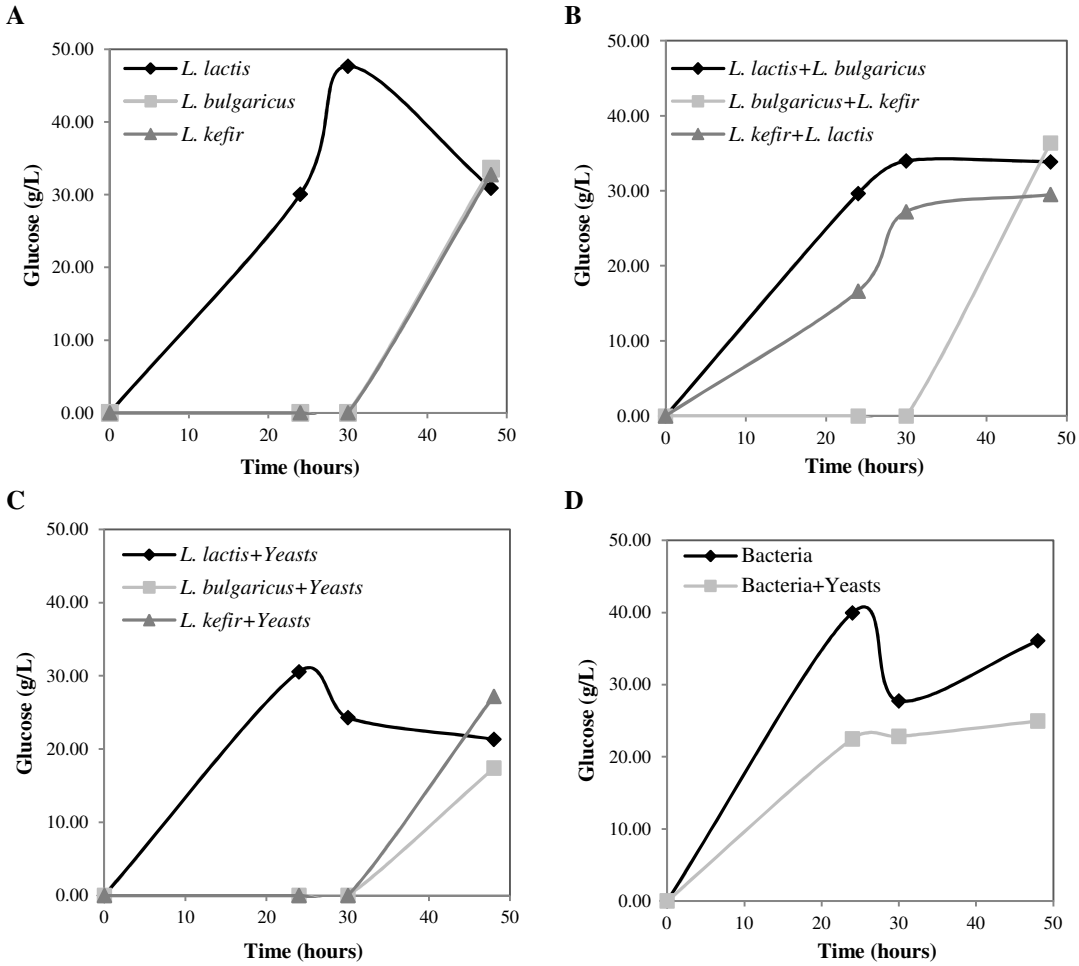


Figure 4.12 – Glucose's concentration along the study, to the strains that exhibit production of EPS. A – Isolated bacteria (*L. lactis* subsp. *lactis*, *L. delbrueckii* subsp. *bulgaricus* and *L. kefir*); B – Mixed bacteria (*L. lactis* subsp. *lactis* + *L. delbrueckii* subsp. *bulgaricus*, *L. delbrueckii* 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.

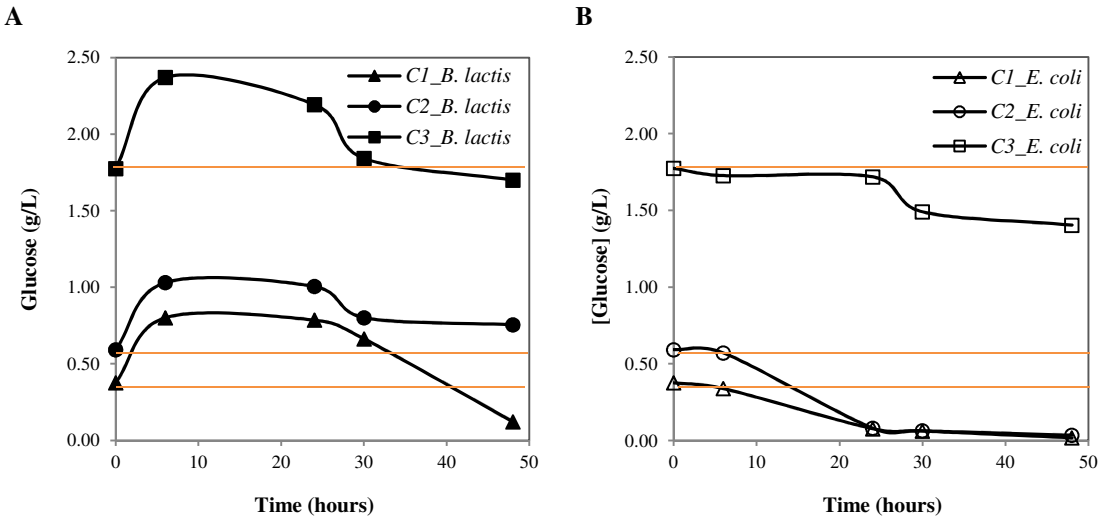


Figure 4.13 – Glucose concentration along the study, to *B. animalis* subsp. *lactis* (A) and *E. coli* (B) to the three different concentrations of EPS. The orange lines mark the value obtained for the solution of AMRS in the initial of the study (time zero). C_1 - half of the sugars concentration present in the original MRS; C_2 – the same concentration of sugars present in the original MRS; C_3 – 10 times higher than C_1.

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 ($C_3=10xC_1$) 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 (C_3).

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*).

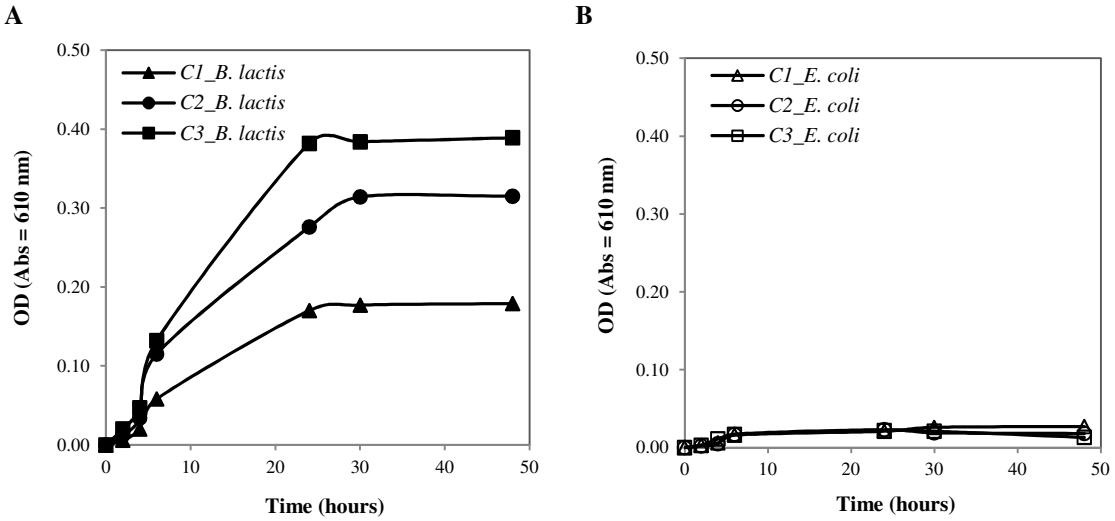


Figure 4.14 – OD observed for *B. animalis* subsp. *lactis* (A) and *E. coli* (B) to the three different concentrations of EPS. C_1 - half of the sugars concentration present in the original MRS; C_2 – the same concentration of sugars present in the original MRS; C_3 – 10 times higher than C_1.

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 *L. lactis* subsp. *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.

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

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 *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 *et al.*, 2000) – or with H_2SO_4 but in different conditions (4 M H_2SO_4, for 8 hours at 105 °C) (Frengova *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.

References

Abraham, A. G., de Antoni, G. L., *Characterization of kefir grains grown in cows' milk and in soya milk*, Journal of Dairy Research, 66. 327-333. 1999.

Adriana, P., Rotar, M.-A., Jimborean, M., Mudura, E., Socaciu, C., *A study on sensory characteristics of a kefir type produced using starter cultures and brewer's yeast*, Journal of Agroalimentary Processes and Technologies, 15:2. 267-272. 2009.

Adriana, P., Socaciu, C., *Probiotic activity of mixed cultures of kefir's Lactobacilli and non-lactose fermenting yeasts*, Bulletin UASVM, Agriculture, 65:2. 329-334. 2008.

Álvarez-Martín, P., Flórez, A. B., Hernández-Barranco, A., Mayo, B., *Interaction between dairy yeasts and lactic acid bacteria strains during milk fermentation*, Food Control, 19. 62-70. 2008.

Aslim, B., Yüksekdağ, Z. N., Beyatli, Y., Mercan, N., *Exopolysaccharide production by Lactobacillus delbrueckii subsp. bulgaricus and Streptococcus thermophilus strains under different growth conditions*, World Journal of Microbiology & Biotechnology, 21. 673-677. 2005.

Assadi, M. M., Pourahmad, R., Moazami, N., *Use of isolated kefir starter cultures in kefir production*, World Journal of Microbiology & Biotechnology, 16. 541-543. 2000.

Badel, S., Bernardi, T., Michaud, P., *New perspectives for Lactobacilli exopolysaccharides*, Biotechnology Advances, 29. 54-66. 2011.

Balabanova, T., Panayotov, P., *Obtaining functional fermented beverages by using the kefir grains*, Procedia Food Science, 1. 1653-1659. 2011.

Baptista, M. E. A., *Determinação de ácidos orgânicos em uvas por cromatografia líquida de alta eficiência com detecção por ultravioleta*, publication in the Educational Development Program for Portugal (PRODEP III), 2004.

Barreteau, H., Delattre, C., Michaud, P., *Production of oligosaccharides as promising new food additive generation*, Food Technology and Biotechnology, 44:3. 323-333. 2006.

Belitz, H.-D., Grosch, W., Schieberle, P., *Carbohydrates*, in Food Chemistry, 4th edition, chapter 4, Springer, Berlin, Germany, 2009.

Beloque, J., Chicón, R., Recio, I., *Quality Control*, in Milk Processing and Quality Management, edited by Tamime, A. Y., 1st edition, chapter 4, Blackwell Publishing, United Kingdom, 2009.

Boels, I. C., van Kranenburg, R., Hugenholtz, J., Kleerebezem, M., de Vos, W. M., *Sugar catabolism and its impact on the biosynthesis and engineering of exopolysaccharide production in lactic acid bacteria*, International Dairy Journal, 11. 723-732. 2001.

Brummer, Y., Cui, S. W., *Understanding carbohydrate analysis*, in Food Carbohydrates: Chemistry, Physical Properties, and Applications, edited by Cui, S. W., 1st edition, chapter 2, CRC Press, Taylor & Francis Group, USA, 2005.

Cerning, J., *Production of exopolysaccharides by lactis acid bacteria and dairy propionibacteria*, Lait, 75. 463-472. 1995.

Cheirsilp, B., Shimizu, H., Shioya, S., *Enhanced kefiran production by mixed culture of Lactobacillus kefiranofaciens and Saccharomyces cerevisiae*, Journal of Biotechnology, 100. 43-53. 2003.

Chen, H.-C., Wang, S.-Y., Chen, M.-J., *Microbiological study of lactic acid bacteria in kefir grains by culture-dependent and culture-independent methods*, Food Microbiology, 25. 492-501. 2008.

Cock, L. S., de Stouvenel, A. R., *Lactic acid production by a strain of Lactococcus lactis subs lactis isolated from sugar cane plants*, Electronic Journal of Biotechnology, 9:1. 40-45. 2006.

Cogan, T. M., Barbosa, M., Beuvier, E., Bianchi-Salvadoris, B., Cocconcelli, P. S., Fernandes, I., Gomez, J., Gomez, R., Kalantzopoulos, G., Ledda, A., Medina, M., Rea, M., Rodriguez, E., *Characterization of the lactic acid bacteria in artisanal dairy products*, Journal of Dairy Research, 64. 409-421. 1997.

Cogulu, D., Topaloglu-Ak, A., Caglar, E., Sandalli, N., Karagozlu, C., Ersin, N., Yerlikaya, O., *Potential effects of a multistrain probiotic-kefir on salivary Streptococcus mutans and Lactobacillus spp.*, Journal of Dental Sciences, 5:3. 144-149. 2010.

DCCC, *Pyruvic Acid*, in Dictionary of Commonly Cited Compounds, http://dccc.chemnetbase.com/AAA00.entry?parentCHNumber=DFR44&exno=DFR44, consulted in 8 of June 2012, 2010.

De Vuyst, L., Degeest, B., *Heteropolysaccharides from lactic acid bacteria*, FEMS Microbiology Reviews, 23. 153-177. 1999.

Duboc, P., Mollet, B., *Applications of exopolysaccharides in dairy industry*, International Dairy Journal, 11. 759-768. 2001.

Dubois, M., Gilles, K. A., Hamilton, J. K., Rebers, P. A., Smith, F., *Colorimetric method for determination of dugars and related substances*, Analytical Chemistry, 28:3. 350-356. 1956.

Fanaro, S., Boehm, G., Garssen, J., Knol, J., Mosca, F., Stahl, B., Vigi, V., *Galacto-oligosaccharides and long-chain fructo-oligosaccharides as prebiotics in infant formulas: a review*, Acta Pædiatrica, 94:449. 22-26. 2005.

Farnworth, E. R., *Kefir – a complex probiotic*, Food Science and Technology Bulletin: Functional Foods, 2:1. 1-17. 2005.

Farnworth, E. R., Mainville, I., *Kefir – a fermented milk product*, in Handbook of Fermented Functional Foods, edited by Farnworth, E. R., 2nd edition, chapter 4, CRC Press, Taylor & Francis Group, Boca Raton, USA, 2008.

Fleet, G. H., *Yeasts in food and beverages: Impact on product quality and safety*, Current Opinion in Biotechnology, 18. 170-175. 2007.

Frengova, G. I., Simova, E. D., Beshkova, D. M., Simov, Z. I., *Exopolysaccharides produced by lactic acid bacteria of kefir grains*, Zeitschrift für Naturforschung, 57. 805-810. 2002.

Garrote, G. L., Abraham, A. G., De Antoni, G. L., *Chemical and microbiological characterisation of kefir grains*, Journal of Dairy Research, 68. 639-652. 2001.

Garrote, G. L., Abraham, A. G., De Antoni, G. L., *Preservation of kefir grains, a comparative study*, Lebensmittel-Wissenschaft und-Technologie., 30. 77-84. 1997.

Gassem, M. A., Schmidt, K. A., Frank, J. F., *Exopolysaccharide production from whey lactose by fermentation with Lactobacillus delbrueckii ssp. bulgaricus*, Journal of Food Science, 62:1. 171-174. 1997.

Ghasemlou, M., Khodaiyan, F., Jahanbin, K., Gharibzahedi, S. M. T., Taheri, S., *Structural investigation and response surface optimisation for improvement of kefiran production yield from a low-cost culture medium*, Food Chemistry. 133:2. 383-389. 2012.

Gobbetti, M., *The sourdough microflora: Interactions of lactic acid bacteria and yeasts*, Trends in Food Science & Technology, 9. 267-274. 1998.

Goršek, A., Tramšek, M., *Kefir grains production – An approach for volume optimization of two-stage bioreactor system*, Biochemical Engineering Journal, 42. 153-158. 2008.

Grobben, G. J., van Casteren, W. H. M., Schols, H. A., Oosterveld, A., Sala, G., Sikkema, J., de Bont, J. A. M., *Analysis of the exopolysaccharides produced by Lactobacillus delbrueckii subsp. bulgaricus NCFB 2772 grown in continuous culture on glucose and fructose*, Applied Microbiology and Biotechnology, 48. 516-521. 1997.

Güzel-Seydim, Z. B., Seydim, A. C., Greene, A. K., Bodine, A. B., *Determination of organic acids and volatile flavor substances in kefir during fermentation*, Journal of Food Composition and Analysis, 13. 35-43. 2000.

Habibi, N., Soleimanian-Zad, S., Zeinoddin, M. S., *Exopolysaccharides produced by pure culture of Lactobacillus, Lactococcus and Yeast isolated from kefir grains by microtiter plate assay: optimization and comparison*, World Applied Sciences Journal, 12:6. 742-750. 2011.

Hattner, J. A., *Digestive health: probiotics and prebiotics for children*, Nutrition Focus, University of Washington, 24:3. 1-8. 2009.

Hertzler, S. R., Clancy, S. M., *Kefir improves lactose digestion and tolerance in adults with lactose maldigestion*, Journal of The American Dietetic Association, 103:5. 582-587. 2003.

Huppertz, T., Kelly, A. L., *Properties and constituents of cow's milk*, in Milk Processing and Quality Management, edited by Tamime, A. Y., 1st edition, chapter 4, Blackwell Publishing, United Kingdom, 2009.

Irigoyen, A., Arana, I., Castiella, M., Torre, P., Ibáñez, F. C., *Microbiological, physicochemical, and sensory characteristics of kefir during storage*, Food Chemistry, 90. 613-620. 2005.

Kearney, N., Stanton, C., Desmond, C., Coakley, M., Collins, J. K., Fitzgerald, G., Ross, R. P., *Challenges Associated with the Development of Probiotic-Containing Functional Foods*, in Handbook of Fermented Functional Foods, edited by Farnworth, E. R., 2nd edition, chapter 2, CRC Press, Taylor & Francis Group, Boca Raton, USA, 2008.

Kimmel, S. A., Roberts, R. F., Ziegler, G. R., *Optimization of exopolysaccharide production by Lactobacillus delbrueckii subsp. bulgaricus RR grown in a semidefined medium*, Applied and Environmental Microbiology, 64:2. 659-664. 1998.

Kouya, T., Misawa, K., Horiuchi, M., Nakayama, E., Deguchi, H., Tanaka, T., Taniguchi, M., *Production of extracellular bifidogenic growth stimulator by anaerobic and aerobic cultivations of several propionibacterial strains*, Journal of Bioscience and Bioengineering, 103:5. 464-471. 2007.

la Rivière, J. W. M., Kooiman, P., *Kefiran, a novel polysaccharide produced in the kefir grain by Lactobacillus brevis*, Archiv für Mikrobiologie, 59. 269-278. 1967.

Latorre-García, L., del Castillo-Agudo, L., Polaina, J., *Taxonomical classification of yeasts isolated from kefir based on the sequence of their ribosomal RNA genes*, World Journal of Microbiology and Biotechnology, 23. 785-791. 2007.

Laws, A. P., Marshall, V. M., *The relevance of exopolysaccharides to the rheological properties in milk fermented with ropy strains of lactic acid bacteria*, International Dairy Journal, 11. 709-721. 2001.

Lopitz-Otsoa, F., Rementeria, A., Elquezabal, N., Garaizar, J., *Kefir: A symbiotic yeasts-bacteria community with alleged healthy capabilities*, Revista Iberoamericana de Micologia, 23. 67-74. 2006.

Madigan, M. T., Mortinka, J. M., Dunlap, P. V., Clark, D. P., *Brock biology of microorganisms*, 12nd edition, chapter 6 and 21, Pearson Benjamin Cummings, San Francisco, USA, 2009.

Magalhães, R., Vicente, C., *Apontamentos da Unidade Curricular Métodos Numéricos e Estatísticos*, Instituto de Ciências Biomédicas Abel Salazar, Intern Publication, 2008.

Marshall, V. M., Cole, W. M., Brooker, B. E., *Observations on the structure of kefir grains and the distribution of the microflora*, Journal of Applied Bacteriology, 57. 491-497. 1984.

Meireles, A.C., Ferreira, C., Bastos, M., Melo, L. M., Afonso, I. M., *Characterisation of microflora present in kefir and analysis of its potential for production of added-value metabolites*, in Book of Abstracts of the International Congress on Promotion of Traditional Food Products, Ponte de Lima, Portugal. 2012.

MetaCyc, *Heterolactic fermentation*, in http://ecocyc.org/META/NEW-IMAGE?type=PATHWAY&object=P122-PWY&detail-level=0&detail-level=1, consulted at 2 of March of 2012, 2007a.

MetaCyc, *Homolactic fermentation*, in http://metacyc.org/META/NEW-IMAGE?type=PATHWAY&object=ANAEROFRUCAT-PWY&detail-level=0, consulted at 2 of March of 2012, 2007b.

MetaCyc, *Mixed acid fermentation*, in http://biocyc.org/META/NEW-IMAGE?type=PATHWAY&object=FERMENTATION-PWY&detail-level=1, consulted at 2 of March of 2012, 2010.

Micheli, L., Uccelletti, D., Palleschi, C., Crescenzi, V., *Isolation and characterisation of a ropy Lactobacillus strain producing the exopolysaccharide kefiran*, Applied Microbiology and Biotechnology, 53. 69-74. 1999.

Miguel, M. G. C. P., Cardoso, P. G., Lago, L. A., Schwan, R. F., *Diversity of bacteria present in milk kefir grains using culture-dependent and culture-independent Methods*, Food Research International, 43. 1523-1528. 2010.

Miller, G. L., *Use of Dinitrosalicylic acid reagent for determination of reducing sugar*, Analytical Chemistry, 31. 426-428. 1959.

Mozzi, F., Vaningelgem, F., Hébert, E. M., Van der Meulen, R., Moreno, M. R. F., de Valdez, G. F., De Vuyst, L., *Diversity of heteropolysaccharide-producing lactic acid bacterium strains and their biopolymers*, Applied and Environmental Microbiology, 72:6. 4431-4435. 2006.

Mullin, W. J., Emmons, D. B., *Determination of organic acids and sugars in cheese, milk and whey by high performance liquid chromatography*, Food Research International, 30:2. 147-151. 1997.

Neri, D. F. M., Balcão, V. M., Costa, R. S., Rocha, I. C. A. P., Ferreira, E. M. F. C., Torres, D. P. M., Rodrigues, L. R. M., Carvalho Jr., L. B., Teixeira, J. A., *Galacto-oligosaccharides production during lactose hydrolysis by free Aspergillus oryzae β-galactosidase and immobilized on magnetic polysiloxane-polyvinyl alcohol*, Food Chemistry, 115. 92-99. 2009.

Nielsen, P. V., Packaging, *Quality Control, and Sanitation of Bakery Products*, in Handbook of Food and Beverage Fermentation Technology, edited by Hui, Y. H., Meunier-Goddik, L., Solvejg Hansen, Å. S., Josephsen, J., Nip, W.-K., Stanfield, P. S., Toldrá, F., 1st edition, chapter 43, Marcel Dekker, Inc., New York, USA, 2004.

Otles, S., Cagindi, O*., Kefir: A Probiotic dairy-composition nutritional and therapeutic aspects*, Pakistan Journal of Nutrition, 2:2. 54-59. 2003.

Petry S., Furlan, S., Crepeau, M.-J., Cerning, J., Desmazeaud, M., *Factors affecting exocellular polysaccharide production by Lactobacillus delbrueckii subsp. bulgaricus grown in a chemically defined medium*, Applied and Environmental Microbiology, 66:8. 3427-3431. 2000.

Piermaria, J. A., de la Canal, M. L., Abraham, A. G., *Gelling properties of kefiran, a food-grade polysaccharide obtained from kefir grain*, Food Hydrocolloids, 22. 1520-1527. 2008.

Rea, M. C., Lennartsson, T., Dilon, P., Drinan, F. D., Reville, W. J., Heapes, M., Cogan, T. M., *Irish kefir-like grains: their structure, microbial composition and fermentation kinetics*, Journal of Applied Bacteriology, 81. 83-94. 1996.

Rimada, P. S., Abraham, A. G., *Polysaccharide production by kefir grains during Whey fermentation*, Journal of Dairy Research, 68. 653-661. 2001.

Rodrigues, K. L., Caputo, L. R. G., Carvalho, J. C. T., Evangelista, J., Schneedorf, J. M., *Antimicrobial and healing activity of kefir and kefiran extract*, International Journal of Antimicrobial Agents, 25. 404-408. 2005.

Sako, T., Matsumoto, K., Tanaka,R., *Recent progress on research and applications of non-digestible galacto-oligosaccharides*, International Dairy Journal. 9. 69-80. 1999.

Sarkar, S., Probiotics, *Prebiotics and synbiotics for infant feeding – a review*, Journal of Microbial & Biochemical Technology, S1:004. 1-9. 2011.

Sauer, M., Porro, D., Mattanovich, D., Branduardi, P., *Microbial production of organic acids: expanding the markets*, Trends in Biotechnology, 26:2. 100-108. 2008.

Scopes, R., *Protein purification: principles and practice*, 3rd edition, chapter 1 and 4, Springer, USA, 1994.

Simões, L. C., Lemos, M., Araújo, P., Pereira, A. M., Simões, M., *The effect of glutaraldehyde on the control of single and dual biofilms of Bacillus cereus and Pseudomonas fluorescens*, Biofouling, 27:3. 337-346. 2011.

Simova, E., Beshkova, D., Angelov, A., Hristozova, T. Frengova, G., Spasov, Z., *Lactic acid bacteria and yeasts in kefir grains and kefir made from them*, Journal of Industrial Microbiology & Biotechnology, 28. 1-6. 2002.

Siritat, D., Jelena, P., *Bacterial inhibition and antioxidant activity of kefir produced from Thai Jamine rice milk*, Biotechnology, 9:3. 332-337. 2010.

Splechtna, B., Nguyen, T., Steinböck, M., Kulbe, K. D., Lorenz, W., Haltrich, D., *Production of prebiotic galacto-oligosaccharides from lactose using β-galactosidases from Lactobacillus reuteri*, Journal of Agricultural and Food Chemistry, 54. 4999-5006. 2006.

Sumner, J. B., *Dinitrosalicylic acid: a reagent for the estimation of sugar in normal and diabetic urine*, The Journal of Biological Chemistry, 47. 5-9. 1921.

Taniguchi, M., Nomura, M., Itaya, T., Tanaka, T., *Kefiran production by Lactobacillus kefiranofaciens under the culture conditions established by mimicking the existence and activities of yeast in kefir grains*, Food Science and Technology Research, 7:4. 333-337. 2001.

Theron, M. M., Lues, J. F. R., *Organic acids and food preservation*, 1st edition, chapter 1 and 2, CRC Press, Taylor & Francis Group, Boca Raton, USA, 2011.

Turpin, W., Humblot, C., Thomas, M., Guyot, J.-P., *Lactobacilli as multifaceted probiotics with poorly disclosed molecular mechanisms*, International Journal of Food Microbiology, 143. 87-102. 2010.

Urdaneta, E., Barrenetxe, J., Aranguren, P., Irigoyen, A., Marzo, F., Ibáñez, F. C., *Intestinal beneficial effects of kefir-supplemented diet in rats*, Nutrition Research, 27. 653-658. 2007.

van den Tempel, T., Jakobsen, M., *Yeasts associated with Danablu*, International Dairy Journal, 8. 25-31. 1998.

Viljoen, B. C., *The interaction between yeasts and bacteria in dairy environments*, International Journal of Food Microbiology, 69. 37-44. 2001.

Walstra, P., Wouters, J. T. M., Geurts, T. J., *Dairy Science and Technology*, 2nd edition, chapter 2, CRC Press, Taylor & Francis Group, New York, USA, 2006.

Welman, A. D., Maddox, I. S., *Exopolysaccharides from lactic acid bacteria: perspectives and challenges*, Trends in Biotechnology, 21:6. 269-274. 2003.

Witthuhn, R. C., Schoeman, T., Britz, T. J., *Characterisation of the microbial population at different stages of kefir production and kefir grain mass cultivation*, International Dairy Journal, 15. 383-389. 2005b.

Witthuhn, R. C., Schoeman, T., Cilliers, A., Britz, T. J., *Impact of preservation and different packaging conditions on the microbial community and activity of kefir grains*, Food Microbiology, 22. 337-344. 2005a.

Xie, N., Zhou, T., Li, B., *Kefir yeasts enhance probiotic potentials of Lactobacillus paracasei H9: The positive effects of coaggregation between the two strains*, Food Research International, 45. 394-401. 2012.

Yaman, H., Elmali, M., Karadagoglu, G., Cetinkaya, A., *Observations of kefir grains and their structure from different geographical regions: Turkey and Germany*, Atatürk Üniversitesi Veteriner Bilimleri Dergisi, 1:1-2. 11-15. 2006.

Yang, Z., Huttunen, E., Staaf, M., Widmalm, G., Tenhu, H., *Separation, purification and characterisation of extracellular polysaccharides produced by slime-forming Lactococcus lactis ssp. cremoris strains*, International Dairy Journal, 9. 631-638. 1999.

Yuksekdag, Z. N., Aslim, B., *Influence of different carbon sources on exopolysaccharide production by Lactobacillus delbruechii subsp. bulgaricus (B3, G12) and Streptococcus thermophiles (W22)*, Brazilian Arquives of Biology and Technology, 51:3. 581-585. 2008.

Zeppa, G., Conterno, L., Gerbi, V., *Determination of organic acids, sugars, diacetyl, and acetoin in cheese by High-Performance Liquid Chromatography*, Journal of Agriculture and Food Chemistry, 49. 2722-2726. 2001.

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

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 ($C_1/C_2/C_3$) [7] ..... 10.0/20.0/100.0 [8]

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.

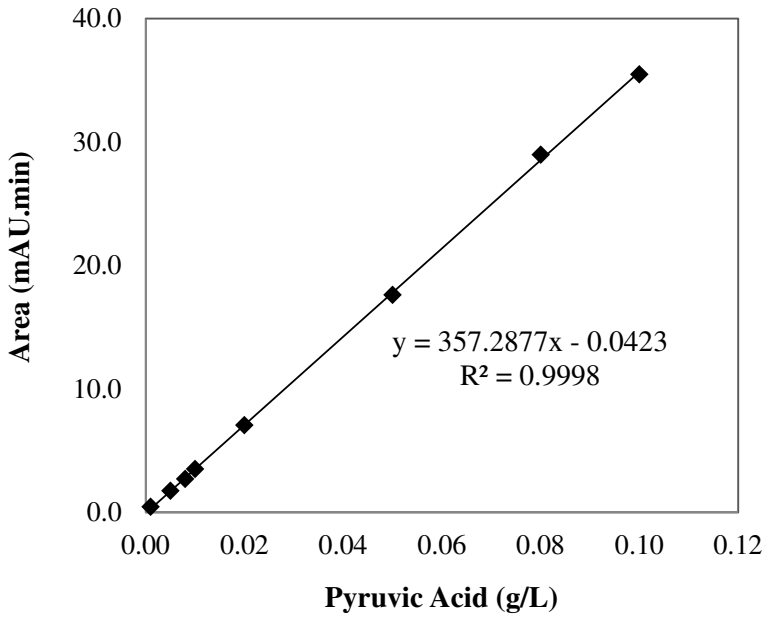


Figure A.1 – Calibration curve for pyruvic acid.

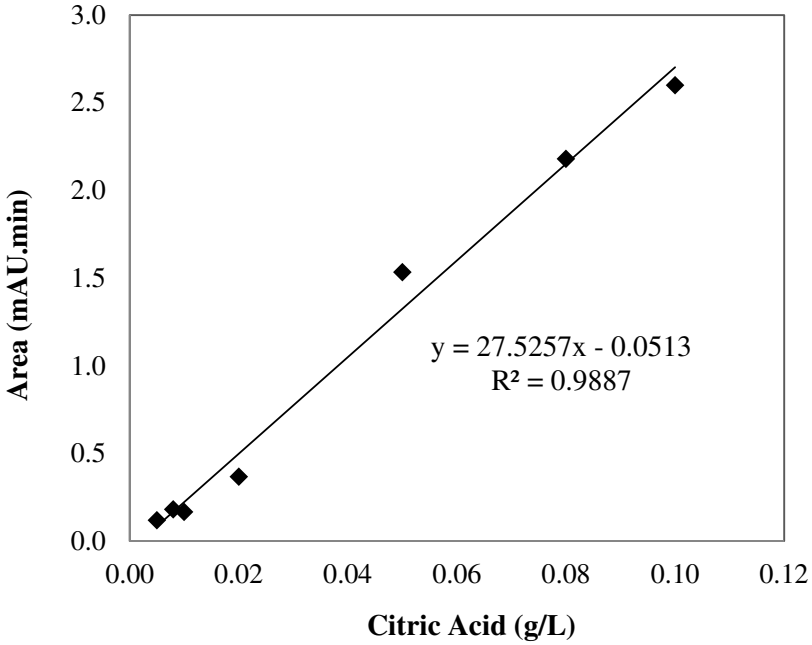


Figure A.2 – Calibration curve for citric acid.

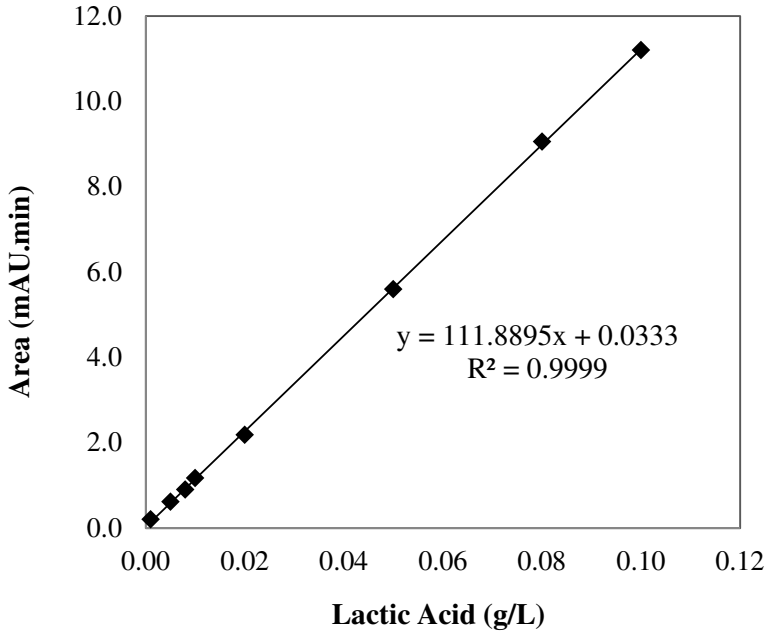


Figure A.3 – Calibration curve for lactic acid.

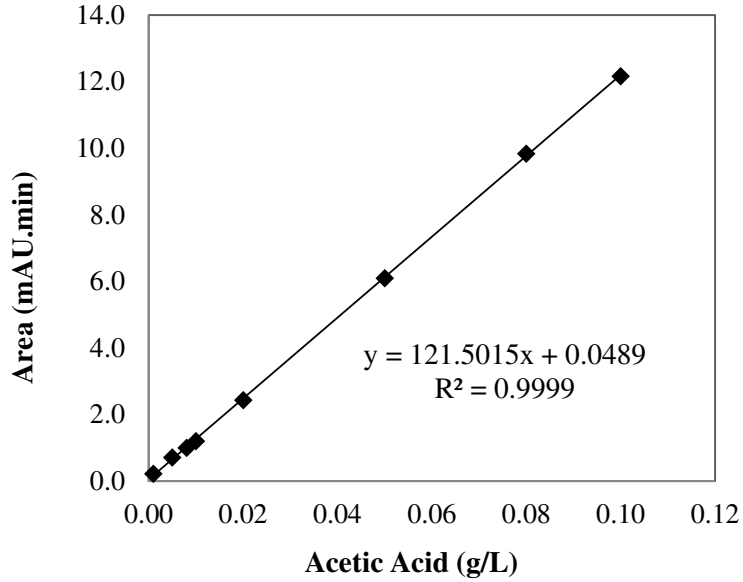


Figure A.4 – Calibration curve for acetic acid.

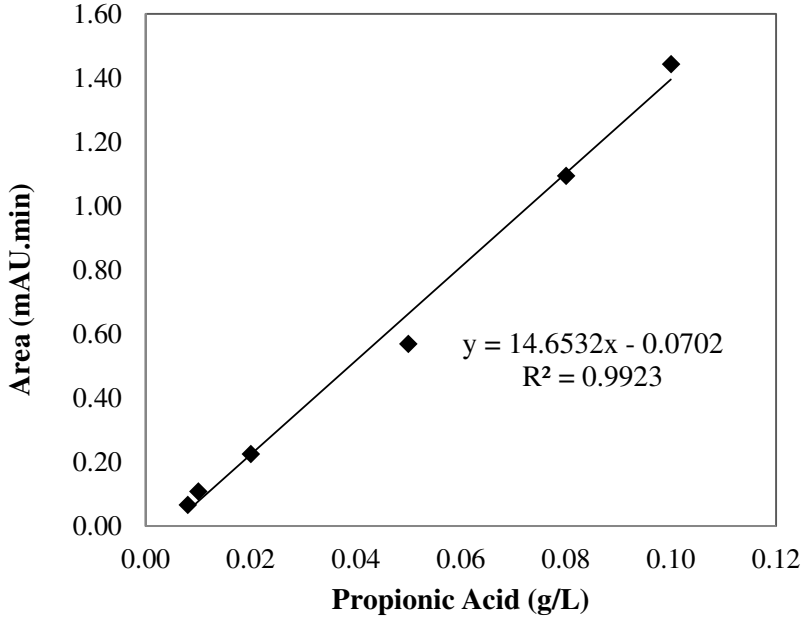


Figure A.5 – Calibration curve for propionic acid.

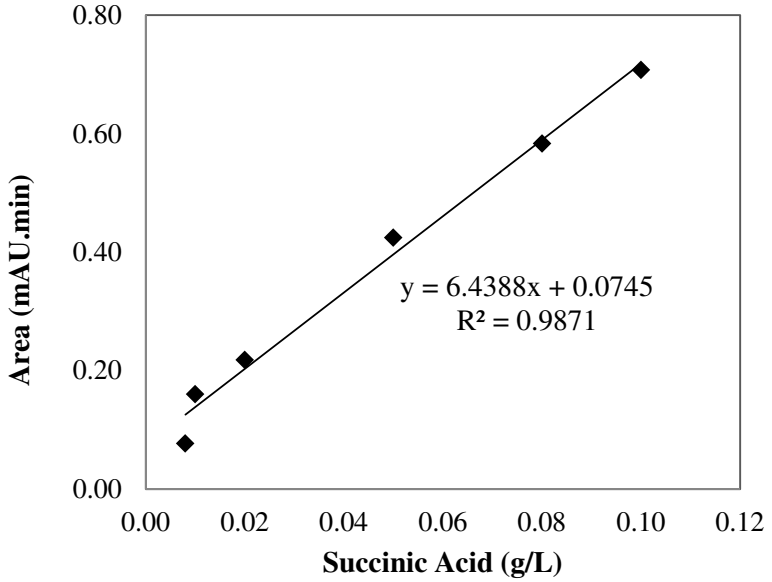


Figure A.6 – Calibration curve for succinic acid smaller concentrations.

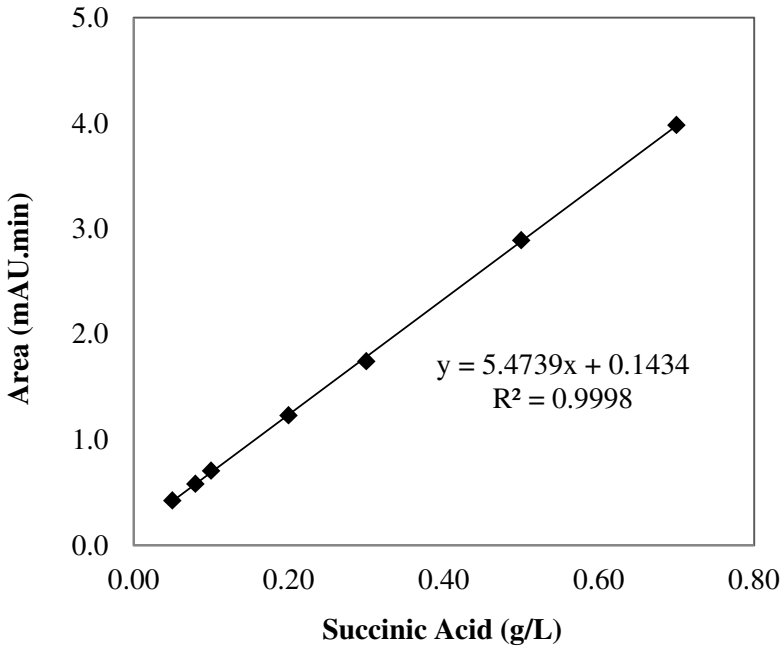


Figure A.7 – Calibration curve for succinic acid higher concentrations.

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.

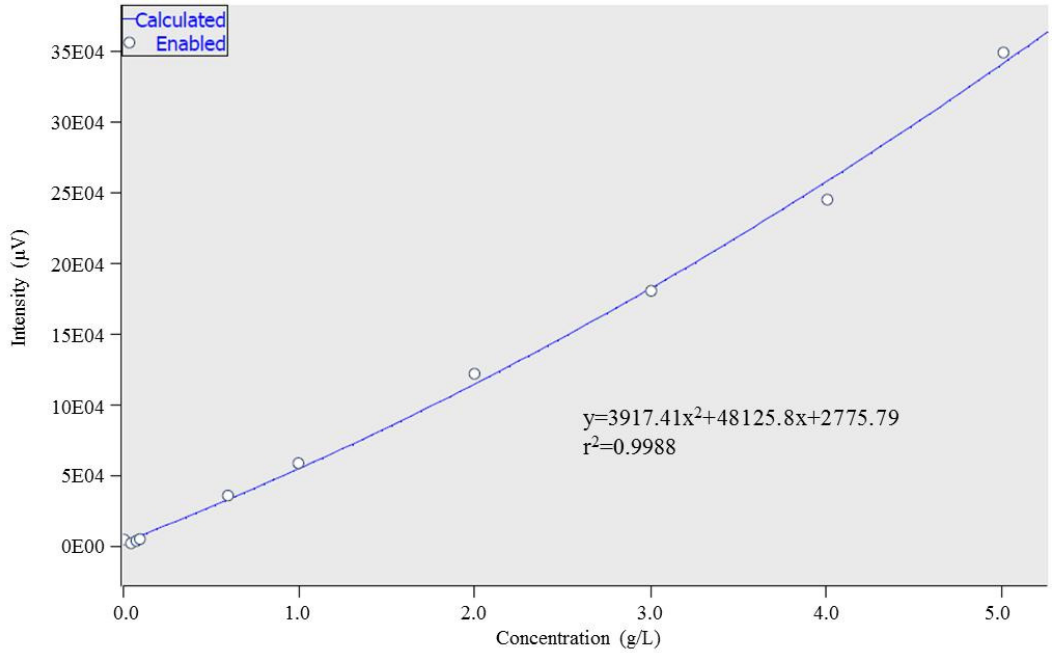


Figure A.8 – Calibration curve for lactose.

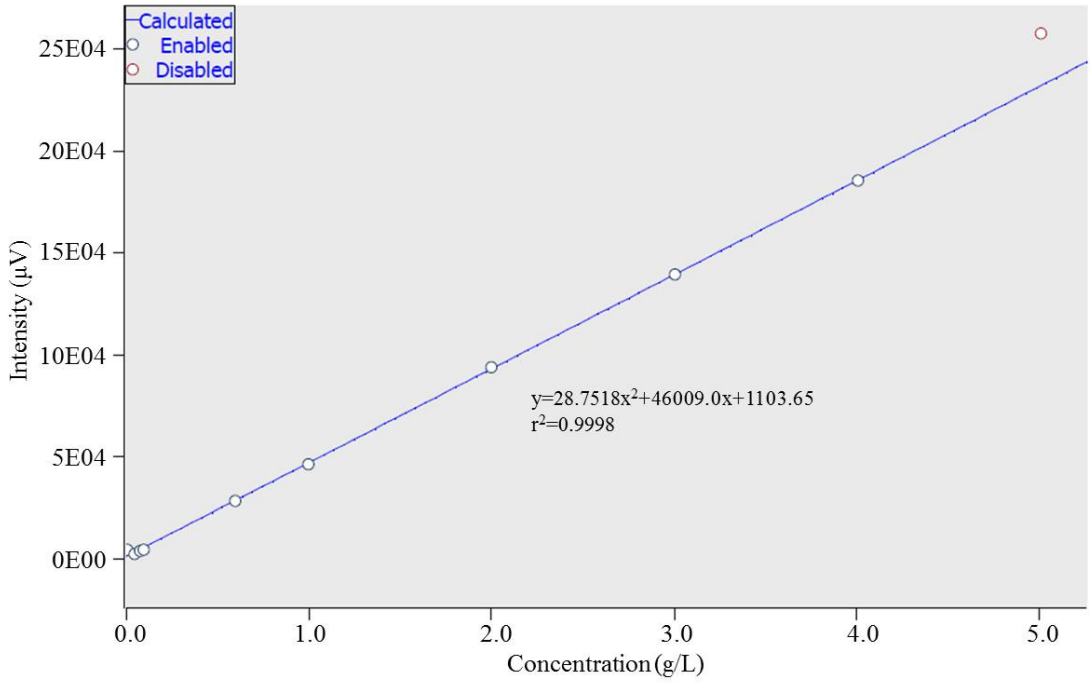


Figure A.9 – Calibration curve for glucose.

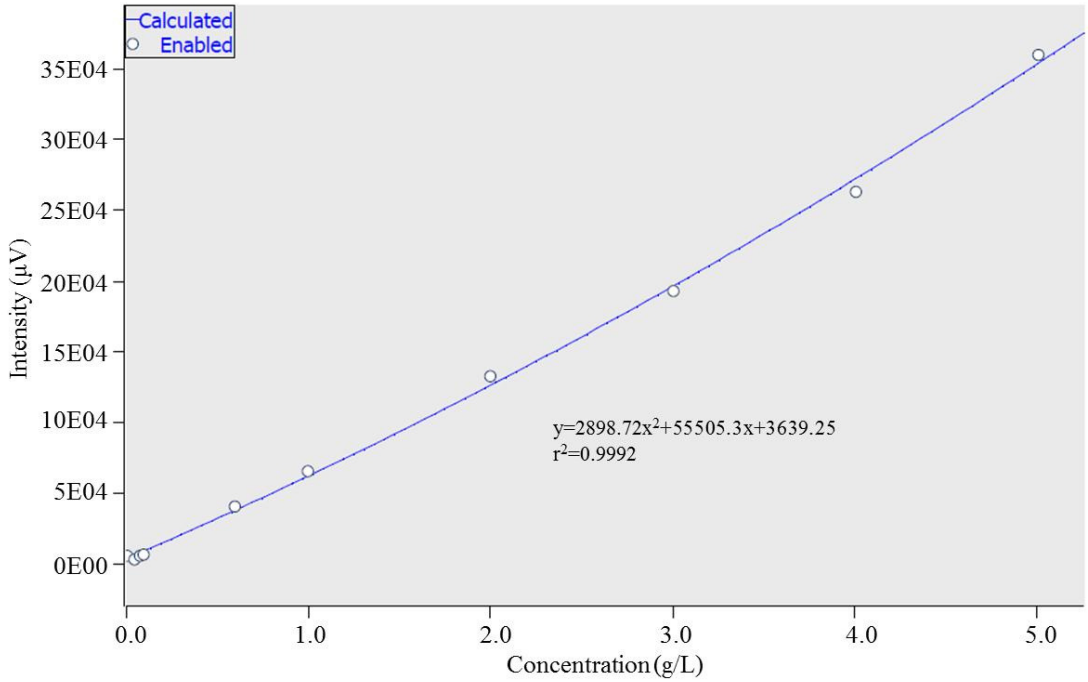


Figure A.10 – Calibration curve for galactose.

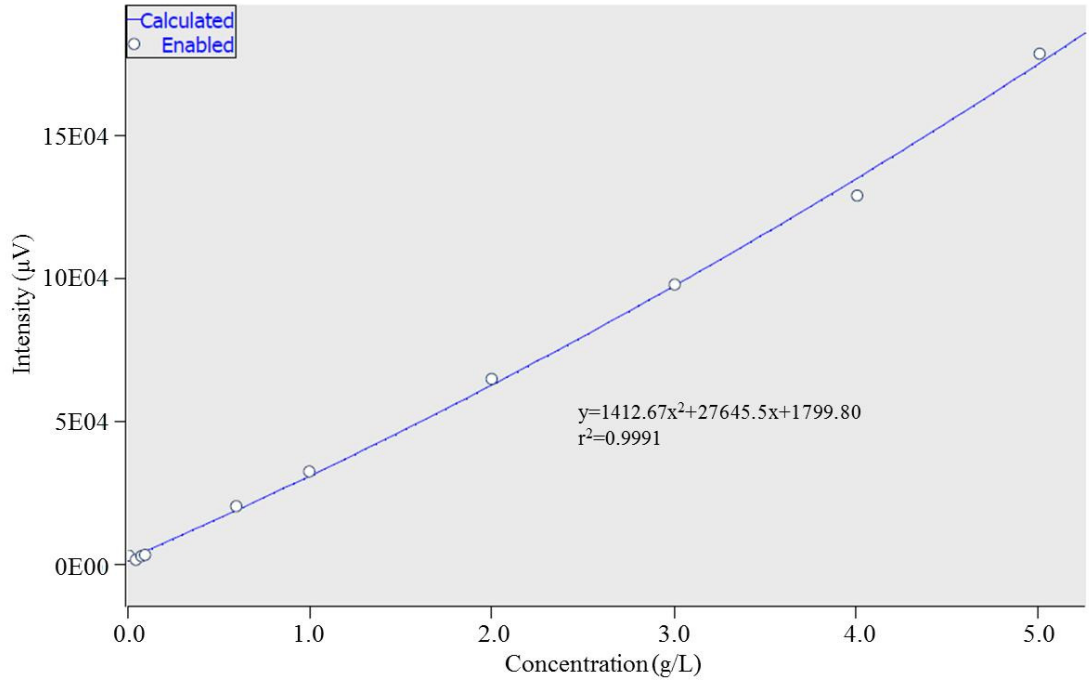


Figure A.11 – Calibration curve for rhamnose.

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.

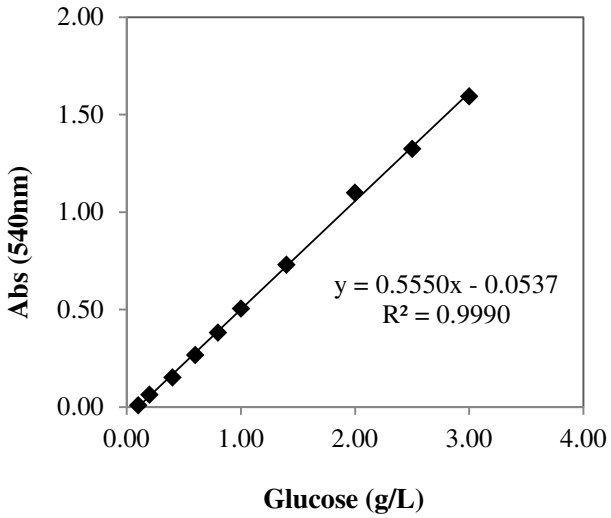


Figure A.12 – Calibration curve for glucose.

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.

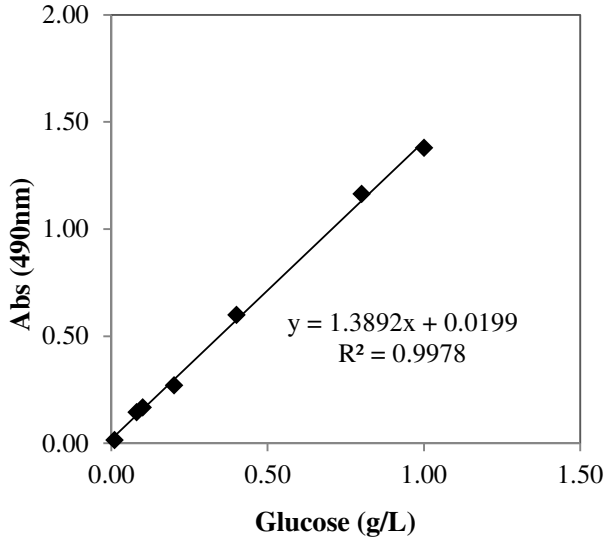


Figure A.13 – Calibration curve for glucose.

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.

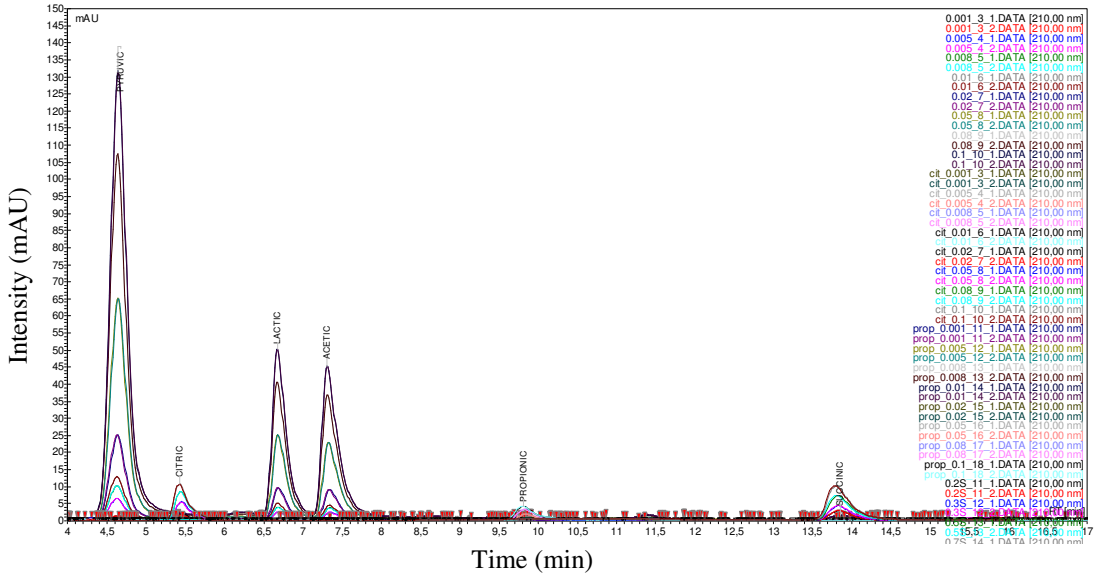


Figure A.14 – Chromatogram obtained for all the organic acids standards.

A.4.2. Carbohydrates Standards

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

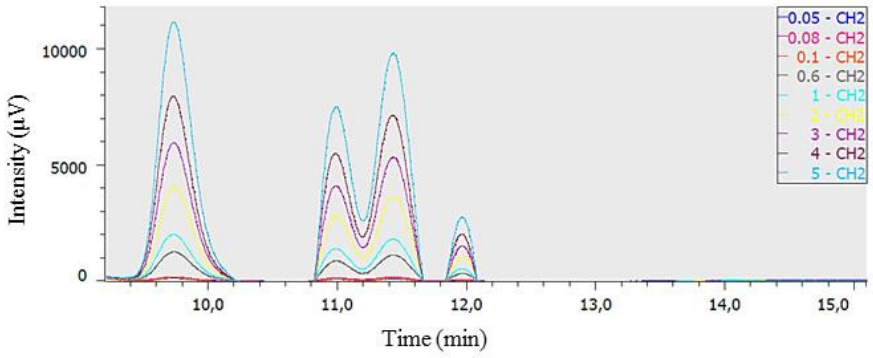


Figure A.15 – Chromatogram obtained for the carbohydrates standards.

A.4.3. Organic Acids Samples

The chromatograms obtained for the samples are shown below.

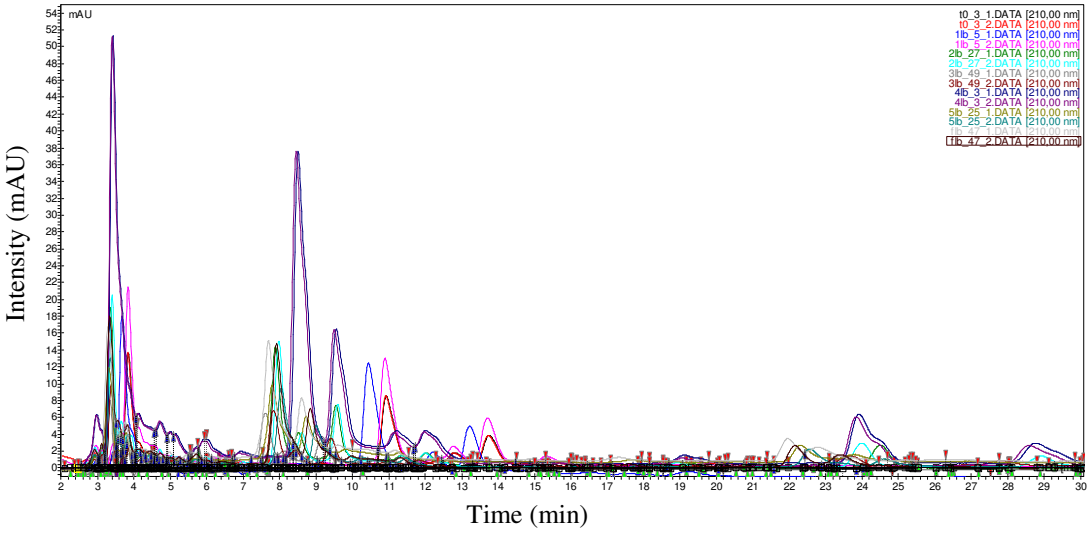


Figure A.16 – Chromatogram obtained for *L. delbrueckii* subsp. *bulgaricus*, for the organic acids.

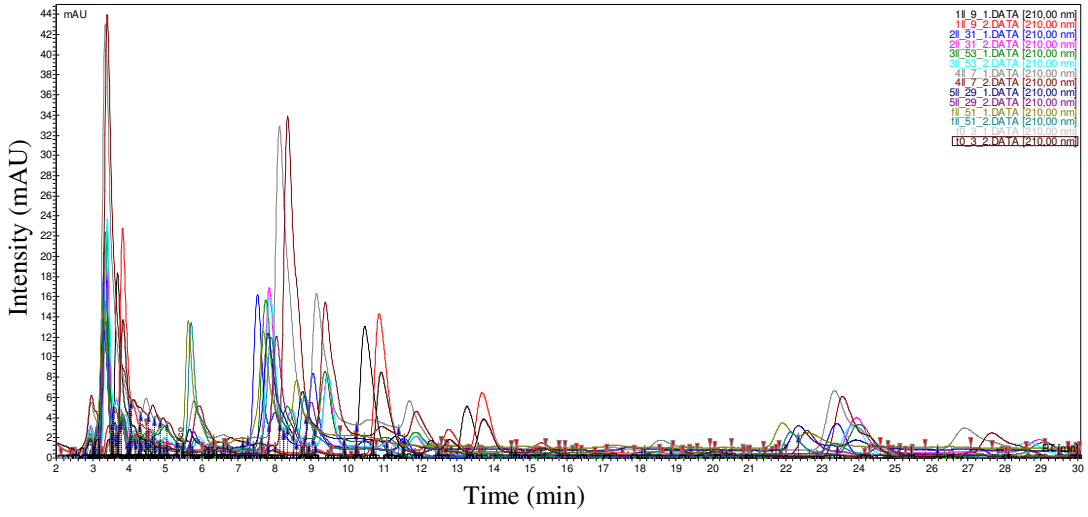


Figure A.17 – Chromatogram obtained for *L. lactis* subsp. *lactis*, for the organic acids.

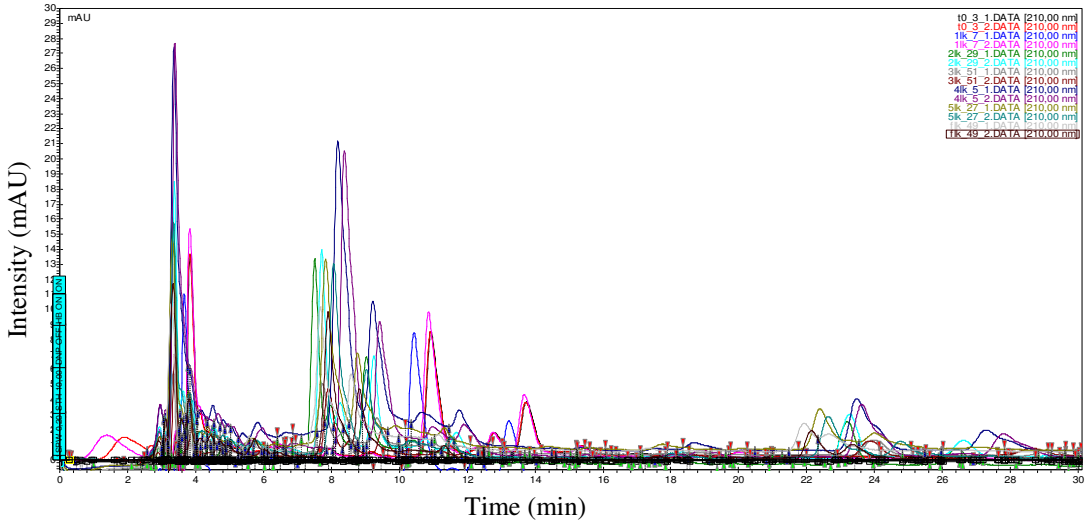


Figure A.18 – Chromatogram obtained for *L. kefir*, for the organic acids.

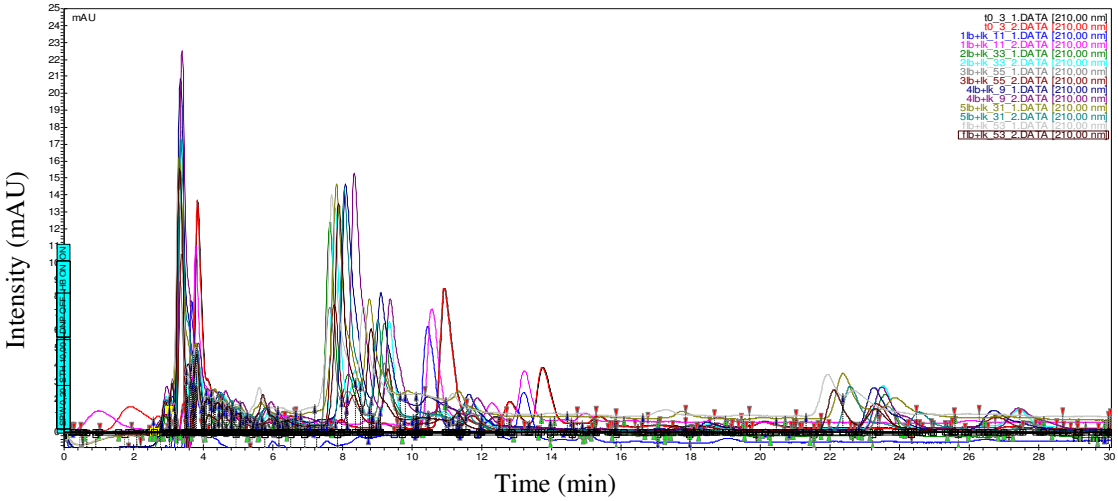


Figure A.19 – Chromatogram obtained for *L. delbrueckii* subsp. *bulgaricus* + *L. kefir*, for the organic acids.

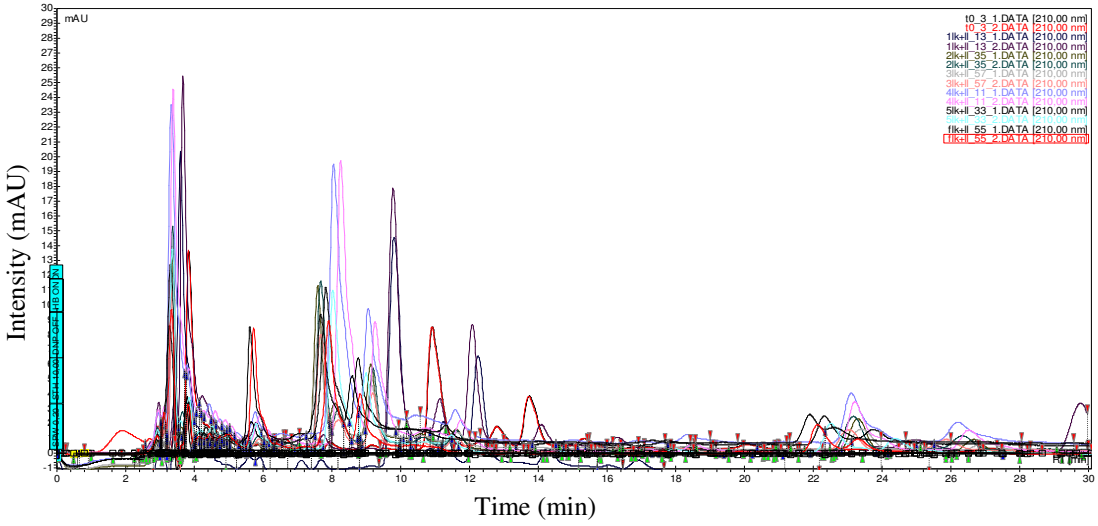


Figure A.20 – Chromatogram obtained for *L. kefir* + *L. lactis* subsp. *lactis*, for the organic acids.

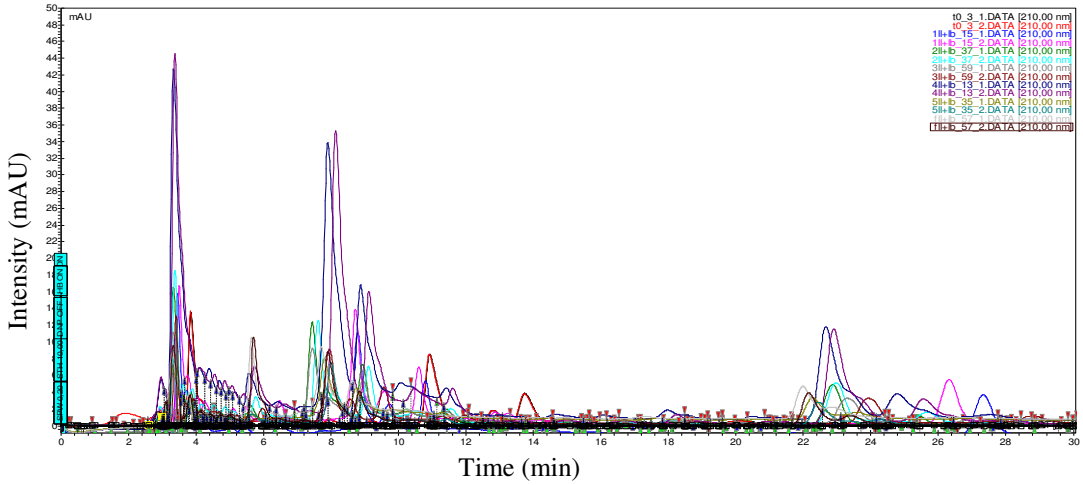


Figure A.21 – Chromatogram obtained for *L. lactis* subsp. *lactis* +*L. delbrueckii* subsp. *bulgaricus*, for the organic acids.

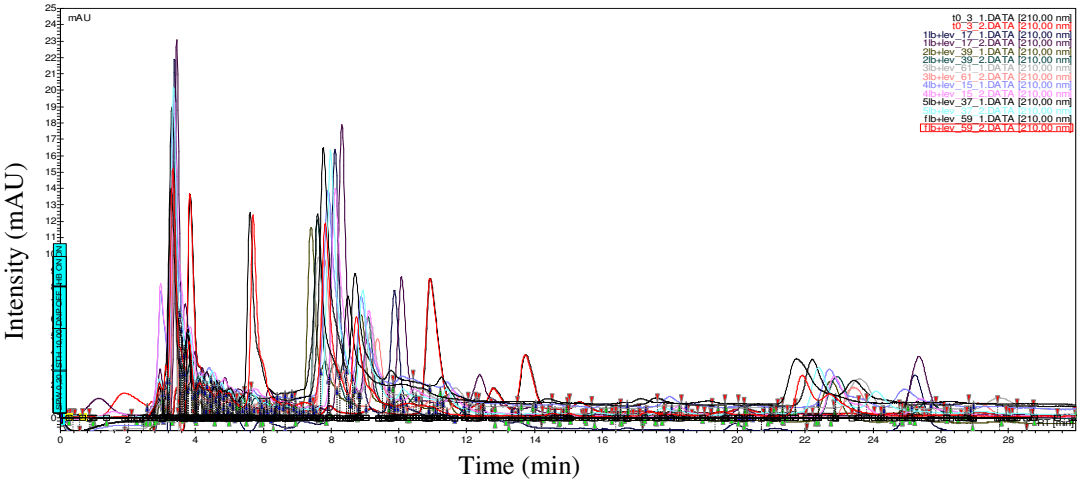


Figure A.22 – Chromatogram obtained for *L. delbrueckii* subsp. *bulgaricus* + yeasts, for the organic acids.

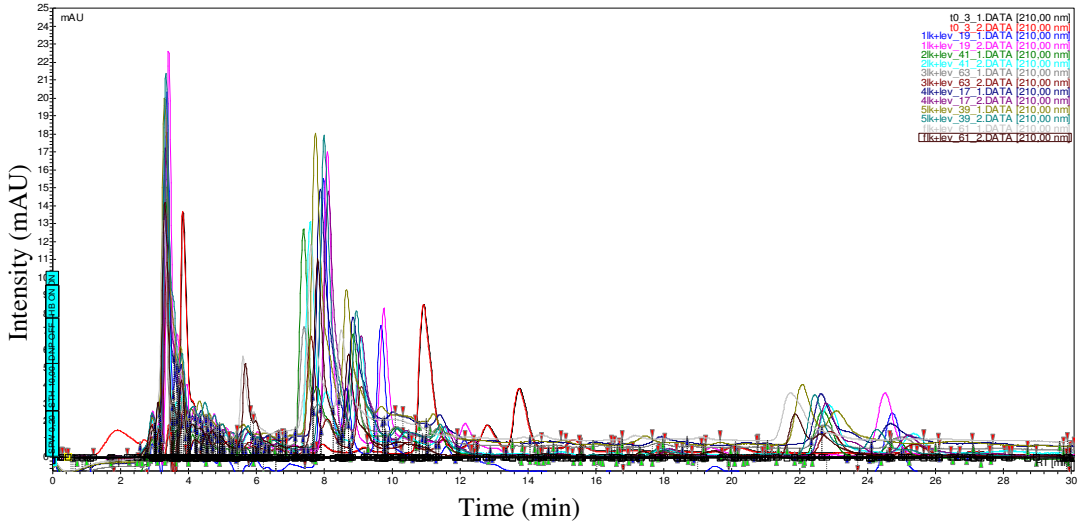


Figure A.23 – Chromatogram obtained for *L. kefir* + yeasts, for the organic acids.

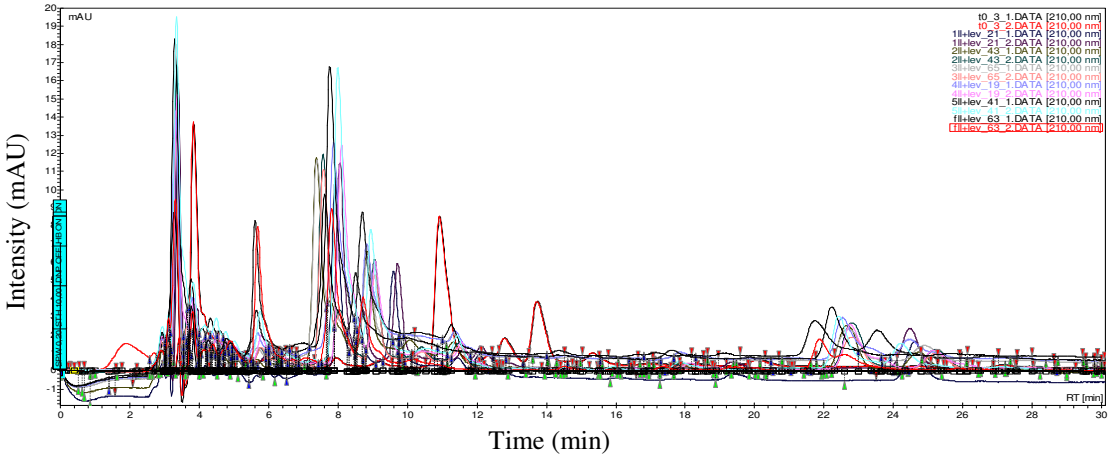


Figure A.24 – Chromatogram obtained for *L. lactis* subsp. *lactis* + yeasts, for the organic acids.

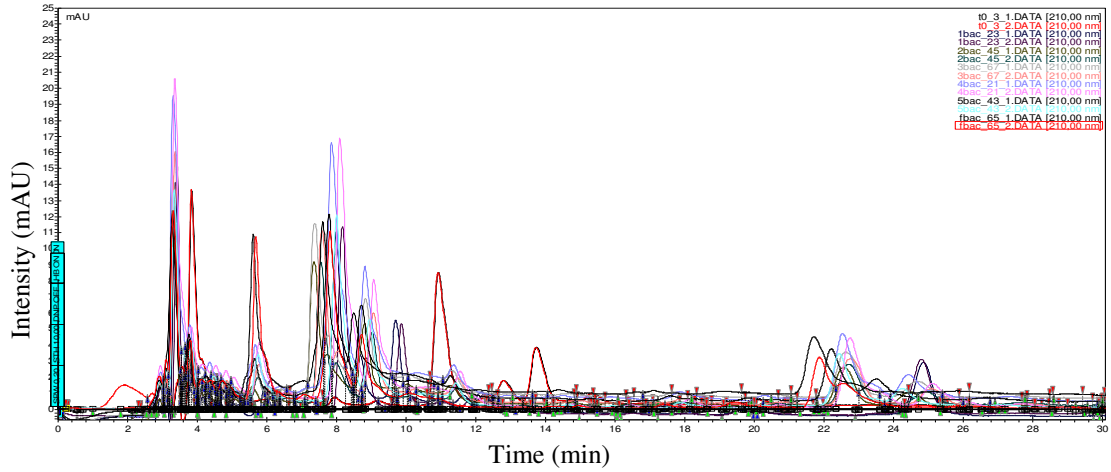


Figure A.25 – Chromatogram obtained for all the bacteria, for the organic acids.

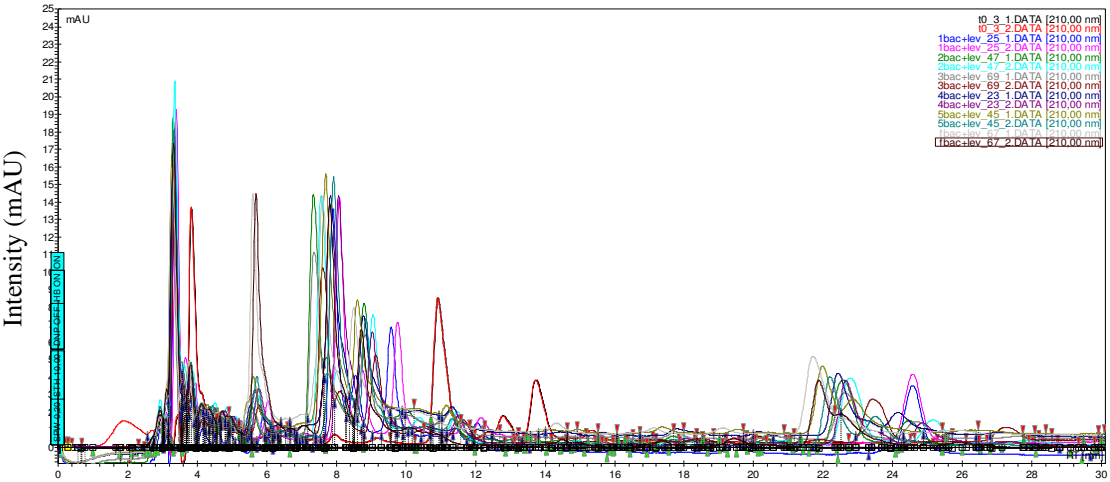


Figure A.26 – Chromatogram obtained for all the bacteria + yeasts, for the organic acids.

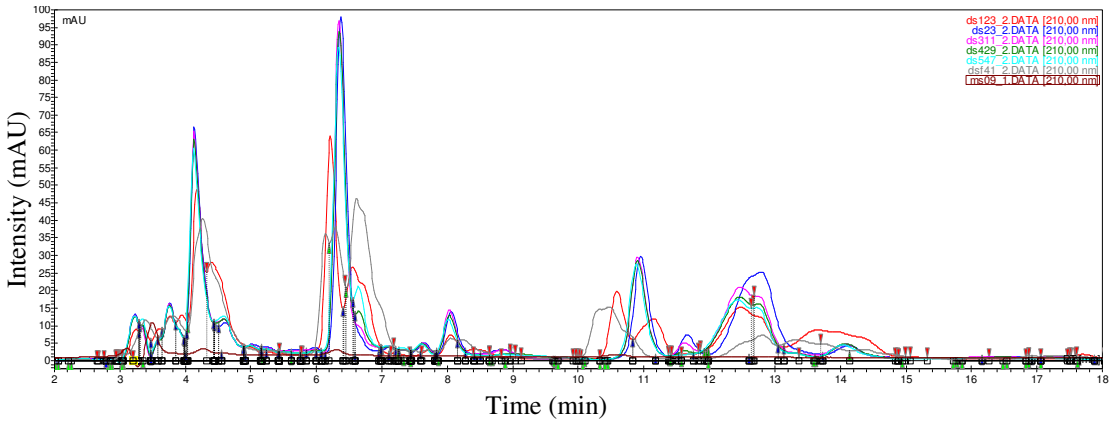


Figure A.27 – Chromatogram obtained for *C. krusei*, for the organic acids.

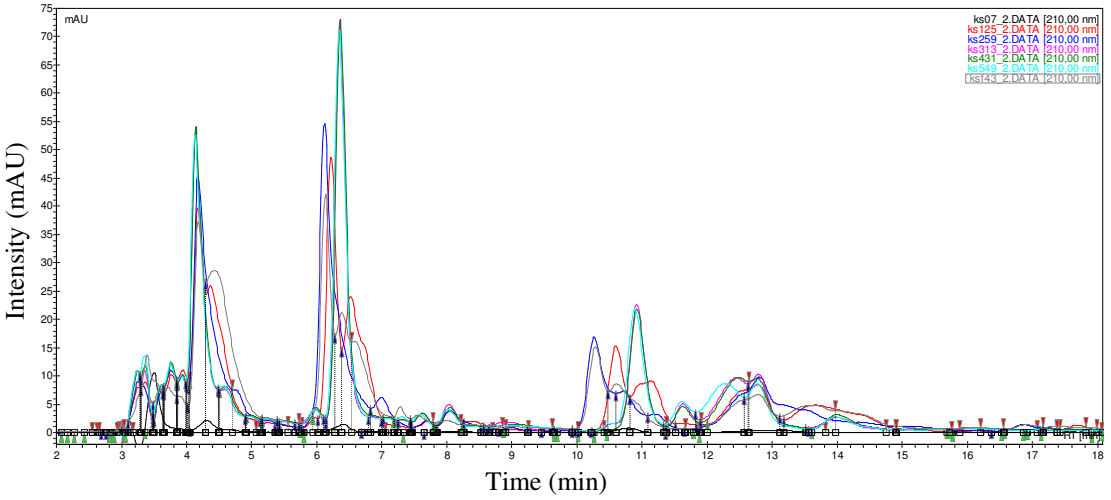


Figure A.28 – Chromatogram obtained for *C. famata*, for the organic acids.

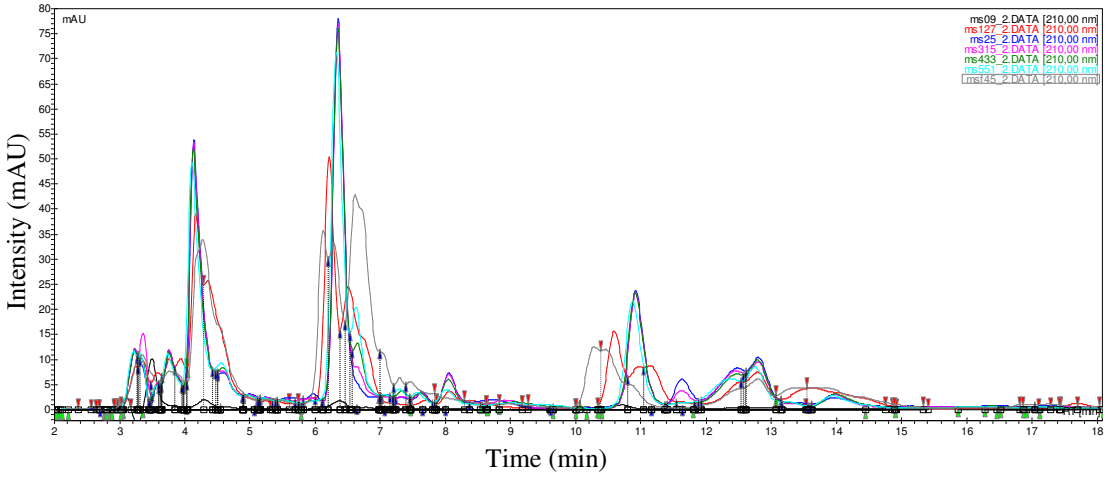


Figure A.29 – Chromatogram obtained for *C. krusei* + *C. famata*, for the organic acids.

A.4.4. Carbohydrates Samples

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

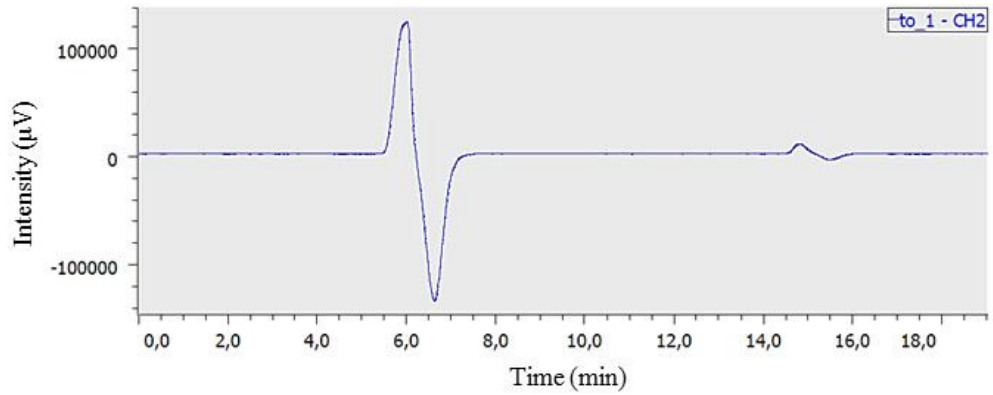


Figure A.30 – Chromatogram obtained for the time zero, for the carbohydrates.

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;

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

$\overline{X}$ - average value that is intended to be verified;

μ - average value, that is used as comparison with $\overline{X}$;

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 > α, accept the null hypothesis (the samples are not significantly different);
   b. If P < α, reject the null hypothesis (the samples are significantly different).