Universidade de Aveiro Departamento de Química
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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biotecnologia, no Ramo de Biotecnologia Alimentar, realizada sob a orientação científica da Doutora Ana Maria Rebelo Barreto Xavier, Professora Auxiliar do Departamento de Química da Universidade de Aveiro e da Doutora Luísa Seuanes Serafim, Professora Auxiliar do Departamento de Química da Universidade de Aveiro.

Dedico este trabalho à minha mãe.

o júri

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agradecimentos

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palavras-chave

Kefir, grãos de kefir, kefirano, exopolissacarídeo, *Lactobacillus kefiranofaciens*, soro de leite.

resumo

O kefir é uma bebida láctea fermentada com propriedades sensoriais únicas e valores nutricionais e terapêuticos elevados. Atualmente, tem havido um aumento tanto no seu consumo como na sua produção em vários países. No entanto, em Portugal, só recentemente é que esta bebida começou a ser fabricada industrialmente. Um estudo detalhado da sua produção deve ser feito com o intuito de ajudar a expandir a implementação do processo industrial de kefir no nosso país. Adicionalmente, o kefirano é um exopolissacarídeo (EPS) de valor acrescentado produzido durante o fabrico de kefir, devido essencialmente à presença de *Lactobacillus kefiranofaciens* nos grãos de kefir, podendo este ser de interesse comercial, uma vez que poderá ter várias aplicações médicas e alimentares. Para produzir kefirano, o soro de leite, subproduto da indústria leiteira, que constitui um problema ambiental significativo devido aos seus elevados volumes produzidos e ao seu alto teor em matéria orgânica, poderá ser usado como fonte de carbono de baixo custo já que contem uma elevada quantidade de lactose.

Neste trabalho, foram estudadas várias condições de fermentação para a produção de kefir através dos grãos usando leite UHT e comparadas no que diz respeito ao pH e às concentrações de lactose, ácido láctico e etanol: concentração inicial dos grãos de kefir (3, 6 e 9% (m/v)), volume inicial de leite (100 e 200 mL), velocidade de agitação (0, 60 e 180 rpm) e temperatura (controlada, 28 ºC, e ambiente, 21-25 ºC). As melhores condições determinadas para a produção de kefir foram também avaliadas usando uma amostra de leite pasteurizado de vaca fornecida pela empresa Lacto Serra.

As melhores condições foram alcançadas com uma concentração inicial de grãos de kefir de 9% (m/v), volume inicial de leite de 200 mL, temperatura controlada de 28 ºC e sem agitação, permitindo a produção desta bebida em aproximadamente 8 h, com ambas as amostras de leite usadas.

Diferentes meios de cultura foram testados e comparados para a produção de kefirano utilizando grãos de kefir e uma cultura pura de *Lactobacillus kefiranofaciens*: meio MRS, amostras de leite UHT e pasteurizado, amostras de soro de leite de vaca (concentrado e pasteurizado) e de ovelha (cru). No final, realizou-se a extração, purificação e quantificação do kefirano.

A produção de kefirano foi a maior quando o meio MRS foi fermentado com grãos de kefir durante 96 h, resultando numa concentração de 1.69 g/L. No entanto, o melhor resultado usando uma amostra de soro de leite foi obtido quando o soro de leite de vaca (concentrado e pasteurizado) foi fermentado através de cultura pura de *Lactobacillus kefiranofaciens* durante 148.5 h, traduzindo-se em 1.16 g/L de kefirano.

Este estudo permitiu mostrar que uma fonte de carbono de baixo custo como o soro de leite pode efetivamente ser usada para produzir um exopolissacarídeo de valor acrescentado como o kefirano. Embora os valores de kefirano obtidos pareçam promissores, muito trabalho pode ainda ser feito no que diz respeito à otimização da produção de kefirano.

keywords

Kefir, kefir grains, kefiran, exopolysaccharide, *Lactobacillus kefiranofaciens*, cheese whey.

abstract

Kefir is a fermented milk beverage with unique sensory properties and high nutritional and therapeutic values. Currently, there is an increase in its consumption and manufacture in many countries, however, in Portugal, only recently this beverage has started to be produced industrially. Thus, a detailed study of kefir production should be done to help expanding the implementation of kefir industrial process in our country. Additionally, kefiran is a value-added exopolysaccharide (EPS) produced during kefir manufacture, mainly due to the presence of *Lactobacillus kefiranofaciens* in kefir grains, which could be of commercial interest, since it may have several food and medical applications. To produce kefiran, cheese whey, a by-product from dairy industry, which represents a significant environmental issue due to its high volumes produced and high organic matter content, could be used as a low-cost carbon source as it contains high amount of lactose.

In this work, several fermentation conditions were studied for kefir production by kefir grains using UHT milk, and compared concerning pH values, lactose, lactic acid, and ethanol concentrations: initial kefir grains concentration (3, 6 and 9% (w/v)), initial milk volume (100 and 200 mL), agitation rate (0, 60 and 180 rpm) and temperature (controlled, 28 ºC, and room temperature, 21-25 ºC). The best conditions determined for kefir production were also assessed using a pasteurized cow milk sample provided by Lacto Serra company.

The best conditions were achieved with 9% (w/v) of initial kefir grains concentration, 200 mL of initial milk volume, controlled temperature of 28 ºC and without agitation, allowing the production of this beverage in approximately 8 h, with both cow milk samples.

Moreover, different culture media were tested and compared for kefiran production by kefir grains and by pure culture of *Lactobacillus kefiranofaciens*: MRS broth medium, UHT and pasteurized cow milk samples, cow cheese whey sample (concentrated and pasteurized) and sheep cheese whey sample (raw). In the end, kefiran extraction, purification and quantification was performed.

Kefiran production was the highest when MRS broth medium was fermented by kefir grains during 96 h, resulting in a concentration of 1.69 g/L. Nevertheless, the best result using a cheese whey sample was obtained when cow cheese whey sample (concentrated and pasteurized) was fermented by pure culture of *Lactobacillus kefiranofaciens* during 148.5 h, providing 1.16 g/L of kefiran.

This study showed that a low-cost carbon source like cheese whey can effectively be used to produce a value-added exopolysaccharide like kefiran. Although kefiran values seem promising, a lot of work can still be done concerning to kefiran production optimization.

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Abbreviations

AAB	Acetic acid bacteria
a_w	Water activity
CFU	Colony-forming units
CLSM	Confocal laser scanning microscopy
CW	Cheese whey
DGGE	Denaturing gradient gel electrophoresis
DNS	3,5-dinitrosalicylic acid
DSC	Differential scanning calorimetry
EPS	Exopolysaccharide
$Ethanol_t$	Ethanol concentration at the time kefir production was accomplished
FTIR	Fourier-transform Infrared
G’	Storage modulus
G’’	Loss modulus
Gal	Galactose
GC-MS	Gas chromatography-mass spectrometry
Glc	Glucose
HPLC	High performance liquid chromatography
LAB	Lactic acid bacteria
$Lactic\ Acid_t$	Lactic acid concentration at the time kefir production was accomplished
$Lactose_t$	Lactose concentration at the time kefir production was accomplished
MRS	Man-Rogosa-Sharpe
ND	Not determined
NEFA	Non-esterified fatty acids
NMR	Nuclear magnetic resonance
pH_t	pH value at the time kefir production was accomplished
$r_{ethanol}$	Ethanol volumetric production rate
$r_{glucose}$	Glucose volumetric consumption rate
$r_{lactic\ acid}$	Lactic acid volumetric production rate
$r_{lactose}$	Lactose volumetric consumption rate

RT	Room temperature
SEM	Scanning electron microscopy
t	Time
TCA	Trichloroacetic acid
TEM	Transmission electron microscopy
TLC	Thin-layer chromatography
UHT	Ultra-high temperature
WVP	Water vapor permeability
X	Biomass
$Y_{biomass/substrate}$	Yield of biomass on substrate
$Y_{ethanol/substrate}$	Yield of ethanol on substrate
$Y_{kefiran/substrate}$	Yield of kefiran on substrate
$Y_{lactic\ acid/substrate}$	Yield of lactic acid on substrate
μ_{max}	Maximum specific growth rate

1. Introduction

1.1. Background

Nowadays, dairy industry faces a large decrease in cow milk consumption. The global increase of food allergies, namely lactose intolerance, and the negative connotation that has been associated to cow milk over the past few years has led to a growing search for plant-based beverages by the consumers, which includes soy, oat, almond, rice, and coconut milk.

Kefir is a probiotic beverage produced through the fermentation of milk, with a yogurt-like appearance, but has unique sensory properties and superior nutritional and therapeutic values. There has been a current increase in kefir consumption and manufacture in many countries, however, in Portugal, only recently this beverage has started to be produced industrially. Therefore, a detailed study and consequent optimization of kefir production should be done to assess the possibility and feasibility of expanding the implementation of kefir industrial process in the country, which could eventually help to overcome this current problem faced by Portuguese dairy industry.

Additionally, kefiran, an exopolysaccharide (EPS) produced during kefir manufacture, is a value-added product which could be of commercial interest, since it may have several food and medical applications. However, more investigation needs to be done to completely understand and optimize its production, purification, and quantification procedures.

1.2. Objectives

The aim of this work was to study the effect of operational conditions in kefir and kefiran production, in order to improve their processes. In this sense, for kefir production by mixed culture (kefir grains), different fermentation conditions were tested and compared using UHT commercial milk: initial kefir grains mass concentration, initial milk volume, agitation rate and temperature. The best conditions determined for kefir production were also evaluated using a pasteurized cow milk sample provided by a dairy industry. In its turn, for kefiran production by kefir grains and by pure culture of *Lactobacillus kefiranofaciens*, different culture media were tested and compared. All the fermentation processes were monitored by determination of pH, consumption of sugars as well as production of lactic acid, acetic acid and ethanol, which were analyzed by high performance liquid

chromatography (HPLC). For kefiran production by pure culture, cell growth was also determined during the fermentation process, by optical density. Lastly, kefiran was also purified and quantified according to the methodologies described in the following literature review.

2. State of the Art

A literature review on kefir and kefiran production is presented in this chapter.

2.1. Kefir

2.1.1. Historical Background and General Properties

Fermentation is one of the oldest and most economical methods used in food production and preservation. Fermented food products play an important role in human diet around the world due to their health benefits [1]. In the case of fermented milk products, the beneficiary health effect on humans was popularized by Elie Metchnikoff (1845-1916), a Russian Nobel laureate in 1908, who worked on the concept of probiotics [2]. Fermentation improves organoleptic qualities of the product, and enhances the mineral bioavailability and the digestibility of proteins and carbohydrates. The trends towards natural (without additives or minimally processed), highly nutritional value, health-promoting and flavor rich foods and beverages have been increased with consciousness of consumers. In this context, traditional Turkish fermented non-alcoholic beverages, like kefir, have been taking great attention from scientists and consumers due to their probiotic characteristics [1].

Kefir is an acidic, viscous and self-carbonated fermented milk beverage with a smooth, slightly foamy body and whitish color, containing reduced concentrations of alcohol [1][3], which can be defined as the yogurt of the 21st century [4]. This beverage is also characterized by its distinct yeast flavor, and an effervescent effect felt in the mouth [5]. Kefir has its origin in the Caucasus mountains in Asia (**Figure 1**), where before 2000 years BC kefir grains were already being traditionally passed from generation to generation among the Caucasus tribes, since they have been considered a source of family wealth [6]. The word kefir is derived from the Turkish word *keyif*, meaning "good feeling", "well-being" or "living well", due to the overall sense of health and well-being generated in those who consume it [6][7].

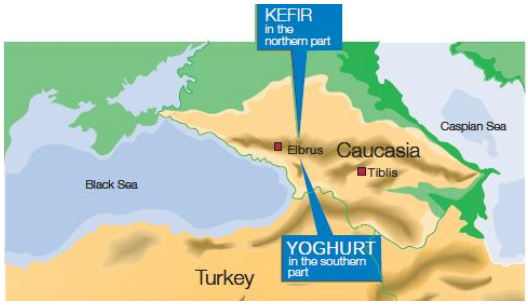

Figure 1. Caucasus mountains, the birthplace of kefir, adapted from [8].

The largest quantity of kefir is consumed in Russia, with an annual total of about five liters per capita [8]. Kefir is also highly consumed in Eastern Europe and Southwest Asia, although it has been gained popularity in various parts of the world including Northern Europe, North America, Japan, Middle East and North Africa [9]. The current increase in kefir consumption in many countries is due to its unique sensory properties and long history associated with beneficial effects on human health [5][6]. These beneficial effects are related to the functional properties of kefir, which have been reported in many studies [10]. Kefir functional properties include antitumoral and anticarcinogenic [11][12], antibacterial and antifungal [13], immunological [14][15], anti-inflammatory [16], antiallergenic [17], antioxidative [18] and hypocholesterolemic effects [19], as well as β-galactosidase activity (reduction of lactose intolerance symptoms) [20][21]. In this way, kefir has been recommended for consumption as a dietetic beverage [22].

2.1.2. Kefir Grains

Kefir is obtained with a fermenting agent called kefir grain, which is a fascinating biological entity [9]. They are small, hard, irregularly shaped granules, with a white to yellowish color and the appearance of miniature cauliflower [23]. Kefir grains have a variable size, from 3 to 35 mm in diameter, and they are also characterized by their elastic, viscous and firm texture (**Figure 2**) [5]. In general, kefir grains are composed of 45.7% mucopolysaccharides, 34.3% total protein (27% insoluble, 1.6% soluble and 5.6% free amino acids), 12.1% ash, 4.4% fat, vitamins B and K, tryptophan, Ca, P and Mg [3]. They are made up of a complex community of different lactic acid bacteria (LAB), acetic acid bacteria (AAB) and yeast species, which grown together symbiotically, embedded in an insoluble protein (casein) and polysaccharide (kefiran) matrix [7][9].

In terms of microbial distribution inside kefir grains, results are somewhat controversial. Several studies by electron microscopy have shown the existence of a wide variation in microbial population between different grains and within the same grain [5]. Some researchers support the hypothesis that yeasts are generally found in the inner and intermediate grain zone, with rod-shaped LAB predominantly on the surface area [24][25]. Conversely, other researchers describe that yeasts are not only found at the core, but also in the outer grain area [26][27][28]. These differences may be due to the grain origin site [25]. Furthermore, Jianzhong *et al.* (2009) and Magalhães *et al.* (2010) have also reported a higher

number of cells observed in the outer grain portion as compared with the inner portion [5][27][28].

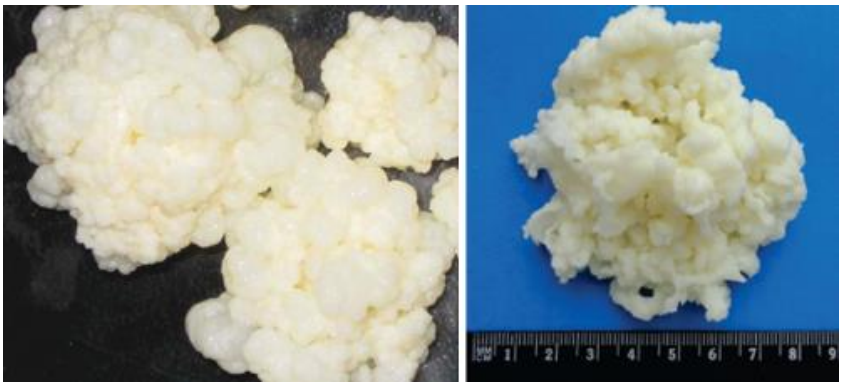

Figure 2. Physical appearance of a typical kefir grain [5][29].

Kefir grains cannot be synthesized artificially. They do not form spontaneously when pure cultures of the typically involved microorganisms are placed together in a test tube, but under proper conditions, kefir grains can apparently be encouraged to form and grow in traditional ways [7]. Kefir was first obtained by fermenting milk in goatskin bags, as it was a way of preserving milk. This led to the first kefir grains and started the long tradition of kefir manufacture [30]. These bags were traditionally hung by the entrances of people's homes, which resulted in the mixture of their content when people entered or left home. Bags could also be carried as people traveled, with the bumpiness of the ride agitating the content [7]. Motaghi *et al*. (1997) tested this hypothesis with some success when they filled a goat-hide bag with pasteurized milk and intestinal flora from sheep, incubating at 24–26 ºC and shaking hourly. After 12 weeks, they were apparently able to obtain kefir grains [31].

Kefir grains can be stored in different ways and for longer periods between their application [9]. They can be preserved wet, dry/lyophilized, or frozen. Kefir grains have a limited shelf life when left wet, since they lose their activity within 8 to 10 days during storage at 4 ºC [1][32]. In turn, lyophilization or drying at room temperature for 36 to 48 h allows maintenance of the activity for 12 to 18 months [3]. However, Garrote *et al.* (1997) observed that freezing at -20 °C or -80 ºC was the best method for grain preservation. Also, they observed that kefir obtained from grains storage at those temperatures had the same microbiota and quality characteristics that kefir produced from unstored kefir grains [33]. Therefore, if stored under favorable conditions, kefir grains can remain stable for many years without losing their activity. The reconstitution process of kefir grains consists of performing

successive incubations in milk, where they slowly re-establish their structure and, subsequently, new grains are formed [3]. Excessive washing and improper utilization alter the microbiota of grains and their viability, as well as the quality of the final product [1].

Since kefir grains are supposed to have developed spontaneously after storing milk in animal-based containers, grain formation may have happened several times and in different locations over the history, so it is not clear whether all can trace their origins back to the Caucasus mountains [34]. Researchers studying kefir often cite the source of their grains as being from private households or local dairies in their various countries, so it is known that grains in active commercial or artisanal use can be found all over Asia (China, Iran, Japan, Taiwan, Tibet), Eurasia (Russia and Turkey) and Europe (Austria, Bulgaria, Denmark, France, Germany, Ireland, Italy, Poland, Portugal, Spain, Sweden), as well as in artisanal use in Africa (South Africa) and South America (Argentina, Brazil) [7]. Many kefir grains show regional differences in microbial profile, grain structure and sensorial properties. These kind of differences may be due to the different sources of the kefir grains, variability of global ambient temperatures and also distinct techniques used during processing [7][32][35].

2.1.3. Microbial Composition and Distribution

Just like in kefir grains, the microbiological profile present in kefir include numerous bacterial species from lactic acid and acetic acid groups, yeasts and filamentous fungi, which also develop complex symbiotic relationships. In this association, the metabolic products of bacteria are used as an energy source for the yeasts, which in turn produce vitamins, amino acids and other essential growth factors that are important for bacteria. This symbiosis allows the maintenance of stability and microbial composition present in both kefir and kefir grains throughout fermentation [3].

It is important to understand and identify the microbial profile present in kefir and its grains since it is directly related to the quality of the probiotic product. Nowadays, many different methodologies are applied to study the microbiota of kefir. The classical approach of culturing microorganisms in nutrient media and identification of isolated cultures is still being performed, although the use of new combined approaches introducing culture-independent methods, such as functional genomics, transcriptomics, proteomics and metabolomics, has allowed identification and characterization of a number of previously

unknown microorganisms in kefir [36][37]. Specifically, analysis of the 16S rRNA gene libraries and/or molecular techniques such as denaturing gradient gel electrophoresis (DGGE) are very useful to evaluate and understand the complex microflora and diversity of strains from the kefir beverage [7].

The microbial composition of kefir varies according to microbiological culture medium, type and composition of milk used, origin of kefir grains, different techniques employed during processing, and storage conditions of kefir and kefir grains. Additionally, the amount of grains added to milk, agitation, and also incubation temperature can influence the extent of acidification and consequently the microbial composition of the final fermented milk [3]. The microbial diversity of kefir described in the literature varies a lot. Witthuhn *et al.* (2004) showed that the population of bacteria in different kefir grains may vary from $6.4x10^4$ to $8.5x10^8$ CFU/g and yeasts from $1.5x10^5$ to $3.7x10^8$ CFU/g [38]. In another study, it was observed that microbial numbers during kefir production may vary between $4.6x10^3$ and $2.6x10^8$ CFU/g [39]. Moreover, Irigoyen *et al.* (2005) studied the microbiological characteristics of kefir during storage, and showed the presence of 10^8 CFU/mL of *Lactobacillus*, 10^5 CFU/mL of *Lactococcus*, 10^6 CFU/mL of yeasts and 10^6 CFU/mL of AAB, after 24 h of fermentation [40]. According to literature, the microbial population in kefir consists primarily of LAB species, comprising 60 to 80% of total microflora, followed by yeasts (10-30%) and AAB species (nearly 10%) [3][41].

Kefir is estimated to have more than 300 different microbial species. Several varieties of LAB, AAB and yeasts have been isolated and identified from kefir and kefir grains using diverse methodologies (**Table 1**). Concerning to LAB species, which are aerotolerant anaerobes [7], many homofermentative and heterofermentative *Lactobacillus spp*. (such as *L. acidophilus* [42][43], *L. brevis* [39][44], *L. casei* [42][44], *L. helveticus* [42][44], *L. kefiranofaciens* [43], *L. kefiri* [41][43] and *L. paracasei* [41]), *Lactococcus spp.* (such as *L. cremoris* [42], *L. lactis* [41][42][43] and *L. raffinolactis* [43]), *Streptococcus spp.* (such as *S. durans* [45] and *S. thermophilus* [43][44][45]) and *Leuconostoc spp.* (such as *L. mesenteroids* [39][43][44][45]) have been reported. On the other hand, AAB species in kefir are represented mainly by *Acetobacter spp.* (such as *A. lovaniensis* [41] *and A. syzgii* [43]). Concerning the yeasts, many lactose-fermenting and lactose-nonfermenting *Saccharomyces spp*. (such as *S. cerevisiae* [41][44], *S. fragilis* [31] and *S. lactis* [31]), *Candida spp*. (such

as *C. kefyr*, *C. krusei*, *C. lambica* [39] and *C. maris* [44]) and *Kluyveromyces spp*. (such as *K. lactis* [41] and *K. marxianus* [44]) have been described.

Table 1. Microbial species found in kefir.

Genus	Species	References
Lactic acid bacteria		
Lactobacillus	*acidophilus*	[42][43]
	brevis	[39][44]
	casei	[42][44]
	helveticus	[42][44]
	kefiranofaciens	[43]
	kefiri	[41][43]
	paracasei	[41]
Lactococcus	*cremoris*	[42]
	lactis	[41][42][43]
	raffinolactis	[43]
Leuconostoc	*mesenteroids*	[39][43][44][45]
Streptococcus	*durans*	[45]
	thermophilus	[43][44][45]
Acetic acid bacteria		
Acetobacter	*lovaniensis*	[41]
	syzgii	[43]
Yeasts		
Candida	*kefyr*	[39]
	krusei	[39]
	lambica	[39]
	maris	[44]
Kluyveromyces	*lactis*	[41]
	marxianus	[44]
Saccharomyces	*cerevisiae*	[41][44]
	fragilis	[31]
	lactis	[31]

Some bacterial contaminants have also been identified, such as *Pseudomonas* spp. [27][46] and members of the *Enterobacteriaceae* and *Clostridiaceae* families [46], when kefir grain microbiota was characterized by pyrosequencing and PCR-DGGE. The presence of these microorganisms may be related with contamination during handling of kefir grains or improper practices adopted during the preparation of the kefir beverage [5].

2.1.4. Chemical and Nutritional Composition

The chemical and nutritional composition of kefir varies widely. The type and volume of milk affect its sensory, chemical and textural properties [23]. Additionally, the origin and composition of the grains used, the time/temperature binomial of fermentation and storage conditions influence its properties [3]. According to the Codex Alimentarius, kefir must contain a minimum of 2.7% of milk protein and 0.6% of lactic acid, and less than 10% of milk fat. The total number of microorganisms in the fermented beverage produced must be at least 10^7 CFU/mL and the yeast number not less than 10^4 CFU/mL. However, no specification for ethanol content has been mentioned [47].

Water content in kefir is the highest (86-90%), followed by sugars (4-6%), protein (3-6%), fat (0.2-3.5%), ash (0.7-1%), with minor amounts of ethanol and lactic acid [23][48][49]. When compared to yogurt, kefir has a similar nutritional base composition, as yogurt contains essentially water (83-88%), followed by sugars (5-6%), protein (2-5%), fat (0.2-6%) and ash (0.4-0.8%) (**Table 2**) [50][51].

Table 2. Comparison between the nutritional base composition of kefir and yogurt.

%	Kefir	References	Yogurt	References
Water	86-90%	[23][48][49]	83-88%	[51]
Sugars	4-6%	[23][48][49]	5-6%	[50][51]
Protein	3-6%	[23][48][49]	2-5%	[51]
Fat	0.2-3.5%	[23][48][49]	0.2-6%	[51]
Ash	0.7-1%	[23][48][49]	0.4-0.8%	[51]
Lactic Acid	0.8-1%	[3]	0.8-1%	[50]
Ethanol	0-2%	[49]	0%	[49]

Concerning to sugars, kefir is a good diet for lactose-intolerant individuals which have the inability to digest significant amounts of lactose, the predominant sugar of milk [52]. Approximately 30% of milk lactose is hydrolyzed by the β-galactosidase enzyme during the fermentation process, converting lactose into glucose and galactose. Furthermore, LAB present in kefir convert glucose into lactic acid, which causes pH reduction and increases its consistency [3]. Kefir shows a similar profile of amino acids when compared to the milk used as the fermentation substrate. However, during fermentation, proteins become easily digestible due to the action of acid coagulation and proteolysis, resulting in levels of ammonia, serine, lysine, alanine, threonine, tryptophan, valine, methionine, phenylalanine and isoleucine higher in kefir compared with unfermented milk [3][49]. Additionally, Ismael

et al. (2011) reported that total amino acid concentration in kefir was higher than that of yogurt, and individual concentration of essential amino acids was 1.5 times higher in kefir [53]. The lipid content (monoacylglycerols, diacylglycerols, triacylglycerols, non-esterified fatty acids (NEFA) and steroids) in kefir depends largely on the type of milk used as the fermentation substrate. In the fermented milk, the presence of NEFA contributes to the improvement of digestibility [3]. Kefir also provides a good source of vitamins and minerals when it is ready for consumption. The vitamin content depends on the quality of the milk used, microorganisms present in kefir grains, and the way of preparation. Kefir possesses vitamins A, B_1, B_2, B_5, biotin, folic acid, B_{12}, C and K in its composition [22][52]. Among the minerals, kefir is a good source of Mg, Ca and P, although minerals such as Zn, Cu, Mn, Fe, Co and Mo are also found [22].

The main products originated during fermentation are lactic acid, CO_2 and ethanol [52]. Lactic acid is the most abundant organic acid after fermentation and is derived from approximately 30% of the original lactose in the starter milk [23]. The lactic acid content of kefir varies between 0.8 and 1.0% (w/v), and its production results in a final pH of 4.2 to 4.6 [3]. The content of carbon dioxide in kefir beverage, which varies between 0.08 and 0.2% (v/v), depends on kefir grains concentration and increases as the level of kefir grains increased in the product [3][29]. Ethanol is produced by yeast during lactose fermentation of starter milk, and its content varies between 0.035 and 2.0% (v/v) [1][3]. Kefir also contains formic acid, acetic acid, propionic acid, butyric acid, pyruvic acid, succinic acid, hippuric acid, acetaldehyde, diacetyl and acetoin, at concentrations below 0.1% (w/v), which are also generated during the fermentation process, as a result of the wide diversity of microorganisms present in kefir grains [23]. Along with lactic acid, these compounds exert a direct influence on the aroma and taste of kefir [29]. Diacetyl and acetaldehyde, which are the major flavoring components, are produced by *Streptococcus Lactis* subsp. *diacetylactis* and *Leuconostoc* sp. [52].

Biogenic amines such as cadaverine, putrescine, spermidine and tyramine are also found in fermented milk as a consequence of LAB activity. The high concentration of bioactive amines is related to the reduction of the sensorial properties of kefir beverage, therefore, these compounds are considered to be an important indicator of its quality and acceptability [3].

2.2. Kefir Production

2.2.1. Type of Milk

The type of milk has a great influence on kefir sensorial and textural properties [48]. Regarding the milk origin, kefir can be produced from any kind of animal milk including goat, sheep, camel, buffalo and mare milk, although cow milk is the most common [1][54]. Amongst these different milk types, kefir produced from goat milk was found to have lower viscosity and sensory properties, when compared to kefir made from cow milk [55]. Wojtowski *et al.* (2003) reported that kefir produced from sheep milk can have more advantageous effect on the health of the consumer than kefir made from goat or cow milk, owing to its lowest contents of medium-chain saturated acids and highest linoleic and α-linolenic acid [56]. Additionally, Kavas (2015) compared some sensorial and chemical properties of kefir made from camel milk and cow milk. Kefir produced from camel milk was perceived as sourer, whereas its other sensorial properties were found to be close to those of cow milk. The cholesterol levels of kefir produced from camel milk were higher when compared to those of kefir produced from cow milk [57]. Kefir can also be prepared using non-dairy beverages such as cocoa-pulp beverage [58], coconut milk [52], rice milk [52], soy milk [52] and walnut milk [59]. However, these non-dairy beverages tend to leave the kefir grains in a weakened state while they ferment and produce non-dairy kefir. After a few fermentation cycles, the grains must be returned to a dairy milk containing fat to fortify the grain [7].

Regarding to the lipid content, kefir can be produced using whole fat, semi-skimmed (low-fat) or skimmed (non-fat) milk. The higher the fat content in milk, the thicker and creamier the kefir [3]. A non-fat choice in kefir production is desirable, since there is an established relationship between many health problems and the consumption of saturated fats and cholesterol. However, non-fat milk results in a kefir with significantly lower sensory quality [7]. In the United States, kefir is usually made with low-fat or whole milk [50].

Finally, the milk used to produce kefir can be thermally treated (pasteurized or sterilized) or not (raw). It is advised to use heat-treated milk to ensure the chemical and microbiological quality of the substrate, since thermal processing of milk reduces the redox potential, eliminates inhibitory substances, prevents hydrolytic rancidity (through inactivation of enzyme lipase) and inactivates pathogenic microorganisms [9]. Several

temperature-time combinations have been recommended for heat-treatment of cow milk previous to kefir production. Suggested heat-treatments were 85 ºC for 25 min [31], 85-90 ºC for 15-20 min, 90-93 ºC for 15 min, 90-95 ºC for 2-3 min, 92 ºC for 20 min, 95 ºC for 10-15 min and 95 ºC for 15 min [9].

2.2.2. Traditional Production vs. Commercial Production

Kefir was first obtained from goat milk with kefir grains in goatskin bag by hanging in the house during winter and outside during summer, as already seen [9]. Initially, kefir was traditionally produced by inoculating milk with kefir grains, or commercially produced by the widely adopted Russian method, which involves the use of bulk milk culture obtained by kefir grains for milk inoculation [5][9]. Currently, kefir can also be produced industrially using freeze-dried pure and mixed cultures isolated from kefir grains or commercial cultures. Food scientists are continually studying modern techniques to achieve the production of a kefir with the same characteristics as those found in the traditionally produced beverage. Thus, there are mainly two methods for kefir manufacture: traditional (artisanal) and commercial (industrial) process [52].

2.2.2.1. Traditional Production

While other fermented milk products are produced by adding a sample of fermented milk as inoculum to fresh milk to produce more fermented milk product (the common fermentation start for yogurts and other traditional fermented milks), traditional kefir requires inoculating fresh milk with the entire kefir grains and allowing fermentation to occur. This is because of the complex symbiotic interactions among the organisms in the kefir grains for their production of kefir, as already seen [7].

The kefir production process by the traditional method is outlined in **Figure 3**. Initially, raw milk is pasteurized and cooled to incubation temperature. Then, thermally-treated milk is inoculated with 2-10% (generally 5%) of active kefir grains (starter culture), for a period between 18-24 h at 20-25 °C [52]. Stirring during incubation is optional, although intermittent stirring for about 10-15 minutes every 2-5 hours is recommended, as the grains tend to sink to the bottom [8].

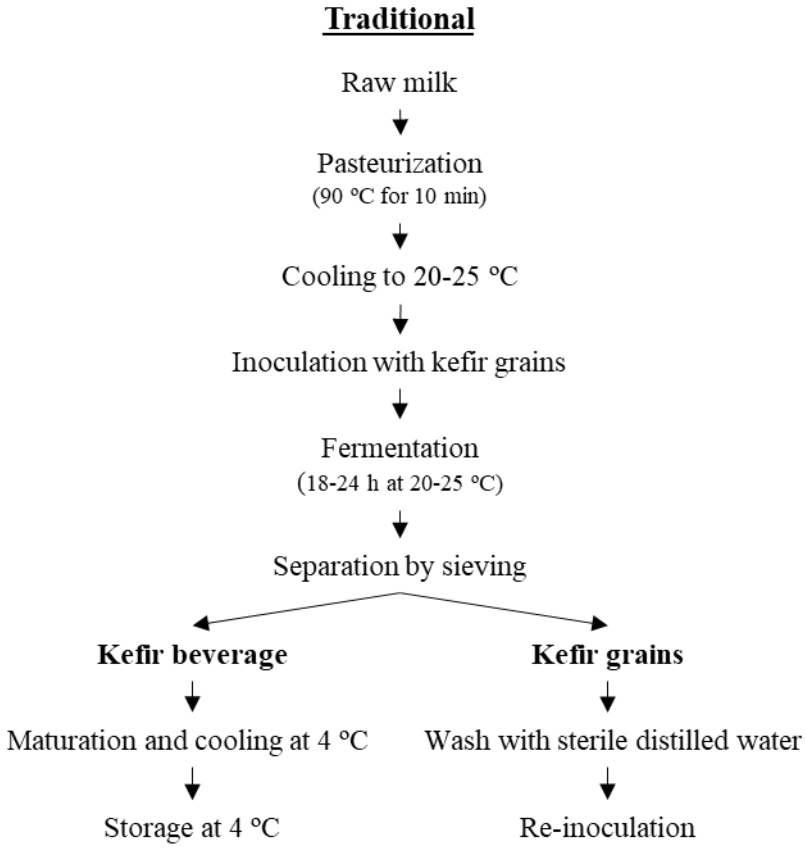

Figure 3. The manufacturing method of the traditional (artisanal) production of kefir, adapted from [1] and [52].

After fermentation, the desired pH is reached (4.2 to 4.6) and the grains are separated from the mother culture by filtering with a sterile sieve. The grains are washed in the strainer with sterile distilled water [8]. Then, they can be dried at room temperature and kept at cold storage for being used and reused as the inoculum for the next and subsequent batches [7][52]. Given the proper environmental conditions, the same grains should be effective for infinite batches of kefir [7]. The microbial population grows nearly 10% per week during incubation, so the grains must be weighed and the excess removed, before being reused [8]. Finally, kefir is stored at 4 ºC for a time period, which stops any further reduction in pH, and then it is ready for consumption [52].

The initial inoculum concentration of the grains (grain/milk ratio) affects the pH, viscosity, final lactose concentration and the microbiological profile of the final product [60]. Agitation during fermentation also influences kefir microbial composition, favoring the development of homofermentative *Lactococcus* spp. and yeast [5]. Finally, incubation at

temperatures above 30 °C stimulates the growth of thermophilic LAB, while being a disadvantage for yeast growth and mesophilic LAB [5].

2.2.2.2. Commercial Production

As already seen, kefir can be industrially produced with the commercial process by the Russian method and with the commercial process using freeze-dried cultures [5].

The Russian method allows the production of kefir on a larger scale, and uses a process of fermentation in series, from the percolate resulting from the first fermentation of the grains (fermented without the grains or mother culture) [5]. This process resembles to traditional method, with one exception: after kefir grain removal, the filtrate (mother culture) is inoculated as a starter culture into pre-treated milk for kefir production, with a dosage of 3-5% of the volume of substrate, instead of using kefir grains. The basic reason for this modification is that kefir grains are bulky and awkward to handle, whereas relatively small volumes of mother culture are easier to control, allowing the production of large quantities of kefir [8].

Nevertheless, both kefir manufacture using traditional process and commercial process by the Russian method sometimes result in unacceptable variations in product quality due to diverse microflora and uncontrolled fermentation [9]. To overcome this problem, freeze-dried pure and mixed cultures for direct use in milk have been developed at culture laboratories and are now commercially available [8]. These cultures contain LAB and/or yeast species, isolated from kefir grains or commercial cultures [1]. Nowadays, the use of freeze-dried cultures is recognized as a promising development in regard to standardization of kefir products and preservation of desirable properties [23].

The kefir production process by commercial method using freeze-dried pure and mixed cultures is outlined in **Figure 4**. Initially, raw milk is pasteurized and cooled to incubation temperature, as already seen for the traditional process [52]. However, before pasteurization, optional procedures can be applied: milk fat content can be standardized, since fat contents of 2.5 to 3.5% are frequently specified, and milk can also be homogenized at 65-70 ºC and 17.5-20 MPa [8]. Then, thermally-treated milk is inoculated with 2-8% of freeze-dried kefir cultures in big tanks, for a period between 18-24h at 18-24 ºC [52]. After fermentation, kefir is stirred in the tank and pumped to the packaging units, which causes the destruction of the original structure of kefir gel [61]. The maturation phase, which can

be performed or not, consists of maintaining the kefir at 12-14 ºC or 3-10 ºC for 24h, to allow microbial growth (essentially yeast), contributing to the specific flavor of the product [52][62]. Omission of this step is associated with development of atypical flavors in kefir [62]. After maturing, kefir is stored at 4 ºC [52].

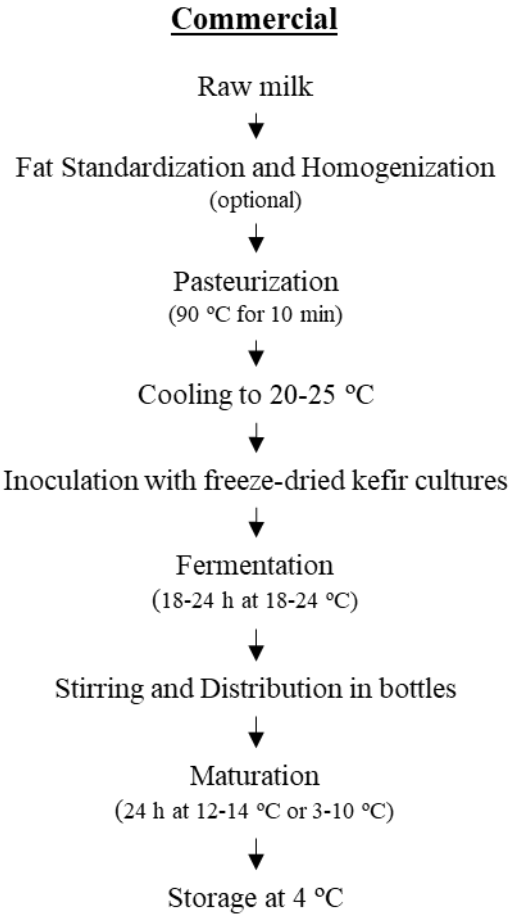

Figure 4. The manufacturing method of the commercial production of kefir using freeze-dried cultures, adapted from [50] and [52].

It is of extreme importance that the product is treated gently when cooled and during subsequent packing. Mechanical agitation in pumps, pipes and filling machines must therefore be minimized. Air entrainment must also be avoided, as air increases the risk of syneresis (whey separation) in the product [8]. In this method, the recovering step of kefir grains is eliminated from the process [3], and with it the risk of reinfecting the culture is reduced [8]. Despite all the advantages of this commercial process, the composition of the final fermented milk beverage presents a lower number and variety of microorganisms than the fermented milk product produced from kefir grains [3].

Regarding the commercial process using freeze-dried mixed cultures, Beshkova *et al.* (2002) proposed two methods of kefir fermentation: one by simultaneous fermentation and one by consecutive fermentation. Therefore, they used a starter culture consisting of bacteria and yeast isolated from kefir grains and two strains commonly used in yogurt manufacturing. Yeast and sucrose were both added to the starter culture of bacteria at the beginning (simultaneous fermentation) or after the lactic acid fermentation step (consecutive fermentation). The two fermentation processes produced kefir with high number of viable *Lactococcus spp.* and *Lactobacillus spp.*, with sensory properties similar to traditional kefir [62].

2.2.2.3. Production Processes Remarks

The traditional process of kefir manufacture is not suitable for large scale production, as grain recovery is laborious and unworkable, and the volumes required would make fermentation irregular [63]. Russian-style kefir is made by taking the traditional kefir beverage, removing kefir grains, and inoculating it into pasteurized milk at a concentration of 3–5%, as already seen. This process can be repeated many times by taking part of the final kefir product and inoculating it into more thermally-treated milk at the same concentration. However, every time inoculation is performed, a change in the microbial composition of the kefir and a decline in the quality of the beverage occurs [7]. Thus, the product loses most of its kefir characteristics after a few fermentation cycles. Any kefir product prepared for widespread commercial distribution would have to be consistent and defined. As grains vary by origin, consistency is hard to control [64]. To overcome this issue, many commercial companies offer freeze-dried kefir starters, which will not form grains. These starters do not seem to remain stable through more than few fermentation cycles, however, they produce a product that is more uniform, making production less laborious and ensuring a longer shelf life of the product, which is desirable at commercial level [65]. As an example, commercial kefir beverage may have a life period of up to 28 days, while a kefir produced with grains should be consumed between 3 to 12 days [5]. Therefore, traditional kefir culturing is at a commercial disadvantage, as the uniformity and shelf life cannot be guaranteed. However, commercial kefir beverage may not present the same therapeutic and probiotic properties existing in traditional kefir [5].

An uniform freeze-dried kefir grain with optimized viability of kefir organisms would be desirable for the commercial market [7]. Chen *et al.* (2009) experimented on making a synthetic kefir grain entrapping bacteria and yeast in two different microspheres in which the entrapment ratio of the strains was based on the distribution ratio found in kefir grains. They prepared yeast and bacterial microspheres, then made kefir using the entrapped culture starter, passing it through 28 fermentation cycles [66].

The main deficiencies in both traditional and commercial kefir manufacturing can be attributed to unpleasant taste and aroma characteristic of yeasts. The latter can be caused by rapid growth of *S. cerevisiae*, accompanied by a typical vinegar aroma. The excessive production of acetic acid can also influence kefir aroma, and occurs due to the intense growth of *Acetobacter spp.* or the presence of *Dekkera spp.* in the product [65].

2.2.3. Commercial Starter Cultures

During the development stages of commercial starter cultures, three essential aspects must be considered: the choice or development of single microbial strains, the blending of the culture strains and the characterization of the developed culture. The blending process consists of mixing three microbial strains in different ratios. However, even if few strains are considered, the number of possible combinations is overwhelming. Therefore, multivariate statistical analysis is needed. This tool allows the assessment of the influences of strains and their ratios on the quality of the dairy product, by mathematical modeling. Subsequently, the starter culture is grown in milk, and the final product is characterized by instrumental measurements and sensory profiling. If the microbial consortium does not provide the desired characteristics, this is either discarded or the mixture of the specific strains is re-adjusted. This process can be repeated several times until the desired characteristics are reached [67].

Starter culture companies, like Chr. Hansen A/S and Danisco Biolacta Sp. z o.o., have made great efforts to develop kefir starter cultures that do not produce grains during the manufacture of kefir. Chr. Hansen A/S has developed and sensory profiled at least three freeze-dried kefir starter cultures containing different yeast species. The developed co-cultures are known as LAF-3 (containing lactose-nonfermenting *Debaryomyces hansenii*), LAF-4 (containing lactose-fermenting *Kluyveromyces marxianus* var. *marxianus*) and LAF-7 (lactose-nonfermenting containing *Candida colliculosa*). These cultures also contain a

consortium of LAB and exopolysaccharide-producing *S. thermophilus*. The cultures were inoculated at two different temperatures (30°C and 35°C), and the kefir products were evaluated after 12 days of storage at 8°C. Kefir developed with LAF-3 cultures resulted in a beverage with mild but very sour taste, and with a high diacetyl content. When developed with LAF-4 cultures, the product presented a high kefir odor and taste, due to the metabolic activity of *Kluyveromyces* species. In turn, kefir beverage developed with LAF-7 cultures had a high acetaldehyde content, which enhanced the fruity note in the product. Therefore, the activity of the chosen yeast strains influenced the typical flavor attribute of the beverage [65].

In the United States, kefir is generally produced by milk inoculation with a kefir starter culture containing *Lactobacillus kefiranofaciens* and *Lactobacillus kefiri* [50]. *Lactobacillus kefiranofaciens* is a homofermentative, facultative anaerobic, gram-positive, slime-forming, nonmotile, capsulated, nonsporeforming, rod-shaped lactic acid bacteria which can ferment glucose, fructose, galactose, sucrose, maltose, lactose, melibiose, and raffinose [68]. On the other hand, *Lactobacillus kefiri* is a heterofermentative, microaerophilic, gram-positive, nonmotile, nonsporeforming, rod-shaped lactic acid bacteria [69]. Other microorganisms can also be employed in commercial kefir manufacture process, such as *Lactobacillus casei*, *Lactobacillus plantarum*, *Streptococcus cremoris*, *Streptococcus diacetylactis*, *Streptococcus lactis*, *Leuconostoc cremoris* and *Saccharomyces florentinus* [9].

2.2.4. Types of Fermentation

Both alcoholic and lactic acid fermentation occur in kefir [1]. Alcoholic fermentation is performed by lactose-fermenting yeast, which produce ethanol and CO_2 from milk sugars. In turn, lactic acid fermentation is performed by LAB, which are primarily responsible for the conversion of the lactose present in milk into lactic acid, resulting in a pH decrease and milk preservation. Lactose-nonfermenting yeasts and AAB also participate in the fermentation processes [5].

Most yeasts metabolize sugars as their main carbon source for energy. In alcoholic fermentation, hexose sugars are metabolized via the glycolysis cycle resulting in the formation of 2 mol of pyruvate, ATP and NADH, per mol of hexose. Pyruvate is later decarboxylated by pyruvate decarboxylase into CO_2 and acetaldehyde, which is finally catalyzed by alcohol dehydrogenase (ADH) to ethanol. Since re-oxidation of NADH to

NAD^+ occurs in the terminal step, the final yield of alcoholic fermentation is 2 mol of ethanol, CO_2 and ATP per mol of hexose [65].

Concerning to lactic acid fermentation, LAB are traditionally classified in two metabolic sub-groups according to the pathway used to metabolize hexose sugars: homofermentative and heterofermentative, each one performing homolactic and heterolactic fermentation, respectively [70]. Initially, lactose is taken up by a specific permease and it is hydrolyzed by β-galactosidase, resulting in galactose and glucose, which are both hexoses [71]. Glucose is phosphorylated by hexokinase to form glucose-6-phosphate, while a three enzyme pathway known as Leloir pathway is required to convert galactose into glucose-6-phosphate [50]. Then, homofermentative LAB dissimilate glucose-6-phosphate through glycolysis, similarly to yeasts in alcoholic fermentation. However, pyruvate is later catalyzed by lactate dehydrogenase to lactic acid, rather than being decarboxylated and converted into ethanol and CO_2. Therefore, homolactic fermentation yields 2 mol of lactic acid and ATP, per mol of hexose. In comparison, heterofermentative LAB have another active pathway, and hexoses are converted to equimolar amounts of lactic acid, ethanol or acetate, and CO_2, resulting in the formation of only 1 mol of ATP per mol of hexose fermented. However, an additional ATP can be produced with the formation of acetate instead of ethanol [70].

Additionally, microbial strains are able to convert the fermented carbohydrates into products other than ethanol, lactic acid, CO_2 and acetate, through alternative metabolic pathways. These metabolic pathways are shown in **Figure 5** [67]. Certain flavoring compounds, such as diacetyl, acetoin and 2,3 butanediol are produced in milk fermented products through the pyruvate catabolism. Initially, pyruvate is converted into acetolactate by acetolactate synthase, and decarboxylation of acetolactate produces acetoin, which can be further reduced to 2,3 butanediol by butanediol dehydrogenase. Furthermore, diacetyl can also be easily produced from acetolactate because the latter is not a stable molecule. In addition, diacetyl can be reduced to acetoin and consequently to 2,3 butanediol by acetoin dehydrogenase and by butanediol dehydrogenase, respectively (**Figure 5., A**). Under changed environmental conditions, pyruvate catabolism can be redirected using other enzymes to yield acetate, acetaldehyde and ethanol. These compounds are formed via a common intermediate, acetyl-CoA, and their production depends mainly on the intracellular redox state. Furthermore, formate can also be obtained, since the conversion of pyruvate to acetyl-CoA catalyzed by pyruvate formate lyase also yield formate (**Figure 5., B**) [67].

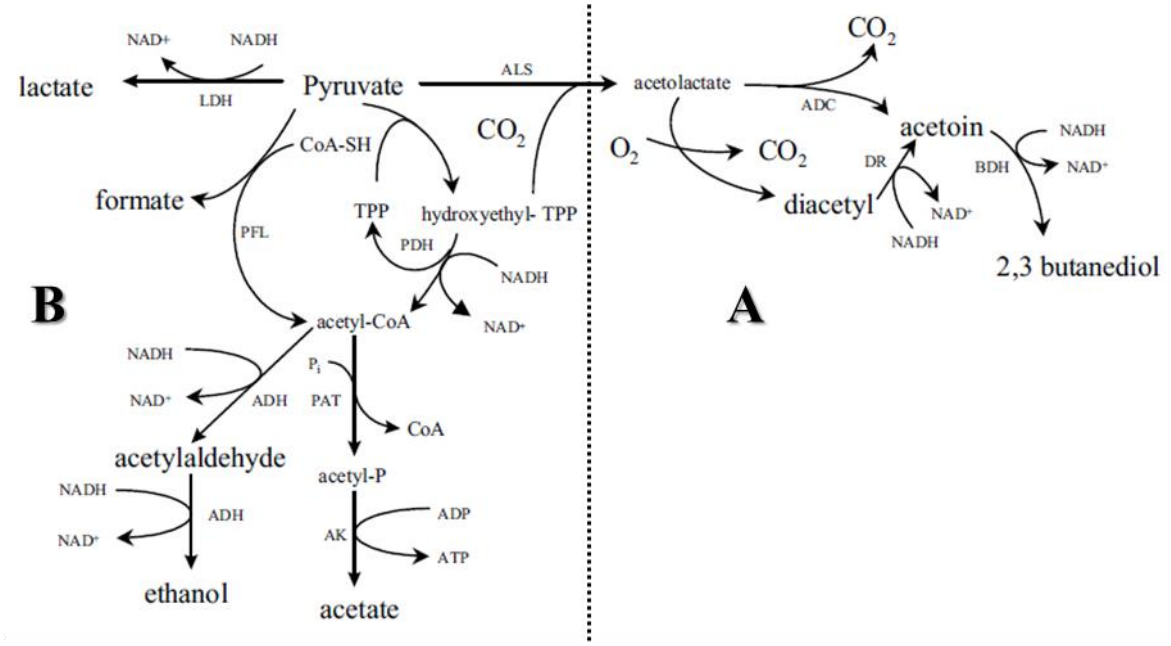

Figure 5. Alternative metabolic pathways, end-products and enzymes involved in pyruvate catabolism that might occur in milk fermented products. ADC, acetolactate decarboxylase; ADH, alcohol dehydrogenase; AK, acetate kinase; ALS, acetolactate synthase; BDH, butanediol dehydrogenase; DR, diacetyl reductase; LDH, lactate dehydrogenase; PAT, phosphotransacetyl transferase; PFL, pyruvate formate lyase; PHD, pyruvate dehydrogenase. Adapted from [67].

2.2.5. Packaging and Storage

Kefir fermentation can continue during storage, causing extremely strong and undesirable products due to the relatively high residual lactose content and the presence of yeasts [1]. Moreover, the CO_2 produced by yeasts and LAB may cause bloating in the product package, a fact that should be considered in the choice of packaging [30]. Therefore, the containers intended for kefir packing must be either strong enough to withstand the buildup pressure, such as glass, or flexible enough to retain the amount of gas produced, such as plastic with an aluminum foil top (which can be found in the market in some of the conventional yogurt packages). Containers should be also impermeable to water and foreign odors. Thus, shelf-life of kefir is dependent on the type of packaging material and varies from 8 to 10 days at 3-4 ºC. Additionally, special containers that have been designed for kefir packing, had lids consisting of three layers that allows the escape of carbon dioxide generated by viable yeast, which prevents bulging and swelling of kefir cups [9].

Concerning to storage, kefir must be conserved at 4 ºC, as already seen. After fermentation, the cooling process of kefir should be done slowly within 10-12 h to ensure the retention of its pronounced aroma and typical taste [72]. Irigoyen *et al.* (2005) reported

that, during refrigerated storage at 5±1 ºC, yeast and AAB counts remained constant, while LAB decreased by 1.5 log units between 7 and 14 days of storage. Additionally, the total fat, lactose, dry matter and pH, also remained constant up to 14 days. The sensory analysis revealed the best acceptability level in the first days, however, the samples were satisfactory until the first week of storage [40]. In turn, Kiliç *et al.* (1999) studied two kefir samples over a 5-day storage period, and concluded that kefir kept under refrigeration should be eaten within 3 days of manufacture, for better sensory qualities [73]. Moreover, Grønnevik *et al.* (2011) found that yeast count in kefir samples increased throughout the storage period of 4 weeks at 5.5-6 ºC [74].

2.3. Kefiran

2.3.1. Structure and Physicochemical Properties

Nowadays, exopolysaccharides (EPSs) produced by LAB have been receiving increasing attention, due to their important applications in food industry, as EPS can impart several functional properties to food and confer health benefits [65][75]. In addition, their *in situ* production in fermented milks is being given "generally recognized as safe" (GRAS) status [7], which allows these EPSs to escape the rigorous toxicological testing [76]. Such characteristics make these EPSs a very interesting alternative to conventional emulsifiers, stabilizers, thickeners, fat substitutes and gelling agents [77].

In general, LAB are able to originate different types of EPSs, which can improve in many ways the texture properties and mouth feel of fermented milk products. These products have been reported to get a higher viscosity and a lower degree of syneresis compared with those obtained with non-EPSs producing cultures [67]. In this sense, there has been a growing research interest towards the production, extraction and purification of EPSs originated by LAB with a high yield, high concentration and high productivity needed for their use in the food and biopharmaceutical industries [75].

The EPS produced by kefir microorganisms is commonly known as kefiran, which is a water-soluble heteropolysaccharide and consists of branched glucogalactan containing approximately equal amounts of D-glucose and D-galactose in the chain sequence [65][78]. Detailed structure of kefiran corresponds to hexasaccharides repeating-units of a backbone composed of (1→6)-linked Glc, (1→2,6)-linked Gal, (1→4)-linked Gal, (1→3)-linked Gal

and (1→4)-linked Glc, with a branch of (2→1)-linked Glc attached to O-2 of (1→2,6)-linked Gal residue (**Figure 6**) [78][79].

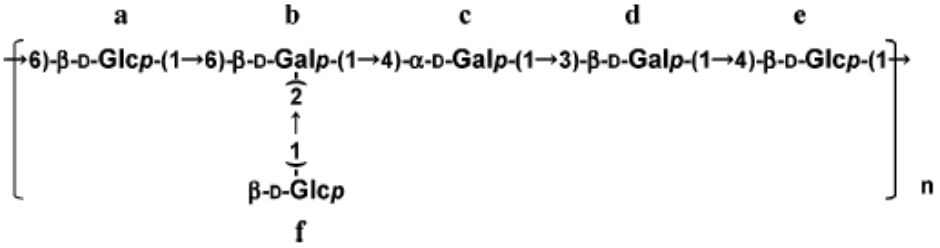

Figure 6. The structure of the repeating unit of kefiran. a, (1→6)-linked Glc; b, (1→2,6)-linked Gal; c, (1→4)-linked Gal; d, (1→3)-linked Gal; e, (1→4)-linked Glc; f, (2→1)-linked Glc [80].

Kefiran structure has been heavily researched [67]. Maeda *et al.* (2004) and Ghasemlou *et al.* (2012) confirmed the structural features of kefiran by a combination of monosaccharide composition analysis, methylation and GC-MS analysis, NMR spectroscopy (^{1}H and ^{13}C) and other methods [79][80]. The data obtained by Maeda *et al.* (2004) indicated that kefiran was composed of glucose and galactose in a relative molar ratio of 1.0:1.05, had an average molecular mass of $7.6x10^5$ Da and a specific optical rotation of $[\alpha]_D$ = +64.5º (c 1.0; H_2O) [80]. In turn, the results obtained by Ghasemlou *et al.* (2012) showed that the polysaccharide was composed of glucose and galactose in a molar ratio of 1.00:1.10, had an average molecular mass of $1.35x10^6$ Da and a specific optical rotation of $[\alpha]_D$ = +64º (c 1.0; H_2O) [79]. Both studies gave similar results and are in agreement with previously reported values.

Additionally, kefiran composition may vary depending on carbon source [81]. Therefore, Wang and Bi (2008) studied the effect of medium composition (varying the carbon source) on the characteristics of kefiran produced by *Lactobacillus kefiranofaciens*. When using lactose and maltose as the sole carbon source, the average molecular mass was $2.4x10^5$ Da and $1.5x10^5$ Da, the glucose and galactose molar ratio was 1:4 and 1:10, and the specific optical rotation was $[\alpha]_D$ = +63º (c 1.0; H_2O) and +68º (c 1.0; H_2O), respectively. Nevertheless, since maltose is a disaccharide made of glucose, and not of galactose, there is no possible explanation for the molar ratios observed in this study, therefore, certainly this is not correct. In addition, these results differ greatly from those reported by Maeda *et al.* (2004) and Ghasemlou *et al.* (2012). Wang and Bi (2008) also claimed that the molecular mass and structure of polysaccharides to be produced must be closely related to their medical and food functions [77].

2.3.2. Kefiran Production

Kefir beverage usually contains around 0.2 to 0.7% of kefiran, whose production is mainly related to the presence of *Lactobacillus kefiranofaciens* and *Lactobacillus kefiri* in kefir grains [1][3]. As previously mentioned, this exopolysaccharide is associated with the co-culture of LAB, yeast and AAB present in the grains, serving as a matrix for the cellular immobilization of these microbial components. Additionally, it has a protective function when the grains are recovered, dried, and re-used for successive milk inoculation [82]. Kefiran may either form an amorphous layer around the cell called capsular kefiran, or be excreted into the medium as broth kefiran. In order to maximize the total production of kefiran, the total amount of both types of this EPS needs to be considered, yet, extraction of capsular kefiran is complicated and the total yield is fairly low [83].

Kefiran is a value-added product which could be of commercial interest, since it may have several food and medical applications due to its functional and physicochemical properties. However, this EPS is still produced on a small scale due to the low productivity of the process comparing with the technologies used for obtaining other EPSs. Thus, the development of effective procedures for mass production and extraction of kefiran by LAB is of significant biotechnological interest, and should be extensively researched [82][84]. In this sense, some methods for kefiran production are currently being developed [82]. One of them involves the determination of the optimal conditions of culturing of kefir grains and the subsequent extraction of kefiran from them [85]. Another method is based on the isolation of the most active EPSs producers from kefir grains and determination of the cultivation conditions that increase kefiran production by those microorganisms [86][87]. Both procedures focus on the optimization of the growth environment, such as the composition of the culture medium (carbon, nitrogen and phosphate sources, vitamins, minerals and growth factors) and the process parameters (temperature, pH, agitation and time of fermentation), since kefiran characteristics and amounts produced are greatly influenced by these factors. Therefore, optimization of these parameters is extremely important for achieving maximal kefiran production [88]. Moreover, metabolic engineering strategies have being also studied, however, they cause modest or negligible effects on EPS yields, due to inherent limitations [89].

OPTIMIZATION OF THE GROWTH ENVIRONMENT

In this context, Dailin *et al.* (2016) focused on maximizing the production of kefiran in semi-industrial scale through the optimization of medium composition using *L. kefiranofaciens* ATCC 8007 cells. Initially, in a small scale, different cultivation media containing different carbon sources, including glucose, lactose, sucrose and starch, were tested and selected for preliminary experimentation to investigate their potential for the production of high yields of kefiran. The best suitable medium produced 0.72 g/L of kefiran and contained 100 g/L sucrose, 10 g/L yeast extract (which is generally known as a source of nitrogen and growth factors), 1 g/L Tween 80, 2 g/L K_2HPO_4 (a source of phosphate), 5 g/L sodium acetate, 2 g/L triammonium citrate, 0.2 g/L $MgSO_4 \cdot 7H_2O$ and 0.05 g/L $MnSO_4 \cdot 5H_2O$. Furthermore, the composition of this medium was optimized in shake flask level, where different concentrations of sucrose (0.0–100 g/L), yeast extract (0.0–14 g/L) and K_2HPO_4 (0.0–2 g/L) were evaluated. The final composition of the optimal medium for kefiran production contained sucrose, yeast extract and K_2HPO_4 at 20.0, 6.0 and 0.25 g/L, respectively, and resulted in the production of 1.29 g/L of kefiran. Finally, in order to evaluate the scalability of the cultivation process, the cell growth and kefiran production were studied in a 16-L pilot scale stirred tank bioreactor under un-controlled pH conditions, using the optimized medium. The maximal cell mass in bioreactor culture reached 2.76 g/L concomitant with kefiran production of 1.91 g/L, after 40 h. In addition, the grow rate and kefiran production rate were 0.051 g L^{-1} h^{-1} and 0.053 g L^{-1} h^{-1}, respectively. Dailin *et al.* (2016) also observed that kefiran was not produced during the first 6 h of cultivation, which was considered as the lag phase where the cells were adapted to the new environment. Kefiran was then produced during the exponential growth phase, showing that kefiran production is associated with the cell growth. However, they concluded that kefiran production is not highly dependent on the amount of biomass achieved by the end of the fermentation, but rather on the type and concentration of nutrients added [87].

Furthermore, Zajšek *et al.* (2013) not only focused on maximizing the production of kefiran through the optimization of medium composition, but also through the optimization of the process parameters. Therefore, they studied the influence of fermentation temperature, agitation rate, and additions of carbon sources, nitrogen sources, vitamins and minerals on the production of kefiran by kefir grains lactic acid bacteria, in a series of experiments using customized milk as fermentation medium. Initially, Zajšek *et al.* (2013) optimized the

process parameters (temperature and agitation rate) during kefiran production from kefir grains. Optimal temperature was found to be 25 ºC and the maximal kefiran production was achieved at an agitation rate of 80 rpm. Higher agitation rates (80-160 rpm) resulted in a lower kefiran content in the grains, since intensive mixing may cause fragmentation of the grains into small pieces, resulting in their damage. Posteriorly, they optimized the composition of the fermentation medium (by addition of carbohydrates, nitrogen sources, vitamins and minerals). Two selected monosaccharides (glucose and fructose) and disaccharides (sucrose and lactose) were tested as the carbon source. Kefiran production was highest when lactose was used, probably due to the consumption of galactose (produced in lactose hydrolysis) required for kefiran biosynthesis. The influence of nitrogen source was studied by testing two organic (tryptone and meat extract) and two inorganic (ammonium nitrate and ammonium chloride) nitrogen sources. Kefiran production was higher using organic nitrogen sources than inorganic nitrogen compounds, however, they found that kefiran production was highest without the addition of any of those nitrogen sources (control experiment). Concerning to vitamins, yeast extract (complex of B vitamins), ascorbic acid (vitamin C), nicotinic acid (vitamin B_3) and thiamine (vitamin B_1) were selected for their research. Maximal kefiran production was achieved when thiamine was supplemented into the basal milk medium. Finally, the optimization of the mineral composition was studied by testing the influence of KCl, $CaCl_2$, $FeCl_3$ and $MgSO_4$. The addition of $FeCl_3$ gave the highest kefiran production, followed by the addition of $CaCl_2$, KCl, and $MgSO_4$. Thus, Zajšek *et al.* (2013) concluded that 5% (w/v) lactose, 0.1% (w/v) thiamine and 0.1% (w/v) $FeCl_3$ led to the maximal production of this EPS, and that good kefir grains growth does not appear to be a determining factor for a high production yield of kefiran [85], as it was later concluded by Dailin *et al.* (2016), like described above [87].

Additionally, Yokoi and Watanabe (1992) studied the optimization of the culture conditions through evaluation of the influence of pH, temperature, and addition of carbon sources, nitrogen sources and other additives, for the effective production of kefiran, using *Lactobacillus* sp. KPB-167B cells isolated from kefir grains. The temperature at 30°C and the pH controlled at 5.0 were the most favorable for kefiran production, when compared to temperatures of 25 ºC and 35 ºC, and pH controlled values of 4.5, 5.5 and 6. Between glucose, sucrose and lactose, the last one was found to be the best chemically defined carbon source for the production of kefiran. Concerning to nitrogen sources, the effects of the

concentration of tryptone, yeast extract, meat extract and triammonium citrate on kefiran production were examined. Concentrations of these four ingredients were adjusted to one-fourth, one-half and twice what they were in the original MRSL medium, and they found that the one containing two-fold nitrogen sources resulted in the highest yield of kefiran. Finally, some additives which could stimulate cell growth were also examined, and Yokoi and Watanabe (1992) found that addition of 5mM of $CaCl_2$ to MRSL medium was effective. Therefore, kefiran was produced in a high yield of 2.04 g/L in 4 days, using a modified MRSL medium containing 10% lactose, 5 mM $CaCl_2$ and double the original concentration of nitrogen sources when the pH was controlled at 5.0 and the temperature at 30 °C [90].

LACTATE ACCUMULATION DURING KEFIRAN PRODUCTION

Nevertheless, the improvement of kefiran yield and productivity is not only dependent of the optimization of the growth environment, as it can also be achieved through the decrease of lactate accumulation during kefiran production process. Lactate accumulation represents a serious problem since it is well known that inhibits the growth of LAB (end-product inhibition), such as *L. kefiranofaciens*, even when medium pH is controlled by adding alkali, resulting in a decline in kefiran productivity because its production is associated with cell growth. Thus, it is expected that the removal of lactic acid from the culture medium might enhance kefiran production [91][92]. Continuous culture systems equipped with a separation membrane [93][94] and/or electrodialyzers [95][96] have been developed for the removal of lactate, however, these separation systems make the entire fermentation process mechanically costly and complex. Therefore, some researchers have studied and developed systems of co-culturing with lactate-assimilating yeast, to easily remove lactate from the culture medium and prevent its accumulation [97][98]. Concerning to kefiran production, Cheirsilp *et al.* (2003) and Tada *et al.* (2007) have already investigated the use of lactate-assimilating yeast *Saccharomyces cerevisiae* to improve the kefiran yield and productivity of kefiran-producing LAB *L. kefiranofaciens* [91][92].

Cheirsilp *et al.* (2003) reported a significant increase of cell growth and kefiran production rates when using co-culture of *L. kefiranofaciens* and *S. cerevisiae*, compared with those in monoculture. Under anaerobic condition (which was achieved by passing CO_2 through the medium during fermentation), the kefiran production rate was 36 mg L^{-1} h^{-1} and 24 mg L^{-1} h^{-1} in the co-culture and monoculture, respectively. Under aerobic condition, a

more intensive interaction between these two strains was observed and higher kefiran production rate (44 mg L^{-1} h^{-1}) was obtained compared with that under the anaerobic condition. They concluded that these results might be due to the fact that yeast could grow easily and produce more growth factors necessary for LAB under the aerobic condition. Moreover, kefiran production was further enhanced by an intermittent addition of fresh medium to the co-culture (fed-batch process), resulting in a final kefiran concentration of 5.41 g/L, achieved at 87 h, thereby attaining the highest productivity of 62 mg L^{-1} h^{-1}. The results obtained by Cheirsilp *et al.* (2003) suggest that co-culture of *L. kefiranofaciens* and *S. cerevisiae* not only reduces the lactate concentration by consumption but also stimulates cell growth and kefiran production of *L. kefiranofaciens* [91].

Tada *et al.* (2007) initially studied the kefiran yield improvement by a batch co-culture of kefiran-producing LAB *L. kefiranofaciens* and lactate-assimilating yeast *S. cerevisiae*. However, lactate accumulation was observed, reaching 33 g/L of concentration in the medium and resulting in only 2.25 g/L of kefiran produced in 97 h. Therefore, to enhance kefiran productivity by preventing lactate accumulation, they conducted a lactose-feeding batch operation with feedforward/feedback control during the co-culture, so that the lactate production rate of *L. kefiranofaciens* was balanced with the lactate consumption rate of *S. cerevisiae*. Lactose feeding was stopped or doubled when pH became lower than 4.95 or higher than 5.05, respectively, because a change in lactate concentration leads to a change in medium pH. With this, lactate concentration was kept at less than 6 g/L throughout the fed-batch co-culture using a 5 L jar reactor and kefiran production was increased to 3.15 g/L in 102 h in the fed-batch co-culture. Consequently, the kefiran yield on lactose basis was increased up to 0.033 g/g in the fed-batch co-culture, whereas that in the batch co-culture was 0.027 g/g. Therefore, Tada *et al.* (2007) concluded that fed-batch co-culture was superior to batch co-culture in terms of lactate removal, kefiran production, and kefiran yield on lactose basis [92].

Concerning to the type of operation, batch or fed-batch processes are the most commonly used for kefiran production studies [83]. However, as it was just seen, fed-batch operation results in a higher kefiran yield and productivity when comparing to batch operation, since fed-batch operation allows a complete control over the lactate concentration in the medium, thus reducing its end-product inhibition effect over *L. kefiranofaciens* [92].

2.3.3. Kefiran Production using cheese whey

The use of industry by-products, namely cheese whey (CW), has also been studied as a substrate for kefiran production [99][100]. Cheese whey is the most polluting waste generated in cheese manufacture. This by-product is a green-yellowish liquid which results from the precipitation and removal of milk casein in cheese making processes [101]. Cheese whey represents about 85-95% of the milk volume and retains 55% of milk nutrients. Among the most abundant of these nutrients are lactose (4.5-5% (w/v)), soluble proteins (0.6-0.8% w/v)), lipids (0.4-0.5% (w/v)) and mineral salts (8-10% (w/v)).

Enormous amounts of this by-product are produced since 9 kg of whey are generated for each kg of cheese made [102]. In 2015, the total worldwide production of whey was estimated at about 180 to 190 million tons/year. Therefore, cheese whey represents a significant environmental and health issue due to its high volumes produced and high organic matter content [103], thus, a solution to this pollution problem has become urgent [102]. Various advanced technologies are in use to tackle this whey management issue, and today major fraction of whey is utilized and transformed into valuable products. However, still a significant amount of whey remains unutilized [103]. Whey can be used as a low-cost carbon source for lactose-consuming microorganisms since it contains high amount of lactose [100], thus, this by-product can be potentially applied for economical raw material for kefiran production.

Cheirsilp and Radchabut (2011) evaluated the feasibility of producing kefiran industrially using lactose extracted from cheese whey. In this study, a co-culture system of kefiran producing *L. kefiranofaciens* JCM 6985 with lactate-assimilating yeast was created to improve the yield and the productivity of kefiran by removing lactic acid from the medium, as already discussed in previous chapter. Firstly, co-cultures of *L. kefiranofaciens* with screened yeast strains were performed to select the suitable yeast that could enhance kefiran production. Then, the optimal culture conditions (whey lactose concentration, nitrogen source and its concentration, initial pH and ratio of *L. kefiranofaciens* to yeast) for kefiran production by the co-culture were determined. Moreover, batch and fed-batch fermentation techniques were carried out in a 2 L bioreactor equipped with aeration and pH control systems, to enhance the kefiran production by the co-culture. The co-culture of *L. kefiranofaciens* with *S. cerevisiae* IFO 0216 gave the highest kefiran production, and

therefore was selected for further optimization. The optimal conditions for kefiran production by the co-culture were: yeast extract 4%; cheese whey lactose 4%; initial pH of 5.5; and inoculum of *L. kefiranofaciens* and *S. cerevisiae* IFO 0216 of $2.1x10^7$ and $4.0x10^6$ CFU/mL, respectively. Scaling up the co-culture in a 2 L bioreactor with dissolved oxygen control at 5% and pH control at 5.5 gave the maximum kefiran production of 2.58 g/L in batch culture and 3.25 g/L in fed-batch culture. Therefore, this study confirmed that cheese whey lactose could be used in fact as a carbon source for kefiran production [100].

2.3.4. Methods Used for EPS Extraction, Purification and Quantification

The use of a suitable culture medium is of extreme importance not only in kefiran production but also in kefiran recovery, since lacking interference of medium components with detection and quantification method is necessary to obtain reliable results [104]. Hence, to culture EPS-producing LAB, complex media such as MRS medium and its modifications are frequently applied [87][92][105]. Additionally, milk or whey-based media, which are more closely resembling to the dairy environment, can also be used [106][107]. However, the presence of other polysaccharides (like glucomannans) rather than kefiran in laboratory or milk media may hamper proper EPS analysis [108]. To correct this problem, results obtained after purification and quantification of EPS could be compared with a similar analysis performed on an uninoculated control [109]. Still, it is more convenient to choose a semi-defined or a chemically defined medium, in which all required nutrients are supplied but interfering compounds are minimal [110][111].

EPS EXTRACTION AND PURIFICATION

For EPS recovery in liquid media, numerous variations of a general procedure based on precipitation and subsequent conditioning can be used. The choice of best method will depend on the food matrix or cultivation medium, the microbial strain, and the degree of accuracy and purity required [104], all of which have been shown to significantly affect the final result [112].

Concerning to the extraction and purification of broth EPS, the culture supernatant is normally obtained after centrifugation and processed as described below. However, if the purpose is to recover capsular EPS, a heating step (at 60–90°C for 15–20 min) can be used to release it from the cells [113][114]. Alternatively, cell pellets can be collected after

culturing and subjected to a first step of sonication or prolonged agitation in 0.5% phenol or 0.05 M EDTA [115].

Initially, pre-treatment steps are recommended before starting the EPS extraction procedure, essentially when using complex media, such as milk-based or peptone-based media [116]. In this context, a first heating step (100 ºC for 15 or 30 min) is often applied to inactivate degradation by endogenous enzymes that may be present in the EPS-containing matrix and can hydrolyze the polymer [107][109]. For milk media, enzyme-inactivating heating can be accomplished in a boiling water bath [85][112]. After heating step, removal of proteins can be carried out. This procedure is usually based on protein precipitation with slowly addition of trichloroacetic acid (TCA) under agitation, in final concentrations of 4 to 20% (w/v), which results in a brilliant impurities removal [85][105][117]. Unfortunately, this treatment can also lead to an undesired co-precipitation of about 50% of the EPSs with the medium proteins, resulting in a lower final recovery. To improve EPS extraction, TCA precipitate should be washed at least once [112]. Alternatively to acidic precipitation, digestion with specific proteases can be performed to precipitate the protein fraction of media, if followed by another heating step for subsequent inactivation of those enzymes [118]. Final step of pre-treatment procedure consists of protein and bacterial cells removal through centrifugation. In addition, subsequent membrane-based filtration (ultrafiltration, diafiltration or both) can be done to further purify the product and remove other contaminants [114][118].

After optional pre-treatment steps described above, EPS precipitation is performed. This procedure is frequently based on one or more steps involving the addition of cold ethanol (4 ºC or below) [105][107][110][111][117], isopropanol [119], acetone [85][114] or a combination of acetone and cold ethanol [118]. However, in some cases, the EPS precipitation step can precede, rather than follow, protein and bacterial cell removal [116]. Following EPS precipitation treatments, the resulting material is resuspended in distilled water, optionally decolorized by treatment with active coal, dialyzed against water for 12 to 48 h at 4 ºC and usually lyophilized [111][117][118]. Dialysis is of extreme importance since it allows the removal of simple carbohydrates present in the material, such as residual lactose, which have been co-precipitated with EPSs during ethanol treatment. It is recommended the used of membranes with a molecular mass cut-off of <8000 Da, as EPS fractions of low molecular mass may otherwise be lost in the dialysis water [112]. However,

its application is somewhat inconvenient since it takes much time and requires specific equipment that makes several samples processing very complicated [120].

Subsequently, partially purified EPS lyophilizate can be further processed by washing of the powder with ethanol or dissolving it with 0.3 M of NaOH followed by centrifugation, to help the elimination of extra contaminants [104][113]. Finally, purification steps involving ion-exchange chromatography, [106] size exclusion chromatography [113] or both [110][111] can also be applied, yet, these procedures are less important when the purpose is only to quantify EPS production [112].

Hence, a general difference can be made between the use of complex and non-complex media, whereby the former requires extensive pre-treatment protocols and the latter requires only a simplified approach performing a basic deproteinization before the centrifugation step (**Figure 7**) [116].

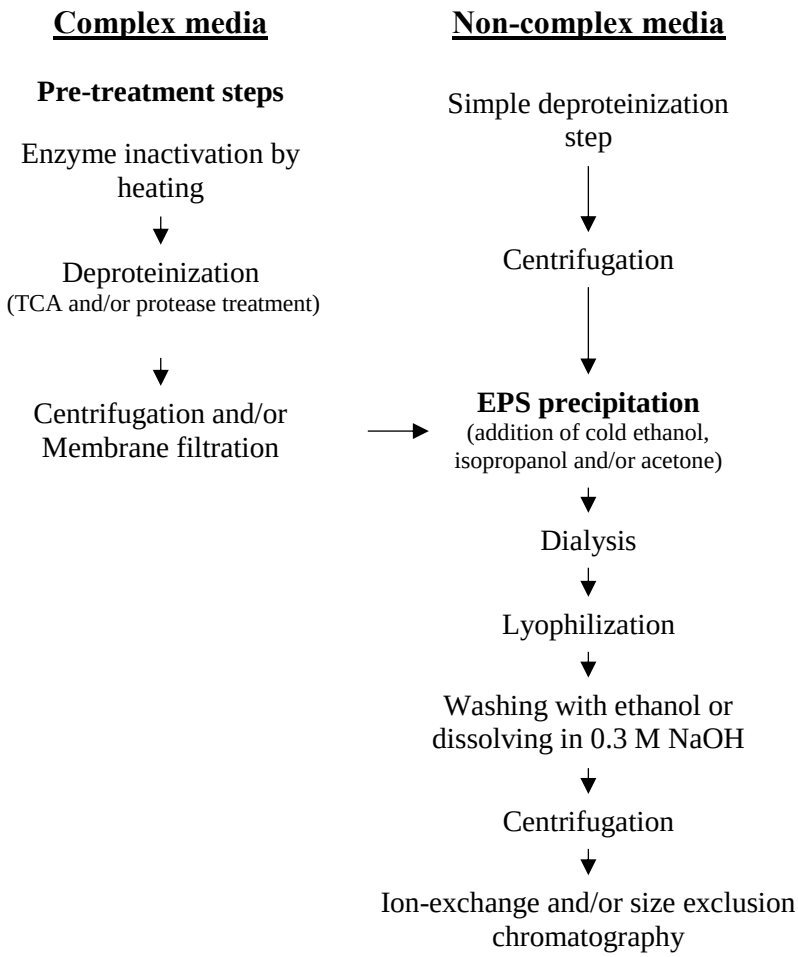

Figure 7. General scheme outlining the extensive and simplified protocols for EPS recovery starting from complex and non-complex media, respectively. Adapted from [116].

EPS QUANTIFICATION

Following EPS extraction and purification, its quantification can finally be performed. Gravimetrics is the most simple and uncomplicated method that can be used to quantify the polymer yields [116]. It consists of measuring the EPS dry mass, for example, after direct drying of isolated polymer or drying of a lyophilized powder, at 37 ºC for 2 days [121][122]. However, this procedure has the disadvantage of taking too much time, when compared to other methods.

Generally, colorimetric methods are the most commonly used, since they are cheap and simple to perform. Yet, they can also lead to serious problems of interference [116]. Phenol-sulfuric acid method from Dubois *et al.* (1956) [123] and anthrone-sulfuric acid method from Ludwig and Goldberg (1956) [124] have being widely applied by several authors to quantify EPS produced by LAB [105][106][111][113][118]. Phenol-sulfuric acid method determines the concentration of total sugar content (both reducing and non-reducing sugars) in a sample, where sugars react to phenol solution under acidic conditions to render an orange-yellow color whose absorbance can be measure at 490 nm [123]. In turn, anthrone-sulfuric method also determines the concentration of total sugars, but yields a blue-green color whose absorbance can be measured at 630 nm [124]. However, both quantification methods do not distinguish contaminating carbohydrates from the specific EPS, which is an undesirable characteristic [116], and may give unreliable results when glucose and galactose molar ration of kefiran is not close to 1:1 [120]. As an alternative for phenol-sulfuric acid method and anthrone-sulfuric acid method, EPS can be hydrolyzed with 2 M sulphuric acid, neutralized with 20% (w/v) NaOH and quantified by reducing sugars determination colorimetric method from Fairbridge *et al.* (1951) [125], as glucose equivalent, which provides reliable results no matter the EPS monosaccharide composition [120].

Furthermore, size-exclusion chromatography can be applied not only to purified EPSs, as already seen, but also for quantification of those polymers, by detection via refractive index measurements in the corresponding elution peak [104].

Nevertheless, the quantification methods mentioned above rely on laborious purification protocols that may be not always straightforward, particularly when production happens within very complex food matrices [116]. Thus, near-infrared spectroscopy (NIR) methods can be applied during fermentation without a prior purification method for rapid

monitoring of exopolysaccharide production [126]. Additionally, EPSs can also be directly monitored in a food matrix on the microstructural level using scanning electron microscopy (SEM) [111] or transmission electron microscopy (TEM) [113], normally after staining with ruthenium red. Alternatively, confocal laser scanning microscopy (CLSM) can be applied, after staining with fluorescent lectin, allowing both qualitatively and quantitatively analysis [127][128].

Recently, Enikeev (2012) developed a new, fast and easy-to-use method for quantitative analysis of EPS in fermented milk products, which seems to overcome the drawbacks previously mentioned related to the deproteinization step with TCA and the dialysis step for simple carbohydrates removal. Instead of using TCA, Enikeev (2012) showed that protein removal can be performed without co-precipitation of EPS by acidification with 12 M HCl for 5 min at 70 ºC, followed by cooling and neutralization with 20% (w/v) NaOH solution and phenolphthalein. Additionally, since proteins are removed at once with this acidification step, the exopolysaccharides can be easily washed from mono- and disaccharides (namely lactose) during EPS precipitation steps with cold ethanol, not requiring the application of dialysis [120].

Finally, to ensure that the purification process was successfully achieved, absence of proteins and other sugars rather than kefiran can be assessed by the Bradford method [129] and qualitative thin-layer chromatography (TLC) on silica gel using n-propanol–acetic acid–water (70:20:10) as the mobile phase, respectively [130].

2.3.5. Biological Properties and Potential Applications

As previously mentioned, the EPSs produced by LAB have attracted considerable interest, since they can exhibit advantageous biological properties (**Table 3**). In the case of kefiran, those properties include immunostimulatory, antibacterial, antifungal [80] and anti-inflammatory activities [131]. Additionally, oral administration of kefiran to mice indicates that it has antitumor activity and a delayed-type hypersensitivity induced by picryl chloride [80][132]. Furthermore, kefiran shows an enhancing effect on the production of interferon β from animal cells, which is suppressed by the stress hormones cortisol and noradrenaline [133], it has been reported to modulate the gut immune system and to protect epithelial cells against *Bacillus cereus* exocellular factors [134] and it also decreases cholesterol blood level

by trapping enterohepatic-circulating cholesterol in the intestine and, therefore, this exopolysaccharide may be therapeutic in the treatment of high cholesterol. Besides, kefiran has various prevention functions where it also acts as a preventive for hepatic disorders (caused by cholesterol and orotic acid) and decreases intestinal histamine concentration [135]. Moreover, kefiran possesses wound healing properties and increases the resistance of lactic acid bacteria to antibiotics and their capacity for intestinal adhesion and colonization [82], thus it may also act as prebiotic, supporting the growth and expression of known probiotic bacteria, namely *Bifidobacterium bifidum* [136]. Furthermore, kefiran also reduces atherosclerosis in rabbits fed with high cholesterol diet [137].

Table 3. Biological properties of kefiran.

Biological properties	References
Immunostimulatory activity	[80]
Antibacterial activity	[80]
Antifungal activity	[80]
Anti-inflammatory activity	[131]
Antitumor activity	[80][132]
Anti-stress properties	[133]
Modulation of the gut immune system	[134]
Protection of epithelial cells against *Bacillus cereus* exocellular factors	[134]
Decreases cholesterol blood level	[135]
Preventive for hepatic disorders	[135]
Decreases intestinal histamine concentration	[135]
Wound healing properties	[82]
Increases the resistance of LAB to antibiotics and their capacity for intestinal adhesion and colonization	[82]
Prebiotic effect on *Bifidobacterium bifidum*	[136]
Reduction of atherosclerosis	[137]

Based on all the beneficial properties mentioned above, possible uses for kefiran include multiple applications in the food industry for gelling, texturizing, rheology and packaging, as well as exploiting its antimicrobial, anti-inflammatory and wound healing properties in medical applications. Nevertheless, kefiran applications studies have focused essentially in food industry, as it may be used as an emulsifier, stabilizer, thickener and gelling agent (therefore, as a food additive), and to produce biodegradable edible films for food packaging [7].

KEFIRAN USE AS A FOOD ADDITIVE

Food industry is always looking for new food ingredients to improve mouthfeel and texture of food, therefore, food gels play an essential role since their application can develop attractive gelled food products [130]. Additionally, the increased demand for natural polymers for numerous industrial applications in recent years has led to a renewed interest in EPS production by microorganisms [138]. Examples of industrially important microbial exopolysaccharides are xanthan, gellan, dextrans, pullulan, yeast glucans and bacterial alginates [139]. Still, physical properties of these polymers are such that they are not suited for all applications and there is a demand for novel materials that give improved rheological characteristic [130]. Thus, kefiran has been investigated as a food additive in this context. Rimada and Abraham (2006) found that this EPS enhances rheological properties of chemically acidified skim milk gels, increasing their apparent viscosity and the storage (G') and loss modulus (G'') of these gels [140].

Piermaria *et al.* (2008) studied some of the physicochemical (intrinsic viscosity, flow behavior and ability to perform cryogels) and gelling properties of kefiran solution. Intrinsic viscosity of kefiran was found to be lower than some polysaccharides used as food additives such as locus bean gum or guar gum, but higher than the intrinsic viscosity of some dextrans. At diluted solutions (<1 g/L) kefiran had a Newtonian behavior, which became pseudoplastic at higher concentrations. Furthermore, they showed that kefiran was able to form gels as a result of cryogenic treatment (freezing, storage in the frozen state for a definite time and defrosting), between concentrations of 5.9 and 14.3 g/L. Kefiran cryogels were translucent and sufficiently cohesive to support their own weight (self-supporting) and had a high water-holding capacity. Additionally, they were found to melt at temperatures about 37 ºC, which demonstrates that kefiran cryogels had the ability to melt at mouth temperature. At those temperatures, both storage and loss modulus fell into good agreement with those obtained for unfrozen samples, indicating that the cryogels revert to normal kefiran solutions after melting. These results suggest that kefiran cryogels could be an interesting alternative for its application in food formulations [130].

In addition, Wang *et al.* (2008) not only studied the physicochemical properties (thermal stability, emulsifying capability and flocculating activity) of kefiran but also compared them with those of xanthan gum, guar gum and locust gum. Thermal properties of

kefiran were analyzed with a scanning calorimeter and the melting points obtained for kefiran, xanthan gum and guar gum were 93.4 ºC, 153.4 ºC and 490.1 ºC, respectively. Thus, the EPS showed a different thermal behavior than the other commercially available gums analyzed. Concerning to emulsifying capability, the purified and partially purified fraction of kefiran retained 88.04% and 84.12% of the emulsification activity after 60 min, respectively. The guar gum and locust gum retained 37.47% and 65.46%, respectively, whereas xanthan gum (which has been widely used in the food industry because of its high emulsifying activity) produced 81.10% emulsion activity after 60 min. Therefore, the purified exopolysaccharide showed better activity when compared with the other commercially available gums analyzed. Flocculating capability test was performed at EPS concentration ranging from 0.1 to 0.6 mg in 5 mg/L dispersion of charcoal-activated carbon. The optimal flocculant concentration in test solution was determined to be 0.4 mg/mL, whereas the optimal flocculant concentration for xanthan gum and guar gum (controls) was 0.3 and 0.5 mg/L, respectively. Hence, kefiran showed a better flocculating activity than guar gum and almost similar to xanthan gum. Wang *et al.* (2008) concluded that kefiran is expected to have a great potential for use as an emulsifier and to be a useful flocculating agent in the food industry [138].

KEFIRAN USE AS A COMPONENT IN BIODEGRADABLE EDIBLE FILMS

Kefiran shows promise as a component in biodegradable edible films. These films are important because environmentally and health-conscious consumers (and therefore the food industry) demand products employing fewer artificial preservatives in their preparation and less petroleum-based products in their packaging, while still insisting on high-quality products that resist spoilage [7]. In recent years, researchers and industry have paid increasing attention to biopolymer-based packaging as a potential alternative to those conventional synthetic polymer-based materials used in food-packaging. Water-soluble polysaccharides (such as starches, chitosan, cellulose derivatives, alginates, carrageenan, pectins and gums), proteins (animal or plant-based) and lipids are usually used for this purpose [141][142]. These materials offer the possibility of creating thin edible films and coatings for covering fresh or processed foods to extend shelf life [141]. Moreover, these edible and/or biodegradable polymer films can be used to cover food surfaces, form a barrier against oxygen, aroma, oil and moisture, prevent quality deterioration of food products,

separate incompatible zones and ingredients or perform as pouches or wraps [143]. Kefiran is an attractive choice over those water-soluble polysaccharides due to its immunomodulation, antibacterial, antifungal, and antitumor properties [143], and according to the literature, it can produce films with satisfactory mechanical properties and good appearance, thus appearing to have excellent potential as film-forming agent [144]. However, films based on kefiran (or any polysaccharide) alone are relatively stiff and brittle, so plasticizers such as water, oligosaccharides, polyols, and lipids are necessary to improve film flexibility, extensibility and handling since they strongly affect the physical properties of biopolymers, reducing intermolecular forces and increasing the mobility of polymer chains [142][145].

Glycerol is the most commonly plasticizer used for edible films, mainly due to its stability and compatibility with hydrophilic bio-polymeric packaging chains [142][143]. Therefore, Piermaria *et al.* (2009) evaluated the ability of kefiran to form films and the effect of glycerol addition at different concentrations on film properties. Rheological characterization of film-forming solutions and physicochemical characterization of films (film transparency and thickness) were performed, and water vapor barrier properties of films were also studied. Kefiran was able to form films at concentrations ranging from 5 to 10 g/kg. All film-forming solutions exhibited a pseudoplastic behavior and glycerol addition did not modify their rheological properties. Kefiran film transparency was within the range of some commonly used synthetic films such as oriented polypropylene and low-density polyethylene. Glycerol addition did not modify this property nor the film thickness. Water vapor permeability (WVP) of kefiran films was lower than those reported for other hydrocolloid films, thus indicating that these films exhibited good water vapor barriers properties. However, at high glycerol concentrations the WVP of the kefiran films increased, since glycerol decreases the attractive forces between polymer chains which allows water molecules to diffuse more easily. Therefore, plasticizers should only be used at minimum amount required to obtain the advantage of enhancing the film. Thus, for kefiran film formulations, Piermaria *et al.* (2009) concluded that the optimum glycerol concentration was 25 g of glycerol per 100 g of polysaccharide [142].

In addition, Piermaria *et al.* (2011) compared the effect of different sugars (glucose, galactose and sucrose) and polyols (glycerol and sorbitol) as plasticizers on kefiran film transparency, opacity, water activity (a_w), water vapor barrier and mechanical properties. All

kefiran films obtained were transparent with very low opacity compared to data previously reported for other films [145][146]. Water activity values of the films were low enough (a_w<0.5) to avoid microbial growth. Yet, the values in unplasticized films were not significantly different to those obtained in plasticized films, which could be due to the fact that kefiran may be able to retain large amounts of water bounded in its structure, acting as a water activity depressant. Plasticizers addition to kefiran films improved both water vapor barrier and mechanical properties. WVP, tensile strength and elastic modulus decreased significantly whereas elongation at break increased in plasticized films, enhancing film flexibility. Regarding the plasticizer type added to kefiran films, the lowest permeability value was obtained with glucose as plasticizer, reducing WVP by 60.65% compared to unplasticized films. On the other hand, the best mechanical properties were obtained with glycerol addition; the elongation at break reached a value 62 times higher than those obtained for the unplasticized film [145].

In a different approach, Montedayen *et al.* (2013) developed new edible composite films by blending corn starch with kefiran and using glycerol as plasticizer. The objective of this study was to characterize their physical, mechanical and water vapor barrier properties. Film-forming solutions of different ratios of kefiran to corn starch (100/0, 70/30, 50/50, 30/70) were cast at room temperature. All films were transparent and homogeneous, easy to handle and not sticky. Increasing starch content from 0% to 50% (v/v) decreased the WVP of films, however, with further starch addition the WVP increased. They were able to prepare films incorporating the strengths of both film producers, with kefiran's good mechanical properties overcoming the weaknesses of starch's mechanical properties. Additionally, the electron scanning micrograph for the composite film was homogeneous, without signs of phase separation between the components. Thus, it was observed that these two film-forming components were compatible, and that an interaction existed between them [143].

KEFIRAN USE IN TISSUE ENGINEERING

Despite several interest in studying kefiran films for food applications, practically no application has been proposed regarding tissue engineering. Only recently, Montesanto *et al.* (2016) evaluated kefiran potential application as scaffold for tissue engineering. Therefore, dense films and porous scaffold from aqueous solutions 2% (w/v) kefiran were prepared and characterized by SEM to assess morphology features, and differential scanning

calorimetry (DSC) to evaluate thermal properties. They observed that porous scaffolds can be produced via freeze-drying, while dense films can be obtained via solvent casting. Scaffolds obtained in this study were with interconnected pore structure and good porosity, which allow diffusion of waste products out of the scaffold and supply of nutrient to the tissue or organ. On the other hand, dense films can be used as support for submerged cultures. Results did not only provided new insights into the foaming methods for producing kefiran scaffolds, but also supplied indications on how to optimize the fabrication parameters to design scaffolds with different morphology and mechanical proprieties, which might address new applications of bioabsorbable scaffolds in tissue regeneration [147].

KEFIRAN MEDICAL APPLICATIONS

As earlier mentioned in this chapter, antimicrobial and anti-inflammatory activity of kefiran has led to the investigation of its potential application as a wound healing agent for topical therapy. In this context, Rodrigues *et al.* (2005) tested both kefiran and kefir gel for antimicrobial and cicatrizing activities using agar diffusion experiments and cicatrizing tests on rats with induced skin lesions and infected with *Staphylococcus aureus*. Concerning to antimicrobial activity, kefiran was able to inhibit the growth of seven bacteria and a yeast. Both the positive control (5 mg/kg neomycin–clostebol emulsion) and kefir gel resulted in a faster reduction of the wound diameter than the negative control (0.9% NaCl). At day 7 of the experiment, the kefir gel-treated wounds were smaller than the clostebol–neomycin emulsion-treated wounds, indicating that animals treated with kefir gel showed better wound healing compared with those treated with the positive control [148]. More recently, Huseini *et al.* (2012) also evaluated the wound healing capacity of kefir gel in the treatment of burns. The healing properties were tested in an animal model with experimental burn and contamination with *Pseudomonas aeruginosa*. After 2 weeks of treatment, the wound area and the percentage of inflammation were reduced in animals treated with kefir gel compared with those treated with silver sulfadiazine cream, which is conventionally used for the topical treatment of burns of second and third degrees. In addition, the percentage of epithelialization and healing in animals treated with kefir gel was also improved [149]. Both studies suggest that kefiran and kefir gel have a good healing capacity, which may result from the synergistic action between their antimicrobial and anti-inflammatory activities [3].

3. Materials and Methods

3.1. Microorganisms

3.1.1. Pure Culture

Lactobacillus kefiranofaciens WT-2B, which was used to produce kefiran in this study, was obtained freeze-dried from Leibniz Institute DSMZ – German Collection of Microorganisms and Cell Cultures. The microorganism was rehydrated, activated and grown under strictly anaerobic conditions as indicated in the catalogue provided. Stock cultures were prepared and stored in 25% glycerol at -80°C. The strain was also maintained at 4 ºC in MRS agar solid medium and MRS liquid medium.

3.1.2. Mixed Culture

Kefir grains, which were used to produce kefir and kefiran in this study, were obtained from Kefiralia (Burumart Commerce S.L.), Spain, immersed in a special liquid to maintain their freshness and properly sealed in a plastic bag. The grains were preserved at 4 ºC in UHT (ultra-high temperature) whole fat cow milk without stirring. During conservation, the medium was changed weekly, and the grains washed with sterile water.

3.2. Culture Media

3.2.1. Man-Rogosa-Sharpe (MRS) broth medium

MRS broth medium (Sigma-Aldrich, St. Louis, MO, USA) was prepared by dissolving it in distilled water, adding 0.1% (v/v) of Tween 80 (Sigma-Aldrich) and adjusting the final pH value to 6.2 ± 0.2. The medium was then sterilized by autoclaving at 121 ºC for 15 minutes (AJC® Uniclave 88). MRS broth medium composition is presented in **Table 4**.

Table 4. MRS broth medium composition.

Component	Concentration (g/L)	
	Liquid Medium	Solid Medium
Agar	---	20.0
Peptone	10.0	10.0
Meat Extract	8.0	8.0
Yeast Extract	4.0	4.0
D(+)-Glucose	20.0	20.0
Dipotassium hydrogen phosphate	2.0	2.0
Sodium acetate trihydrate	5.0	5.0
Triammonium citrate	2.0	2.0
Magnesium sulfate heptahydrate	0.2	0.2
Manganous sulfate tetrahydrate	0.05	0.05

3.2.2. Milk

Different milk samples were used in this work, namely UHT whole fat cow milk (acquired commercially - Mimosa, Lactogal Produtos Alimentares S.A., Portugal) and raw cow milk (provided by Lacto Serra – Comercialização e Fabrico de Lacticínios, Lda. Aguiar da Beira, Portugal). Nutritional composition of the UHT milk is shown in **Table 5**. The raw cow milk sample was pasteurized at 90 ºC for 15 minutes, as described by Guzel-Seydim *et al.* (2011) [150], and therefore, it is referred as "pasteurized milk sample" during this work.

Table 5. Nutritional composition of UHT milk according to commercial information.

Component	Concentration (g/L)
Total Fat	36
Saturated Fat	23
Total Carbohydrate	49
Sugars	49
Protein	33
Salt	1

3.2.3. Cheese Whey

Six different cheese whey samples were used in this work and were supplied by Lacto Serra – Comercialização e Fabrico de Lacticínios, Lda. Aguiar da Beira, Portugal (**Table 6**).

Table 6. Cheese whey samples provided by Lacto Serra.

Sample Number	Cheese Whey
CW1	Sheep (Normal, raw)
CW2	Sheep (Ultrafiltrated, raw)
CW3	Sheep (Concentrated, ultra and nanofiltrated, raw)
CW4	Cow (Normal, pasteurized)
CW5	Cow (Concentrated, pasteurized)
CW6	Unknown (Concentrated)

Before being used, all cheese whey samples were deproteinized according to the procedure of Ahn *et al.* (2000), with some modifications [151]. Cheese whey was autoclaved at 121 ºC for 20 minutes (AJC® Uniclave 88), followed by decantation and centrifugation at 5000 rpm for 1 h under refrigeration (Megafuge 16R centrifuge, Thermo Scientific) in sterile 50 mL Falcon centrifuge tubes, for removal of the precipitated protein aggregates. After centrifugation, the resulting supernatant was decanted again, to ensure protein removal.

In order to choose the best cheese whey sample for kefiran production, pH value, conductivity and reducing sugars content were determined for all samples.

3.3. Study of Kefir production by Mixed Culture

Kefir grains were inoculated in UHT whole fat cow milk using 250 mL glass jars (previously autoclaved at 121 ºC for 20 minutes) and incubated for about 24 h in batch mode. At regular time intervals, samples were taken (3.5 mL each time) to determine pH and analyze lactose, glucose, lactic acid, acetic acid and ethanol concentrations by HPLC. Assays with different fermentation conditions were performed to evaluate the effect of initial kefir grains concentration (3%, 6% and 9% (w/v)), initial milk volume (100 and 200 mL), agitation rate (0, 60 and 180 rpm) and temperature (controlled, 28 ºC, and room temperature, 21-25 ºC) in kefir production, as summarized in **Table 7**. In the end, the best conditions determined for kefir production using UHT milk were also evaluated using the pasteurized cow milk sample provided by Lacto Serra. For each condition tested, two assays were performed, in duplicate (so that it was possible to calculate the mean and the standard deviation), one starting 10 h after the other, to better follow the fermentation process along 24 h straight.

Table 7. Assays with the different conditions tested for kefir production using UHT milk.

Assay	Initial Kefir Grains Concentration % (w/v)	Initial Milk Volume (mL)	Agitation Rate (rpm)	Temperature (ºC)
1	3	200	0	28
2	6	200	0	28
3	9	200	0	28
4	9	100	0	28
5	9	200	60	28
6	9	200	180	28
7	9	200	0	21-25

At the end of each assay, kefir grains were separated from the medium by filtration using a metal household sieve and washed with sterile water. Then, kefir grains mass was determined by weighting (Sartorius BP 3100 S). All the procedures were performed in a laminar flow chamber (BBH4 Braun Horizontal), to ensure sterile conditions.

3.4. Study of Kefiran production by Mixed Culture

Kefiran production by mixed culture was studied using 100 mL of five different media: MRS broth medium, UHT and pasteurized cow milk, cheese whey samples CW1 and CW5. The assays were made in duplicate (so that it was possible to calculate the mean and the standard deviation) in 250 mL glass jars (previously autoclaved at 121 ºC for 20 minutes) and were inoculated with kefir grains in order to obtain an initial concentration of 10% (w/v). The jars were incubated at 28 ºC without agitation for 96 h. At regular time intervals, a 3.5 mL sample was taken to determine pH and to later analyze lactose, glucose, lactic acid, acetic acid and ethanol concentrations by HPLC. After fermentation, the kefir grains were separated from the fermented medium by filtration using a metal household sieve, and the remaining medium was stored at -20 ºC to further extraction, purification and quantification of kefiran. The kefir grains were washed with sterile water and their mass was determined (Sartorius BP 3100 S). All the procedures were performed in a laminar flow chamber (BBH4 Braun Horizontal), to ensure sterile conditions.

3.5. Study of Kefiran production by *Lactobacillus kefiranofaciens*

3.5.1. Inoculum preparation

The inoculum was prepared by transferring 1.2 mL of a liquid stock culture of *L. kefiranofaciens* WT-2B into 10 mL of MRS broth medium and incubating at 30 ºC with an agitation rate of 180 rpm along 40 h (until the end of the exponential phase), under anaerobic conditions.

3.5.2. Assays

Each fermentation was carried out in duplicate (allowing to calculate the mean and the standard deviation), by transferring 5 mL of the inoculum into 45 mL of medium and incubating at 30 ºC with an agitation rate of 180 rpm for 54 h or 148.5 h, under anaerobic conditions. Those conditions were achieved by purging with N_2 the culture medium inside 120 mL encapsulated vials (previously autoclaved at 121 ºC for 20 minutes), followed by addition of reducing agent L-cysteine-HCl 10% (w/v) solution to depress and poise the redox potential at optimum levels. Two different media were tested: MRS broth medium and cheese whey sample CW5. At regular time intervals, samples were taken to determine pH

and optical density at 620 nm, and to later analyze lactose, glucose, lactic acid, acetic acid and ethanol concentrations by HPLC. The remaining culture medium was stored at -20 ºC to further extraction, purification and quantification of kefiran.

All the procedures were carried out in a laminar flow chamber (BBH4 Braun Horizontal) and working near an open flame, to ensure sterile conditions. At the end of each assay, a microbial contamination test was also performed in MRS agar plates.

3.6. Kefiran extraction, purification and quantification

Extraction, purification and quantification of kefiran was performed based on the procedures of Rimada and Abraham (2003), with some modifications [112]. Initially, 10.0 mL of the remaining fermentation medium were heated at 100 ºC for 15 minutes in a boiling water bath, to avoid degradation by endogenous enzymes, followed by slowly addition of 1 mL of 20% (w/v) TCA solution, for protein precipitation. After storage at 4 ºC for 24 h, protein and bacterial cells removal was achieved through refrigerated centrifugation at 5000 rpm for 20 minutes and 4 ºC (Megafuge 16R centrifuge, Thermo Scientific). Then, 4 volumes of cold absolute ethanol (4 ºC) were added to the resulting supernatant for kefiran precipitation. After storage at -20 ºC for 24 h and centrifugation (5000 rpm, 15 minutes, 4 ºC), kefiran pellet was resuspended in 5.00 mL of hot distilled water, treated again with 4 volumes of cold absolute ethanol (4 ºC), stored at 4 ºC for 24 h and centrifuged once more (5000 rpm, 15 minutes, 4 ºC). Next, kefiran pellet was resuspended in 10.0 mL of hot distilled water, dialyzed for 48 h against 4 changes of distilled water (Molecular weight cut-off 6000-8000 Da, Spectra/Por) and lyophilized. Kefiran quantification was achieved by measuring the dry mass of the lyophilized fraction. In the end, the purified kefiran fractions were tested for the absence of lactose, glucose and other compounds by HPLC, following the analytical method described in **Chapter 3.7.5.**. Additionally, to confirm the presence of kefiran after extraction, purification and quantification procedures, acid hydrolysis of kefiran fractions was performed: 200 µL of H_2SO_4 72% solution were added to 3 mg of each sample and incubated at room temperature for 3 h, followed by addition of 1.00 mL of distilled water and incubation at 120 ºC for 1 h. After acid hydrolysis, pH value was adjusted to 1, and samples were then analyzed by HPLC.

3.7. Analytical Methods

3.7.1. pH

The pH was determined in samples with a pH meter Hach sensION^{TM}+ MM340.

3.7.2. Conductivity

Conductivity values of milk and cheese whey samples were determined using a conductivity meter Russell Model RL105 equipped with a Sentek electrode.

3.7.3. Determination of Reducing Sugars Content

Reducing sugars content was determined by DNS method, described by Miller (1959) [152], where 3,5-dinitrosalicylic acid (DNS) is reduced to 3-amino-5-nitrosalicylic acid, passing from yellow to reddish orange. Initially, deproteinized cheese whey samples were centrifuged at 13000 rpm for 8 minutes (Eppendorf MiniSpin), after a dilution of 1:100. In DNS method, 1 mL of DNS reagent was added to 1.00 mL of supernatant of each sample, then, samples were heated to 100 ºC for 5 minutes and cooled in ice to stop the reaction. Next, 10.0 mL of distilled water were added, and absorbance was finally measured at 540 nm using a Shimadzu UVmini-1240 spectrophotometer. The calibration curve for quantification was done using glucose with concentrations between 0 and 1 g/L (**Appendix 1**).

3.7.4. Determination of Biomass Concentration

L. kefiranofaciens biomass was monitored during fermentation by measuring optical density at 620 nm with a Shimadzu UVmini-1240 spectrophotometer (Turbidimetric Method), which was converted into concentration using a calibration curve that relates biomass dry weight with optical density (**Appendix 2**). Biomass dry weight was determined in triplicate, by filtering 10.0 mL of sample from a previously prepared inoculum with a 0.45 µm membrane filter (Whatman, ME 25/21 ST), and washing with distilled water. Membranes were then dried at 100 ºC until constant weight (Gravimetric Method). To obtain the calibration curve, optical density from different dilutions (1:100 to 1:2) of the inoculum used to determine biomass dry weight was measured.

3.7.5. Determination of Substrates and Metabolites Concentration

Lactose, glucose, lactic acid, acetic acid and ethanol concentrations were measured by high performance liquid chromatography (HPLC). Initially, samples were centrifuged at 13000 rpm for 8 minutes (Eppendorf MiniSpin) for biomass removal, and the supernatant was collected and stored at -20 ºC. Before HPLC analysis, 500 µL of supernatant were treated with 25 µL Carrez Reagent 1 and 25 µL Carrez Reagent 2, as described by Indyk *et al.* (1996) [153], and centrifuged again at 13000 rpm for 8 minutes (Eppendorf MiniSpin) for removing of any interfering compounds, namely proteins and fats (except for samples from assays with MRS medium). After a dilution of 1:5, 1:12 or 1:30 (depending on the sample), the resulting supernatant was filtered off with 0.2 µm filters (VectaSpin Micro, Whatman) at 8000 rpm (Eppendorf MiniSpin) for 20 minutes. Finally, 10 µL of filtrate were injected in a LaChrom Elite HPLC chromatograph (Hitachi) equipped with a L-2130 pump (Hitachi), a L-2200 autosampler injector (Hitachi), a Gecko 2000 oven (Cluzeau Info Labo) at 65 ºC, a Rezex™ ROA – Organic Acid H^+ (8%) column (Phenomenex), and a L-2490 Refractive Index (RI) Detector (Hitachi), using H_2SO_4 0.005 N as eluent with a flow rate of 0.5 mL/min. A calibration curve was done using standard solutions with defined concentrations of lactose, glucose, lactic acid, acetic acid and ethanol (0-5 g/L) for quantification of the analyzed compounds.

3.8. Calculations

3.8.1. Volumetric rates

Lactose volumetric consumption rate, $r_{lactose}$ (g L^{-1} h^{-1}), and glucose volumetric consumption rate, $r_{glucose}$ (g L^{-1} h^{-1}), were determined from the beginning of the fermentation until substrate exhaustion, using the following equation:

$$-r_S = \frac{Substrate\ Concentration_{initial} - Substrate\ Concentration_{final}}{Time_{final} - Time_{initial}} \qquad \textbf{Equation (1)}$$

Lactic acid volumetric production rate, $r_{lactic\ acid}$ (g L^{-1} h^{-1}), and ethanol volumetric production rate, $r_{ethanol}$ (g L^{-1} h^{-1}), were determined from the beginning of the assay until the maximum product concentration was reached, using the following equation:

$$r_P = \frac{Product\ Concentration_{final} - Product\ Concentration_{initial}}{Time_{final} - Time_{initial}}$$ **Equation (2)**

3.8.2. Yields

The yield of lactic acid on substrate, $Y_{lactic\ acid/substrate}$ (g/g), the yield of ethanol on substrate, $Y_{ethanol/substrate}$ (g/g), and the yield of kefiran on substrate, $Y_{kefiran/substrate}$ (g/g), were determined from the beginning of the assay until the maximum product concentration was reached, using the following equation:

$$Y_{P/S} = \frac{Product\ Concentration_{final} - Product\ Concentration_{initial}}{Substrate\ Concentration_{initial} - Substrate\ Concentration_{final}}$$ **Equation (3)**

The yield of biomass on substrate, $Y_{biomass/substrate}$ (g/g), was determined from the beginning of the assay until the maximum biomass concentration was obtained, using the following equation:

$$Y_{X/S} = \frac{Biomass\ Concentration_{final} - Biomass\ Concentration_{initial}}{Substrate\ Concentration_{initial} - Substrate\ Concentration_{final}}$$ **Equation (4)**

3.8.3. Maximum Specific Growth Rate

Maximum specific growth rate, μ_{max} (h^{-1}), was calculated by the determination of the slope during the exponential phase, using the following expression:

$$\ln([X_t]) = \mu_{max} t + \ln([X_0])$$ **Equation (5)**

Where t stands for time (h), $[X_t]$ for biomass concentration during the fermentation time (g/L), and $[X_0]$ for initial biomass concentration (g/L).

4. Results and Discussion

4.1. Study of Kefir Production by Mixed Culture

In order to improve kefir production process, several assays using kefir grains and UHT milk with different operational conditions were performed. Those conditions include the initial concentration of kefir grains, medium volume, agitation rate and temperature. In the end, kefir production was also assessed using pasteurized cow milk sample provided by Lacto Serra company, with the best conditions determined.

4.1.1. Milk Samples Characterization

To study kefir production, UHT whole fat cow milk was chosen, based on the literature, as it seems to provide better sensorial and textural properties to kefir [7][9][50]. In addition, the use of pasteurized cow milk supplied by Lacto Serra was also studied, as it was intended by the company. Before kefir production assays, both UHT and pasteurized cow milk samples were chemically characterized.

According to **Table 8**, pasteurized milk presented a slightly higher lactose content, 53.64 g/L, when compared to UHT milk, 48.74 g/L. In its turn, lactic acid concentrations, 0.44-0.50 g/L, and pH values, 6.42-6.51, were similar in both samples, as lactic acid is known to be responsible for the pH value in milk and fermented milk beverages [3]. Neither ethanol nor acetic acid were detected, thus, all the results obtained were close to the reference values found in the literature [50]. Glucose concentrations observed were minimal (less than 0.2 g/L), and, therefore, despised in comparison with lactose concentrations obtained.

Table 8. Lactose, lactic and acetic acids, and ethanol concentrations, and pH values in UHT and pasteurized cow milk samples used in this work, and reference values for those chemical parameters [50].

Chemical Parameters	UHT Milk	Pasteurized Milk	Reference Values for Cow Milk
Lactose (g/L)	48.74	53.64	50
Lactic Acid (g/L)	0.503	0.436	0-0.5
Acetic Acid (g/L)	0	0	0
Ethanol (g/L)	0	0	0
pH value	6.42	6.51	6.6-6.7

4.1.2. Effect of Initial Concentration of Kefir Grains

To study the effect of initial concentration of kefir grains, three different assays were performed with 3%, 6%, and 9% (w/v) of kefir grains. The results obtained are shown in **Figure 8**, **Figure 9**, **Figure 10** and in **Table 9**. Since kefir is a complex mixture of sugars, organic acids and ethanol, the definition of a kefir beverage is based on the ranges of concentration of its main components. According to the literature, a natural kefir contains approximately 40 g/L of sugars, 8-10 g/L of lactic acid, and 0.35-20 g/L of ethanol. Also the pH is used to define a kefir beverage and is usually between 4.2 and 4.6 [3][23]. Thus, these values were used as a reference to perceive and identify, during the different assays, the moment at which kefir was produced. According with these values, for the assay with 3% (w/v) of kefir grains (**Figure 8**), the medium composition corresponded to kefir in about 22 h of fermentation, when lactose and lactic acid contents were 41.2 and 7.72 g/L, respectively, and pH value was 4.29. At the same time, ethanol concentration was 1.18 g/L. For the assay with 6% (**Figure 9**), kefir production could be considered as accomplished around 9 h of fermentation, when the concentrations of lactose, lactic acid and ethanol were 38.8, 8.51 and 1.66 g/L, respectively, and a pH value of 4.35 was obtained. In the assay with 9% of kefir grains (**Figure 10**), kefir was obtained after 8 h of fermentation, when lactose and lactic acid content reached 36.1 and 7.50 g/L, respectively, and pH value was 4.37. At 8 h of fermentation, ethanol content was between 2.72-3.65 g/L.

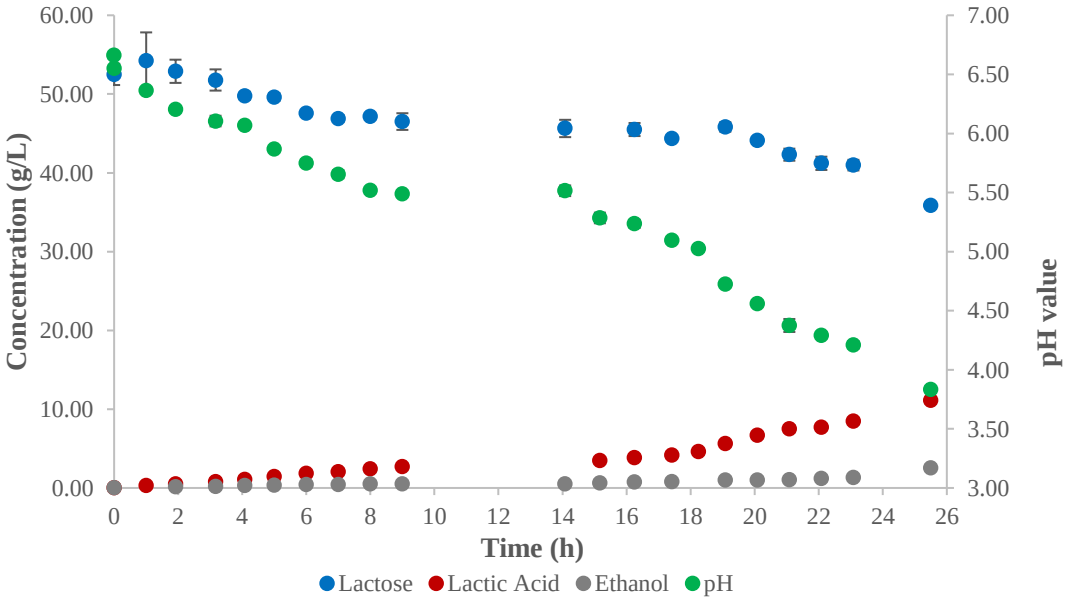

Figure 8. Lactose, lactic acid and ethanol concentrations, and pH value obtained during the assay with 3% (w/v) of kefir grains concentration.

Table 9. Chemical parameters, kefir grains mass variations, volumetric rates and yields obtained in every assay performed concerning to kefir production study and optimization. Kefir grains mass variation values are presented with the standard deviation. $Lactose_t$, lactose concentration at the time kefir production was accomplished; $Lactic\ Acid_t$, lactic acid concentration at the time kefir production was accomplished; $Ethanol_t$, ethanol concentration at the time kefir production was accomplished; pH_t,, pH value at the time kefir production was accomplished.

Parameters	**Initial Concentration of Kefir Grains**			**Initial Milk Volume**	**Agitation Rate**		**Temperature Control**	**Milk Type**
	3% (w/v)	**6% (w/v)**	**9% (w/v)**	**100 mL**	**60 rpm**	**180 rpm**	**No (21-25 ºC)**	**Pasteurized milk**
$Lactose_t$ (g/L)	41.2	38.8	36.1	42.8	36.7	41.6	42.5	42.9
$Lactic\ Acid_t$ (g/L)	7.72	8.51	7.50	7.62	7.83	7.86	8.65	8.74
$Ethanol_t$ (g/L)	1.18	1.66	2.72-3.65	1.19	2.81	1.40	4.39	2.13
pH_t	4.29	4.35	4.37	4.51	4.42	4.64	3.70	4.44
Time to produce Kefir (h)	**22**	**9**	**8**	**5**	**6**	**5**	**15**	**8**
Kefir Grains Mass Variation (%)	46.2±1.7	32.0±1.7	4.77±5.20	37.3±1.6	19.9±6.4	8.98±2.50	22.5±2.0	14.3±4.6
$r_{lactose}$ (g/L.h)	0.65	1.07	1.83	1.93	1.90	2.50	1.29	1.77
$r_{lactic\ acid}$ (g/L.h)	0.43	0.88	0.63	1.08	1.04	1.07	0.56	0.70
$r_{ethanol}$ (g/L.h)	0.10	0.25	0.11	0.36	0.57	0.54	0.26	0.35
$Y_{lactic\ acid/substrate}$ (g/g)	0.67	0.82	0.35	0.56	0.48	0.47	0.43	0.54
$Y_{ethanol/substrate}$ (g/g)	0.15	0.23	0.05	0.19	0.30	0.26	0.20	0.27

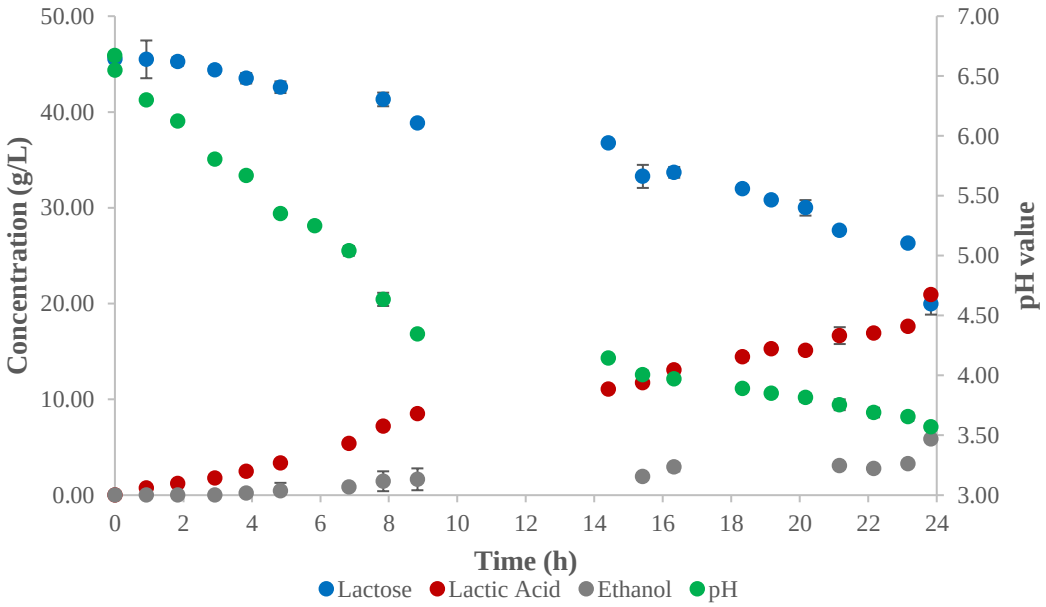

Figure 9. Lactose, lactic acid and ethanol concentrations, and pH value obtained during the assay with 6% (w/v) of kefir grains concentration.

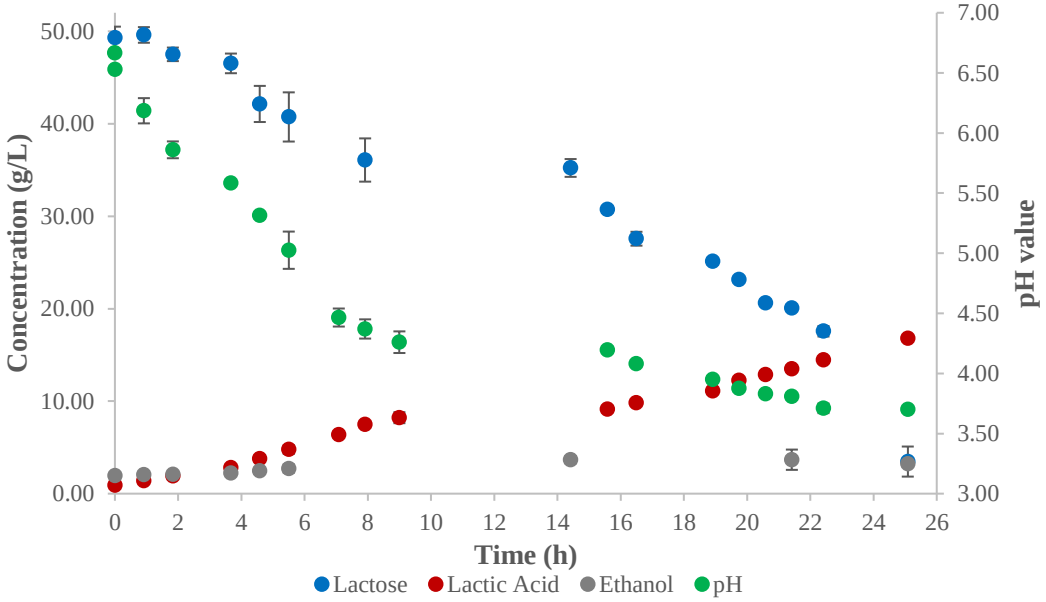

Figure 10. Lactose, lactic acid and ethanol concentrations, and pH value obtained during the assay with 9% (w/v) of kefir grains concentration.

As would be expected, an increase on the initial kefir grains mass concentration resulted in a decrease on the time required for kefir production. However, when increasing the initial concentration from 6% to 9% (w/v), the fermentation process was reduced in only 1 hour, thus, the decrease on the time required for kefir production would eventually stagnate. For that reason, an additional assay with a kefir grains concentration higher than 9% (w/v) was not carried out.

Kefir grains mass was also determined at the end of each assay, to better understand the effect of the different conditions tested in its variation during the fermentation process. According to **Table 9**, an increase on the initial concentration of kefir grains resulted in a lower increase of its own mass during fermentation, since with 3% (w/v) kefir grains increased 46.2%, with 6% (w/v) 32.0%, and with 9% (w/v) 4.77%. Thus, at lower concentrations, the grains appear to have a greater capacity to grow in milk. Yet, the assay performed with an initial concentration of kefir grains of 9% (w/v), should not be considered fully reliable, as the washing step of kefir grains with distilled water was not performed before the beginning of the fermentation, which may have resulted in the determination of a lower kefir grains mass increase. Therefore, the results of kefir grains mass variation presented in **Table 9** were obtained without performing a standard and correct procedure to measure kefir grains mass, hence, they might not be so significant. Kefir grains should have been washed with distilled cold water, every time they were separated from the fermented product by filtration using a household sieve and before initiating each fermentation process, and in addition, they should also have been dried carefully using paper toweling, as described by Zajšek *et al.* (2013) [85], which did not happened. Due to lack of time, this assay was not repeated.

Volumetric rates and yields were determined based on the lactose, lactic acid and ethanol concentrations during fermentation (**Table 9**). Lactose volumetric consumption rate values ($r_{lactose}$) showed that lactose was consumed more rapidly with higher kefir grains concentration. Although the assay with 9% (w/v) presented a higher $r_{lactose}$, 1.83 g/L.h, it did not show the highest volumetric production rates ($r_{lactic\ acid}$ and $r_{ethanol}$) when compared with the other two assays. In fact, the highest $r_{lactic\ acid}$, 0.88 g/L.h, and $r_{ethanol}$, 0.25 g/L.h, were obtained with an initial concentration of 6% (w/v), which also resulted in the highest lactic acid and ethanol yields on substrate, 0.82 g/g and 0.23 g/g, respectively. Additionally, no differences were observed on the appearance of the beverages produced under these conditions, thus an initial mass concentration of 9% (w/v) was chosen for the following assays, since resulted in a faster kefir fermentation, which is of industrial interest.

4.1.3. Effect of Initial Milk Volume

To evaluate the effect of the initial milk volume in kefir production, an assay with an initial kefir grains concentration of 9% (w/v) and an initial milk volume of 100 mL was performed, in order to compare with the previous assay done with 200 mL. This assay was

carried out to assess the impact of the headspace volume in kefir production, as the amount of oxygen available during fermentation may influence the fermentation process, since different types of aerobic and anaerobic microorganisms are present in kefir grains [7][9]. As flasks with 250 mL of total volume were always used in these assays, the use of 100 mL of initial milk volume resulted in a headspace volume 3 times higher, 150 mL, than when was used 200 mL, with only 50 mL of headspace volume. When using 100 mL of initial milk volume (**Figure 11** and **Table 9**), kefir was obtained in approximately 5 h of fermentation, when lactose and lactic acid content were 42.8 and 7.62 g/L, respectively, and pH value was 4.51. In the same period, ethanol concentration was 1.19 g/L. In this assay, kefir production was accomplished faster when compared to assay with an initial kefir grains concentration of 9% (w/v) and an initial milk volume of 200 mL.

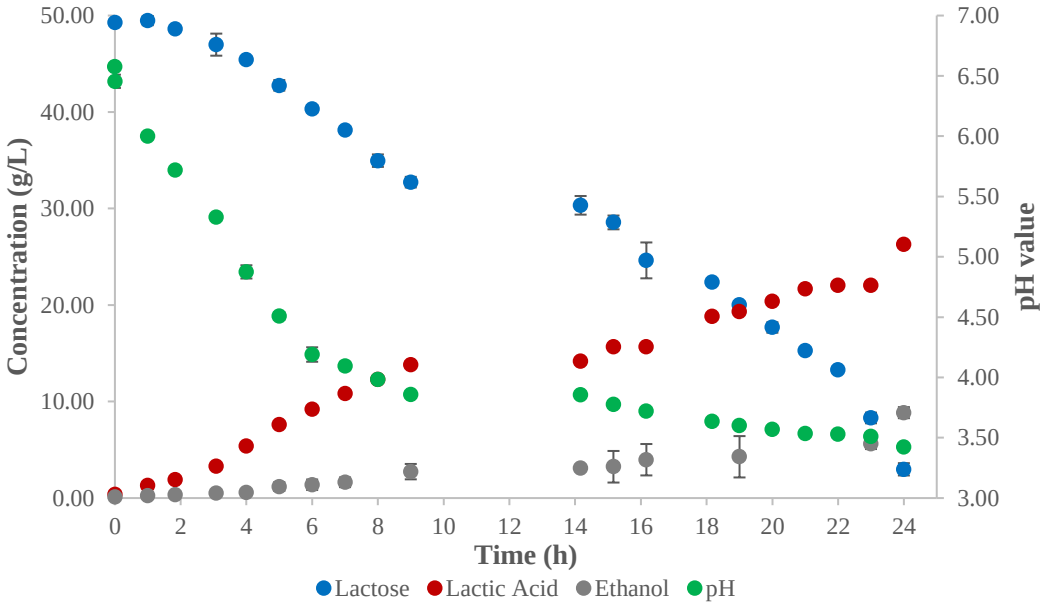

Figure 11. Lactose, lactic acid and ethanol concentrations, and pH value obtained during the assay with 100 mL of initial milk volume.

Kefir grains mass variation was also determined at the end of this assay. According to **Table 9**, it seems that using an initial milk volume of 100 mL results in a significant higher increase of kefir grains mass, 37.3%, when compared to the use of 200 mL, 4.77%. Nevertheless, the assay with an initial kefir grains concentration of 9% (w/v) and an initial milk volume of 200 mL may not be reliable for comparison in terms of kefir grains mass variation, as discussed in **Chapter 4.1.2.**. Volumetric rates and yields were also determined for this assay based on the lactose, lactic acid and ethanol concentrations during fermentation, and were compared with those determined previously for the assay where 200

mL of initial milk volume were used (**Table 9**). Lactose volumetric consumption rate values ($r_{lactose}$) showed that lactose was consumed slightly faster when 100 mL of initial milk volume were used, which is in accordance with the results previously discussed, since kefir was obtained in less time with those conditions. Additionally, the highest volumetric production rates, $r_{lactic\ acid}$, 1.08 g/L.h, and $r_{ethanol}$, 0.36 g/L.h, were also obtained when 100 mL of initial milk volume were used, which also resulted in the highest lactic acid and ethanol yields on substrate, 0.56 g/g and 0.19 g/g, respectively.

Moreover, considering that a significant amount of medium was removed in different proportions during fermentation when used 100 and 200 mL of initial milk volume and 9% (w/v) of initial concentration of kefir grains, since samples of 3.5 mL were taken almost every hour, a volume adjustment was performed in both assays to better understand the impact of the headspace volume and the influence of the amount of oxygen available during fermentation in kefir production. According to **Table 10**, it can be observed that a lower initial milk volume (100 mL), and therefore, a higher headspace volume (150 mL), resulted in the production of less ethanol, 0.951 g/L, but more lactic acid, 6.58 g/L, when compared to the use of 200 mL of milk volume, 2.16 and 6.19 g/L, respectively, which could be related to the presence of a higher amount of oxygen available and, therefore, the favoring of the aerobic microorganisms from kefir grains microflora during fermentation.

Table 10. Lactose consumption and lactic acid and ethanol production at the time kefir was produced, before and after volume adjustment.

Chemical Parameters	**Before Adjustment**		**After Adjustment**	
	100 mL (5 h)	200 mL (8 h)	100 mL (5 h)	200 mL (8 h)
Lactose Consumption (g/L)	6.52	13.2	4.11	11.5
Lactic Acid Production (g/L)	7.22	6.59	6.58	6.19
Ethanol Production (g/L)	1.05	2.31	0.951	2.16

The results obtained might indicate that the usage of 100 mL of initial milk volume rather than 200 mL (that is, a 150 mL headspace volume rather than 50 mL) allowed a faster kefir production process. However, the differences observed between both assays concerning the time to obtain kefir were very likely a result of the sampling process: when using an initial milk volume of 100 mL, there is a more pronounced decrease in total milk volume during fermentation, and consequently, a higher increase in kefir grains concentration, which eventually leads to a faster kefir production. That was the case

observed in this study, as kefir was obtained in only 5 h with 100 mL of initial milk volume, comparing to the 8 h needed to produce kefir when 200 mL of initial milk volume were used (**Table 9** and **Table 10**). Yet, this situation would not be expected in an industrial environment, as sampling volume would be totally neglected. Therefore, it cannot be concluded that a higher headspace provided better results, thus an initial milk volume of 200 mL was chosen for the following assays, as it has already been used in the first trials. To better understand the effect of headspace volume in kefir manufacture process, assays with the same initial milk volume but with different flasks volumes should be carried out.

4.1.4. Effect of Agitation Rate

To evaluate the effect of the agitation rate in kefir production, two different assays with an initial concentration of kefir grains of 9% (w/v), initial milk volume of 200 mL, and an agitation rate of either 60 or 180 rpm were performed. When using an agitation rate of 60 rpm (**Figure 12** and **Table 9**), kefir production was accomplished in around 6 h of fermentation, obtaining a product with lactose, lactic acid and ethanol concentrations of 36.7, 7.83 and 2.81 g/L, respectively, and a pH value of 4.42. In its turn, when using an agitation rate of 180 rpm (**Figure 13** and **Table 9**), kefir was obtained in about 5 h of fermentation, when lactose and lactic acid contents were 41.6 and 7.86 g/L, respectively, and pH value was 4.64. In the same period, ethanol concentration was 1.40 g/L.

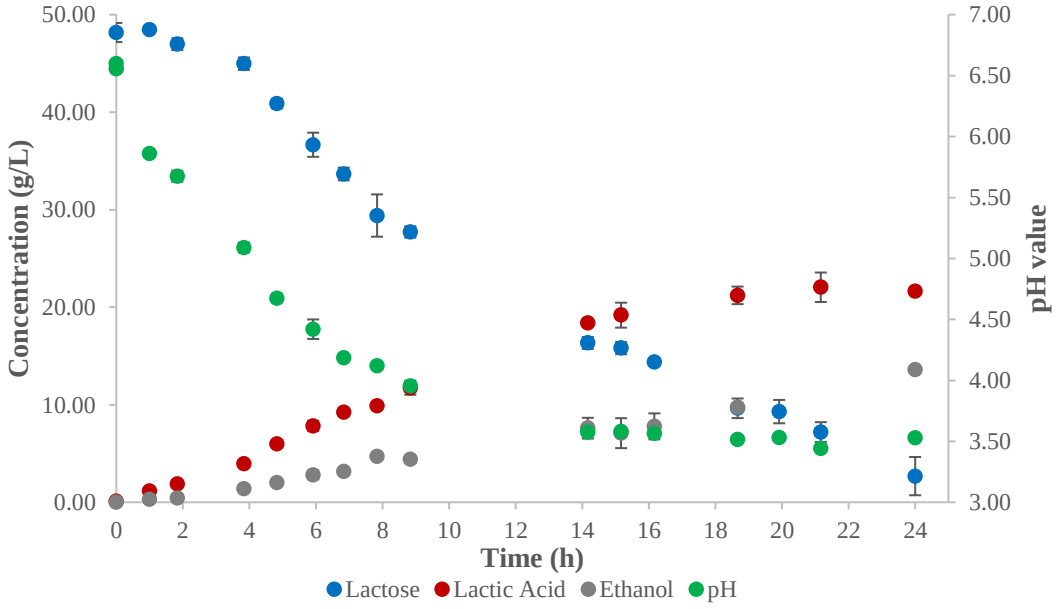

Figure 12. Lactose, lactic acid and ethanol concentrations, and pH value obtained during the assay with 60 rpm of agitation rate.

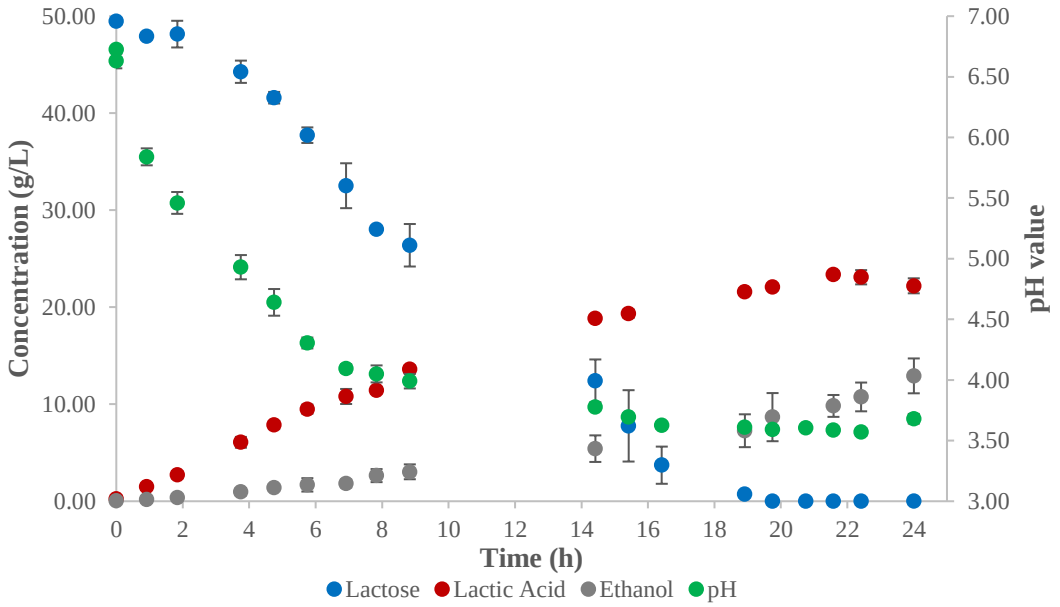

Figure 13. Lactose, lactic acid and ethanol concentrations, and pH value obtained during the assay with 180 rpm of agitation rate.

As would be expected, an increase on the agitation rate resulted in a decrease on the time required to obtain kefir, since without agitation kefir production was accomplished in approximately 8 h, with 60 rpm around 6 h, and with 180 rpm in only 5 h. Moreover, kefir grains mass variation was also determined at the end of these assays. According to **Table 9**, when increasing the agitation rate from 0 to 60 rpm, there is a higher increase of kefir grains mass, 4.77% to 19.9%. However, when the agitation rate was increased to 180 rpm, a lower increase of kefir grains mass was detected, 19.9% to 8.98%, thus, suggesting that their mass might be positively influenced by lower agitation rates, but negatively influenced when higher agitation rates are applied.

Volumetric rates and yields were also determined for these assays based on the lactose, lactic acid and ethanol concentrations during fermentation, and were compared with those determined previously for the assay where a static process was used (**Table 9**). Lactose volumetric consumption rate values ($r_{lactose}$) showed that lactose was consumed more rapidly with the increase of agitation rate, confirming that the fermentation process occurred faster. However, both assays with agitation rates of 60 and 180 rpm showed similar $r_{lactic\ acid}$, 1.04 and 1.07 g/L.h, and $r_{ethanol}$, 0.57 and 0.54 g/L.h, respectively, and significantly higher when compared to the values obtained without agitation, 0.63 and 0.11 g/L.h, respectively. Similar lactic acid and ethanol yields on substrate were also obtained between both assays with

agitation rates of 60 and 180 rpm, which were also higher than those obtained without agitation.

The results obtained may be because the presence of agitation led to a more homogeneous distribution of the kefir grains in the fermentation medium, improving the mass transfer and the mixing capacity, thus allowing a higher contact between lactose and other components of milk and the microorganisms, accelerating the fermentation process. It would be expected to assume that kefir production would be more efficient under conditions in which agitation is applied. However, the beverages obtained in the agitated process presented visual differences when compared with those obtained in the static process (**Figure 14, A**): kefir produced with 60 rpm consisted of a heterogeneous mixture with suspended particles (**Figure 14, B**), and in the assay at 180 rpm, some precipitation occurred (**Figure 14, C**). The precipitate observed corresponds to butter, as milk fat can easily suffer precipitation due to its low density, forming cream on the surface of the beverage (oil-in-water emulsion), which, in turn, results in butter when severely agitated (water-in-oil emulsion) [8]. Those visual differences are undesired in organoleptic terms, as the development of suspended particles changes the typical smooth and texture of the beverage, and the formation of butter results in a low-fat kefir, with is known to have a significantly lower sensory quality, as the higher the fat content, the thicker and creamier is the beverage [3][7]. Therefore, despite the occurrence of a faster fermentation with agitation, the static process was preferred.

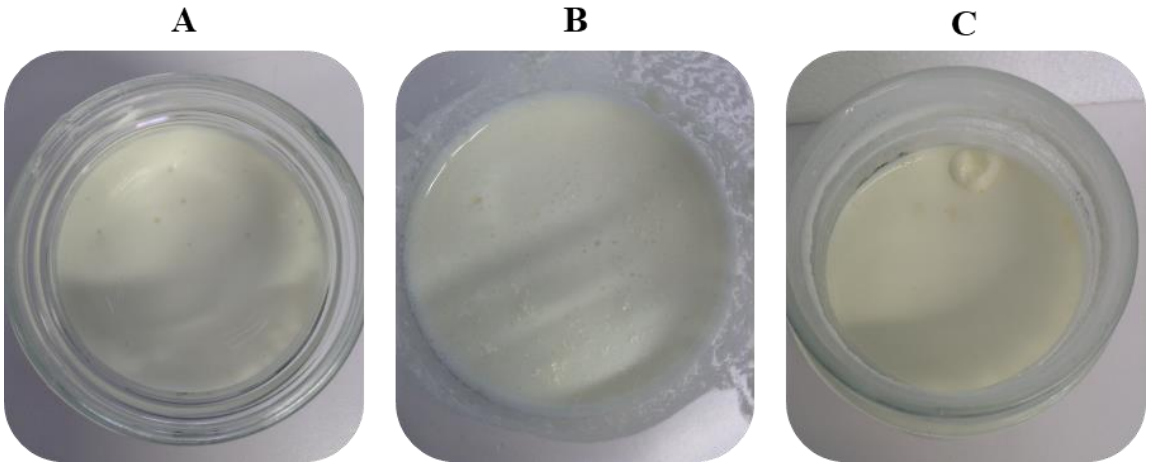

Figure 14. Appearance of kefir beverages produced with different agitation rates. A – kefir produced without agitation (0 rpm), with a typical visual aspect; B – kefir produced with 60 rpm, consisting of a heterogeneous mixture of suspended particles; C – kefir produced with 180 rpm, with a precipitate formation.

4.1.5. Effect of Temperature

To evaluate the effect of temperature on the production of kefir, an assay with an initial concentration of kefir grains of 9% (w/v) and initial milk volume of 200 mL, without agitation and at room temperature was performed and compared with the assay under similar conditions but with temperature controlled at 28 ºC. The choice of not controlling the temperature was based on the possibility of decreasing the costs associated to energy consumption in kefir production, which could be of industrial interest.

Along the assay, room temperature oscillated between 21.0 and 25.0 ºC (**Figure 15** and **Table 9**). Kefir production was accomplished in 15 h of fermentation, obtaining a product with lactose and lactic acid contents of 42.5 and 8.65 g/L, respectively, and a pH value of 4.39. At 15 h of fermentation, ethanol content was 3.70 g/L. Moreover, kefir grains mass variation was also determined at the end of this assay. According to **Table 9**, it seems that using an uncontrolled temperature, which oscillated between 21-25 ºC, results in a significant higher increase of kefir grains mass, 22.5%, when compared to the use of a controlled temperature of 28 ºC, 4.77%. Nevertheless, the assay with an initial kefir grains concentration of 9% (w/v), initial milk volume of 200 mL, without agitation and with controlled temperature of 28 ºC may not be reliable for comparison in terms of kefir grains mass variation, as discussed in **Chapter 4.1.2.**.

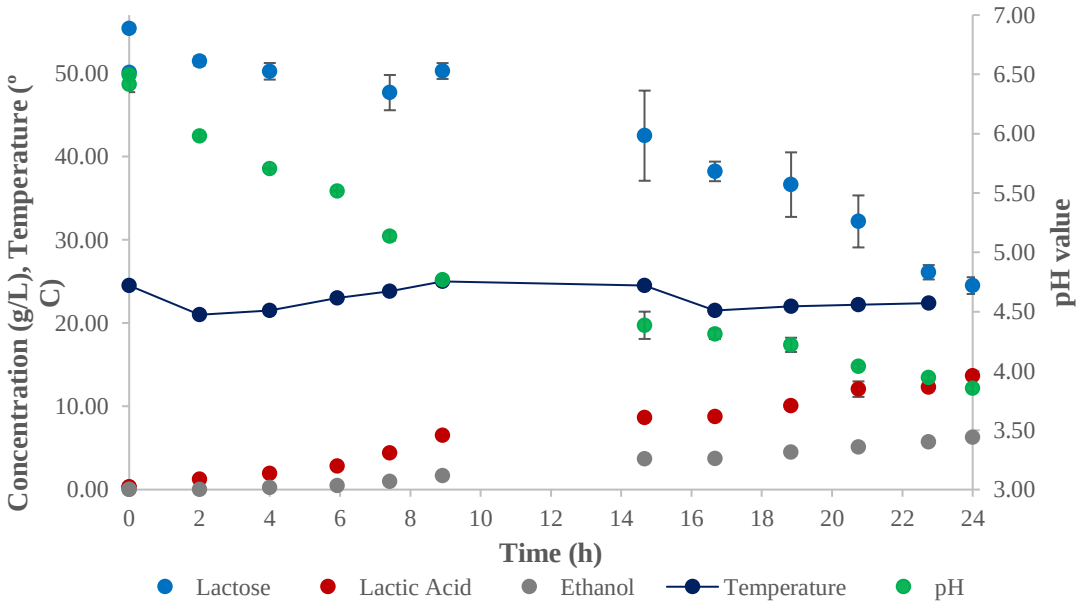

Figure 15. Lactose, lactic acid and ethanol concentrations, pH value, and temperature variation determined during the assay at room temperature (RT).

Volumetric rates and yields were also determined for this assay based on the lactose, lactic acid and ethanol concentrations during the fermentation (**Table 9**). Lactose volumetric consumption rate values ($r_{lactose}$) showed that lactose was consumed faster at a higher temperature, which was in accordance with the results previously presented, since kefir was obtained in less time with those conditions. Additionally, the highest $r_{lactic\ acid}$, 0.63 g/L.h, and $r_{ethanol}$, 0.26 g/L.h, were obtained when a controlled temperature of 28 ºC and an uncontrolled room temperature were used, respectively. This can be justified since higher temperatures may favor lactic acid fermentation and, in turn, lower temperatures may favor alcoholic fermentation. Nevertheless, the highest lactic acid and ethanol yields on substrate, 0.43 and 0.20 g/g, respectively, were obtained both with uncontrolled room temperature.

A decrease on temperature resulted in an increase on the time required for kefir production, since with controlled temperature of 28 ºC kefir production was accomplished in approximately 8 h and with uncontrolled room temperature of 21-25 ºC in around 15 h, almost twice the time required to obtain this beverage. In this case, although using an uncontrolled room temperature would presumably decrease the energetic costs associated with the usage of a controlled temperature during the process, the difference on the time required to obtain kefir between both assays is very significant to considerer the use of an uncontrolled room temperature, rather than a controlled temperature of 28 ºC, in an industrial process. Therefore, and considering that no differences were observed on the appearance of the beverages produced under these conditions, controlled temperature of 28 ºC was chosen for the following assay, since resulted in a faster kefir fermentation.

4.1.6. Kefir Production from Pasteurized Milk

The best operational conditions for kefir production from UHT milk were determined: an initial concentration of kefir grains of 9% (w/v), initial milk volume of 200 mL, controlled temperature at 28 ºC, and without agitation. These conditions were also tested with pasteurized milk, which will be the most probable medium to be used in industrial kefir production [9].

When using pasteurized milk (**Figure 16** and **Table 9**), kefir production was accomplished after 8 h of fermentation, obtaining a product with lactose, lactic acid and ethanol concentrations of 42.9, 8.74 and 2.13 g/L, respectively, and a pH value of 4.44.

Moreover, kefir grains mass variation was also determined at the end of this assay. According to **Table 9**, it seems that the use of pasteurized milk resulted in a relatively higher increase of kefir grains mass, 14.3%, when compared to the use of UHT milk, 4.77%. This may be because pasteurized milk is less processed than UHT milk, and therefore, more natural, a characteristic that could be more favorable to the development of kefir grains. Nevertheless, the assay with UHT milk which gave the best results for kefir production, may not be reliable for comparison in terms of kefir grains mass variation, as discussed in **Chapter 4.1.2.**.

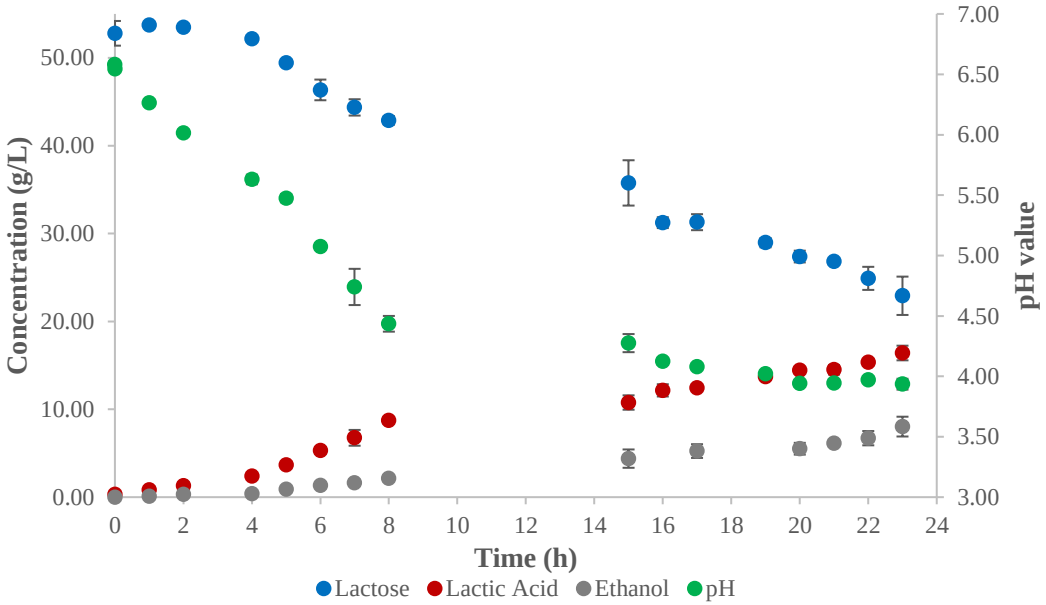

Figure 16. Lactose, lactic acid and ethanol concentrations, and pH value obtained during fermentation when using pasteurized milk sample.

Volumetric rates and yields were also determined for this assay based on the lactose, lactic acid and ethanol concentrations during fermentation, and were compared with those determined previously for the assay where UHT milk was used (**Table 9**). Lactose volumetric consumption rate values ($r_{lactose}$), 1.83 and 1.77 g/L.h with UHT and pasteurized milk, respectively, showed that lactose was consumed approximately at the same speed with both milk samples, which is in accordance with the results previously presented, since in those assays kefir was obtained at the same time. Still, the highest volumetric production rates, $r_{lactic\ acid}$, 0.70 g/L.h, and $r_{ethanol}$, 0.35 g/L.h, were obtained when using pasteurized milk, which also resulted in the highest lactic acid and ethanol yields on substrate, 0.54 g/g and 0.27 g/g, respectively.

Considering that no differences were observed on the appearance of the beverages produced and no significant changes were detected on the time required to obtain kefir, since its production was accomplished in around 8 h of fermentation in both assays, pasteurized milk can be used for kefir production.

While other kefir production studies found in the literature needed between 16 to 48 h to produce this beverage, the results obtained in this work reduced that time to only 8 h, when using an initial concentration of kefir grains of 9% (w/v), initial milk volume of 200 mL, controlled temperature at 28 ºC and without agitation. For example, Beshkova *et al.* (2002) used 5.5% (w/v) of bacterial and yeast starter cultures or 3% (w/v) of kefir grains at 22 ºC to obtain kefir, a process that took between 16 to 22 h. The kefir beverages were produced with lactic acid and ethanol concentrations between 8.18-8.37 g/L and 2.5-4.8 g/L, respectively, and pH values around 4.40-4.50, and were in agreement with those obtained in the present work (**Table 9**) [62]. Moreover, Chen *et al.* (2009), inoculated 10% (w/v) of kefir grains into pasteurized milk at 20 ºC, during 24 h of incubation. Although kefir was produced with a pH value of 4.34, lactic acid and ethanol concentrations were lower than would be expected, 4.79 and 0.50 g/L, respectively, thus resulting in very low values of $r_{lactic\ acid}$, 0.20 g/L.h, and $r_{ethanol}$, 0.02 g/L.h, when compared with those observed in the present study (**Table 9**) [66]. Additionally, when Garrote *et al.* (1998) fermented milk with an initial concentration of kefir grains of only 1 to 5% (w/v), at 20 ºC, 48 h were needed to obtain pH values between 4.59 to 4.15, respectively [60]. According to these studies, it can be observed that the initial concentration of kefir grains and the temperature have a crucial role in kefir production.

Moreover, Pop *et al.* (2014) studied the optimization of kefir grains biomass production using milk as culture media. Different fermentation times (4, 8, 12, 16, 24, 33, 48 and 72 h), temperatures (20, 25, 28 and 32 ºC) and agitation rates (100, 125, 150 and 200 rpm) were tested and the results showed that the best incubation parameters were 24 hours at 25 ºC with an agitation rate of 125 rpm. According to **Table 9**, these results can be related with those obtained in the present work, as kefir grains mass variation was higher when a lower temperature (21-25 ºC) and agitation rate (60 rpm) were applied, when compared with a higher temperature (28 ºC) and agitation rate (180 rpm), respectively, also during 24 h of fermentation. In addition, Pop *et al.* (2014) observed that the growth rate of kefir grains is

directly affected by the type of milk, which could explain the differences found between the use of UHT and pasteurized milk, 14.3% and 4.77%, respectively [154].

In every assay performed in this chapter, glucose concentrations were minimal (less than 0.2 g/L), and, therefore, despised in comparison with lactose concentrations obtained. In addition, acetic acid production was not detected. Nevertheless, Magalhães *et al.* (2011) also observed the absence of acetic acid during 24 h of milk fermentation when producing kefir [155].

4.1.7. Comparison with Commercial Kefir Samples

To evaluate possible differences between the kefir beverages produced and those already commercialized in Portugal, three different commercial kefir products, Kefir Vigor Natural, a national kefir with natural flavor, Kefir Vigor Manga Maracujá, a national kefir with mango and passion fruit flavor, and Andechser Natur Bio Kefir, an organic foreign kefir with natural flavor, were acquired and chemically compared with the kefir produced in this study. In those samples, acetic acid was not detected, similarly to the beverages obtained in this work. Nutritional composition of the three commercial kefir samples and photos of their packages are presented in **Appendix 3** and **Appendix 4**, respectively.

Lactic acid content in kefir is of extreme importance since it provides a pleasant taste to the beverage and inhibits the development of undesirable or pathogenic microorganisms. In its turn, pH value also strongly affects the quality of the product, and its variation is associated to lactic acid production during fermentation, since the increase of lactic acid concentration acidifies the beverage, reducing its pH value [156]. According to **Table 11**, lactic acid contents and pH values of the kefir beverages obtained in the present work are similar to the values obtained for commercial samples.

Concerning to total sugar content, Kefir Vigor Natural (39.29 g/L) and Andechser Natur Bio Kefir (38.56 g/L) values were close to 40 g/L, as in the beverages obtained in this work, kefir from UHT milk (36.08 g/L) and from pasteurized milk (42.88 g/L) . However, for Kefir Vigor Manga Maracujá, total sugar content, 111.1 g/L, was almost 3 times higher than the expected. This resulted from the addition of concentrated mango and passion fruit juices (1.4 and 0.7%, respectively) which are rich in sucrose, fructose and glucose, as only 31.4 g/L of the total sugar content corresponded to lactose.

Additionally, ethanol, which could be also produced during the fermentation process of kefir due essentially to the presence of yeasts like *Saccharomyces cerevisiae* [156], was not detected in these commercial kefir samples. The absence of ethanol could be related with the fact that, namely, in kefir beverages of Vigor brand, there is an indication that the starter culture used to produce kefir did not contain yeasts (**Appendix 4**).

Table 11. Lactose, other sugars and lactic acid concentrations, and pH values in commercial kefir samples and in kefir beverages obtained in this work.

Chemical Parameters	Kefir Vigor Natural	Kefir Vigor Manga Maracujá	Andechser Natur Bio Kefir	Kefir from UHT milk	Kefir from Pasteurized Milk
Total Sugars (g/L)	39.29	111.1	38.56	36.08	42.88
Lactose	32.8	31.4	37.8	36.08	42.88
Other Sugars	6.49	79.7	0.76	0	0
Lactic Acid (g/L)	9.19	8.08	10.96	7.50	8.74
pH value	4.23	4.24	4.45	4.37	4.44

4.2. Study of Kefiran Production

4.2.1. Cheese Whey Samples Characterization

According to the literature, to be used as substrate for the production of kefiran, cheese whey should have a high reducing sugars content, which would consist essentially of lactose, the preferred substrate to obtain kefiran [85][90]. Also the medium should have a pH value close to 5.0, which seems to be the optimal value of pH for kefiran production [90][92]. A low salt content and, therefore, a low conductivity, are also desirable, since high salt concentrations could be inhibitory to the fermenting microorganisms [157]. Finally, a reduced protein content is also important in order to simplify the pre-treatment step. The six cheese whey samples provided by Lacto Serra (**Table 7**) were submitted to a pre-treatment and, then, they were characterized regarding the mentioned parameters.

According to **Table 12**, there are no significant differences between pH values since they oscillate only between 5.4 and 6.0. Concerning to reducing sugars content, samples CW1, CW2 and CW4 showed really low concentrations, less than 45 g/L, when compared to CW3, CW5 and CW6, more than 100 g/L, which could be related to the fact that this three samples were concentrated by the dairy industry during their processing. Additionally,

samples CW1 and CW2 also showed the highest conductivity values (7.3 mS), which is undesired, and considering that sample CW4 has the lowest reducing sugars concentration (26 g/L) despite having the lowest conductivity (3.4 mS), this three samples were excluded.

Table 12. Reducing sugars concentration, conductivity and pH value of the cheese whey samples provided by Lacto Serra, after pre-treatment.

Cheese Whey Sample	pH	Reducing Sugars Concentration (g/L)	Conductivity (mS)
CW1	6.04	34.5	7.32
CW2	5.90	43.4	7.34
CW3	5.61	105.5	6.24
CW4	6.17	26.4	3.39
CW5	5.38	138.5	5.24
CW6	5.56	111.2	5.86

Moreover, the qualitative protein content of each cheese whey sample can be assessed through their physical appearance after autoclaving, which can be visualized in **Figure 17**. Samples CW1, CW2 and CW6 showed the highest protein content, as precipitated protein aggregates are observed in higher quantity. Therefore, sample CW6 was also excluded.

Figure 17. Physical appearance of cheese whey samples, after autoclaving, with the precipitated protein aggregates. From left to right: CW1, CW2, CW3, CW4, CW5, CW6.

Thus, CW3 and CW5 were determined as to be the best cheese whey samples provided by Lacto Serra to produce kefiran. CW3 seemed to have the lowest protein content, since this sample was obtained after ultra and nanofiltration steps performed in the dairy industry. CW3 had also a high reducing sugars concentration, around 105 g/L, and CW5, which despite having a higher protein content than CW3, had the highest reducing sugars concentration, almost 140 g/L. However, unfortunately, cheese whey sample CW3 was not available when kefiran production assays were performed. Hence, one of the other excluded samples was chosen to replace CW3. Due to availability reasons and in order to test a sample from a different origin (sheep milk), CW1 was chosen to be tested for kefiran production.

4.2.2. Study of Kefiran Production by Mixed Culture

In order to study kefiran production process by kefir grains, five different media were fermented over 96 h, under the conditions mentioned in **Chapter 3.4.**, which were defined considering previous studies found in the literature. Those media include MRS broth medium, UHT and pasteurized milk, and cheese whey samples CW5 and CW1. After the production, extraction and purification of kefiran, its quantification was achieved at the end of each assay by measuring the dry mass of the lyophilized fractions.

MRS BROTH MEDIUM

Kefiran concentration was the highest when MRS broth medium was used, 1.69 g/L, which is a surprisingly result, considering that this medium had the lowest initial substrate content, when compared to the other four media used (**Table 13**). In addition, the yield of kefiran on substrate ($Y_{kefiran/substrate}$) was also the highest, 0.1030 g/g. This could be related to the fact that MRS broth contains glucose instead of lactose, which is a sugar more easily metabolized. Also, MRS broth is a semi-synthetic fermentation medium specifically designed to cultivate *Lactobacillus* species, namely *Lactobacillus kefiranofaciens* and *Lactobacillus kefiri*, which can be found in kefir grains and are known as the main responsible for kefiran production [1][3]. MRS broth provides the best conditions for the development of both microorganisms and, consequentially, resulted in the production of more kefiran. However, MRS medium is the most expensive of the five tested, and therefore, the least economically viable to produce kefiran.

Table 13. Chemical parameters, kefir grains mass variations, volumetric rates, yields and kefiran concentrations obtained in every assay performed concerning to kefiran production study by kefir grains. Kefir grains mass variation values are presented with the standard deviation.

Parameters	MRS	UHT Milk	Pasteurized Milk	CW5	CW1
Kefiran Production (g/L)	1.69±0.08	0.725±0.075	1.28±0.07	0.890±0.050	0.670±0.030
$Lactose_i$ (g/L)		48.7	53.6	130.3	44.7
$Lactose_f$ (g/L)		1.36	1.66	76.9	1.26
Lactose Consumption (g/L)		47.4	52.0	53.4	43.5
$Glucose_i$ (g/L)	16.1				
$Glucose_f$ (g/L)	0				
Glucose Consumption (g/L)	16.1				
Lactic Acid Production (g/L)	15.6	19.5	27.3	41.4	29.6
Ethanol Production (g/L)	3.39	13.4	13.1	9.87	8.76
pH_i	5.36	6.42	6.51	5.26	5.17
pH_f	4.03	3.53	3.54	3.15	3.03
Kefir grains mass variation (%)	-12.7±2.3	11.7±4.4	9.23±2.44	15.4±4.0	-8.08±0.47
$r_{lactose}$ (g/L.h)		0.90	0.99	0.56	0.45
$r_{glucose}$ (g/L.h)	0.58				
$r_{lactic\ acid}$ (g/L.h)	0.55	0.48	0.52	0.43	0.31
$r_{ethanol}$ (g/L.h)	0.12	0.25	0.23	0.21	0.18
$Y_{lactic\ acid/substrate}$ (g/g)	0.95	0.53	0.53	0.77	0.68
$Y_{ethanol/substrate}$ (g/g)	0.21	0.27	0.23	0.18	0.21
$Y_{kefiran/substrate}$ (g/g)	0.1030	0.01543	0.02502	0.01667	0.01541

During fermentation, (**Figure 18** and **Table 13**), all the initial glucose, 16.1 g/L, was consumed, resulting in the production of 15.6 and 3.39 g/L of lactic acid and ethanol, respectively. However, the highest lactic acid and ethanol concentrations were achieved in just 28 h of fermentation, as after that their content declined. In its turn, glucose exhaustion was observed after 52 h of fermentation. Additionally, pH values ranged from 5.36 to 4.03, as a result of glucose consumption and the increase in lactic acid concentration during the process, since pH variation is associated to lactic acid and CO_2 productions, as already discussed in **Chapter 4.1.7.**. As verified in previous assays of kefir production, acetic acid production was not detected.

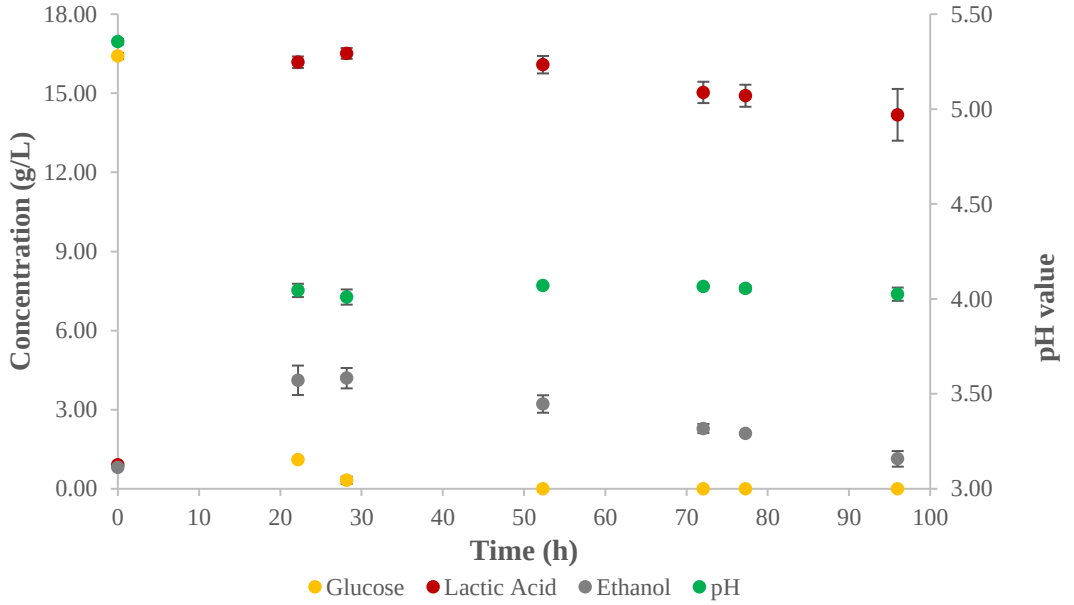

Figure 18. Glucose, lactic acid and ethanol concentrations, and pH value obtained during the assay with MRS broth medium, for kefiran production by kefir grains.

Kefir grains mass was also determined at the end of each assay, to better understanding the effect of the different media tested in its variation during the fermentation process. According to **Table 13**, when using MRS broth medium, the worst mass variation of the five media tested was observed, as mass decreased by 12.7%, which could be related to the fact that this medium had the lowest initial substrate content, only 16.1 g/L. In addition, the matrix of MRS broth medium might not be ideal for kefir grains development, considering that, after fermentation, they acquired a brown color, rather than keeping their typical white to yellowish color.

Volumetric rates and yields were also determined based on the lactose, glucose, lactic acid and ethanol concentrations during the fermentations performed (**Table 13**). With MRS broth medium, the highest $r_{lactic\ acid}$ was obtained, 0.55 g/L.h, which also resulted in the highest yield of lactic acid on substrate, 0.95 g/g. However, it was also observed the lowest $r_{ethanol}$, 0.12 g/L.h, which allowed obtaining a yield of ethanol on substrate of 0.21 g/g. In its turn, glucose volumetric consumption rate ($r_{glucose}$) was 0.58 g/L.h.

MILK SAMPLES

After MRS broth, the best result regarding kefiran concentration was obtained using pasteurized milk, 1.28 g/L, almost twice the concentration obtained with UHT milk, only 0.725 g/L (**Table 13**). This may be because pasteurized milk, besides having a higher initial

lactose concentration than UHT milk, it is also less processed, and therefore, more natural, a characteristic that could be more favorable to the development of *Lactobacillus* species capable of producing kefiran. In addition, the yield of kefiran on substrate was also the second highest, 0.02502 g/g, when pasteurized milk was used, and only 0.01543 g/g, when UHT milk was used. Nevertheless, although being less expensive than MRS broth, milk is an essential good and for ethical reasons would not make any sense to use it as fermentation medium to produce kefiran, on an industrial scale.

When UHT milk sample was used as medium (**Figure 19** and **Table 13**), 47.4 g/L of lactose were consumed during the 96 h of fermentation, resulting in the production of 19.5 and 13.4 g/L of lactic acid and ethanol, respectively. However, the highest lactic acid and ethanol concentrations, and the lowest lactose concentration, were all achieved in only 52 h of fermentation, as after that their content remained constant (as the case of lactose and ethanol) or declined (as the case of lactic acid). Furthermore, pH values ranged from 6.42 to 3.53. In this assay, the 96 h of fermentation almost cause substrate exhaustion since only 1.36 g/L of lactose left over. Also, acetic acid production was not detected.

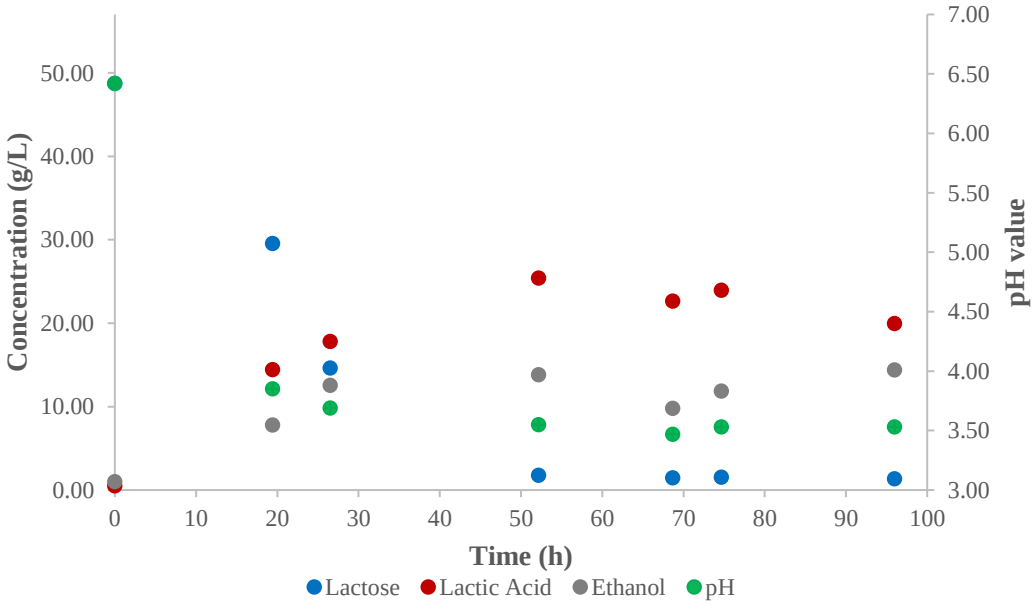

Figure 19. Lactose, lactic acid and ethanol concentrations, and pH value obtained during the assay with UHT milk, for kefiran production by kefir grains. One of the replicates was not considered, as its results were meaningless, and so there is no standard deviation.

In its turn, 52.0 g/L of lactose were consumed during the 96 h of fermentation, when pasteurized milk sample was used as medium (**Figure 20** and **Figure 13**), resulting in the production of 27.3 and 13.1 g/L of lactic acid and ethanol, respectively. Nevertheless, the

highest lactic acid and ethanol concentrations, and the lowest lactose concentration, were all achieved also in just 52 h of fermentation, as after that their content remained constant. Moreover, pH values ranged from 6.51 to 3.54. In addition, the 96 h of fermentation almost cause substrate exhaustion, since only 1.66 g/L of lactose left over. When compared to MRS broth, it can be observed that lactic acid and ethanol production was higher when milk samples were used as medium, which could be related to the differences between the initial substrate content of MRS broth and the milk samples, as UHT and pasteurized milk samples contain 48.7 and 53.6 g/L, respectively, concentrations three times higher than the one found in MRS broth, 16.1 g/L. Also, acetic acid production was not detected.

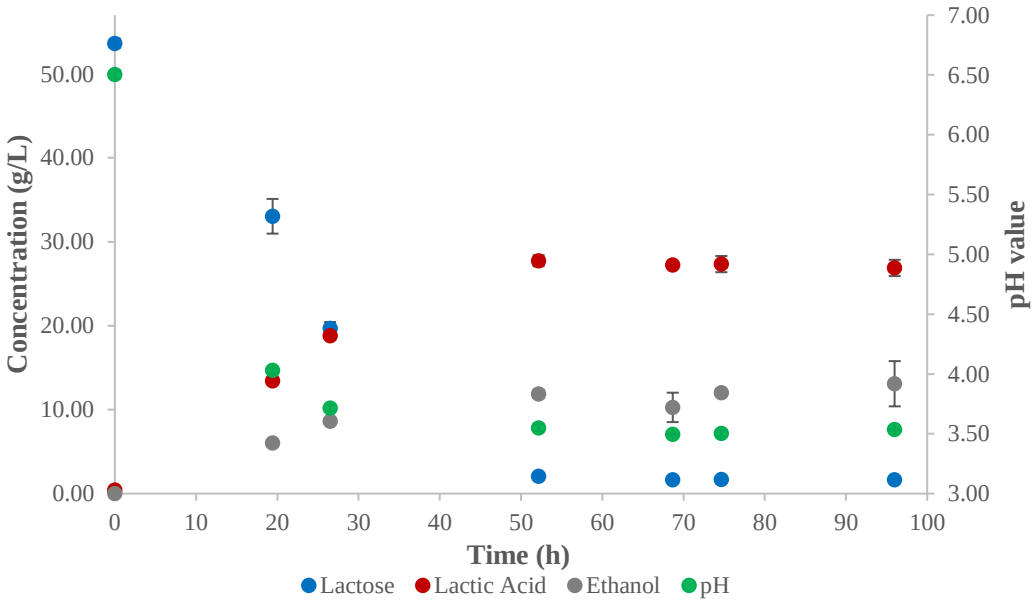

Figure 20. Lactose, lactic acid and ethanol concentrations, and pH value obtained during the assay with pasteurized milk, for kefiran production by kefir grains.

Kefir grains mass was also determined. According to **Table 13**, the use of UHT and pasteurized milk samples resulted in a mass increase of 11.7% and 9.23%, respectively. Since the differences in mass increase and initial lactose content, 48.7 and 53.6 g/L, respectively, were not so significant between both milk samples, it can be in fact presumed that the initial substrate content might have a direct influence on the grains mass variation, as already mentioned above in this chapter, when discussed the mass decrease of 12.7% obtained with the use of MRS broth, which has an initial substrate content three times lower than those found in milk samples. 16.1 g/L. In addition, contrary to what was observed with MRS broth, the milk samples had the ideal matrix for kefir grains development, as they were

first obtained spontaneously when a milk sample was placed in goatskin bags and shaken [30].

Volumetric rates and yields were also determined and, as it can be observed in **Table 13**, the values obtained for both milk samples are quite similar. Lactose volumetric consumption rate values ($r_{lactose}$) showed that lactose was consumed more rapidly when UHT and pasteurized milk samples were used as medium, 0.90 and 0.99 g/L.h, respectively, which is in accordance with the results previously discussed, since in those media, lactose reached minimum concentration in only 52 h of fermentation (**Figure 19** and **Figure 20**). In addition, the highest $r_{ethanol}$ values were obtained with both UHT and pasteurized milk, 0.25 and 0.23 g/L.h, which also resulted in the highest $Y_{ethanol/substrate}$ values, 0.27 and 0.23 g/g, respectively. Nevertheless, $r_{lactic\ acid}$ values were lower when compared to MRS broth, 0.48 and 0.52 g/L.h, for UHT and pasteurized milk, respectively, resulting in the lowest $Y_{lactic\ acid/substrate}$, 0.53 g/g, for both samples.

CHEESE WHEY SAMPLES

The real interest of this study was to evaluate and compare with other fermentation media the ability to produce kefiran using cheese whey samples, which is a by-product that represents a significant environmental and health issue due to its high volumes produced and high organic matter content [103]. The use of a very cheap fermentation medium and rich in lactose, to produce kefiran, could be interesting and economically viable for industrial purposes. After MRS broth and pasteurized milk, the best result was obtained using a cheese whey sample, CW5, 0.890 g/L, which could be possibly related to the high initial substrate concentration present in this sample, 130 g/L, when compared to the others (**Table 13**). Moreover, the worst result was obtained using sample CW1, 0.670 g/L, which has an initial substrate concentration of 44.7 g/L, almost three times lower than the one in sample CW5. Additionally, it can also be observed in **Table 13** that the yield of kefiran on substrate varied according to kefiran concentration, as the lowest $Y_{kefiran/substrate}$ was obtained when using sample CW1 as medium, 0.01541 g/g, followed by UHT milk, 0.01543 g/g, sample CW5, 0.01667 g/g, pasteurized milk, 0.02502 g/g, and finally MRS broth, 0.1030 g/g, which resulted in the highest kefiran yield on substrate.

When sample CW5 was used as medium (**Figure 21** and **Table 13**), 53.4 g/L of lactose were consumed during the 96 h of fermentation, resulting in the production of 41.4 and 9.87 g/L of lactic acid and ethanol, respectively. However, the highest ethanol concentration was achieved in 44 h of fermentation, as after that its content remained constant. Additionally, pH values ranged from 5.26 to 3.15. In this assay, the 96 h of fermentation were not enough to almost cause substrate exhaustion, an incident that happened when the other media were used, as it is shown and discussed throughout this chapter. This may be because sample CW5 has a very high substrate concentration, 130 g/L, when compared to the other media used, less than 55 g/L, thus requiring more time to consume all the lactose present in the sample. Also, acetic acid production was not detected.

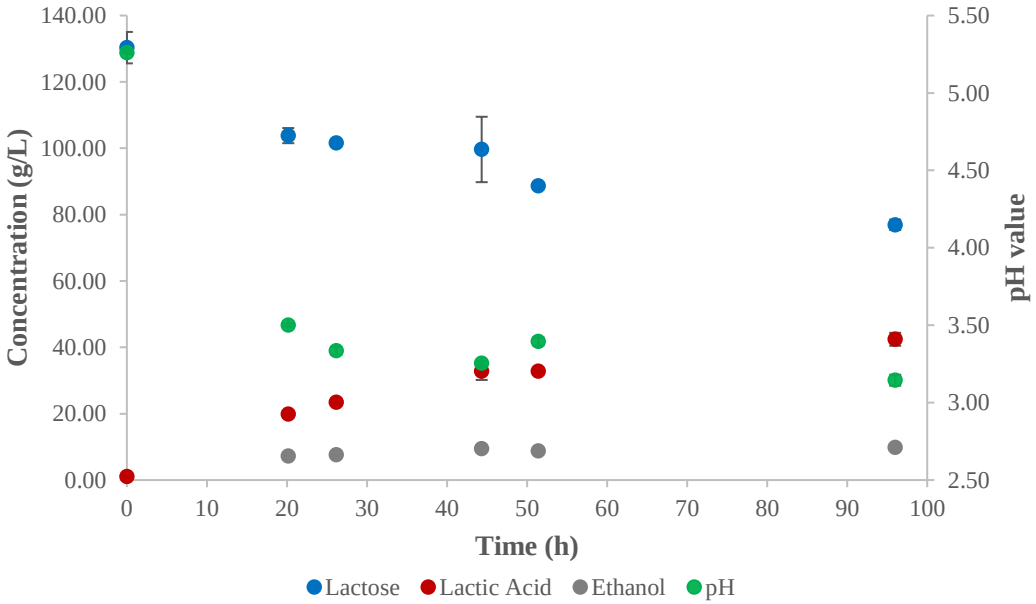

Figure 21. Lactose, lactic acid and ethanol concentrations, and pH value obtained during the assay with sample CW5, for kefiran production by kefir grains.

In its turn, 43.5 g/L of lactose were consumed when sample CW1 was used as medium (**Figure 22** and **Table 13**), resulting in the production of 29.6 and 8.76 g/L of lactic acid and ethanol, respectively. Nevertheless, the highest ethanol concentration was achieved in 51 h of fermentation, as after that its content remained constant. Moreover, pH values ranged from 5.17 to 3.03. In this assay, the 96 h of fermentation almost cause substrate exhaustion, since only 1.26 g/L of lactose left over. When compared to sample CW5, a lower lactose consumption and, consequently, a lower lactic acid and ethanol production were observed

with CW1. These results may be related to the differences between the initial substrate content present in both samples, as in sample CW5, 130 g/L, lactose concentration is almost three times higher than in sample CW1, 44.7 g/L. In addition, when comparing CW5 and CW1 samples with UHT and pasteurized milk samples, a higher lactic acid production was observed, 41.4, 29.6, 19.5 and 27.3 g/L, respectively. In fact, this might be because homolactic fermentation may be favored in detriment of alcoholic and heterolactic fermentation, which could also explain the achievement of a lower ethanol production, 9.87, 8.76, 13.4 and 13.1 g/L, respectively. Furthermore, it can also be observed that the higher the substrate content initially present in the sample, the greater the consumption of it during fermentation, as sample CW5, pasteurized milk, UHT milk, sample CW1 and MRS broth had an initial substrate content of 130, 53.6, 48.7, 44.7 and 16.1 g/L and resulted in a substrate consumption of 53.4, 52.0, 47.4, 43.5 and 16.1 g/L, respectively. Also, acetic acid production was not detected.

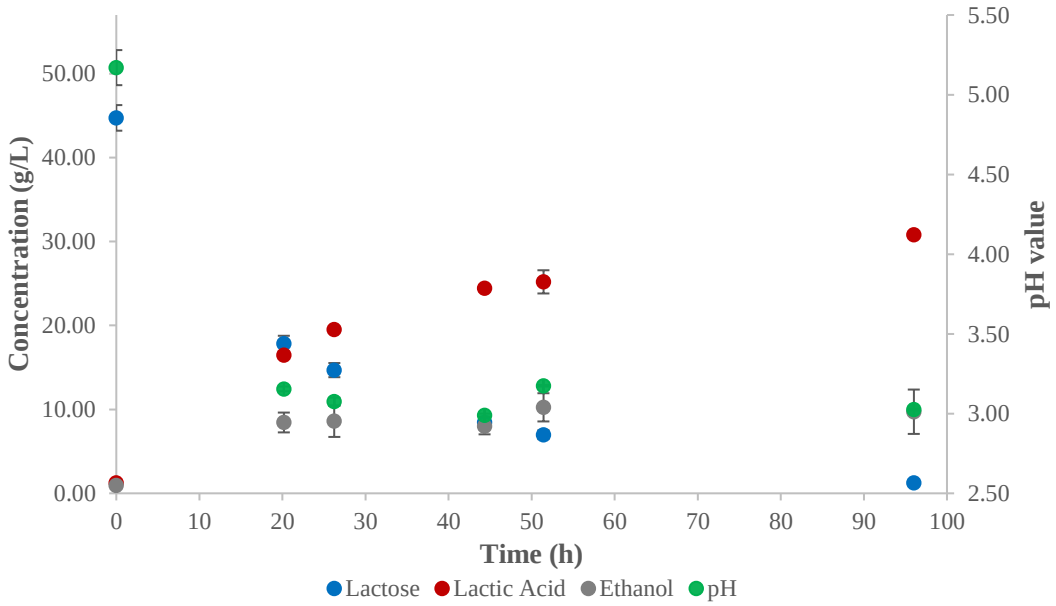

Figure 22. Lactose, lactic acid and ethanol concentrations, and pH value obtained during the assay with sample CW1, for kefiran production by kefir grains.

Kefir grains mass was also determined. According to **Table 13**, the use of CW5 and CW1 samples resulted in a mass increase of 15.4% and decrease of 8.08%, respectively. This difference might be related with their initial substrate content, as sample CW5, 130 g/L, has a lactose content almost three times higher than sample CW1, 44.7 g/L. Thus, initial substrate content seems to be an important feature in kefir grains development. However, when comparing CW5 and CW1 samples with UHT and pasteurized milk samples, it can be

noticed that CW1 resulted in an undesired decrease of mass, although having an initial lactose content, 44.7 g/L, close to those observed in both milk samples, 48.7 and 53.6 g/L, respectively. As discussed above in this chapter, milk has the ideal matrix for kefir grains development, when compared to cheese whey, therefore, it could explain the decrease of mass observed with CW1. Nevertheless, it is important to note that these results were obtained without performing a standard and correct procedure to measure kefir grains mass, which also occurred with the kefir production assays previously performed, thus, they might not be so significant, as already discussed in **Chapter 4.1.**. Volumetric rates and yields were also determined. According to **Table 13**, when samples CW5 was used, a higher $r_{lactose,}$ 0.56 g/L.h, and $r_{lactic\ acid}$, 0.43 g/L.h, were observed, which also resulted in a higher $Y_{lactic\ acid/substrate}$, 0.77 g/g, when compared with CW1 sample, 0.45 and 0.31 g/L.h, and 0.68 g/g, respectively. Still, $r_{ethanol}$ and $Y_{ethanol/substrate}$ were similar between both cheese whey samples, 0.21 and 0.18 g/L.h, and 0.18 and 0.21 g/g, with CW5 and CW1, respectively. Moreover, when comparing CW5 and CW1 samples with UHT and pasteurized milk samples, it can be noticed that the highest volumetric production rates, $r_{lactic\ acid}$ and $r_{ethanol}$, were obtained when UHT and pasteurized milk samples were used as medium.

4.2.3. Study of Kefiran Production by *Lactobacillus kefiranofaciens*

In order to study kefiran production process by *Lactobacillus kefiranofaciens*, two different media, MRS broth and cheese whey sample CW5, were fermented over 54 and 148.5 h, respectively, under the conditions mentioned in **Chapter 3.5.**, which were defined considering previous studies found in the literature. After the production, extraction and purification of kefiran, its quantification was achieved at the end of each assay by measuring the dry mass of the lyophilized fractions.

MRS BROTH MEDIUM

According to **Table 14**, kefiran concentration was only 0.755 g/L with MRS broth, although when mixed culture was used, the highest amount of kefiran was obtained with the same medium (**Table 13**). This result may be due to the differences on the glucose consumption, since with mixed culture all the initial glucose content was consumed during fermentation, 16.1 g/L (**Table 13**), which was twice the glucose consumed by pure culture, 7.38 g/L (**Table 14**). Moreover, the similarity between the yield of kefiran on substrate (Y

$_{kefiran/substrate}$) values observed with both kefir grains and pure culture, 0.1030 and 0.1059 g/g, respectively, can justify the hypothesis suggested to explain those differences in kefiran concentrations (**Table 13** and **Table 14**).

Table 14. Chemical parameters, rates, yields and kefiran concentrations obtained in the assays performed concerning kefiran production study by *Lactobacillus kefiranofaciens*.

Parameters	MRS	CW5
Kefiran Production (g/L)	0.755±0.005	1.16±0.20
$Lactose_i$ (g/L)		131.4
$Lactose_f$ (g/L)		116.1
Lactose Consumption (g/L)		15.3
$Glucose_i$ (g/L)	15.1	
$Glucose_f$ (g/L)	7.77	
Glucose Consumption (g/L)	7.38	
Lactic Acid Production (g/L)	4.92	23.9
Biomass Increase (g/L)	0.990	ND
pH_i	5.50	5.11
pH_f	4.21	3.49
$r_{lactose}$ (g/L.h)		0.18
$r_{glucose}$ (g/L.h)	0.24	
$r_{lactic\ acid}$ (g/L.h)	0.16	0.21
μ_{max} (h^{-1})	0.113	ND
$Y_{lactic\ acid/substrate}$ (g/g)	0.66	1.16
$Y_{biomass/substrate}$ (g/g)	0.14	ND
$Y_{kefiran/substrate}$ (g/g)	0.1059	0.06714

During the 54 h of fermentation (**Figure 23** and **Table 14**), the 7.38 g/L of glucose that were consumed resulted in the production of 4.92 g/L of lactic acid and biomass increase of 0.990 g/L. However, no significant variations on the glucose concentration were observed during the first 10 h of fermentation. As expected, ethanol production was not detected, since *Lactobacillus kefiranofaciens* is a homofermentative lactic acid bacteria, thus, performing only homolactic fermentation, where ethanol is not produced [68]. Additionally, pH values ranged from 5.50 to 4.21. In **Figure 23**, it can also be observed that glucose, acid lactic and pH variations are associated to biomass increase during the process. In this assay, the 54 h of fermentation did not cause substrate exhaustion, as 7.77 g/L of glucose left over, which was around half the initial glucose content presented in this medium, 15.1 g/L. Also, acetic acid production was not detected.

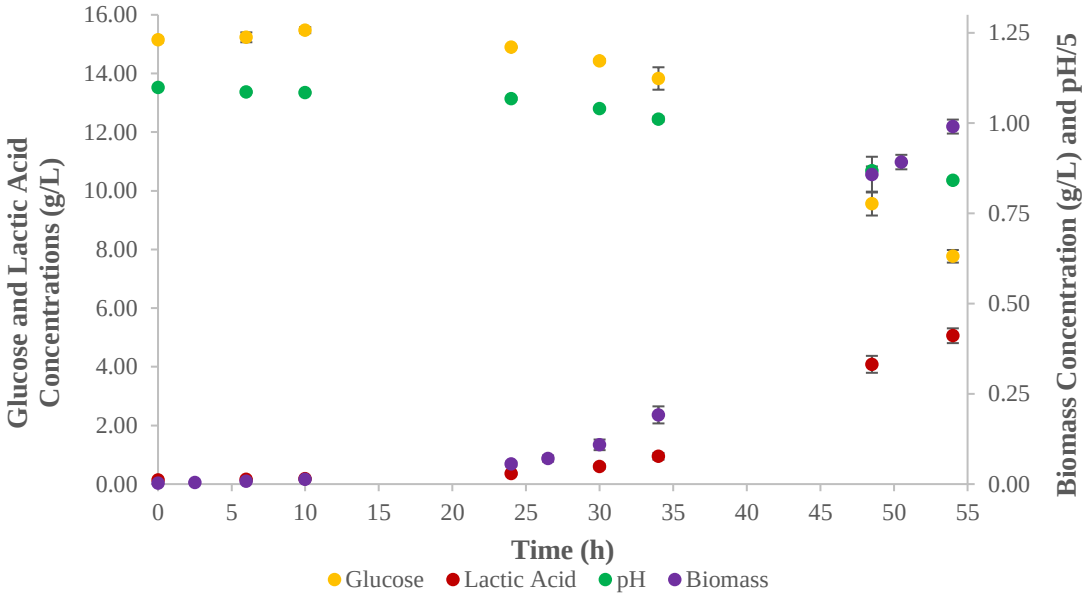

Figure 23. Glucose, lactic acid and biomass concentrations, and pH value obtained during the assay with MRS broth medium, for kefiran production by pure culture.

Rates and yields were determined based on the lactose, glucose, lactic acid and biomass concentrations during the fermentations performed (**Table 14**). With MRS broth medium, the lowest volumetric lactic acid production rate ($r_{lactic\ acid}$) was obtained, 0.16 g/L.h, which also resulted in the lowest yield of lactic acid on substrate ($Y_{lactic\ acid/substrate}$), 0.66 g/g. In its turn, glucose volumetric consumption rate ($r_{glucose}$) was 0.24 g/L.h. Moreover, a yield of biomass on substrate of 0.14 g/g and a maximum specific growth rate, μ_{max}, of 0.113 h^{-1} were also observed. It can be said that a high μ_{max} was obtained in this study, considering that, for example, Tada *et al.* (2007) only observed a μ_{max} three times lower, 0.036 h^{-1}, for batch pure culture, using the same strain and modified MRSL medium [92].

CHEESE WHEY SAMPLE CW5

According to **Table 14**, when using *Lactobacillus kefiranofaciens*, kefiran concentration was the highest with cheese whey sample CW5, 1.16 g/L, which was an interesting result, considering that cheese whey is a low-priced by-product that represents a significant environmental issue [103], and therefore, it is the most economically viable medium of those tested in this study to produce kefiran on an industrial scale. This result may be due to the differences on the substrate consumption, since 15.3 g/L of lactose were consumed when sample CW5 was used, which was twice the glucose consumed during fermentation with MRS broth medium, 7.38 g/L. In addition, the yield of kefiran on substrate

($Y_{kefiran/substrate}$) did not vary according to kefiran concentration, as the lowest $Y_{kefiran/substrate}$ was obtained when using sample CW5 as medium, 0.06714 g/g, and the highest with MRS broth, 0.1059 g/g, contrary to what was observed previously in **Chapter 4.2.2., Table 13**. This could be related to the differences on the substrate consumption between both assays and already explained above, which has a direct influence on the $Y_{kefiran/substrate}$ value (**Chapter 3.8.2**, **Equation (3)**). Additionally, the fact that MRS broth contains glucose instead of lactose, which is a sugar more easily metabolized, could also help to understand those $Y_{kefiran/substrate}$ values, since a faster glucose metabolization could have resulted in a faster kefiran production. Moreover, when comparing both assays performed with sample CW5, it can be noticed that kefiran production by pure culture resulted in a higher kefiran concentration, 1.16 g/L, than the one obtained by kefir grains, 0.890 g/L (**Table 13**).

When cheese whey sample CW5 was used as medium (**Figure 24** and **Table 14**), the 15.3 g/L of lactose that were consumed resulted in the production of 23.9 g/L of lactic acid. Nevertheless, no significant variations on the lactose concentration were observed during the first 28 h of the process, and the lowest substrate content was achieved in 124.5 h of fermentation, as after that its concentration remained constant. As expected, ethanol production was not detected, because of the homofermentative capacity of *Lactobacillus kefiranofaciens* [68]. In addition, higher substrate consumption and, consequently, higher lactic acid production were observed, when compared to the assay performed with MRS broth medium. This could be related with the differences on the initial substrate content between both media, since sample CW5 had 131 g/L of lactose initially, which was almost nine times higher than the initial glucose concentration found in MRS broth, 15.1 g/L. Yet, when comparing both assays around the same time of fermentation, 52.5 to 54 h, substrate consumption and lactic acid production were also higher in CW5 medium, 8.05 and 8.60 g/L, respectively. In addition, pH values ranged from 5.11 to 3.49. Also, acetic acid production was not detected. According to **Figure 24**, the 148.5 h of fermentation did not cause substrate exhaustion, as 116.1 g/L were still present in the medium, which was around 88% of the initial lactose content presented in this medium, 131.4 g/L. No values regarding biomass concentration were obtained, as optical density at 620 nm was not measured during this assay. Nevertheless, it should have been assessed to better understand the adaptation and growth capacity of *Lactobacillus kefiranofaciens* in the cheese whey sample.

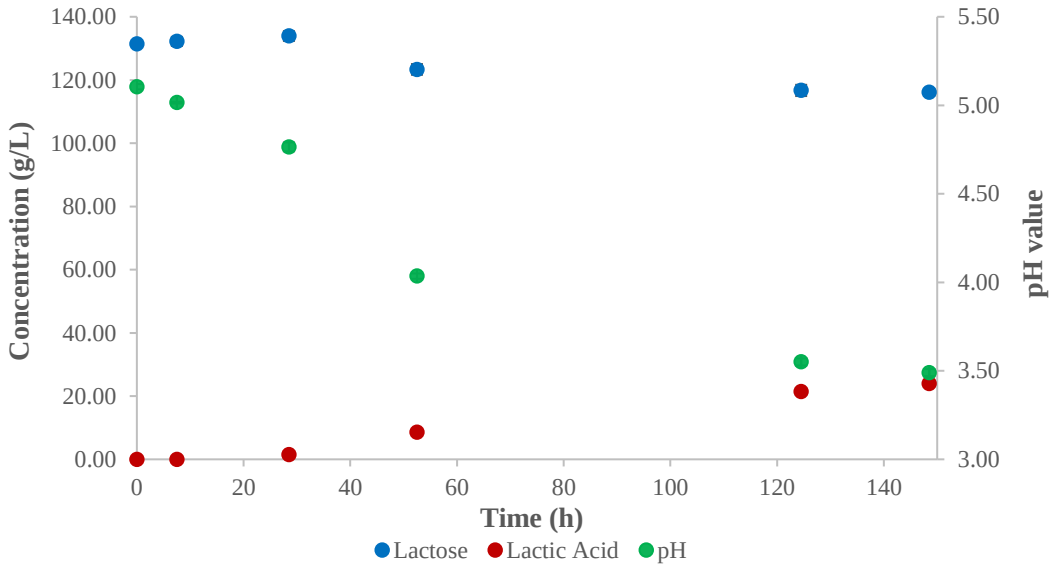

Figure 24. Lactose and lactic acid concentrations, and pH value obtained during the assay with cheese whey sample CW5, for kefiran production by pure culture.

Rates and yields were also determined. According to **Table 14**, lactose and glucose volumetric consumption rate values ($r_{lactose\ and}\ r_{glucose}$) showed that the fermentation substrates were consumed slightly slower when cheese whey sample CW5 was used as medium, although total substrate consumption was higher at the end of the fermentation. However, volumetric lactic acid production rate values ($r_{lactic\ acid}$) showed that lactic acid was produced faster when sample CW5 was used, 0.21 g/L.h, which also resulted in a higher yield of lactic acid on substrate ($Y_{lactic\ acid/substrate}$), 1.16 g/g. Moreover, when comparing the $Y_{lactic\ acid/substrate}$ values observed in both **Table 13** and **Table 14**, it can be said that the production of kefiran by *Lactobacillus kefiranofaciens* using sample CW5 resulted in the highest $Y_{lactic\ acid/substrate}$ value, 1.16 g/g.

When comparing all the kefiran concentration values obtained in this study (**Table 13** and **Table 14**) with those found in the literature (**Chapter 2.3.2.**), it can be noticed that the values obtained in this work are slightly below expectations, as for example, Dailin *et al.* (2016) obtained 1.91 g/L of kefiran when using an optimized semi-synthetic medium containing sucrose, yeast extract and K_2HPO_4 at 20.0, 6.0 and 0.25 g/L, respectively, and working with a semi industrial scale stirred tank bioreactor with a fermentation volume of 8 L [87], Yokoi and Watanabe (1992) obtained 2.04 g/L when using an optimized semi-synthetic modified MRSL medium containing 100 g/L of lactose and 5 mM of $CaCI_2$, and working with a bioreactor with a fermentation volume of 1.5 L [90], Tada *et al.* (2007) obtained 3.15 g/L when using a semi-synthetic modified MRSL medium containing 100 g/L

of lactose and working with a bioreactor with a fermentation volume of 2 L, and under a fed-batch coculture operation [92], and Cheirsilp *et al.* (2003) obtained 5.41 g/L when using a modified semi-synthetic MRSL medium containing 100 g/L of lactose and working with a bioreactor with a fermentation volume of 3 L, and under a fed-batch coculture operation [91]. The same applies to kefiran production using cheese whey as medium, since Cheirsilp and Radchabut (2011) obtained a maximum kefiran production of 2.58 g/L in batch culture and 3.25 g/L in fed-batch culture, when using a semi-synthetic modified MRS-whey lactose medium containing 76.5 g/L of whey lactose and a fermentation volume 20 times higher than the one used in this study [100]. Nevertheless, although the results observed in this work were lower than those observed in the literature, they were obtained only with natural media, in the case of milk and cheese whey samples, or semi-synthetic medium without modification, in the case of MRS broth, and without any optimization of the operational conditions and the growth environment, thus they still can be considered quite satisfactory.

To assess the absence of lactose, glucose, other sugars, lactic acid and ethanol after extraction, purification and quantification, kefiran fraction obtained with mixed culture and sample CW5 was dissolved and analyzed by HPLC, since it was the medium that remained with the highest sugars (76.9 g/L of lactose) and lactic acid (42.4 g/L) concentrations after the 96 h of fermentation. As would be expected, no sugar, lactic acid and ethanol content was detected, thus confirming that the extraction and purification process performed was effective in separating those remaining chemical compounds from the fermented medium. Additionally, to confirm the presence of kefiran after extraction, purification and quantification procedures, acid hydrolysis of the same sample used to assess the absence of sugars, lactic acid and ethanol was performed, and then analyzed again by HPLC. Since the HPLC column used was not able to separate glucose from galactose, a single peak was observed in the region where glucose and galactose are both eluted at the same time, thus, it can be concluded that there are strong indications of the presence of kefiran, as its chemical structure consists of a branched glucogalactan containing approximately equal amounts of those sugars [65][78]. More analytical tests like GC-MS, Size-exclusion Chromatography, NMR or FTIR Spectroscopy, could be done in future works to better understand the chemical structure of kefiran. In addition, the Bradford method should be also performed to assess the absence of proteins in the lyophilized fractions [129], so that it can be guaranteed that the lyophilized fractions obtained consist solely of kefiran.

5. Conclusion

This work focused on the study of the operational conditions and the use of different milk and cheese whey samples in kefir and kefiran production processes. Concerning to kefir, it was concluded that the best conditions for its production were achieved with 9 % (w/v) of initial kefir grains concentration, 200 mL of initial milk volume, controlled temperature of 28 ºC and without agitation, allowing the production of this beverage in approximately 8 h, with both UHT and pasteurized milk samples, despite kefir production was accomplished in just 5 to 6 h when an initial milk volume of 100 mL was used or when an agitated process was performed. Nevertheless, it was noticed that the differences on the time required to obtain kefir between the use of 100 mL or 200 mL were very likely a result of the sampling process. In addition, the beverages obtained in the agitated process had undesired precipitated fractions, and therefore, were rejected. Lastly, the analysis of three commercial kefir beverages by HPLC allowed to observe that there were no significant differences that could not be explained between the kefir produced in this work and those already commercialized in Portugal.

Moreover, concerning to kefiran, the aim of this study was to assess its production using cheese whey, a by-product from the cheese industry that represents a significant environmental problem, and compare it with kefiran production using other fermentation media. In this sense, samples CW1 and CW5, with 34.5 and 138.5 g/L of reducing sugars, respectively, were chosen out of the six different cheese whey samples initially provided by Lacto Serra. Nevertheless, kefiran production was the highest when MRS broth medium was fermented by kefir grains during 96 h, resulting in a concentration of 1.69 g/L. This might be related to the fact that MRS broth is a fermentation medium specifically designed to cultivate *Lactobacillus* species, like *Lactobacillus kefiranofaciens* and *Lactobacillus kefiri*, which are known as the main responsible for kefiran production and can be found in kefir grains. In its turn, the best result using a cheese whey sample was obtained when CW5 was fermented by pure culture of *Lactobacillus kefiranofaciens* during 148.5 h, providing a kefiran concentration of 1.16 g/L. Although these kefiran values were lower to those obtained in previous studies found in the literature, they seem promising considering that a lot of work can still be done concerning to kefiran production optimization. Thereby, this study helped to show that a low-cost carbon source like cheese whey can effectively and

efficiently be used to produce a value-added exopolysaccharide like kefiran. Lastly, the presence of kefiran and the absence of lactose, glucose, other sugars, lactic acid and ethanol in the lyophilized fractions obtained after extraction and purification was successfully confirmed by HPLC. Nevertheless, the absence of proteins should also be assessed so that it can be guaranteed that those fractions consist solely of kefiran.

6. Further Work

The study of kefir and kefiran production is far from finished. When these products are obtained by mixed culture, a standard and reformulated procedure to measure kefir grains mass should be carried out, so that significant conclusions can be taken from their variations during fermentation. Besides assessing the production of this beverage by kefir grains, kefir production should be also tested using pure culture of *Lactobacillus kefiranofaciens* or commercial starter cultures. In addition, conducting a sensorial analysis for all the beverages obtained would also be of extreme importance, as it might help to better understand the influence of the different conditions tested in the final product.

Concerning to kefiran, it is important to continue the optimization of its production, using both kefir grains and pure culture of *Lactobacillus kefiranofaciens*, in terms of how different fermentation conditions, like pH, initial inoculum concentration, temperature, substrate content, agitation rate, and nutrient concentration can influence the process. Similar to what was done in this work for kefiran production by mixed culture, it would be essential to test other fermentation media when working with pure culture, like different cheese whey samples, or even other synthetic or semi-synthetic media cheaper than MRS broth. Additionally, also identifying whether or not kefiran production follows *Lactobacillus kefiranofaciens* cell growth might be of great importance, as the process duration may be eventually reduced based on that information. Furthermore, assessing kefiran production by co-culture of *L. kefiranofaciens* and *S. cerevisiae* could also increase kefiran yields as it might reduce effectively lactic acid accumulation during fermentation, which is a well-known compound that inhibits the growth of LAB.

Another important aspect would be the optimization of the time required for kefiran extraction, purification and quantification, as it is currently a time-consuming process. In addition, a more exhaustive characterization of the chemical structure of kefiran is also an interesting work that should be done, using other analytical techniques like GC-MS, Size-exclusion Chromatography, NMR or FTIR Spectroscopy, considering that it could help to better understand the potential applications of this exopolysaccharide.

Finally, scale-up studies would be essential for both kefir and kefiran production processes, so it could be assessed the possibility of their industrial implementation.

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Appendix

Appendix 1 – DNS Calibration Curve

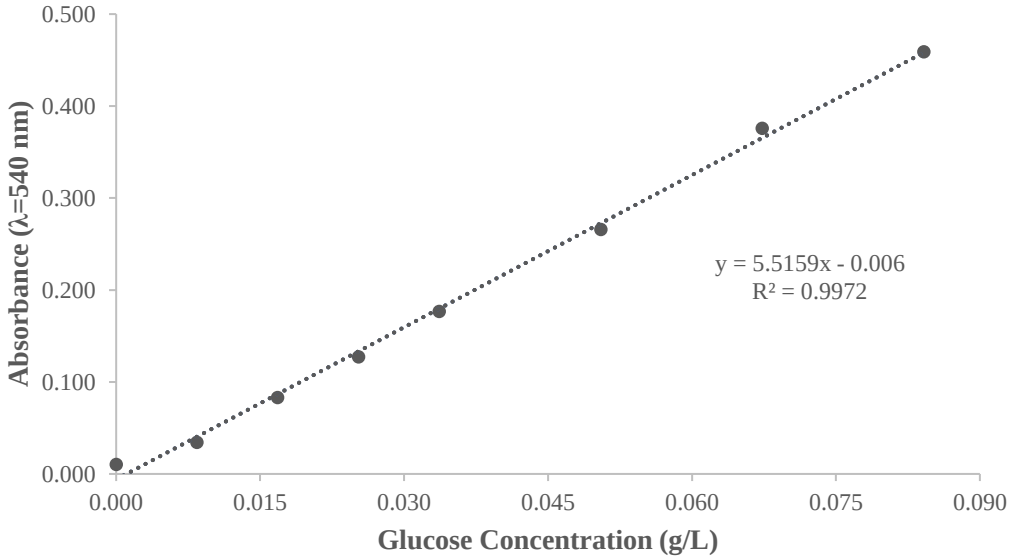

Figure 25. DNS calibration curve which relates absorbance at 540 nm with glucose concentration.

Appendix 2 – Calibration Curve for Optical Density and Biomass Concentration

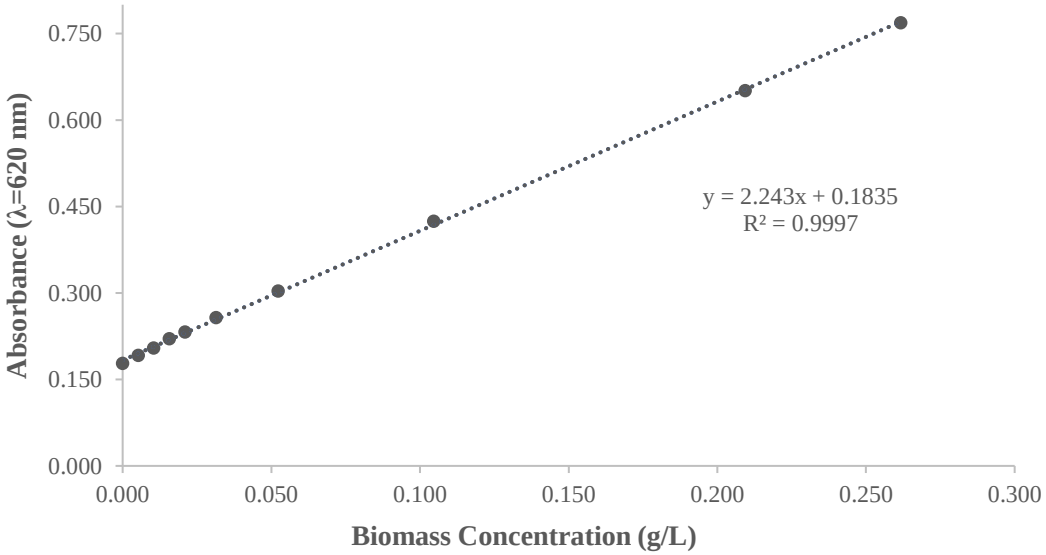

Figure 26. Biomass calibration curve which relates absorbance at 620 nm with biomass concentration.

Appendix 3 – Nutritional Composition of Commercial Kefir Samples

Table 15. Nutritional composition of the three different commercial kefir samples.

Component	Concentration (g/L)		
	Kefir Vigor Natural	Kefir Vigor Manga Maracujá	Andechser Natur Bio Kefir
Total Fat	17	16	15
Saturated Fat	12	11	10
Total Carbohydrate	40	120	41
Sugars	40	110	41
Protein	32	30	34
Salt	1.1	1.3	1.3

Appendix 4 – Photos of Commercial Kefir Samples Packages

Figure 27. Commercial kefir products analyzed. From left to right: Kefir Vigor Natural, Kefir Vigor Manga Maracujá, Andechser Natur Bio Kefir. In kefir beverages of Vigor brand, it can be observed the following indication "leite fermentado sem leveduras", which stands for "milk fermented without yeasts".