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PROTEOMIC CHARACTERIZATION OF KEFIR MILK BY TWO DIMENSIONAL ELECTROPHORESIS FOLLOWED BY MASS SPECTROMETRY

TIPO TESI: sperimentale

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List of abbreviations

Name	Abbreviation
$(NH_4)_2SO_4$	ammonium sulphate
°C	Celsius degree
2-DE	two-dimensional gel electrophoresis
ACE	angiotensin-converting enzyme
ADH	alcohol dehydrogenase
ATP	Adenosine triphosphate
BBF	bromophenol blue
BF	before Christ
ca.	circa
CaCo2	cells heterogeneous human epithelial colorectal adenocarcinoma cells
CEP	cell envelope - associated proteinase
cfu/g	colony forming unit per gram
cfu/ml	colony forming unit per milliliter
CHAPS	3-[(3-Cholamidopropyl) dimethylammonio]-1-propanesulfonate
CO_2	carbon dioxide
DDT	dithiothreitol
DHFR	dihydrofolate reductase
DPPH	1,1-diphenyl-2-picrylhydrazyl
EMP	Embden- Meyerhof-Parnas
EPS	extracellular polysaccharides
EPSs	exopolisaccharides
g	gram

GMP	glycomacropeptide
h	hours
H2O2	hydrogen peroxide
HCl	hydrochloride
HPLC	High-performance liquid chromatography
IEF	Isoelectric focusing
Ig	immunoglobulin
IL-	Interleukin
IPG	immobilized pH gradient
Kcal	kilocalories
kDa	kiloDalton
LAB	lactic acid bacteria
LC	liquid chromatography
LC-MS	liquid chromatography-mass spectometry
MF	microfiltration
mg	milligram
mg/kg	milligram per kilogram
mg/ml	milligram per milliliter
mM	millimolar
MRS	de Man, Rogosa, Sharpe
MS	mass spectrometry
MW	molecular weight
NADH	nicotinamide adenine dinucleotide (reduced form)
NEFA	non-esterified fatty acids
NK	natural killer
NLAB	non-lactic acid bacteria
nm	nanometer

NPN	non-protein nitrogen
PAGE	polyacrylamide gel electrophoresis
PCR	polimearase chain reaction
PCR-DGGE	electrophoresis in denaturing gradient gel
PDC	pyruvate decarboxylase
PepT1	Peptide transporter 1
PER	protein efficiency ratio
pI	isoelectric point
RAPD-PCR	randomly amplified polymorphic DNA-PCR
rDNA	ribosomal DNA
rpm	revolutions per minute
SDS	sodium dodecyl sulfate
subsp.	Subspecies
TGF-α	growth factor-α
TGF-β1	growth factor-β1
TN	total nitrogen
TNF-α	Tumor necrosis Factor Alpha
v/v	volume/volume
VBNC	viable but not culturable cells
W/v	weight/volume
α	alpha
β	beta
κ-CN	κ -casein

Chapter 1
INTRODUCTION

1.1. Origin and history

The origins of fermented milk beverages are ancient and date back to the domestication of certain mammals livestock animals such as cow, goat and sheep. It is safe to assume that the first types of products were made accidentally by the fermentation of milk stored at room temperature (~20–30°C). Due to variations in the climatic and environmental conditions in different parts of the world, specific strains of micro-organism became dominant in these products and, as a consequence, specific types of fermentations evolved and became distinct in a given region. The production of fermented milk was known to the ancient Greeks and Romans. The Greek historian Herodotos (485–425 BC) reported that a refreshing drink produced from mare's milk was popular among the Ghets tribes. According to Caucasian legend, Mahomet (ca. 570–632) forbade to spread kefir preparation secretes. This legend may be the reason why the method of kefir preparation has kept a mystery for a long time (Arihara, 2014; Wszolek et al., 2007).

Thus, kefir is one of the oldest fermented milk beverages, and the technology of its production and the use of specific starter cultures has developed through the ages.

Its origin can be traced back to the northern Caucasus, Tibetan or Mongolian mountains areas where it has been produced by a traditional method. Traditional kefir was made spontaneously in skin and animal hides bags that naturally contain micro-organisms, including yeasts. In this wild bag, the milk was poured daily, and a natural fermentation took place continuously because a new amount of fresh beverage was added when kefir being consumed. It was customary to hang the bag near the door, and everyone who came in or out had to push or kick the bag to mix the liquid (Arihara, 2014).

The microorganisms form a thin layer and then clusters on the surfaces of the containers due to the high temperature. In 1997 Motaghi et al. suggested that kefir grains can be produced by using the same traditional method of handling milk. They washed a goatskin bag with sterile water, and then they poured pasteurised milk and the intestinal flora extracted from the gastrointestinal tract of sheep. That mix was kept at 24–26°C for 48 h and periodically was blended. After the milk

coagulation, much of the fermented milk was replaced with a fresh one. This procedure was repeated for 12 weeks. Gradually, a polysaccharide layer is grown on the surface of the hide. The coating was removed aseptically from the hide and propagated in pasteurised cow's milk where grains were formed (0.5–3.2 cm in diameter) and added several times to fresh cow's milk. (Wszolek et al., 2007).

"Kefir" term derived from the word "kef", which means 'pleasant taste' in Turkish and it is also known as kefyr, kephir, kefer, kiaphur, knapon, kepi or kippi. Anyway, the terms are referred to 'well-being' or 'living well', due to the overall sense of health and well-being it generated in those who consume it (Arslan, 2015; Leite et al., 2013; Rosa et al., 2017).

Kefir is considered one of the first fermented foods that may have been produced by accident. However, fermentation of foods such as milk became a popular method of preservation. The fermentation of milk in skin bags as a way of preserving milk led to the production of the first kefir grains and started the long tradition of kefir producing (Arihara, 2014).

To date, kefir is characterized by its distinct flavour, typical of yeast, and an effervescent effect felt in the mouth. The finished product has high acidity and varying amounts of alcohol and carbon dioxide. This drink differs from other fermented dairy products because derived from the symbiotic association of bacteria and yeast (Arihara, 2014; Leite et al., 2013). While it has been widely consumed in Russia and central Asia Countries such as Kazakhstan, Kyrgyzstan for centuries, it is currently increasingly popular in European countries, Japan and the United States due to its nutritional and therapeutic effects (Arslan, 2015).

1.2. Kefir grains

These grains are composed of microorganisms immobilized on a polysaccharide and protein matrix, where several species of bacteria and yeast coexist in symbiotic association. Kefir grains can be defined as small cauliflower florets or cooked rice, having irregularly shaped, viscous, white to yellowish, lobed, having firm texture and slimy appearance. They are variable in size, from 0.3 to 3.5 cm in diameter (Ahmed et al., 2013; Leite et al., 2013; Rosa et al., 2017). When the grains are added in milk, they grow and multiply to form new ones (Wszolek et al., 2007).

These grains are a good source of lactic acid bacteria, acetic acid bacteria, and yeast cells embedded in a matrix composed by caseins and complex sugars. Yeasts and bacteria in kefir grains include *Leuconostoc mesenteroides, Lactobacillus helveticus, Lactobacillus brevis,*

Lactobacillus plantarum, Lactobacillus kefiranofaciens, Lactobacillus kefir, Kluyveromyces lactis, Kluyveromyces marxianus, Saccharomyces lipolytica, Kazachstania aerobia, and *Pichia fermentans* are recognized (Ahmed et al., 2013; Wang et al., 2008, 2009).

In general, kefir grain consists of 4.4% fat, 12.1% ash, 45.7% mucopolysaccharide, 34.3% total protein (27% insoluble, 1.6% soluble and 5.6% free amino acids), vitamins B and K, tryptophan, Ca, P and Mg. In kefir grains, the external portion is composed almost exclusively by bacteria, predominantly Bacillus. By contrast, the inner part of the grain contains yeasts, and the interface between the inner and outer portions show a mixed composition, colonized by bacteria with long polysaccharide filaments, yeasts and fungi (Rosa et al., 2017). The microorganisms in kefir grains can produce weak organic acids, antibiotics, and several types of bactericide; all these substances shown a lethal effect on pathogenic micro-organisms (Ahmed et al., 2013).

There are several methods to store kefir grains. The wet grains are preserved at 4°C for 8-10 days. Besides, the lyophilized and dried grains are kept at room temperature for 36 to 48 h maintaining their activity for 12 to 18 months (Leite et al., 2013; Rosa et al., 2017). Garrote et al. in 1997 observed that freezing at –20°C represents the best method for grain preservation. Wszolek et al. in 2006 proposed a conventional method of drying at 33°C or vacuum drying to preserve the grains. However, if stored under favourable condition, the kefir grains remain stable for many years without losing their activity. Despite the unfavourable conditions, the grains can slowly re-establish their structure and, subsequently, new kefir grains are formed (Rosa et al., 2017).

Kefir production by kefir grains in an industrial scale is quite difficult to perform due to the microbiological diversity of grains, the handling and storage conditions. Also, the ratio of specific compounds (i.e. lactic acid, ethanol, carbonyl compounds) defining kefir aroma may differ from one industrial plant to another leading to the appearance of unstandardized products (Barukčić et al., 2017).

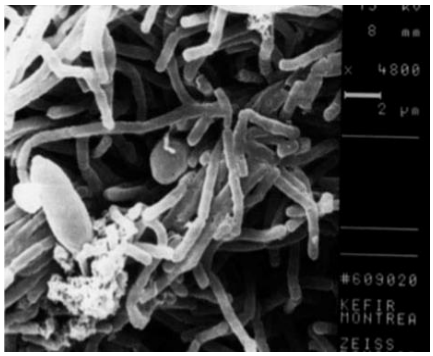
A)  B) 

Figure 1. A) Macroscopic structure of kefir grains; B) Electron micrograph of kefir grain (Arslan, 2015).

1.2.1. Kefiran

Some species of LAB (lactic acid bacteria) are known to produce extracellular polysaccharides (EPS) material, which contributes to the texture of the grain. The EPS produced by the kefir micro-organisms is commonly known as "kefiran". It is a water-soluble compound and consists of branched glucogalactan containing equal amounts of D-glucose and D-galactose able to bind water, denatured proteins, and a small amount of fat (Ahmed et al., 2013; Wszolek et al., 2007). Although the EPS produced by the LAB can affect the rheological properties of the product such as improve the texture and mouth-feel, they can also exhibit advantageous biological properties, such as immunostimulatory, antimutagenic, anticancer, anti-ulcer activities, and act as a prebiotic compound (Wszolek et al., 2007).

Additionally, kefiran exhibits inhibitory effects on rotavirus and epithelium protection (Ahmed et al., 2013).

Early kefir investigators suggested that kefiran was produced by *Lactobacillus kefir*, *Streptococcus mutans*, *Leuconostoc mesenteroides*, and/or *Streptococcus cremoris* since all are capable of producing extracellular polysaccharides. More recent investigations suggest that *Lactobacillus kefiranofaciens* is the primary producer of the exopolysaccharide matrix of the kefir grain (Guzel-Seydim et al., 2009).

1.3. Microbial composition

The microbial diversity of kefir described in the literature is extremely variable, and it is estimated to include more than 300 microbial strains. The microbial composition of kefir is affected by numerous factors such as the microbiological culture medium, the origin of kefir grains, different

techniques employed during processing, varying room temperatures, type and composition of milk used, storage conditions of kefir and kefir grains. Additionally, the amount of grain added to the milk, agitation, and the incubation temperature can affect the acidification and consequently, the microbiological composition of the final fermented milk (Rosa et al., 2017).

In kefir, lactic acid bacteria (LAB) are primarily responsible for the conversion of the lactose naturally present in milk to lactic acid, which results in a pH decrease and milk preservation. The starting microorganism proportion in the grains differs from those present in the final product. This difference is associated with the fermentation process conditions such as fermentation time, temperature, degree of agitation, type of milk, grain/milk inoculum ratio and microorganism distribution (Leite et al., 2013).

The symbiosis between microorganisms allows the maintenance of stability, so that during the fermentation cycle, the microbiological profile of kefir grains and kefir remains unaltered despite the variations in the milk quality and microbial contamination (Rosa et al., 2017). Because of the microbial symbiotic association present in the grains, the growth and survival of individual strains are dependent on the presence of each other. Often, when microorganisms are isolated from the grains, they do not grow well in milk and show reduced biochemical activity (Farnworth and Mainville, 2008).

Different methodologies have been applied to study the microbiota of kefir. However, the classical approach of culturing micro-organisms in nutrient media (universal and selective) followed by the identification of isolated cultures is still being performed. Unfortunately, these methods are prevented by the inability of certain microorganisms to grow on synthetic growth media (Bourrie et al., 2016; Rosa et al., 2017; Taş et al., 2012). Nowadays, the use of molecular techniques, including functional genomics, transcriptomics, proteomics and metabolomics, combined to culture-dependent methods, are encouraging to identify the microbial composition (Taş et al., 2012). With metagenomic analyses, also death cells will be detected and this should be taken into consideration since they would not be actively involved in the fermentation (Bourrie et al., 2016). The PCR (polymerase chain reaction) technique, combining to electrophoresis in denaturing gradient gel (PCR-DGGE) has resulted appropriate to analyze complex microbial association (Leite et al., 2013). Also, PCR with sequence analysis of 16S rDNA (for bacteria) and 18S rDNA (for yeast) have used for the identification and analysis of microflora (Taş et al., 2012). Further identifications of kefir grains microbiota can be achieved using RAPD-PCR (randomly amplified polymorphic DNA-PCR) to determine the similarities and differences among the inhabitant strains (Taş et al., 2012).

The microbial composition of kefir grains comprised 65 to 80% of Lactobacillus and Lactococcus, and the remaining portion is completed by yeasts (Rosa et al., 2017). Ahmed et al. in 2013 estimated that the bacterial population in kefir ranged between 6.4 × 104 – 8.5 × 108 cfu/g, and for yeasts ranged between 1.5 × 105–3.7 × 108 cfu/g. Moreover, Ahmed et al. (2013), according to Irigoyen et al. (2005) quantified the main micro-organism after 24 hours of fermentation. Their enumeration reported the following results; lactobacilli (108 cfu/ml), lactococci (105 cfu/ml), yeasts (106 cfu/ml), and acetic acid bacteria (106 cfu/ml) (Ahmed et al., 2013).

Anyway, kefir has variable and complex microflora predominantly consisting of defined and undefined species of yeast and bacteria in varying amounts (Ahmed et al., 2013). Many researchers identified bacterial, yeast and fungal species into the kefir milk and kefir grains as below reported.

1.3.1. Kefir Bacteria

The bacterial genera most commonly found in kefir belong to lactic acid bacteria (LAB) order such as Lactobacillus, Lactococcus, Streptococcus, and Leuconostoc. Other common bacteria found in kefir belong to Acetobacter and Bifidobacteria genera, as shown in **Table 1a** and **Table 1b**.

Scientific data reported that kefir grains from different countries/origin contain diverse microbiota even if the LAB and yeast contents of kefir grains are similar (Kök-Tas et al.,2012).
However, *Lactococcus* subsp. *lactis* and *Streptococcus thermophilus* were determined by Simova et al. (2002) as the predominant species in kefir grains (53–65%) and kefir samples (74–78%). In addition to LAB, acetic acid bacteria such as *Acetobacter lovaniensis* (8.91%) (Magalhaes et al., 2011) and *Acetobacter syzygii* are present, although in a lower amount.
Enterococcus faecalis, *Enterococcus faecium*, *Lactobacillus acidophilus*, L. *casei*, Leuconostoc and Lactococcus genera have been also identified by other researches (Witthuhn et al. 2004) (Ahmed et al., 2013).

In some countries, the presence of some species is considered undesirable and has received less attention, even if it is assumed to play an essential role in both the microbial community and the sensory characteristics of the final product (Leite et al., 2013).

1.3.1.1. Lactic acid bacteria

According to their morphology, these micro-organisms can be classified into cocci and rod shape. Additionally, according to the growth temperature range, they can be distinguished in mesophiles and thermophiles, able to growth at 20–30ºC and 37–45ºC, respectively (Tamime et al.,2006).

The group of lactic acid bacteria (LAB) from many years occupies a central role in the fermentation process of fermented foods and beverages. LAB can produce antimicrobial substances, sugar polymers, sweeteners, aromatic compounds as health-promoting properties, so are defined as probiotics (Bourrie et al., 2016).

LAB produce several natural antimicrobial compounds including organic acids (lactic acid, acetic acid, formic acid, phenyl lactic acid, caproic acid), carbon dioxide, hydrogen peroxide, diacetyl, ethanol, bacteriocins, reuterin, and reutericyclin. Some of them contribute to the aroma and prevent mould spoilage in the final product. Bacteriocins released by LAB are low-molecular-weight peptides with an antibacterial action against Gram-positive bacteria. They can be applied for food preservation because of their microbiological, physiological and technological advantages. Besides, many bacteriocins are active towards foodborne pathogens such as *Clostridium botulinum, Staphylococcus aureus,* and *Listeria monocytogenes* (Leroy & De Vuyst, 2004).

Anyway, the group of LAB including Lactobacillus, Lactococcus, Streptococcus, Leuconostoc genera, in kefir accounts from 37% to 90% of the total microbial community present (Bourrie et al., 2016).

According to their metabolism, different lactobacilli are distinguished in categories.

Obligatory homofermentative lactobacilli ferment the hexoses contained in the product almost exclusively to lactic acid (>85%) through the Embden- Meyerhof-Parnas (EMP) pathway to produce energy and to equilibrate the redox balance. The homofermentative lactobacilli possess fructose-1,6-bisphosphate-aldolase but lack phosphoketolase enzyme, and therefore, neither gluconate nor pentoses are fermented (Leroy & De Vuyst, 2004; Holzapfel et al., 1995). However, pyruvate can lead to the generation of many other metabolites such as acetate, ethanol, diacetyl, and acetaldehyde. In this way, LAB produce volatile substances that contribute to the typical flavour of certain fermented products such as kefir. (Leroy & De Vuyst, 2004). Homofermentative LAB, including Lactobacillus species, such as *L. delbrueckii* subsp. *bulgaricus, L. helveticus, L. kefiranofaciens* subsp. *kefiranofaciens, L. kefiranofaciens* subsp. *kefirgranum* and *L. acidophilus;*

Lactococcus spp. such as *L. lactis* subsp. *lactis* and *L. lactis* subsp. *Cremoris*, *L. gasseri*, *L. casei-Pediococcus* and *Streptococcus thermophilus* have been identified in both kefir grains and fermented beverage (Leite et al., 2013; Holzapfel et al., 1995; Wszolek et al., 2007).

Heterofermentative Lactobacilli included in LAB category are classified in facultatively and obligatory heterofermentative.

Facultatively heterofermentative lactobacilli ferment almost exclusively hexoses to lactic acid by the EMP pathway. The organisms possess both aldolase and phosphoketolase enzymes, and therefore, not only ferment hexose but pentoses too. Additionally, obligated heterofermentative lactobacilli ferment hexoses by the phosphogluconate pathway yielding lactate, ethanol (or acetic acid) and carbon dioxide in equimolar amounts. Pentoses also enter this pathway and may be fermented (Holzapfel et al., 1995).

Heterofermentative LAB, including *L. kefiri, L. parakefiri, L. fermentum* and *L. brevis* have also been identified in kefir grains and the fermented beverage (Leite et al., 2013).

The proportions of species can also differ among grains and milk. For example, L. *lactis* subsp. *lactis*, and *S. thermophilus* levels are generally much more significant in the fermented kefir than in the kefir grains. The levels of these species increase further in kefir made using kefir as an inoculum. Indeed, the total increase observed has been as much as 30% in some cases (Simova et al., 2002). The reason for this increase during fermentation of milk may be due to the rise in temperature created by the active fermentation. The Lactobacillus entrapment within kefir grains can be another explanation. *L. kefiranofaciens, L. kefiri*, and *L. parakefiri* are the most common species of Lactobacillus found in kefir. Other micro-organisms genera have been identified in these grains. However, they typically represent less than 10% of the community, including Leuconostoc, Lactococcus, Lactobacillus genera (Bourrie et al., 2016).

Microbial genera	Microbial species	
Lactobacillus	*Lactobacillus kefir*	*Lactobacillus johnsonii*
	Lactobacillus kefiranofaciens	*Lactobacillus rhamnosus*
	Lactobacillus delbrueckii	*Lactobacillus rossiae*
	Lactobacillus helveticus	*Lactobacillus sakei*
	Lactobacillus casei	*Lactobacillus reuteri*
	Lactobacillus kefiri	*Lactobacillus kalixensis*
	Lactobacillus brevis	*Lactobacillus rapi*
	Lactobacillus paracasei	*Lactobacillus diolivorans*
	Lactobacillus parakefir	*Lactobacillus parafarraginis*
	Lactobacillus plantarum	*Lactobacillus gallinarum*
	Lactobacillus satsumensis	*Lactobacillus crispatus*
	Lactobacillus curvatis	*Lactobacillus otakiensis*
	Lactobacillus fermentum	*Lactobacillus instestinalis*
	Lactobacillus viridescens	*Lactobacillus amylovorus*
	Lactobacillus acidophilus	*Lactobacillus pentosus*
	Lactobacillus gasseri	*Lactobacillus salivarius*
	Lactobacillus kefirgranum	*Pediococcus claussenii*
	Lactobacillus parakefiri	*Pediococcus damnosus*
	Lactobacillus parabuchneri	*Pediococcus halophilus*
	Lactobacillus garvieae	*Pediococcus pentosaceus*
	Lactobacillus buchneri	*Pediococcus lolii*
	Lactobacillus sunkii	

Table 1a. List of bacterial species found in kefir grains and milk using both culture-dependent and culture-independent techniques (Bourrie et al., 2016).

Microbial genera	Microbial species	
Lactococcus	*Lactococcus lactis subsp. Lactis* *Lactococcus lactis subsp. cremoris* *Lactococcus garvieae*	*Lactococcus lactis subsp. lactis biovar diacetylactis*
Streptococcus	*Streptococcus salivarius subsp. Thermophilus* *Streptococcous thermophilus*	*Streptococcus durans*
Leuconostoc	*Leuconostoc spp.* *Leuconostoc mesenteroides subsp. Mesenteroides* *Leuconostoc mesenteroides subsp. Cremoris*	*Leuconostoc mesenteroides* *Leuconostoc pseudomesenteroides*
Acetobacter	*Acetobacter spp.* *Acetobacter sicerae*	*Acetobacter orientalis* *Acetobacter lovaniensis*
Bifidobacterium	*Bifidobacterium spp.* *Bifidobacterium breve* *Bifidobacterium choerinum*	*Bifidobacterium longum* *Bifidobacterium pseudolongum*

Table 1b. List of bacterial species found in kefir grains and milk using both culture-dependent and culture-independent techniques (Bourrie et al., 2016).

1.3.1.2. Non-Lactic acid bacteria

Concerning the non-lactic acid bacteria (NLAB) that have been associated with kefir, it is notable that culture-dependent (De Roos & De Vuyst, 2018) and independent methods have revealed Acetobacter as one of the dominant genera present in grains (Bourrie et al., 2016).

The growth of Acetic acid bacteria mainly occurs under aerobic conditions, leading to increased acetic acid concentrations, which might be undesirable. Under anaerobic conditions, they remain in a VBNC (viable but not culturable cell) state. Then, when oxygen becomes available, they restart their functioning and start to grow (De Roos & De Vuyst, 2018). *Acetobacter* spp. is not commonly isolated from kefir as an essential micro-organism, even if some studies have found Acetic acid bacteria in large quantities in kefir grains (Bourrie et al., 2016). *Acetobacter aceti* and *Acetobacter rasens* represent the main Acetic acid bacteria found in kefir milk. Angulo et al. (1993) considered them to be contaminants, while Koroleva (1988) reported that their presence

is desirable (Wszolek et al., 2007). As shown in **Table 1b**, other Acetobacter species are found in kefir by researches around the world.

The incorporation of Bifidobacteria genera of micro-organism into fermented milk and other fermented dairy products is commonly performed. There are some Bifidobacterium strains, which are widely used in probiotic milk products in combination with different LAB. They include strains of *B. breve, B. pseudolongum, B. animalis* subsp. *lactis, B. bifidum* (Prasanna et al., 2014).

The ability of some Bifidobacteria to produce EPSs (exopolysaccharides) enhance the viscosity of fermented dairy products resulting in a positive effect in industrial application. EPSs reduce interactions between protein aggregates, changing the rheological properties of fermented milk products. Furthermore, EPS could increase the water holding capacity of fermented milk products (Prasanna et al., 2014).

1.3.2. Kefir yeasts

In addition to the large and variable bacterial population in kefir grains, there is an abundant yeast population that exists in a symbiotic relationship with the bacteria as shown in **Table 1c**. Three genera of yeasts are commonly isolated from kefir grains or milk: *Saccharomyces*, *Kluyveromyces*, and *Candida* (Bourrie et al., 2016), Like kefir bacteria, the profile of yeasts is different in kefir grains when compared to the final kefir product. The properties of yeasts found in kefir grains are variable. For example, some of the yeasts found in kefir grains are capable of fermenting lactose (Farnworth, 2005).

The primary yeast able of fermenting lactose found in kefir and kefir grains are *Kluyveromyces marxianus, Kluyveromyces lactis* var. *lactis, Candida kefyr, Debaryomyces hansenii* e *Dekkera anomala*. At the same time, the non-lactose fermenters include *Saccharomyces cerevisiae, Torulaspora delbrueckii, Pichia fermentans, Kazachstania unispora, Saccharomyces turicensis, Issatchenkia orientalis* and *Debaryomyces occidentalis* (Leite et al., 2013; Taş et al., 2012).

Many different species of *Saccharomyces* have been isolated from kefir. However, *S. cerevisiae* and *S. unisporus* are the most commonly present in many varieties.

Kluyveromyces make up the majority of the lactose utilizing yeast population, including *K. marxianus* and *K. lactis* species (Bourrie et al., 2016).

The *Candida* population include mainly the most prevalent species such as *C. holmii* and *C. kefyr*. Outside of these three genera, only *Pichia* has been identified as *Pichia fermentans* by both culture-dependent and culture-independent method (Bourrie et al., 2016).

As fermentation progresses the proportions of some yeast species change with non-lactose fermenting yeasts, such as Saccharomyces, decreasing, whereas lactose utilizing *K. marxianus* and *K. lactis* show a similar distribution between grain and kefir (Simova et al., 2002).

Similar to the bacterial population in kefir grain, the yeast component varies considerably in grains when are analyzed by culture-independent techniques.

It has been indeed possible to identify a reduced concentration of yeasts such as *Kazachstania* including *Kazachastania aerobia* (Rosa et al., 2017), *Kluyveromyces*, and *Naumovozyma* (Bourrie et al., 2016).

Sequencing based approaches have also identified others yeast species that had not previously been associated with kefir, such as *Dekkera anomala*, *Issatchenkia orientalis*, and *Pichia fermentans*. They have even shown that, in some grains, the yeast population is dominated by a mix of these other species (Bourrie et al., 2016). Rosa et al. in 2017 also identified other yeast species such as *Lachanceae meyersii*, *Yarrowia lipolytica* present in kefir and kefir grains in more significant numbers.

Yeast and fungal species	
Zygosaccharomyces spp.	*Kazachstania exigua*
Candida kefyr	*Naumovozyma spp.*
Candida lipolytica	*Cryptococcus humicolus*
Saccharomyces cerevisiae	*Geotricum candidum*
Candida holmii	*Kazachstania servazzii,*
Torulaspora delbrueckii	*Kazachstania solicola*
Saccharomyces unisporus	*Kazachstania aerobia*
Candida friedrichii	*Saccharomyces cariocanus*
Kluyveromyces lactis	*Kluyveromyces marxianus var. lactis*
Pichia fermentans	*Saccharomyces humaticus*
Issatchenkia orientalis	*Candida sake*
Kluyveromyces marxianus	*Yarrowia lipolytica*
Saccharomyces turicensis	*Dipodascus capitatus*
Dekkera anomala	*Trichosporon coremiiforme*
Candida inconspicua	*Dioszegia hungarica*
Candida. maris	

Table 1c. List of yeast and fungal species found in kefir grains and milk using both culture-dependent and culture-independent techniques (Bourrie et al., 2016).

1.3.3. Interactions between kefir microorganisms

The complex interactions between yeast and bacteria and their interdependence in kefir grains are not entirely understood. However, if the bacteria are separated from the grain, yeast will not grow as efficiently. Due to its high capacity to metabolize lactose, the genus *Lactococcus* tends to grow faster than yeast in milk. Tamime in 2006 noted the ability of genus *Lactococcus* to hydrolyze lactose, producing lactic acid and a suitable environment for yeast growth. Moreover, yeasts generally synthesize complex B vitamins and hydrolyze milk proteins, using oxygen to produce carbon dioxide and ethanol. Species such as *Debaryomyces hansenii* and *Yarrowia lipolytica* consume the lactic acid formed by LAB, raising the pH and stimulating bacteria growth. The production of vitamin B by Acetobacter *spp*. also contribute to the growth of other microorganisms present in kefir grains (Leite et al., 2013).

The microbial disposition in kefir grains has always stirred the curiosity of researches which have theorized several hypotheses about it.

A group of researchers supports the hypothesis that yeasts are generally found in the inner and intermediate grain portion, accompanied to rod-shaped bacteria and rare Lactococci

predominantly on the surface area. By contrast, some Authors (Jianzhong et al., 2009; Magalhães et al.,2011) have reported a lower number of cells observed in the inner grain portion as compared with the outer part. Also, some Authors have postulated that fibrillar material found in the grain may be the polysaccharide kefiran. In general, rod-shaped bacteria were observed both in the inner and the outer grain portions, whereas yeasts are most frequent in the outer part. Cocci are noted in the outer portion of one of the grains. In all probability, the granular material observed corresponded to particles of coagulated milk adhered to the surface of the grain and the fibrillar material to the polysaccharide kefiran, spread over all parts of the grains. However, studies have shown that a wide variation in microbial population occurs between different grains and within the same grain (Leite et al., 2013).

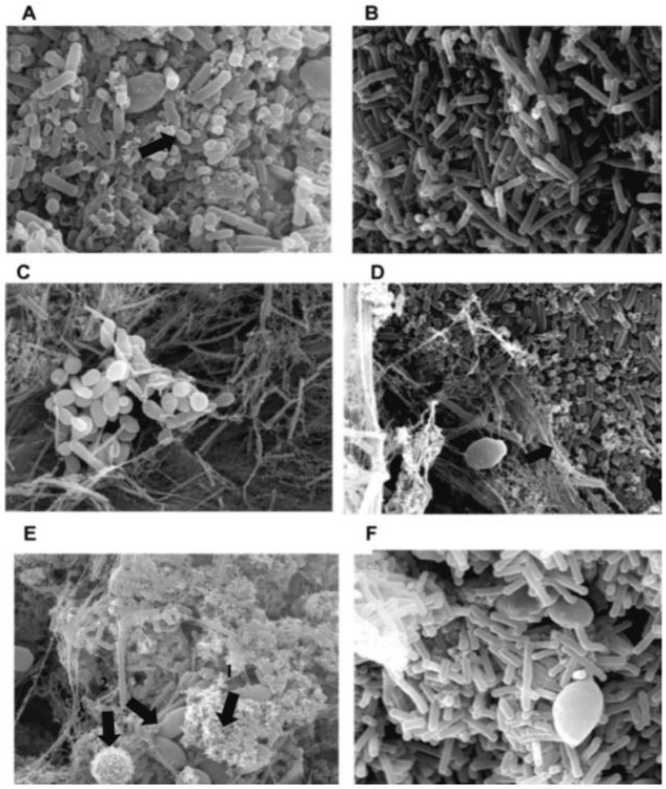


Figure 2. Scanning electron microscopy of Brazilian kefir grain microbiota. A, C, E: outer grain portions, B, D, F: inner grain portions. Arrows Micrography A:cocci; Micrography D: fibrillar material-kefiran polysaccharide; Micrography E: arrow 1-granularmaterial-coagulated protein, arrow 2 - different yeast species (Leite et al., 2013).

1.4. Fermentation process

Fermentation has been used in foods for thousands of years to extend the shelf life and improve the nutritional values in product. Today this biochemical process is commonly used in vegetables, fruits, cereals, meat, milk, fish and consumed fermented products.

Fermented dairy products include a wide range of fermented foods and provide several beneficial effects on human health as investigated by Metchnikoff last century.

Approximate fermentation time during kefir production is mostly between 16 and 24 h. More precisely, at 25 °C the fermentation process needs from 10.5 to 12 h, while at process temperature of 35 °C it spends shorter (approximately 9 h). The shortest fermentation time (approximately 9 h at 35 °C) has observed in samples produced by kefir grains in prevalence. By contrast, samples produced by starter culture are characterized by the longest fermentation time (from 11 to 12 h), regardless of the fermentation temperature (Barukčić et al., 2017).

The fermentation process performed by lactic acid bacteria and yeasts results in the production of carbon dioxide, alcohol, and aromatic molecules that give kefir distinctive organoleptic properties. Their release results in typical tart flavor and slightly carbonated flavour because of the natural presence of CO_2 (Hertzler & Clancy, 2003).

1.4.1. Fermentation of Lactic acid bacteria

Marshall and Tamime in 1997 assumed that the mechanisms responsible for the production of flavour compounds are the result of metabolism (i.e. the catabolism of carbohydrate and nitrogen utilisation), which is necessary for the micro-organisms to grow efficiently and to achieve the maximum populations (Wszolek et al., 2007).

Generally, the main activity of lactic acid bacteria is the breakdown of the carbohydrates present in milk to lactic acid for the generation of energy required for biomass synthesis. Lactic acid bacteria are not equipped with the enzymes necessary for respiration, and they are, therefore, unable to perform oxidative phosphorylation. The energy demand is then satisfied exclusively through substrate-level production of adenosine triphosphate (ATP) or the equivalent of ATP. The usually rapid conversion of carbohydrate to metabolic products by the starter cultures is simultaneous with the generation of energy required for growth. The energy-generating pathways of lactic acid bacteria include different pathways and two types of fermentations, homolactic and

heterolactic fermentation, based on the identity of the main products formed (Tamime et al., 2006).

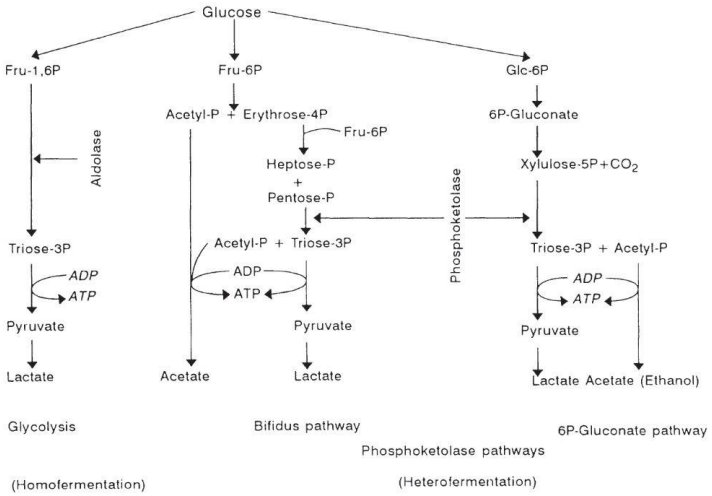


Figure 3. Schematic presentation of the main pathways of hexose fermentation in lactic acid bacteria (Holzapfel et al., 1995)

Homolactic fermentation takes place in two steps. In the first step, called glycolysis or Embdene-Meyerhofe-Parnas (EMP) pathway, glucose is transformed into pyruvic acid which is subsequently reduced to lactic acid by the reducing power previously produced in the form of NADH (Castillo Martinez et al., 2013; Leroy & De Vuyst, 2004). LAB may either form D(-) or L(+)-lactic acid, or a racemic mixture of the two isomers. The EMP pathway of glycolytic homofermentation is characterized by the key role of aldolase, as compared to phosphoketolase serving as a key enzyme in the phosphoketolase pathway in glycolytic heterofermentation, as shown in **Figure 3** (Holzapfel et al., 1995).

Thus, lactic acid is obtained from glucose as the exclusive product according to the overall equation:

Glucose → 2 Lactic Acid + 2 ATP

The microorganisms that use only this pathway for the consumption of carbohydrates are called obligatory homofermentatives. Homolactic fermentation should theoretically yield 2 mol of lactic acid per mole of consumed glucose with a theoretical yield of 1 g of product per g of substrate. Still, the experimental yields are usually lower (0.74-0.99 %) because a portion of the carbon source is used for biomass production (0.07-0.22 %)(Castillo Martinez et al., 2013).

Also, homolactic fermenter bacterial strains can convert the fermented carbohydrate into products other than lactate. Alternative end-products of pyruvate catabolism include formate, acetylaldehyde, ethanol, acetate, acetoin, and 2,3 butanediol as shown in **Figure 4** (Tamime et al., 2006).

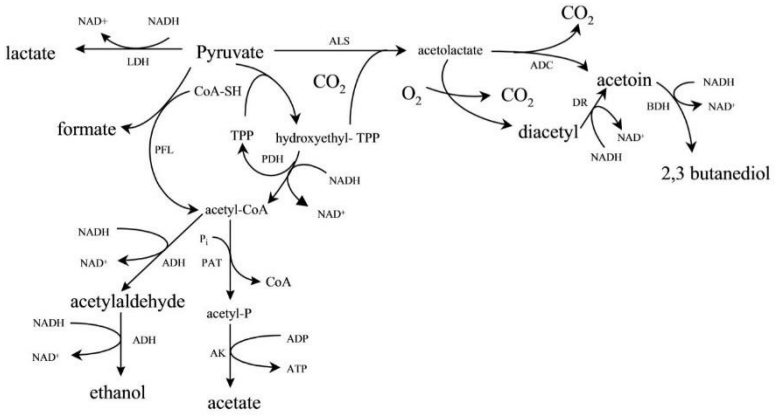


Figure 4. Alternative end-products of pyruvate catabolism – products found extracellularly are given in large letters (Tamime et al., 2006).

Heterolactic fermentation is characterized by the formation of co-products such as carbon dioxide, ethanol and/or acetic acid in addition to lactic acid as the end product of fermentation (**Figure 3**). The first step of glucose degradation, which is called the pentose-phosphate pathway (included in phosphoketolase pathway), leads to glyceraldehyde 3-phosphate, acetyl phosphate and carbon dioxide. Glyceraldehyde 3-phosphate enters the glycolysis pathway through which it is converted into lactic acid. Also, acetyl-phosphate is converted into acetic acid and/or ethanol according to the overall equations:

Glucose → Lactic acid + CO_2 + Ethanol + ATP

Glucose → Lactic acid + CO_2 + Acetic acid + 2 ATP + 2 NADH

The relationship between the amounts of acetic acid and ethanol, which reduces the theoretical yield to 0.50%, depends on the ability of the microorganism to re-oxidize the NADH generated in the early stages of the process along with its energy requirements. Microorganisms that use only this metabolic pathway for the consumption of carbohydrates are called obligatory heterofermentative (Castillo Martinez et al., 2013).

Certain carbonyl/flavouring compounds, such as acetate, diacetyl, acetoin and 2,3 butanediol, and carbon dioxide (**Figure 4**) are produced in milk through the metabolism of citrate. While acetoin and butanediol are tasteless and not involved in flavour, diacetyl is an important flavour component in buttermilk and sour cream, produced by mesophilic starter cultures (Tamime et al., 2006).

1.4.2. Fermentation of yeasts

According to Walker (1998), yeasts can metabolize a wide range of organic substrates, which will provide the cells essential carbon and energy by the alcoholic fermentation process. Most yeasts metabolize sugars as their primary carbon source to release energy (Walker, 1998). Ten reactions are required to produce pyruvate from glucose. In the final step of alcoholic fermentation of sugars, the yeasts re-oxidize NADH to NAD by the consecutive action of the enzyme pyruvate decarboxylase (PDC) that decarboxylates pyruvate to acetaldehyde, and alcohol dehydrogenase (ADH), that reduces acetaldehyde to ethanol (Wszolek et al., 2007). Below the alcoholic fermentation reaction is reported.

$$C_6H_{12}O_6 + 2\ P + 2\ ADP \rightarrow 2\ C_2H_5OH + 2\ CO_2 + 2\ ATP + 2\ H_2O$$

Saccharomyces spp. (i.e. aerobic and anaerobic fermentation) and *Kluyveromyces spp.* (i.e. anaerobic fermentation) can metabolize a wide range of sugars besides glucose, such as lactose, galactose, melibiose and fructose (Wszolek et al., 2007).
Also, the principal mode of sugar catabolism by *Candida, Pichia* and *Torulopsis* species is the anaerobic fermentation. *Candida*, *Saccharomyces* and *Kluyveromyces* species possess minimal extracellular proteolytic activity (Wszolek et al., 2007).

However, yeast metabolism in kefir is not well established. It may result in different types of degradation of the milk components, all of which can potentially contribute to the flavour of the product. The hydrolysis of milk constituents by the yeasts in the kefir grains and/or the starter culture includes both the fat/lipids hydrolysis and the protein hydrolysis (Wszolek et al., 2007). Lipolysis of the fat component in milk results in the formation of free fatty acids, which can be precursors of flavour compounds to be formed in kefir. These compounds may include methyl ketones, alcohols, lactones and esters. Lipolysis and decarboxylation of free fatty acids lead to the formation of methylketones, especially 2-nonanone and 2-heptanone, which are known as

blue cheese flavours (Marshall & Tamime, 1997). The yeasts are also able to degrade casein to small peptides and free amino acids; the latter are converted to alcohols, aldehydes, volatile acids, esters and sulphur-containing compounds (especially methionine, which is the precursor for volatile aroma compounds). Examples of sulphur compounds are dimethyldisulfide and dimethyltrisulfide, which are regarded as essential components for a cheesy flavour. Branched-chain amino acids are precursors of aroma compounds, such as isobutyrate, isovalerate, 3-methylbutanal, 2-methylbutanal and 2-methylpropanal (van Kranenburg et al., 2002).

1.5. Kefir production

Traditionally, kefir was produced in animal skin bags where the fermentation process occurred thanks to the kefir grains and periodically fresh mammalian milk addition. Even if kefir produced from bovine milk is the most commonly used, it can be produced from whole, semi-skimmed or skimmed milk, pasteurized from bovine, goat, sheep, camel or buffalo milk (Rosa et al., 2017; Wszolek et al., 2007).

The current methods for the production of kefir are:

- the traditional method based on the use of kefir grains;

- the industrial process using a kefir starter culture.

A significant difference in processing involves the choice of using kefir grains or a kefir grain-free extract as a starter culture. Starter culture companies have made great efforts to develop kefir starter cultures that do not produce grains during the kefir manufacture and, as a consequence, the characteristics of the product are different from a traditional product. These developed kefir starter cultures make the production of the product less laborious and will ensure a longer shelf-life of the kefir (Wszolek et al., 2007). According to specific analysis, the microbial profiles of the grains and the mother culture used (percolate taken from the grains) can affect the final product characteristics. As a result of the ultimate complexity of micro-organisms activities performed in fermented milk, the kefir production must start from kefir grains (Farnworth, 2005).

1.5.1. Production of kefir grains and starter cultures

The preparation stages for the production of kefir grains and a mother starter culture are discussed

below.

The kefir grains are generally supplied in 50 g portions and are preserved in an isotonic salt solution. The proliferation of the kefir grains is obtained daily in skimmed milk, which is heated to 95°C for 30 min, cooled to 18–20°C, and inoculated with kefir grains and incubated for 20–24 h. Finally, it is ripened for 7–8 h at 10–12°C to facilitate the growth of the yeasts. The grains are then strained using a sterile sieve, and the fermented kefir without the kefir grains (mother starter culture) is inoculated into freshly prepared milk (Wszolek et al., 2007). However, as the mass density of the grains increases every time they are grown in milk, the low activity grains are removed and discarded once or twice a week (Wszolek et al., 2007).

The standard ratio of kefir grains to milk has to be maintained to preserve the right quality product. Koroleva et al. in 1988 assumed that the use of a high proportion of grains (e.g. 1: 20, grain to milk ratios) accelerates the acidification rate. Koroleva and co-authors also reported that increasing the proportion of milk from 1:20 to 1:50, maintains the balance, the activity, and the growth of the micro-organisms in the kefir grains (Wszolek et al., 2007).

Additionally, several micro-organisms (bacteria, yeasts, moulds or combinations of these) are employed in the manufacture of fermented milk products as starter cultures (Tamime et al., 2006). The main functions of microbial starter cultures adopted in dairy products may be summarized as:

• product preservation due to the fermentation process resulting in an extended shelf-life and enhanced safety;

• bacteriocins production with antimicrobial effect, which increases food preservation;

• enhancing of the perceived sensory properties of the product (e.g. due to the production of organic acids, carbonyl compounds and partial hydrolysis of the proteins and/or fats);

• rheological properties of fermented milk products improvement (i.e. viscosity and firmness);

• consumer dietetic/functional/nutraceutical properties improvement (Tamime et al., 2006).

Three main aspects are considered during the development stages of starter culture strains in dairy product manufacturing. They are summarized as the choice or development of a single bacterial strain, followed by the blending of the culture strains, and finally the characterization of the developed culture (Tamime et al., 2006).

One of the most critical stages in preparing a kefir starter culture is the handling of the kefir grains. Several researchers, including Koroleva (1988) and Polish Standards (Anon., 2002) do not recommend rinsing the kefir grains if they are transferred daily to freshly prepared milk. Alternatively, the grains can be delicately rinsed with pasteurized milk or sterile water, and stored at < 6°C or frozen. Rinsing the kefir grains is a common practice in the industry that can lower the counts of lactococci bacteria, yeasts, and the biomass of the grains (Wszolek et al., 2007).

To date, to ensure a correct kefir making, many starter culture companies offer dairy manufacturers freeze-dried kefir cultures containing, in part, some of the micro-organisms present in traditional kefir grains (Wszolek et al., 2007).

1.5.2. Traditional method

The traditional method of kefir making is performed by adding kefir grains directly, as a starter, to fresh milk. In-home production, the fermentation temperature and process time are not rigidly controlled. The final product cannot be used to inoculate new milk to produce kefir because the original balance of microorganisms in the grains has been disrupted (Arihara, 2014). To make authentic kefir, a variable amount of kefir grains (2 to 10%) are added to previously fresh milk and incubated with stirring for a period between 18-24 h at 25°C in a partially closed container. Generally, the fermentation and simultaneously the stirring are carried out at 20 to 25°C. Regardless, the enhancing of homofermentative lactococci and yeast development is achieved by the stirring process.

A maturation step, carried out at 8 to 10°C for 15 to 20 h, is often added. The grains are sieved and can be used for a new fermentation or kept (for 1-7 days) in fresh milk where may increase in size by up to 2% of the original to form new biomass, which allows continuous production of grains eventually added to a fermentation substrate. Meanwhile, kefir beverage is stored at 4 °C, ready for consumption. (Guzel-Seydim et al., 2009 (Arihara, 2014; Leite et al., 2013). During the cooling step, alcoholic fermentation leads to the accumulation of CO_2, ethanol as well as vitamin B complex products, which confer the typical characteristics of kefir milk (Rosa et al., 2017).

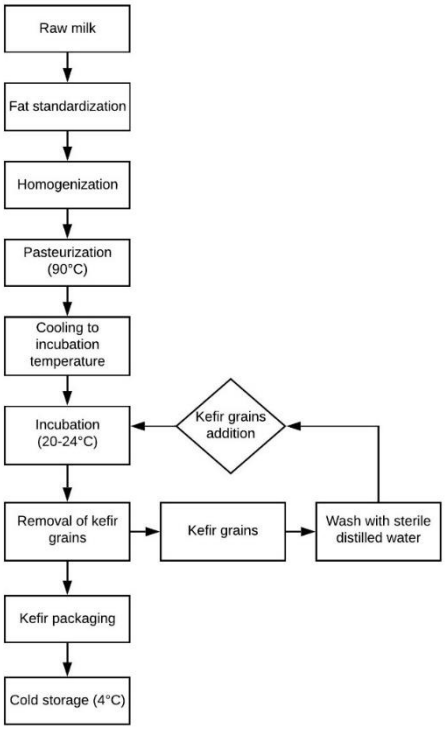


Figure 5. Production scheme for making authentic kefir (Guzel-Seydim et al., 2009).

1.5.3. Industrial process

The properties of kefir (chemical, physical, and organoleptic) were initially challenging to be duplicated in large-scale production. Much of the kefir produced in industrial practice was not considered authentic kefir because it was not incubated with the grains, resulting in less quality of the product. Indeed, large-scale production of kefir has been hindered by problems about the kefir grains and product consistency. Initially, the set method was used to produce kefir. In this procedure, inoculated milk was filled into bottles, fermented at a controlled temperature until the formation of a coagulum, and then cooled. However, the kefir produced was lower quality product compared to that made on a smaller scale by traditional methods.

Currently, kefir is provided by a stirred process where fermentation, coagulum formation, agitation, ripening and cooling all occur in one vessel. The chemical, organoleptic, and microbiological characteristics of the final product depends on the type of milk used. Also, the source of grains, the preparation of the mother culture (the percolate portion after the removal of grains), the length of fermentation, the inclusion of a cooling step, and the inclusion of a maturation step, affect the final properties of the product (Arihara, 2014). Regardless, the

industrial-scale production rarely utilizes kefir grains for fermentation. Instead, it uses starter cultures of microbes that have been isolated from kefir or kefir grains to provide more consistent products (Bourrie et al., 2016).

Much of the commercial production of kefir involves the use of lyophilized or freeze-dried (Rosa et al., 2017) starter cultures containing LAB or and yeast. In this way, the activated starter culture is added to homogenized and pasteurized milk. Then, after the fermentation process at 25°C for 20–24 h, the product is stored at refrigeration temperatures. Meanwhile, the pH gradually drops to 4.6 and fermentation process ends in approximately 20–24 h, which gives sufficient time for the formation of taste and aroma substances when an inoculation rate of 2–5% kefir culture is used (Guzel-Seydim et al., 2009).

The currently used method permits to produce a final fermented product with different characteristics compared to those obtained by the set method.

1.5.3.1. Manufacturing stages of commercial kefir

Firstly, the milk is warmed at 65°C, homogenized, heated at 95°C for 5 min, cooled to 19°C in summer or to 22°C in winter and inoculated with the starter culture at a rate of 3–4 % using a bulk starter culture.

For set-type kefir production, the inoculated milk is packaged in glass bottles or semi-rigid containers, fermented for 10–12 h at 19–22°C, cooled to 9°C and ripened for 1–3 days. The product is then cooled slowly and stored at 6°C before dispatch for distribution and retailing.

By contrast, stirred kefir is fermented in tank for the same duration adopted in the manufacture of set kefir, but the fermented product is cooled to 15°C, packaged and then ripened at 9°C for only 15 h and stored at 6°C.

The manufacturing stages differ in the following steps.

- Set kefir: the inoculated and packaged milk is fermented to pH 4.6–4.7, cooled to < 10°C and ripened for 15–20 h.

- Stirred kefir: the fermented product is cooled to 15–20°C, packaged, followed by slowly cooling to < 10°C, and ripened for 15–20 h. By filling the kefir when it is warm, the packaging seal collapses and, when the CO_2 is released during the ripening stage, the seal regains its original

form rather than blowing up the container (Wszolek et al., 2007).

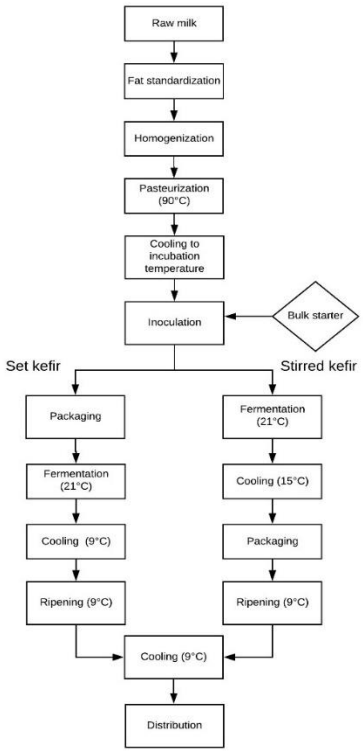


Figure 6. General scheme for the industrial production of kefir using mother culture as a starter (Wszolek et al., 2007).

1.6. Chemical and nutritional composition of kefir

Researchers highlight a significant variation in the composition of fermented kefir products. Most of these variation arises due to changes in the source, the fat content of milk, the nature of the grains or cultures and the manufacturing process of kefir product (Ahmed et al., 2013). Regarding the chemical composition, moisture is the predominant constituent (90%), followed by sugars (6%), fat (3.5%), protein (3%) and ash (0.7%) (Rosa et al., 2017).

The reported microbiological and chemical composition of kefir varies widely also because of the source of kefir grains used during the production affects the final product. Also, different types of milk (various species, various levels of fat), as well as its volume, affect kefir's sensory, chemical and textural properties. Moreover, the different production methods (commercial, artisan) and the storage conditions can change the final product composition (Arihara, 2014; Arslan, 2015). It has been also observed that storage temperature at 48°C may reduce the ethanol, and acetoin

concentration in the product, and increase the acetaldehyde content (Ahmed et al., 2013; Arihara, 2014).

The ingredients most commonly measured as indicators of quality are CO_2, proteins, lipids, lactose, ethanol, and lactic acid.

Öner, Karahan, and Çakmakci in 2010, investigated the properties of kefir produced using various milk origin (bovine, ovine and caprine) and culture types (kefir grain and commercial starter culture). They reported that starter culture type, storage period and mammalian species significantly affected the pH (Arihara, 2014), which generally ranged between 4.2- 4.6 based on the kefir chemical composition (Arslan, 2015).

Table 2 is a summary of the composition of kefir reported in the literature (Otles and Cagindi, 2003).

The significant products formed during fermentation are CO_2, lactic acid, and ethanol (Ahmed et al., 2013; Arihara, 2014). The amounts of CO_2 and ethanol produced during the fermentation of kefir depends on the production conditions.

The CO_2 content in fermented kefir product is dependent on kefir grain and increases as the level of kefir grain increases in the product. The desired carbon dioxide concentration may be up to 1.98 g/L followed by the simultaneous production of ethanol in small amounts (Ahmed et al., 2013), between 0,5 and 2,0% (v/v) (Rosa et al., 2017).

L (+)-lactic acid is the most abundant organic acid after fermentation and is derived from approximately 25% of the original lactose in the starter milk. Magalhaes et al. in 2011 investigated the content of lactic acid in Brazilian kefir. Results have shown its content in a range between 1.4 - 17.4 mg/ ml (Arslan, 2015).

Other products formed during fermentation are acetic acid, pyruvic acid, hippuric acid, propionic acid, butyric acid diacetyl, acetoin, and acetaldehyde. Diacetyl and acetaldehyde are aromatic compounds which along to the other compounds, improve taste and aroma in the product (Ahmed et al., 2013; Arihara, 2014). Diacetyl is produced during the fermentation stage by *Streptococcus lactis* subsp. *diacetylactis* and Leuconostoc sp. (Otles & Cagindi, 2003). In Magalhaes et al. investigation, the content of acetic acid can increase from 2.10 to 2.73 mg/ml (Magalhães et al., 2011).

Additionally, appreciable amounts of macroelements and microelements were also observed in kefir (Ahmed et al., 2013). These chemical compounds, including proteins, sugars, vitamins, lipids and other minor compounds can improve the nutritional value of the final product.

Components	100 g	Components	100 g
Energy	65 kcal	**Vitamins (mg)**	
Fat (%)	3.5	A	0.06
Protein (%)	3.3	Carotene	0.02
Lactose (%)	4.0	B1	0.04
Water (%)	87.5	B2	0.17
		B6	0.05
Milk acid (g)	0.8	B12	0.5
Ethanol	0.9	Niacin	0.09
Lactic acid	1	C	1
		D	0.08
Essential amino acids (g)		E	0.11
Tryptophan	0.05		
Phenylalanin+tyrosine	0.35	**Mineral content (g)**	
Leucine	0.34	Calcium	0.12
Isoleucine	0.21	Phosphor	0.10
Threonine	0.17	Magnesium	12
Methionine+cystine	0.12	Potassium	0.15
Lysine	0.27	Sodium	0.05
Valine	0.22	Chloride	0.10

Table 2. Chemical Composition and nutritional values of kefir (Otles and Cagindi, 2003).

1.6.1. Nutritional value of kefir

The high nutritional value of kefir (**Table 2**) is attributed to the presence of balanced chemical substances such as protein, fat, minerals, vitamins, and the fermentation products able to increase its nutritional profile (Ahmed et al., 2013). The protein, fat, and mineral content of kefir is similar to that of the milk from which it is made, and therefore kefir has an inherently high nutritional value as a source of protein and calcium (Arihara, 2014).

The nutritional composition of kefir varies widely and is influenced by milk composition, the origin and composition of the grains used, the time/temperature of fermentation and storage conditions (Rosa et al., 2017). Kefir also has a reputation as being palatable with high digestibility, allowing a large consumption without intestinal disturbance (Arihara, 2014).

Regardless, FAO/WHO provides the “Standard for fermented milk”. It is redacted in 2018 and includes the chemical composition and microbiological quality of kefir as follows:

• Protein content*: not less than 2.7 %;

• Fat level: not more than 10 %;

• Titratable acidity: not less than 0.6 %;

• Bacterial count in 1 g: not specified;

• Yeast count: not less than 10^4 cfu/g

*Protein content is 6.38 multiplied by the total Kjeldahl nitrogen determined.

1.6.2. Protein content

The fermentation process leads to the denaturation of milk proteins and hydrolysis of some of them, resulting in smaller peptides that are more susceptible to digestion by gastric and intestinal juices. (Arihara, 2014). The levels of ammonia, serine, lysine, alanine, threonine, tryptophan, valine, lysine, methionine, phenylalanine and isoleucine are higher in kefir compared to unfermented milk. This is because free amino acids increase during the fermentation and storage. According to Liutkevičius & Šarkinas, the essential amino acid content in kefir is in descending order: lysine (376mg/ 100g); isoleucine (262mg/100g); phenylalanine (231mg/100g); valine (220mg/100g); threonine (183mg/100g); methionine (137mg/100g); and tryptophan (70mg/100g) (Arslan, 2015; Rosa et al., 2017). Also, kefir has a superior biological value (protein efficiency ratio — PER) than milk, which could be due to its better protein digestibility. Also, as a result of bacterial metabolism, both the total nitrogen (TN) and the non-protein nitrogen (NPN) in kefir are higher than those in the milk produced from (Arihara, 2014).

1.6.3. Lactose content

The lactose from milk is degraded to lactic acid during the fermentation process, which causes pH reduction and increase in kefir consistency. Approximately 30% of milk lactose is hydrolyzed by the β-galactosidase (EC 3.2.1.23) enzyme, hydrolyzing lactose into glucose and galactose. This particular property makes kefir an excellent beverage for lactose-intolerant individuals, who have an insufficient activity in their intestine of the enzyme β-galactosidase (Arihara, 2014).

1.6.4. Lipid content

The lipid content monoacylglycerols, diacylglycerols and triacylglycerols, non-esterified fatty acids (NEFA) and steroids in kefir can vary depending on the type of milk used in the fermentation. In fermented milk, the presence of NEFA contributes to the improvement of intestinal digestibility (Rosa et al., 2017).

According to Alm (1982a), only minor differences in the fat content and composition (mono-, di-, and triglycerides, free fatty acids, and steroids) of kefir compared to the starting milk are demonstrated. The fact that free fatty acids were found in all the fermented milk products analyzed (including kefir) indicated a disruption of the fat molecules in milk during fermentation. Also, it is assumed that this effect could result in an increase in the digestibility of milk fat in fermented products compared to other fat sources (Arihara, 2014).

1.6.5. Vitamin content

The vitamin content depends on the quality and origin of the milk used, micro-organisms present in the kefir grains, and the way of preparation. Kefir provides vitamins B1, B2, B5, C, A, K, and carotene (Arslan, 2015; Rosa et al., 2017). According to Liutkevicius and Sarkinas (Liutkevicius & Sarkinas, 2004) kefir contains vitamins B 5, B 2 and B 1 at approximately 3, <5 and <10 mg/kg, respectively.

Kneifel and Mayer in 1991 reported the vitamin profiles of kefir made from milk of different species. They found vitamin concentration enriched by >20% with thiamine in ovine milk, pyridoxine in all ovine, caprine, equine milk, and folic acid in all ovine, caprine, bovine milk (Arslan, 2015).

Commercial kefir is commonly produced starting from bovine milk. It is generally considered a good source of most water-soluble vitamins, except for ascorbic acid (C) and cobalamin (B 12).

Several investigators have measured the amount of vitamins in kefir to evaluate whether the fermentation process changes their level compared to milk. The investigation reported the decrease of cobalamin (B12) content in kefir during the fermentation and maturation stages. Also, Panic et al. in 1963, followed by Alm in 1980, used marketable kefir grains to produce a fermented product characterized by an increase in folic acid (B9) content compared to the starting milk, as well as, vitamin B6 and biotin reduction. Additionally, Bossi et al. in 1986 reported declines in vitamin A, thiamin, riboflavin, nicotinamide, and vitamin C in laboratory-prepared kefir compared to the starting milk. Regardless, the source of the milk may influence the growth of particular microorganisms which, in turn, determine the final vitamin content of kefir. Besides, specific bacteria can also be added to the initial mother culture to increase the levels of folic acid and vitamin B12 (Arihara, 2014).

1.6.6. Mineral content

Among the minerals, kefir is a good source of calcium and magnesium (Arslan, 2015; Rosa et al., 2017). Phosphorus, which is the second most abundant mineral in the human body and is

essential for the utilization of carbohydrates, fats and proteins for cell growth, maintenance and energy, is also abundant in kefir. Liutkevicius and Sarkinas (2004) identified the macro-element composition in kefir grain as: potassium, 1.65%; calcium, 0.86%; phosphorus, 1.45%; and magnesium, 0.30%, while the micro-elements found were: (mg/kg) copper, 7.32; zinc, 92.7; iron, 20.3; manganese, 13.0; cobalt, 0.16; and molybdenum, 0.33 (Arslan, 2015).

1.6.7. Others components

Even though lactic acid, CO_2, and ethanol are the main fermentation products, kefir also contains formic, propionic and succinic acids, aldehydes, traces of acetone and isoamyl alcohol, and a variety of folates (Rosa et al., 2017).

Lactic acid is found in all fermented dairy products as a result of the action of homo- and heterofermentative microorganisms. Lactic acid can exist in either the L(+) or D-(–) isomeric forms and as a 50/50 DL racemic mixture. L-(+)-lactic acid is completely metabolized by the body, while D-(–)-lactic acid is excreted more slowly by the body, and the excess D(–)-lactic acid accumulation can cause metabolic disturbances. Regardless, Kefir contains almost exclusively L-(+)-lactic acid. Kefir also includes a variety of microorganisms that have the potential to aid lactose digestion (Arihara, 2014).

Others product derived from the LAB activity are the biogenic amines such as putrescine, cadaverine, spermidine and tyramine. The high levels of biogenic amines determine the loose of sensorial properties of fermented milk and are considered to be an essential indicator of quality and acceptability. The high concentration of bioactive amines in fermented products, especially putrescine, cadaverine, agmatine and N-methyl putrescine, as well as monoamines such as penicillamine and histamine are positively correlated with an inharmonious bitter taste (Rosa et al., 2017).

1.7. Bioactive Peptides in kefir milk

Recently, milk proteins have attracted more attention in the biological and medical research community not only because of their nutrient's value but also for proteins containing potential bioactive sequences. (Hebert al., 2010). Bioactive peptides are hidden within the primary sequences of casein and whey proteins, released by the proteolytic activity performed during the gastrointestinal digestion or during the food processing (Gobbetti et al., 2013; Hebert et al., 2010). Therefore, peptides with physiological effects are found mainly in fermented milk and cheeses. Antimicrobial, immunomodulatory, antithrombotic, opioid, antioxidant activities, enhancement of mineral absorption and/or bioavailability, and blood pressure-lowering effects are some of the

biological activities attributed to milk-derived peptides c. The size of bioactive peptide sequences known to possess beneficial health properties may vary from two to twenty amino acid residues (Mohanty et al., 2016).

Also, specific milk-derived peptides are commercially produced and used as dietary supplements in functional foods, personal care products or drugs (Hebert et al., 2010).

1.7.1. Generation of Bioactive Peptides

There are several ways that encrypted peptides can be released from milk proteins:

1- enzymatic hydrolysis with digestive enzymes such as pepsin, trypsin, and chymotrypsin;
2- fermentation of milk with proteolytic starter cultures;
3- proteolysis by enzymes derived from proteolytic microorganisms (e.g., *Lactobacillus helveticus* and *Lactobacillus delbrueckii* subsp. *lactis*) (Gobbetti et al., 2013; Hebert al., 2010; Mohanty et al., 2016).

During the gastrointestinal digestion the release of the bioactive peptide may occur by the action of digestive enzymes like pepsin, trypsin or chymotrypsin. Firstly, the dietary proteins undergo denaturation in the presence of hydrochloric acid (HCl) into the stomach. The acid activates pepsinogen and converts it into its active form, pepsin. Pepsin acts on proteins to metabolize them to amino acids. Gastrointestinal digestion permits the following action of the enzymes present in the small intestine such as pepsin, trypsin or chymotrypsin, which are responsible for protein hydrolysis. Several bioactive peptides (antibacterial, immunomodulatory, anti-hypertensive and opioid peptides) are known to be released from casein and/or whey proteins by gastrointestinal digestion (Mohanty et al., 2016). Some other proteolytic enzymes such as alcalase, thermolysin, may be utilized with pepsin and trypsin to simulated gastrointestinal digestion. They have also been employed to release various bioactive peptides, including CCPs, ACE inhibitory, anti-bacterial, anti-oxidative, immunomodulatory and opioid peptides (Mohanty et al., 2016).

Additionally, in milk fermentation processes, the cell envelope - associated proteinase (CEP) is the critical enzyme involved in the first step of casein degradation and inhibit the LAB growth in milk, thereby ensuring successful fermentation (Hebert al., 2010).

Also, several lactic acid bacteria (LAB) (e.g. *Lactococcus lactis, Lactobacillus helveticus*) have been reported to release bioactive peptides during the fermentation process. This system provides a wide number of distinct intracellular peptidases, including endo-peptidases, amino-peptidases, di-peptidases, and tri-peptidases showing antimicrobial, immunomodulatory, antioxidative and ACE-inhibitory properties (Mohanty et al., 2016).

1.7.2. Fate of Bioactive Peptides

The luminal digestion of proteins (generated by different ways) results in free amino acids and 3 to 6 amino acid peptides, which could have biological activity. Subsequent brush-border peptidases, including enterokinase and dipeptidases, at the surface of the epithelial cells, digest peptides into dipeptides, tripeptides and free amino acids. However, specific peptides are resistant to the action of proteolytic enzymes and remain intact in the intestinal tract, producing local effects (Hebert al., 2010).

Therefore, following digestion, bioactive peptides can produce local effects in the gastrointestinal tract or be absorbed through the intestine to enter intact into the blood circulation and exert systemic effects. Different transport systems for the absorption of peptides are known. Oligopeptides are transported by endocytosis, although it has been reported that more than 90% of the transported peptides are hydrolyzed in the absorptive cells. Besides, oligopeptides could be passively transported via paracellular pathways, which is known to be the primary mechanism for transport of intact peptides. Dipeptides and tripeptides are actively transported via a specific transport system that exists in the brush - border membrane, the Peptide transporter 1 (PepT1). Whichever the mechanism used, a variety of peptides would reach the portal circulation, exerting diverse physiological functions (Hebert et al., 2010).

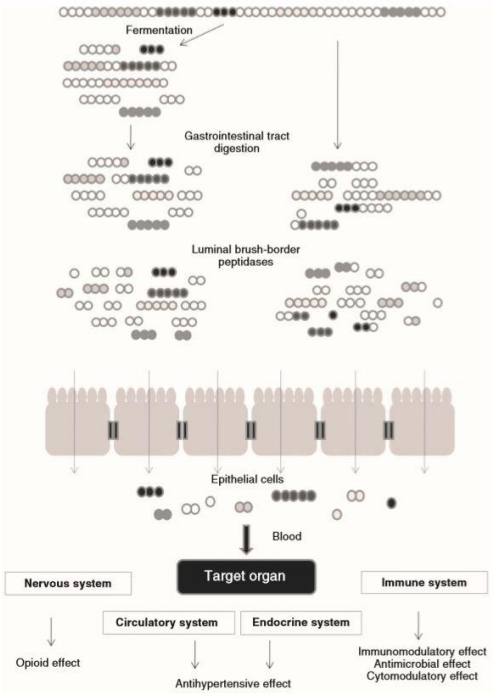


Figure 7. The fate of peptides released from precursor proteins by fermentation and/or gastrointestinal digestion (Hebert al., 2010).

1.7.3. Opioid effect

The caseins and whey proteins are potential sources of opioid peptides. Milk derived peptides, generated by hydrolysis of precursor proteins, are atypical opioid peptides. If these peptides resist the hydrolysis by intestinal brush–border enzymes, they can exert direct effects on specific gastrointestinal target receptors. Alternatively, they can be absorbed intact into the blood circulation and subsequently perform their effect after reaching endogenous opioid receptors (Hebert et al., 2010).

The α- and β-casomorphins and lactorphins act as opioid agonists, contrary casoxins act as opioid antagonists (Gobbetti et al., 2013). Endogenous opioid agonist peptides may regulate the growth and function of cells involved in the central nervous system. Also, β-casomorphins are transported across mucosal membranes of neonates that regulate physiological responses, resulting in calmness and sleep in infants (Mohanty et al., 2016). Also, casomorphins may produce analgesia, influence postprandial metabolism by stimulating the secretion of insulin and somatostatin hormones and may influence gastrointestinal absorption of nutrients by prolonging the gastrointestinal transit time and exerting an anti-diarrhoeal effect (Gobbetti et al., 2013). By

contrast, the opioid antagonists can suppress the agonist activity of enkephalin, a peptide with painkilling properties. (Mohanty et al., 2016).

1.7.4. Anti-hypertensive effect

Some evidence indicates that probiotic bacteria or their fermented products play an essential role in controlling blood pressure. The anti-hypertensive effects have been observed in experimental and clinical studies, although the data are limited and controversial. Quirós et al. in 2005 assumed kefir ability to inhibit the activity of the angiotensin-converting enzyme (ACE) through the action of bioactive peptides released by caseins during the milk fermentation process (Rosa et al., 2017). According to Maeda et al. (2004), the antihypertensive activity observed in their study is also due to the ability of kefiran to inhibit ACE activity.

The ACE has been associated with the renin-angiotensin system, which regulates the peripheral blood pressure. The ACE converts angiotensin I to angiotensin II that show a potent vasoconstrictor ability, and it also stimulates aldosterone in the kidney to retain more liquid in the body, resulting in the blood pressure increasing (Ahmed et al., 2013). Additionally, ACE-inhibitory peptides also inhibit the breakdown of bradykinin, a hormone that has vasodilating action, contributing to the decrease in blood pressure (Gobbetti et al., 2013; Rosa et al., 2017). ACE inhibition mainly results in an antihypertensive effect but may also influence different regulatory systems involved in immune defense and nervous system activity (Ahmed et al., 2013; Gobbetti et al., 2013).

1.7.5. Antithrombotic effect

Jollès et al. (1982) followed by Fiat et al. (1993) showed similarities between the clotting of blood and milk and between human fibrinogen γ-chain and the bovine κ-casein, both involved in limited proteolysis.

In the final step of blood clotting, thrombin hydrolyses fibrinogen into an insoluble fibrin clot and fibrinopeptides, whereas in milk, chymosin cleaves κ -casein (κ-CN) to form para κ - casein and glycomacropeptide (GMP). Moreover, the fibrinogen γ-chain is essential for platelet aggregation because its 400 to 411 sequence can bind the glycoprotein receptors on platelets. The dodecapeptide of fibrinogen and the 106 to 110 sequence of the κ-CN and related sequences show

functional homologies. It has been shown that the peptide, called casoplatelin, produced by trypsin hydrolysis action on κ-CN has antithrombotic activity by inhibiting fibrinogen binding to platelets (Gobbetti et al., 2013).

1.7.6. Immunomodulatory effect

The interest in the study of the relationship between nutrition and immunity has increased in the last years. The hypothesis that consumption of specific foods may reduce susceptibility to the establishment and/or progression of immunological diseases has been reported (Hebert et al., 2010). Peptides released during milk fermentation by LAB have been found to modulate the proliferation of human lymphocytes, to regulate the production of certain cytokines and to stimulate the phagocytic activities of macrophages. In this way, immunomodulatory peptides can exert an essential effect on the development of the immune system in newborn infants (Korhonen et al., 2005).

Also, many immunomodulatory peptides contained in fermented milk products can stimulate the macrophage phagocytic activity, antibody synthesis, (Mohanty et al., 2016) suppress the Th2 immune response, increase the production of cytokines, and stimulate the secretion of IgG and IgA by B-lymphocytes in the intestinal lumen. These bioactive compounds can also promote the cell-mediated immune response against infections and intracellular pathogens (Rosa et al., 2017).

In more details, caseins hydrolyzed by *Lactobacillus GG* and digestive enzymes (pepsin and trypsin), has been reported to yield compounds possessing both stimulating and suppressing effects on lymphocyte proliferation. Whereas αs1-casein, β-caseins and α-lactalbumin (Gobbetti et al., 2013; Hebert et al., 2010) suppress the proliferation of lymphocytes, κ-casein stimulates its proliferation. Also, it has been shown that immunopeptides obtained from αs1-casein, β-casein, and α-lactalbumin protect mice against *Klebsiella pneumoniae* infection (Gobbetti et al., 2013). Additionally, cytomodulatory peptides produced from casein may inhibit cancer cell growth by stimulating the activity of immune- cells. Also, glycomacropeptide (GMP) and its derivatives have been shown to have essential immunomodulatory functions, including immune suppressive effects on the production of IgG antibodies (Mohanty et al., 2016). Moreover, some other peptides derived from α-lactalbumin are also used in immune therapy of human immune deficiency virus infection (Mohanty et al., 2016). Also, it has been suggested that immunomodulatory milk peptides may alleviate allergic reactions in atopic humans and enhance mucosal immunity in the gastrointestinal tract (Korhonen et al., 2005).

1.7.7. Anticarcinogenic effects

The reduction in cancer risk can be attributed to the presence of some polysaccharides and bioactive compounds, such as specific proteins and peptides, present in kefir. The bioactive compounds of kefir can prevent cancer initiation or suppress the initiated tumour growth by inhibition of certain enzymes avoiding the conversion of procarcinogens to carcinogens compounds. The possible anticancer effect of milk kefir can be considered systemic since its regular consumption has potential in cancer prevention in the gastrointestinal tract, breasts and lungs. (Rosa et al., 2017).

The other mechanism by which cancer initiation process slows down is the activation of the immune system (Ahmed et al., 2013).

1.8. Health effect of kefir

Kefir has a wide spectrum of essential health benefits, including physiological, prophylactic and therapeutic properties. These effects are the result of a wide variety of bioactive compounds released from different processes (previously described) and the highly diverse microbiota, which act both independently or synergistically to influence the health benefits (Rosa et al., 2017).

1.8.1. Kefir as probiotic

Fermented dairy products are considered one of the most common sources of probiotic bacteria. Lactobacillus and Bifidobacterium species are the most commonly used probiotics, which are often implemented in dairy products in combination with other LAB (Collado, 2009).

A probiotic is defined as a microbial preparation containing live and/or dead cells, including their metabolites, used to improve the health of both animals and humans through the modulation of the intestinal microbiota. The numbers of micro-organisms in kefir are large enough (>107 cfu/g) to be considered a probiotic beverage. During the fermentation process, microbial metabolites and/or degraded milk constituents may be produced, providing beneficial effects to human health (Arihara, 2014). Several well-characterized strains of Lactobacilli and Bifidobacteria are available for human use to reduce the risk of gastrointestinal infections. It is also essential to recognize that not all Lactic acid bacteria and Bifidobacteria are probiotics, so their beneficial effects need to be scientifically demonstrated (Collado, 2009).

Some of the beneficial effects of probiotic consumption include the improvement of intestinal tract health through the regulation of microbiota and stimulation and development of the immune

system, synthesizing and enhancing the bioavailability of nutrients, reducing symptoms of lactose intolerance and reducing the risk of certain diseases. Still, they may modify the environmental gut pH, inhibit pathogens through the production of antimicrobial compounds, compete for pathogen binding to the receptor sites. Also, they can produce nutrients and growth factors, stimulating immunomodulatory cells. The effects of probiotics in the gut are well documented. Additionally, new evidence supports the use of probiotics in the prevention and treatment of several diseases, including atopic diseases, immune disorders, obesity, diabetes, and some cancers (Collado, 2009).

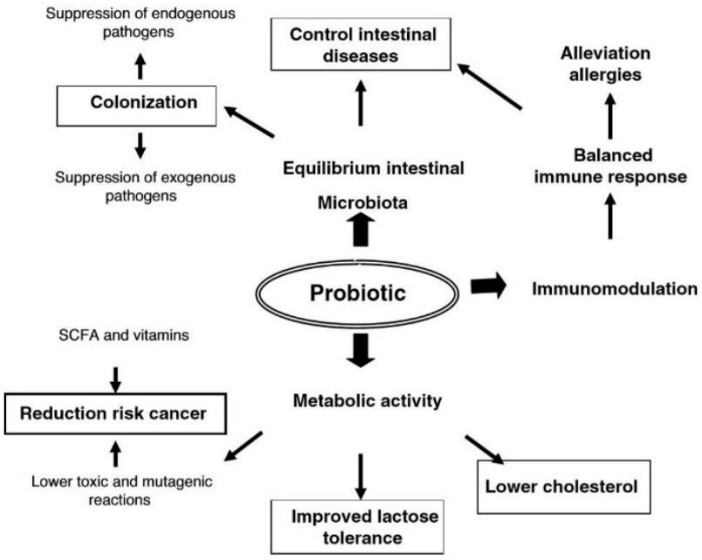


Figure 8. Beneficial effects of probiotics on human health (Collado, 2009).

1.8.2. Effect on lactose intolerance consumers

Lactose maldigestion is the inability to completely digest lactose, the primary carbohydrate source in all mammalian milk. Lactose maldigestion affects approximately 75% of the world's adult population. It occurs most often as the result of a genetically programmed decrease in intestinal lactase activity after childhood. Lactose maldigestion simply describes the incomplete digestion of lactose. Lactose intolerance, on the other hand, describes the presence of gastrointestinal symptoms such as abdominal pain, flatulence, bloating, nausea, or diarrhoea resulting from lactose maldigestion. In recent years, it has been documented that a much smaller percentage of individuals who maldigest lactose develop clinical symptoms. Although lactose intolerance

indeed remains much less common than lactose maldigestion, lactose intolerance is a significant problem worldwide. Since milk and dairy products contain high concentrations of lactose, the use of fermented dairy foods has long been employed as a strategy to overcome lactose intolerance (Hertzler & Clancy, 2003).

Intestinal absorption of lactose requires hydrolysis of this disaccharide by the intestinal β-galactosidase to form galactose and glucose that are subsequently absorbed in the small-intestinal mucosa. This enzyme is naturally present in kefir grains thanks to the microbial release from specific probiotic strains during the fermentation process. Moreover, fermented products such as kefir are characterized by a delayed gastric emptying, which helps in lactose digestion (Ahmed et al., 2013; Rosa et al., 2017). According to Alm (1982b), after the fermentation period, kefir has a reduction of 30% in the content of lactose, compared with the unfermented milk, providing better comfort for individuals with lactose intolerance disorders (Rosa et al., 2017).

1.8.3. The effect in the Immune system

There is a strict relationship between nutrition and the immune system. Fermented products produced by LAB have potential in improving specific or nonspecific immune responses both in animals and human models. These probiotic microorganisms have two-way actions, either by stimulating live microbial cells directly or indirectly through their secondary metabolites such as bioactive compounds (Ahmed et al., 2013).

Vinderola et al. in 2005 have shown the increase of IgA and IgG cells in the small intestine of host regularly subjected to the fermented dairy product administration, as well as increases of IL-4, IL-10, IL-6, and IL-2 amount in the lamina propria cells (Bourrie et al., 2016).

In more details, strains of *L. kefiri* induced lower TNF-α (Tumor necrosis Factor Alpha) release combined to higher IL-10/IL-12 release, resulting in much more reduction on the pro-inflammatory response. Also, other Lactobacilli isolated from kefir can inhibit the production of pro-inflammatory cytokines, promoting anti-inflammatory cytokine production. Further investigation into the mechanisms of protection against colitis showed that *L. kefiranofaciens* M1 decreased the production of pro-inflammatory cytokines IL-1β and TNF-α, while increasing the production of IL-10 in vivo (Bourrie et al., 2016).

Also, yeasts from kefir have shown immunomodulatory activities. *K. marxianus* B0399 has been shown to adhere to Caco-2 cells, that co-incubated with lipopolysaccharide (LPS) provide a significant decrease in the secretion of IL-10, IL-12, IL-8, and IFN- γ (Bourrie et al., 2016).

Additionally, the high level of Bifidobacterium and obligate heterofermentative lactobacilli (*L. acidophilus, L. delbrueckii, and L. helveticus*) in the gut of infants have been associated with a lower incidence of allergic disease later in life. In this regard, kefir and kefiran have been observed to exert these effects on the gut microbiota in animal trials (Bourrie et al., 2016).

1.8.4. Control of plasma glucose by kefir

The regular consumption of probiotics can improve blood sugar levels. This effect has been attributed mainly to the probiotic ability to positively modulate the composition of the intestinal microbiota and reduce intestinal permeability, oxidative stress and inflammation. The regular intake of probiotics can reduce the number of Gram-negative bacteria in the intestinal lumen, and therefore lower the amount of lipopolysaccharide (LPS). Besides, probiotics can improve intestinal barrier function leading to the reduction of intestinal permeability. The absorption of lower amounts of LPS may, therefore, diminish the low-grade chronic inflammatory process characteristic of diabetes. Moreover, lower LPS may restore the function of insulin receptors leading to better control of blood glucose. We can thus conclude that kefir might be used in the prevention of diabetes (Rosa et al., 2017). Also, water and methanol-soluble fractions of kefiran were observed suitable in management of Type II diabetes. (Ahmed et al., 2013).

1.8.5. Healing action of kefir

Some of the novel benefits provided by the regular kefir consumption include the healthier skin, improvement of eczema, atopic dermatitis and burns, healing of scars, and rejuvenation. Rodrigues et al. in 2005 tested the scar ability of a 70% kefir and kefiran gel in skin wounds infected with *Staphylococcus aureus*. The treatment with kefir and kefiran for seven days provided a protective effect on connective tissue, significantly improving tissue healing compared with treatment with 5mg/kg neomycin–clostebol emulsion. The study also reported the ability of kefir grains and gels prepared with kefir culture to reduce the percentage of inflammation compared with those treated with silver sulfadiazine cream, used for the topical treatment of burns of second and third degrees (Rosa et al., 2017). One such factor contributing to making healthier wounds in

the skin is connected to the antimicrobial ability of kefir against some bacteria and fungi. Another possible factor is the ability to modulate the immune system and recruit immune cells, improving the healing process (Bourrie et al., 2016).

1.8.6. Antimicrobial properties of kefir

Studies in the early twentieth century reported the positive effect on the life expectancy exerted by the regular consumption of yoghurt, or other fermented milk products containing LAB, able to inhibit the growth of some pathogenic micro-organisms. Since then, antifungal and antibacterial activities of probiotics like kefir have been extensively studied (Rosa et al., 2017).

Antibacterial properties of kefir are related to a combination of several factors, including the competition for available nutrients, the action of organic acids (lactic and acetic acids), H_2O_2, acetaldehyde, CO_2, ethanol and bacteriocins produced during the fermentation process (Ahmed et al., 2013; Arihara, 2014; Rosa et al., 2017). All these compounds are useful towards preservation of food products and reduction in foodborne pathogens. Also, they exhibit some effects similar to those of nutraceuticals, preventing gastrointestinal disorders and vaginal infections (Ahmed et al., 2013).

It is widely demonstrated that kefir grains have shown higher antibacterial activity than kefir. Additionally, kefir has been shown to lose its intrinsic inhibitory effect after lyophilization and reconstitution in distilled water or milk (Arihara, 2014; Rosa et al., 2017).

It is reported that kefir exerts bactericidal effects against both Gram-negative and Gram-positive bacteria, showing more potent activity in the latter (Ahmed et al., 2013; Rosa et al., 2017). The antagonistic action has been observed against *Shigella spp., Staphylococcus, Helicobacter pylori, Escherichia coli, Enterobacter aerogenes, Proteus vulgaris, Bacillus subtilis, Micrococcus luteus, Listeria monocytogenes, Streptococcus pyogenes* and also against the yeast *Candida albicans* (Rosa et al., 2017). Silva et al. in 2009 also reported antimicrobial activity of kefir against *Salmonella typhi* and *Shigella sonnei*. The results reported can be compared with the antibacterial action of ampicillin and gentamicin (Rosa et al., 2017).

Recently, studies on several sources of kefir grains highlighted the ability of Lactococcus genera to produce three types of bacteriocins. One such bacteriocin has shown a narrow spectrum, inhibiting only lactococci. Another kind inhibits strains of *Lactobacillus casei, Lactobacillus helveticus, and Pediococcus pentosaceus*. The last bacteriocin type has named lacticin 3147 and

is produced by *Lactococcus lactis* strain DPC3147 (Arihara, 2014). It inhibits a wide spectrum of bacteria, including specific Lactococcus, Leuconostoc and Pediococcus strains. Also, it inhibits bacterial species such as *B. cereus, B. subtilis, C. sporogenes, C. tyrobutyricum, Enterococcus faecium, E. faecalis, L. innocua, L. monocytogenes, S. aureus,* and *C. difficile* (Bourrie et al., 2016), and *Streptococcus thermophilus.* Others Lactobacillus species such as *Lactobacillus acidophilus* and *Lactobacillus kefiranofaciens*, as well as some *S. thermophilus* strains have shown antimicrobial activity against a whole range of pathogenic organisms including *E. coli, L. monocytogenes, S. aureus, S. typhimurium, S. enteritidis, S. flexneri, P. aeruginosa*, and *Y. enterocolitica*, when tested using an agar spot test (Bourrie et al., 2016).

The killing action of kefir against *Yersinia enterocolitica, Listeria innocua*, *Salmonella enteritidis*, and *Staphylococcus aureus, Bacillus cereus, Listeria monocytogenes* (ATCC 7644), and *E. coli* (ATCC 8739) has also been revealed (Ahmed et al., 2013). The inhibitory effect on *E. coli* has been attributed to the combined effect of acid compounds and hydrogen peroxide (Arihara, 2014).

Also, kefir has been shown to have antifungal activity against a variety of fungi, yeasts, and moulds (Arihara, 2014). The concentration of kefir from 7 to 10% (w/w) caused the complete inhibition of Aspergillus flavus sporulation, and consequently, the production of aflatoxin B1. Additionally, the organic acids produced during the fermentation of kefir can change the molecule aflatoxin B1, by converting it into less toxic forms such as aflatoxicol aflatoxin B and B2. In this context, kefir appears as a safe alternative for food preservation, protecting against poisoning from aflatoxin B1 (Rosa et al., 2017).

1.8.7. Hypocholesterolemic effect of kefir

Cardiovascular disease (CVD) is one of the leading causes of death in the western world, combined with the high levels of serum cholesterol being a significant risk factor for the disease. Diet can play a significant role in the management of serum cholesterol levels and thus, the risk of contracting CVD (WHO, 1982).

Zheng et al. (2013) reported the *L. acidophilus* LA15, *L. plantarum* B23, and *L. kefiri* D17 ability to lower serum total cholesterol, LDL, and triglyceride levels. Also, the three strains have shown an improvement in faecal cholesterol and bile acid secretion (Bourrie et al., 2016).

The consumption of probiotic dairy products, including kefir, has been proposed as a strategy to reduce levels of circulating cholesterol. Some mechanisms are proposed to justify these findings:

- The LAB inhibits the absorption of exogenous cholesterol in the intestine due to binding and incorporation of cholesterol by the bacterial cells. Therefore the high count of LAB present in kefir may directly or indirectly reduce cholesterol in the medium by up to 33% (Rosa et al., 2017).

- Probiotic bacteria increase the production of short chain fatty acids, which would decrease circulatory cholesterol levels either by inhibiting hepatic cholesterol synthesis or by redistributing cholesterol from the plasma to the liver (Arihara, 2014). Among the different SCFA produced, propionate reduces the production of cholesterol by inhibiting hydroxymethylglutaryl CoA (HMG-CoA) reductase activity. Additionally, plasma cholesterol is redistributed to the liver, where the synthesis and secretion of bile acids are increased since the activity of the 7α-hydrolase enzyme is stimulated (Rosa et al., 2017).

- Another possible mechanism involves the deconjugation of bile acids, which may be increased in the large intestine, caused by the bile salt hydrolase (BSH) enzyme released by specific micro-organisms, including some Bifidobacteria strain. BSH deconjugates bile acids and, as deconjugated bile salts are less soluble and less efficiently reabsorbed from the intestinal lumen, this leads to increased bile salt excretion in the faces (Zhuang et al., 2012). This mechanism is simultaneously associated with the conversion of cholesterol to bile salts to replace those lost through the excretion resulting in a cholesterol-lowering effect (Bourrie et al., 2016). Also, Liu et al. in 2012 reported the ability of *K. marxianus* (strains K1 and M3) to excrete the bile salt hydrolase (BSH) activity resulting in bloodstream cholesterol-lowering (Liu et al., 2012).

1.8.8. Antioxidative activity of kefir

Dietary components play a significant role in protecting the body against oxidative damage. Kefir contains a series of components that have excellent antioxidant activity (Ahmed et al., 2013). The authors reported the great ability of kefir to bind the 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical and superoxide radicals, besides the inhibition of linoleic acid peroxidation. In this way, the antioxidative activity of kefir can reduce DNA damage, enhancing its anticarcinogenic potential. According to the strong relationship between the increased concentration of free radicals and the increased risk of chronic diseases development, kefir consumption should be

encouraged since it is a natural source of antioxidant compounds and enhances the activity of enzymes involved in the antioxidant systems (Rosa et al., 2017).

1.8.9. Anticarcinogenic activity of kefir

The reduction in cancer risk can be attributed to the presence of some polysaccharides in kefir as well as the bioactive compound released (Rosa et al., 2017). Scientific studies reported that the consumption of fermented milk might play an essential role in the modulation of carcinogenesis.

In vivo-experiments reported the ability of water-soluble polysaccharides contained in kefir to protect against pulmonary metastasis, whereas the water-insoluble polysaccharide fraction could inhibit melanoma metastasis (Rosa et al., 2017).

Also, Khoury et al. reported the ability of kefir to inhibit proliferation and induce apoptosis in HT 29 and Caco2 colorectal cancer cells. In these cultured cells, kefir can induce cell cycle arrest at the G1 phase, decrease mRNA expression of transforming growth factor-α (TGF-α) and transforming growth factor-β1 (TGF-β1), indicating its pro-apoptotic effect in vitro (Rosa et al., 2017).

Additionally, the beneficial effect provided by Lactic acid bacteria can enhance cancer therapy results. Lactic acid bacteria might modulate immune parameters, including T cell, natural killer (NK) cell, and macrophage activity, which are essential for hindering tumour development. Activated macrophages, NK cells, and some T lymphocyte subpopulations (e.g., CD4, TH1 cells) can secrete tumour necrosis factor (TNF-α), which can induce apoptotic and necrotic forms of tumour cell lysis (Je-Ruei et al., 2002).

Chapter 2
OBJECTIVE

According to the aforementioned attractive properties of kefir bioactive compounds, this research aimed to obtain a proteomic profile of kefir, by two-dimensional gel electrophoresis (2DE) and mass spectrometry (MS). As a result, milk-derived bioactive peptides with a positive impact on human health in vitro were identified and described.

Chapter 3
MATERIALS AND METHODS

3.1. Materials used, reagents and buffers

The reagents and materials used in this work came from the following Companies: Oxoid Limited; Becton Dickinson; Merck; GE Healthcare; Bio-Rad.

- Commercial kefir from bovine milk produced in organic farming was used.
  The microbial population identified and reported in product label belong to the following genera: *Lactobacillus* spp., *Lactococcus* spp., *Leuconostoc* spp., *Saccharomyces* spp., *Acetobacter* spp. The mean nutritional value for 100 g of the product was: fat, 3.5 g; carbohydrates, 3.5 g; proteins, 3.3 g; lactose, 3.5 g; calcium, 120 mg. The energetic value was 245 kJ.

- Rehydration solution: 8M urea; 2% (w/v) 3-[(3-Cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS); 65mM dithiothreitol (DTT); 0.001% (w/v) bromophenol blue; 0.5% (v/v) IPG buffer, pH range 3-10.

- 4xResolving gel buffer (1000 mL): 181.5 g 1.5 M Tris-HCl pH 8.8; 750 mL of ultrapure H_2O

- SDS-equilibration buffer: 50mM Tris-HCl, pH 8.8; 6M urea; 30% glycerol; 2% SDS; 65 mM DTT and few grains of bromophenol blue

- SDS-electrophoresis buffer: 6.04 g of Tris base; 28.8 g of Glycine; 2 g of SDS; 2 L ultrapure H_2O

- Sample buffer (40 ml): 10 ml 0.5 M Tris HCL pH 6.8; 8 mL glycerol; 16 mL 10 % SDS; 0.937 mL 40 % Acryl/Bis; 4 mL β-mercaptoethanol; 200 μl 0.05% BBF; 2 mL H_2O

- Agarose sealing solution: 0.5 g of agarose; 50 mL of ultrapure H_2O; few grains of bromophenol blue

- Staining solution: 0.1% Coomassie Brilliant Blue R250 in 50% methanol and 10% acetic acid

- De-staining solution: 5% acetic acid in 10% methanol and 85% ultrapure H_2O

- Marker Bio-Rad low range (phosphorylase b, 97.4 kDa; bovine serum albumin, 66.2 kDa; ovalbumin 45.0 kDa; carbonic anhydrase, 31 kDa; soybean trypsin inhibitor, 21.5 kDa; lysozyme, 14.4 kDa)

3.2. Sample preparation

In order to get successful results in the proteomic analysis, the first aim has regarded the correct preparation of a sample. The sample under investigation was subjected to several steps that had led to the protein purification, as shown in the diagram, **Figure 9**. Indeed, removal of non-protein compounds from the sample was essential to achieve high-quality results.

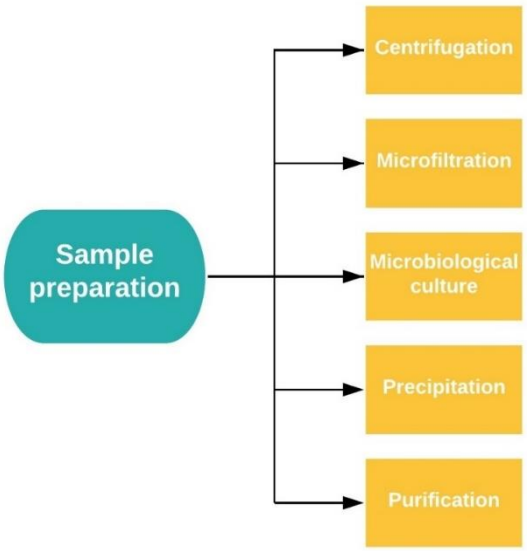


Figure 9. Sample preparation in individual steps

3.2.1. Centrifugation

Centrifugation is a biomechanical process that uses centrifugal forces to separate particles of different masses or densities dispersed in a solution. When the tube, with a mixture of proteins or other particles, rotates at high speed, the particle suspension is rotated in a centrifuge. Pellet is formed by most massive particles at the bottom of the tube. Thus, the incompatible particles remain mostly in the supernatant and can be removed from the pellet by pipetting.

Centrifugation is applied to separate components, including micro-organisms, fat globules and insoluble components that show different densities compared to the dispersing phase.

3.2.2. Microfiltration

An alternative to centrifugation for the removal of micro-organisms and somatic cells is the use of microfiltration (MF), which has met with some success. This method is particularly adapted to the removal of bacteria from skimmed milk, as the size of the micro-organisms is in the same range as fat globules (Gésan-Guiziou, 2010).

Regardless, the use of both centrifugation and microfiltration can lead to the efficient removal of micro-organisms.

In practice, 50 ml of kefir was centrifuged at 13000g for 20 minutes to allow the separation of the gross components. Then the supernatant obtained after the centrifugation process was filtered through a 0.22µ filter to remove micro-organisms before to be subjected to the two-dimensional electrophoresis analysis (filtered kefir).

3.2.3. Microbiological culture

To ensure that all the yeasts and bacteria were removed from the kefir, a microbiological analysis was carried out. Each agar plate was divided into two parts: one for the filtered kefir and the other for the unfiltered kefir.

Both filtrate and unfiltered kefir were spread onto MRS (de Man, Rogosa, Sharpe) Agar plates for the isolation and cultivation of *Lactobacillus* spp.

MRS Agar is a selective culture medium designed to favour the growth of *Lactobacilli* genera. It contains sodium acetate, which suppresses the growth of many competing bacteria, although some other micro-organisms belonged to Lactobacillales order, like *Leuconostoc* and *Pediococcus*, may grow (Man, 1960). The MRS agar typically contains (w/v):

- 1.0 % peptone

- 1.0 % beef extract
- 0.4 % yeast extract
- 2.0 % glucose
- 0.5 % sodium acetate trihydrate
- 0.1 % polysorbate 80
- 0.2 % dipotassium hydrogen phosphate
- 0.2 % triammonium citrate
- 0.02 % magnesium sulfate heptahydrate
- 0.005 % manganese sulfate tetrahydrate
- 1.0 % agar
- pH adjusted to 6.2 at 25°C

The meat extracts and peptone provide sources of carbon, nitrogen, and vitamins for overall bacterial growth. Also, the yeast extract contains vitamins and amino acids specifically required by *Lactobacilli*.

Also, the filtrate and unfiltered kefir were spread onto Sabouraud Glucose Agar plates for the isolation of saprophytic or pathogenic yeasts and moulds. This medium was initially used for the cultivation of dermatophytes. Currently, Sabouraud Glucose Agar is used for the isolation and cultivation of all fungi. The peptones contained are sources of nitrogenous growth factors. Also, glucose provides an energy source for the growth of micro-organisms. Notably, the high glucose concentration provides an advantage for the growth of the fungi while most bacteria do not tolerate the high sugar concentration (Sutton, 2003). Besides, the low pH is optimal for fungi, but not for many bacteria. Sabouraud Agar is commercially available and typically contains:

- 40 g/L glucose
- 10 g/L peptone
- 20 g/L agar
- pH 5.6

After the cultivation process, all the plates were incubated in aerobiosis at 35°C for 2-3 days.

3.2.4. Precipitation

For mass purification of proteins, the first common step in protein isolation is precipitation with ammonium sulphate $(NH_4)_2SO_4$. The separation is accomplished by the gradual addition of ammonium sulfate to form a protein precipitate.

Usually, in a solution containing proteins, three main protein-water interactions are founded. The ion hydration between charged side chains (e.g., Asp, Glu, Lys), hydrogen bonding between polar groups and water (e.g., Ser, Thr, Tyr, and the main chain of all residues), and hydrophobic hydration (Val, Ile, Leu, Phe). When salt is added to the solution, the surface tension of the water increases, resulting in increased hydrophobic interaction between protein and water. Each protein reduces its surface area in order to minimize the contact with the solvent, as shown by folding phenomena (the folded conformation is more compact than the unfolded one) resulting in protein precipitation (Wingfield, 2001). Ammonium sulfate, $(NH_4)_2SO_4$, is often used because of its high solubility, which allows for solutions of very high ionic strength and low price (Duong-ly & Gabelli, 2014).

According to the effect as mentioned earlier, the total proteins of the filtered kefir were precipitated by the addition of 90% ammonium sulfate to the filtrate sample maintaining it in ice and slow agitation during the performed process.

Subsequently, the sample was centrifuged at 13000 rpm for 30 minutes in order to precipitate the total proteins. The kefir protein pellet was then resuspended with 1 ml of 50 mM Tris HCl, pH 7.5 and the protein content was determined by the Bradford assay (Bradford, 1976).

3.2.5. *Purification*

Subsequently, 1 mg of total kefir proteins (resuspended in1 ml of 50 mM Tris HCl, pH 7.5) was treated with 2D-Clean-Up (GE-Healthcare), according to the manufacturer's instructions, and the final protein precipitate was dissolved in a 350 mL rehydration solution (see section 3.4.2.: Isoelectric focusing (IEF) of kefir sample).
The 2D-Clean-Up represents an effective method to remove impurities. The reagents quantitatively precipitate proteins while leaving interfering substances such as detergents, salts, lipids, phenolics and nucleic acids in solution. Thus, sample preparation with this kit results in proper isoelectric focusing and minimal streaking produced by eventual contaminants.

3.3. Bradford assay

The Bradford protein assay is a procedure for the determination of total protein concentrations in solutions. It relies on the change in absorbance based on the binding of the dye Coomassie Blue G250 to arginyl and lysyl residues of proteins. This specificity can lead to variation in the response of the assay among different proteins and this is the major drawback of the assay. The assay is based on the observation that the absorbance maximum for an acidic solution of Coomassie Brilliant Blue G-250 shifts from 465 nm to 595 nm when binding to protein occurs. Both hydrophobic and ionic interactions stabilize the anionic form of the dye, causing a visible colour change., within the linear range of the assay, the more protein present, the more Coomassie binds. A set of standards is created from a stock of protein whose concentration is known. The Bradford values obtained for the standard are then used to fit a standard curve to which the unknown values obtained can be compared to determine their concentration. A calibration curve is developed using Bovine Serum Albumin (BSA) 0.1 mg/ml as the protein standard. Below, the protocol is reported.

1. Prepare a stock solution of 0.1 mg/ml BSA.
2. The stock BSA solution was diluted to span the sample range in the following manner:

	Bovine Serum Albumin (BSA) (µL/ml)	**Water (µL/ml)**	**Coomassie blue G250 (µL/ml)**
Blank	0	800	200
2	20	780	200
4	40	760	200
6	60	740	200
8	80	720	200
10	100	700	200

Table 3. Standard BSA solution preparation

3. To each tube add 200 µL of Coomassie blue G250 and mix gently by vortexing.
4. Use reagent blank to zero the spectrophotometer. Transfer the samples to disposable cuvettes and measure the absorbance at 595 nm.

Samples can be subjected to spectrophotometer analysis from 2 - 60 minutes after addition of Coomassie as Bradford reagent.

3.4. Two-dimensional electrophoresis

Two-dimensional (2D) gel electrophoresis is a powerful and practical proteomics tool, capable of simultaneously separating and resolving complex protein mixtures and enabling the visualization and identification of several thousand proteins on a single gel. This resolution is achieved by separating proteins based on their isoelectric points in the first dimension through the Isoelectric focusing (IEF) and then using sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) to separate them based on their apparent size in the second dimension (Wu, 2006).
The technique is also useful in detecting proteolytic cleavages that result in protein species with altered molecular weight (MW) and isoelectric point (pI) (Friedman et al., 2009).
Depending on the gel size and pH gradient used, 2D analysis can resolve more than 5000 proteins simultaneously (2000 proteins routinely) and can detect 1 ng of protein per spot. Furthermore, it delivers a map of intact proteins, which reflects changes in protein expression level, isoforms or PTM (Post-translational modification). Indeed, 2D gel experiments are particularly powerful for visualizing protein isoforms that result from post-translational modification including phosphorylation, glycosylation or limited proteolysis and which can be readily located in 2D gels as distinct spot trains in the horizontal and/or vertical axis of the 2D gel (Friedman et al., 2009; Görg et al., 2004).

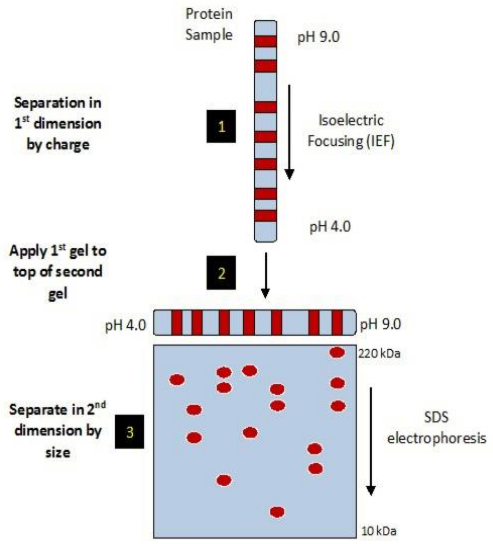


Figure 10. Scheme of two-dimension electrophoresis based on the horizontal and vertical separation according to differences in pI and molecular weight, respectively. 1. Proteins are first separated by isoelectric focusing. 2. IPG-strip is then laid horizontally, and 3. the proteins are separated by SDS polyacrylamide gel electrophoresis. (kendricklabs.com).

3.4.1 Isoelectric focusing (IEF)

Isoelectric focusing (IEF) is an electrophoretic separation method which separates amphoteric molecules such as proteins and peptides according to their charge as defined by the pKa values of proton-accepting sites within a molecule. For proteins and peptides, these sites can be found in the free amines and carboxylic acids located at the N- and C-ends as well as on the side chains of arginine, lysine, histidine, aspartic acid, and glutamic acid residues (Friedman et al., 2009).

The protein separation by migration in a variable pH environment is achieved with an applied electric field. In an alkaline environment, the proteins have a negative charge and therefore migrate to the anode. By contrast, in the acid environment, the total charge is positive, and they move to the cathode. At a pH equal to the isoelectric point, pI, the molecule is neutral and focuses at the point where the total charge of the protein is equal to zero (Trebunova & Zivcak, 2018). The isoelectric point is a specific physicochemical parameter of an amphoteric molecule defined as the pH value where a molecule is in a net neutral charge state. Through isoelectric focusing, the protein separation is achieved. Proteins with different isoelectric points are in fact distributed differently throughout the gel **(Figure 11)** (Friedman et al., 2009; Trebunova & Zivcak, 2018)**.**

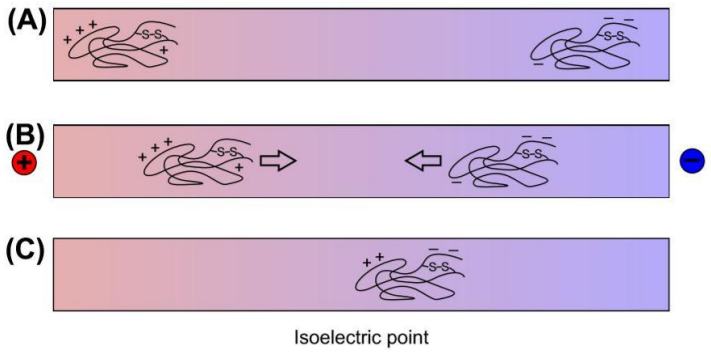


Figure 11. Isoelectric focusing. (A) The same protein placed at various places of an IPG strip and the charge it obtains (left side, low pH; right side, high pH). (B) Protein movement due to electric current application. (C) Protein in its isoelectric point (Ciborowski & Silberring, 2016).

In this technique, a pH gradient is created using a mixture of acids and bases of organic nature with a low molecular weight called ampholytes. The mixture is distributed in an electric field generated through the gel.

The sample has to be adequately prepared and conditioned with a zwitterionic detergent, and carrier ampholytes to improve the solubility of proteins and inhibit the potential interactions between them.
The sample is then applied to the IEF gel where the electric field will perform the separation. Modern IEF methods for 2D gel electrophoresis use a thin polyacrylamide gel as a molecular sieve that contains an immobilized pH gradient (IPG).
At the end of an IEF run, the proteins become highly concentrated at their isoelectric points, resulting in high sensitivity for their detection (Friedman et al., 2009).

3.4.1.1. Immobilized pH gradients

A significant improvement in 2D gel electrophoresis methodology came from the introduction of an IPG (immobilized pH gradient) within a polyacrylamide matrix for IEF. The IPG strip technology has facilitated the methodology and highly increased the reproducibility within a laboratory and across different working groups, and is now considered to be the method of choice for IEF.
The IPG gels are dried for long-term preservation and can be rehydrated as necessary shortly before use. The primary benefit of IPGs is the absence of the cathodal drift resulting in fixing the gradient into the matrix. The main application of IPGs is IEF under denaturing conditions,

applied in the first dimension of high-resolution two-dimensional electrophoresis (Friedman et al., 2009).

Commercial IPG strips are now available with a wide variety of strip differing in lengths and pH gradients to guarantee the optimal protein separation. In particular, pH gradients are available from a vast range (e.g., pH 3–11), to medium ranges (e.g., pH 4–7, pH 7–11), to restricted ranges (e.g., pH 4–5, pH 5.5–6.5). Strip lengths also vary from as short as 7 cm to as long as 24 cm. Strips typically are 3 mm in width, with an average thickness of approximately 0.5 mm (Friedman et al., 2009).

These strips are highly advantageous because, after gel formation, all polymerization catalysts and unreacted compounds are washed away, providing the low electrical conductivity needed for IEF (Pergande & Cologna, 2017).

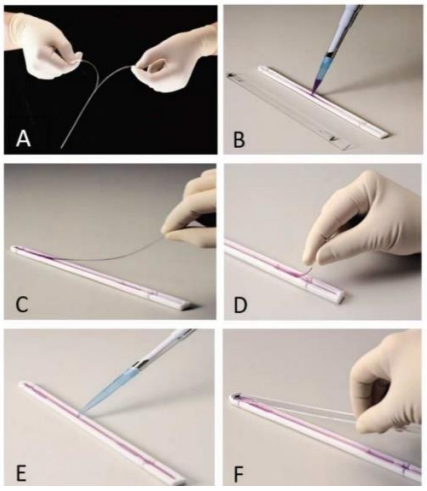


Figure 12. IPG strip preparation. A: remove the protective film, B: Apply rehydration solution to the strip, C: wet the entire length of IPG strip in rehydration solution by placing IPG strip in strip holder (gel facing down), D: gently lay entire IPG strip in the strip holder, placing the end of IPG strip over cathode electrode. E: protein sample can be applied at sample application well following the rehydration step if the protein sample was not included in the rehydration solution, F: place cover on strip holder (Trebunova & Zivcak, 2018).

3.4.2. Isoelectric focusing (IEF) of kefir sample

The precipitated kefir protein samples coming from the treatment by the 2D-Clean-up kit were prepared for the first dimension (isoelectric focusing) as follows:

1. The precipitated proteins were solubilized in 360 µL of IPG strip Rehydration buffer.

2. The immobilized pH gradient (pH range 3-10) was obtained through the Immobiline Drystrip gel (18 cm)
3. The solubilized sample was put in the strip holder and the dry strip was placed in the strip holder upside down. The strip should be completely covered by the sample avoiding the formation of bubbles between the sample and the gel.
4. The strip was covered with a proper "cover oil" to prevent the sample evaporation during the run
5. The IPGphor isoelectric focusing cell (GE-Healthcare) was programmed:

- rehydratation stage for 12 h at 20°C without voltage.
  Then, the focusing stage was performed at 20 °C in 3 run steps:
- the first step, a voltage of 500 V for one hour;
- the second step, a voltage of 1000 V for one hour;
- the third step, a voltage of 8000 V for 4 hours (50 mA per strip).
- The total time for the first dimension, using a 18 cm strip was 18 hours

After the IEF, the strips were incubated with the SDS equilibration buffer for 15 min. at room temperature in gentle agitation to confer the negative charge to the proteins and prepare them to the second dimension.

Eventually, the strip can be stored at -20 °C.

3.4.3. *The second dimension*

The second dimension of 2D electrophoresis separates proteins according to their molecular weight (MW). This separation takes place in a polyacrylamide gel matrix, which acts as a molecular sieve. The appropriate mesh in this sieve is made of the polymerized acrylamide, cross-linked by bis-acrylamide. The size of pores in the polyacrylamide gel depends on the percentage amounts of their components. A lower percentage of acrylamide (larger pore size) allows the separation of high molecular weight proteins. By contrast, a higher acrylamide content (smaller pore size) is more suitable for resolving smaller proteins. However, for separation of a complex mixture of proteins with a wide range of MW, the use of gradient gels is recommended. In gradient gels, pore sizes decrease along the gradient and allow to achieve a proper protein separation for both large and small proteins (from 0.5 to 300 kDa) on the same gel (Ciborowski & Silberring, 2016).

The separation process in the second dimension is based on protein migration caused by the application of an electric current through a polyacrylamide gel. During this process, large proteins with high molecular weights are not able to migrate through the polyacrylamide sieve for a long distance and stay at the cathode end of the gel. Smaller proteins, with lower molecular weights, migrate easily through the pores and travel toward the anode end of the gel. It is essential to highlight that proteins may have various shapes and can migrate through the gel with various speeds, despite their molecular weight. For example, proteins with an elongated shape may stay at the cathode end of the gel, even though they are not so "heavy." Alternatively, they might have a relatively high molecular weight, but their structure may be compressed and thus may travel through the gel faster (Ciborowski & Silberring, 2016).

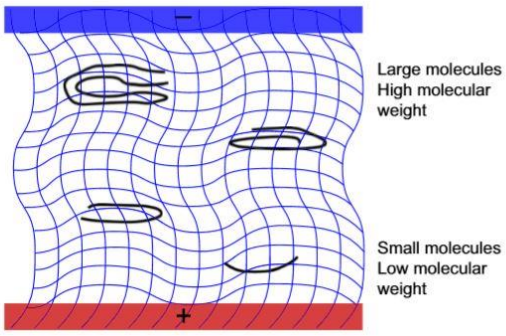


Figure 13. Proteins of different masses travelling through a polyacrylamide gel; (-) cathode; (+) anode (Ciborowski & Silberring, 2016).

3.4.3.1 Separation principle

Since SDS confers to the proteins a negative charge, the separation occurs according to the apparent molecular mass (commonly referred to as molecular weight).

When SDS is added to a protein solution in excess, the proteins form anionic micelles with a constant negative net charge per mass unit, destroying the tertiary and secondary structures. The disulfide bonds between cysteines have to be cleaved with a reducing agent like dithiothreitol (DTT) to achieve a complete unfolding (Friedman et al., 2009). Thus, after reduction and alkylation in buffers performed by SDS, all proteins show a similar, rod-like structure.

SDS is a molecule that possesses a negative charge at the end of its hydrophobic dodecyl group. When SDS is added to the reduction and alkylation buffer, its hydrophobic chain interacts with nonpolar amino acids, and the complete protein gains a strong negative charge, which masks its

intrinsic charge. Moreover, SDS binds to the protein in proportion to its size, meaning that biomolecules with various masses gain the same charge density at their surface and may move in the electric field with the same mobility. This phenomenon guarantees separation almost exclusively due to the protein molecular mass, regardless of its pI value or shape (Ciborowski & Silberring, 2016).

Generally, polyacrylamide gels are prepared by co-polymerizing acrylamide with methylene bis-acrylamide, a cross-linking agent, in the presence of ammonium persulfate that acts as a catalyst, and TEMED (N-tetramethylenediamine).

TEMED catalyzes the decomposition of ammonium persulfate, resulting in the formation of the corresponding free radical. This reactive molecule attacks the double bonds of the acrylamide leading to the gel polymerization. the gel porosity depends on the concentration of acrylamide and methylene bis-acrylamide used in the gel preparation.

Generally, SDS PAGE is composed of a blending of two gels:

- **Stacking gel;** it has large-sized pores that allow the proteins to migrate freely and get stacked at the interface between stacking gel and running gel. It consists of 4% polyacrylamide, 0.125 M pH 6.8 Tris / HCl buffer containing SDS.

- **Running gel;** represents a matrix able to separate each protein. It consists of 7.5-20% polyacrylamide, 0.375 M pH 8.8 Tris / HCl buffer containing SDS.

3.4.3.2. The second dimension of kefir sample

The SDS-PAGE gel with 15% acrylamide was prepared to achieve the pore sizes of the gel able to allows the separation of proteins with a molecular mass between 10000 and 100000. The use of Agarose Sealing Solution, containing the Bromophenol Blue, is useful to keep the IPG-strips on the top of the gel and to monitor the electrophoretic run. Indeed, the Bromophenol Blue dye is composed of smaller peptides that achieve faster the bottom of the gel.

The molecular mass determinations were based on the markers Bio-Rad low range (phosphorylase b, 97.4 kDa; bovine serum albumin, 66.2 kDa; ovalbumin 45.0 kDa; carbonic anhydrase, 31 kDa; soybean trypsin inhibitor, 21.5 kDa; lysozyme, 14.4 kDa).

The second dimension was performed in a Protean II apparatus (Bio-Rad), and three independent 2DE experiments were performed.

The 15% SDS-PAGE was prepared according to the following protocol:

- 35 mL of 12.5% acrylamide
- 17.5 mL 4x Resolving buffer
- 0.7 mL 10% SDS
- 16.45 mL of ultrapure water
- 700 µl of ammonium persulfate
- 50 µl of TEMED.

1- Overlap the two glasses and pour the SDS-PAGE 15% gel between them and remove any bubbles. The proper electrophoretic comb is then inserted between the two glasses to lead at a proper polymerization.
2- SDS-electrophoretic buffer was added to the electrophoretic chamber.
3- 15 µl of the markers Bio-Rad low range were loaded into the proper electrophoresis well.
4- The SDS-equilibrated IPG-strip coming from the first dimension is inserted on the electrophoretic apparatus.
5- The agarose sealing solution was placed on the top of the IPG-strip loaded, to keep the IPG-strip on the electrophoretic gel
6- The electrophoresis was run at 12 mA overnight. The run ends when the dye strip is on the bottom of the gel.
7- At the end of the electrophoretic course the gel was stained with the staining solution

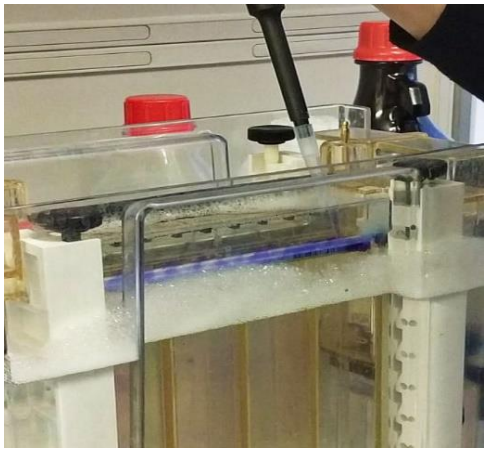
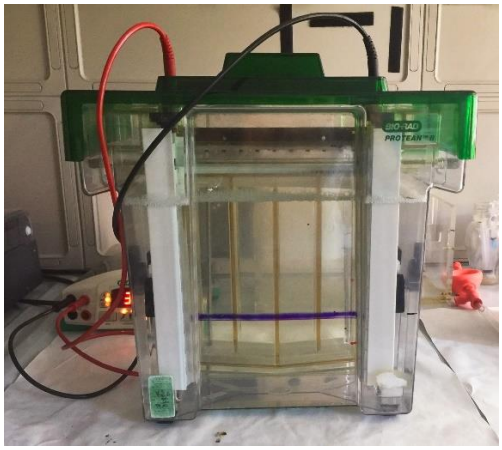
A)  B) 

Figure 14. A) Placement of the agarose sealing solution on the IPG-strip loaded. B) The Bromophenol Blue highlight the electrophoretic run.

3.4.4. Gel Staining

Gel staining allows the visualization of the proteins, an ideal dye should bind to the proteins noncovalently and in proportion to their concentration. It should possess a wide dynamic range and be sensitive enough to visualize low abundant proteins. The dye should be compatible with the next steps of analysis (mass spectrometry in this case). There is no perfect staining method for 2D gel electrophoresis. For proteomics purposes, the most popular is Coomassie Brilliant Blue that is able to detect a minimum of 10 ng protein, and its dynamic range covers two orders of magnitude. It is preferred when relative amounts of protein are to be determined because it binds stoichiometrically to proteins (Ciborowski & Silberring, 2016).

According to the consequence as mentioned above, after the electrophoresis kefir proteins were stained for 1 h with 0.1% Coomassie Brilliant Blue R250 in 50% methanol and 10% acetic acid.

3.5. Sample preparation for Mass-Spectrometry

3.5.1 Proteins viewing and PDQuest analysis

The gels were de-stained, scanned at 600 dpi resolution, and the gel images were analyzed using PDQuest software (Bio-Rad) in order to calculate for each spot, the normalized quantity, the isoelectric point (pI), and the molecular mass. The quantity of each spot was normalized using the local regression model (LOESS). After the generation of a master gel, based on a

representative gel for each type of sample, the spots were detected and matched automatically by the software. The spot detection and matching were also edited manually. Finally, a spot quantity table containing all matched spots was generated.

Therefore, the PDQuest software provides a table containing information including the isoelectric point, the MW and the normalized quantity. The pIs were determined using a linear 4/7 distribution, and molecular mass determinations were based on the markers Bio-Rad low range (phosphorylase b, 97.4 kDa; bovine serum albumin, 66.2 kDa; ovalbumin 45.0 kDa; carbonic anhydrase, 31 kDa; soybean trypsin inhibitor, 21.5 kDa; lysozyme,14.4 kDa). Target protein gel spots were manually excised (1 mm in diameter) from the 2-DE for MS identification.

In order to convert the normalized quantity obtained from the PDQuest analysis to the amount of protein (micrograms), a pure standard-standard protein (dihydrofolate reductase, DHFR, purity> 98%) was loaded in different quantities (from 0.5 to 50 micrograms) in a 15% electrophoresis gel in the presence of SDS (15% SDS-PAGE). At the end of the electrophoretic course the proteins bands were analyzed by the PDQuest software to define the normalized quantity.

Thus, the normalized quantity of each DHFR band calculated from PDQuest software was correlated with the corresponding quantity (in micrograms) of proteins, obtaining a straight calibration line reporting the quantity of protein (in micrograms) on the x-axis and the normalized quantity in y-axis calculated by the PDQuest program.

The equation of the straight line obtained can be used to calculate the quantity in micrograms of a protein spot obtained with a two-dimensional electrophoresis gel.

3.5.2. *In-gel digestion of proteins*

In-gel digestion of proteins isolated by gel electrophoresis provides the base for mass spectrometry (MS)-driven proteomics analysis. Shevchenko et al. in 2007, implemented a protocol to increase the speed and sensitivity of the analysis (Shevchenko et al., 2007).

Primarily, chemical and keratin reduction are recommended. Thus, wearing gloves at all times and rinse them occasionally as they readily accumulate static charge and attract dust and pieces of hair and wool.

1. Excise protein bands (spots); Rinse the entire slab of a one- or two-dimensional gel with water for a few hours, put a plastic tray with the gel onto a lightbox and excise bands (spots) of interest with a clean scalpel.
2. Cut excised bands (spots) into cubes (ca. 1 x 1 mm).
3. Transfer gel pieces into a microcentrifuge tube and spin them down on a bench-top microcentrifuge.
4. Distain gel pieces excised from Coomassie-stained gels;

   - Add about 100 ml of 100 mM ammonium bicarbonate/acetonitrile (1:1, vol/vol) and incubate with occasional vortexing for 30 min, depending on the staining intensity.

   - Add 500 ml of neat acetonitrile and incubate at room temperature with occasional vortexing, until gel pieces become white and shrink and then remove acetonitrile. Although the bulk of Coomassie staining should be removed, it is not necessary to de-stain the gel pieces completely.
5. Samples are now ready for in-gel digestion. Alternatively, they can be stored at -20 °C for a few weeks.
6. Saturate gel pieces with trypsin;

- Add enough trypsin buffer to cover the dry gel pieces (typically, 50 ml or more, depending on the volume of a gel matrix) and leave it in an ice bucket or a fridge.
- After about 30 min, check if all solution was absorbed and add more trypsin buffer, if necessary. Gel pieces should be covered entirely with trypsin buffer.

7. Leave gel pieces for another 90 min to saturate them with trypsin and then add 10–20 ml of ammonium bicarbonate buffer to cover the gel pieces and keep them wet during enzymatic cleavage. Although after about 30 min dried gel pieces do not absorb any more buffer, the yield of tryptic peptides increases substantially while extending the incubation time, presumably because of slow diffusion of the enzyme into a polyacrylamide matrix.
8. Digestion; place tubes with gel pieces into an air circulation thermostat and incubate samples overnight at 37 °C for analyses performed at the limit of instrument sensitivity, which require maximal peptide recovery.
9. Extract peptide digestion products; add 100 ml of extraction buffer (1:2 (vol/vol) 5% formic acid/acetonitrile) to each tube and incubate for 15 min at 37 °C in a shaker. For samples with a much larger (or smaller) volume of the gel matrix, add the extraction buffer such that the approximate ratio of 1:2 between volumes of the digest and extraction is achieved.

3.6. LIQUID CHROMATOGRAPHY– MASS SPECTROMETRY (LC-MS/MS)

One of the main strengths of 2DE and related IEF techniques involve complementarity with mass spectrometry (MS). When used together, this approach can reduce proteome complexity with enough resolution that a single protein can be deeply analyzed (Pergande & Cologna, 2017). The are several possible experimental IEF methodologies coupled with mass spectrometry, including the combination to liquid chromatography (LC), adopted in kefir proteins investigation. Regardless, Mass spectrometry (MS) plays a crucial role in analyte identification and characterization.

While the image obtained from the gel-based experiment provides information about the protein composition, the combination of other analytical tools provides a complete determination of the proteins. It is now common practice to perform 2D electrophoresis analysis in order to separate proteins and visualize spots while also obtaining quantitative information, followed by excision of the spot and downstream mass spectrometry analysis (Pergande & Cologna, 2017).

An important application area of LC-MS is the characterization and analysis of peptides and proteins in term of molecular mass determination, amino-acid sequencing, determination of nature and position of chemical and post-translational modifications of proteins, and the investigation in protein tertiary and quaternary conformations (Niessen, 2019). MS is the fastest and cheapest method of protein identification. Single protein species can be identified by peptide mass fingerprinting using this technology. The proteins are first "in-gel digested" with a highly specific endoproteinase including trypsin, and the resulting peptides are extracted, mixed with a matrix, spotted on a target, and analyzed by MS. Then peptides with different molecular weights (m/z ratio) are measured accurately by the mass spectrometer (accurate to at least 0.01Da). This analytical tool also provides a list of the identified peptide masses producing a 'fingerprint', which determine its uniqueness. Submitting the peptide list to a protein-searching database permit to achieve a proper identification of protein under investigation (Chevalier, 2011).

3.6.1. LC-MS/MS analysis

These analyses were performed in collaboration with the HPLC-MS Laboratory, University of Camerino. Target protein gel spots were in-gel digested with trypsin following the protocol described by Shevchenko and co-workers, extracted, and subjected to MS identification.

The tryptic peptides were resuspended in 100 mL of 0.1% (v/v) formic acid and injected into a reversed-phase chromatography connected to an HPLC Agilent Technologies 1100 Series. The column effluent was analyzed by MS using an electrospray ion trap mass spectrometer (Agilent Technologies LC/MSD Trap SL) operating in positive ion mode over the mass range 300-2200 amu (atomic mass units).

MS operating conditions were set as follows:

1. nebulizer pressure, 70 psi;
2. draining gas flow, 12 L/min;
3. drying gas temperature, 300°C;
4. capillary voltage, 3.5 kV.

Obtained spectra were extracted and analyzed by the MASCOT software (www.matrixscience.com) with the following search parameters: database, NCBInr taxonomy: Mammalia; enzyme, trypsin; peptide tolerance, 1.2 Da; MS/MS tolerance, 0.6 Da and allowance of one missed cleavage. (Vincenzetti et al., 2015).

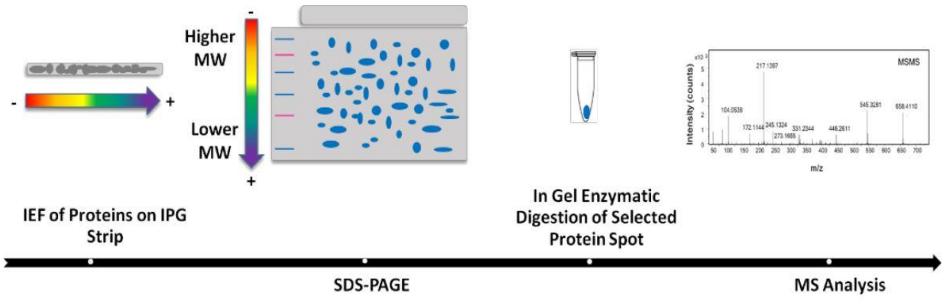


Figure 15. Proteins are resolved by two-dimensional gel electrophoresis (2DE) and visualized with a stain. Spots are excised, subjected to proteolytic digestion, and the resulting solution is analyzed via mass spectrometry (Pergande & Cologna, 2017).

Chapter 4
RESULTS AND DISCUSSION

4.1. Microbiological culture

In the Petri dish portion where the filtrated kefir sample was spread, no lactobacilli neither mould were grown. On the other hand, some colonies of lactobacillus (**Figure 16**) and moulds are grown on the portion of the unfiltered sample. Therefore, the obtained results show the need to perform the filtration step to ensure the removal of any micro-organisms contained in kefir sample.

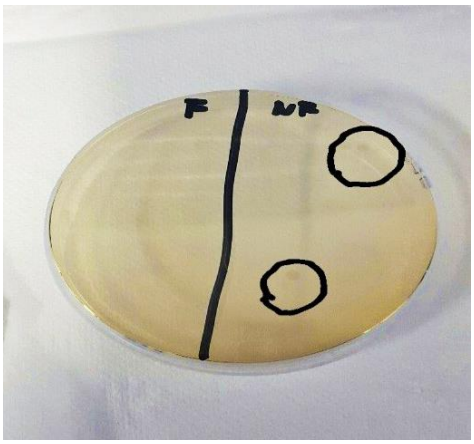


Figure 16. Sabouraud Glucose Agar plates reporting both filtrate (F) and unfiltered (NF) samples.

4.2. Bradford analysis

According to the Bradford method, all tubes for the calibration line were prepared. The Absorbance values (Abs) on the y-axis were plotted against the BSA concentration values on the x-axis. resulting in a straight calibration line, and its related equation was generated (**Figure 17)**. Also, tubes containing filtered kefir milk in dilution factors of 1:10 and 1:100 have been subjected to spectrophotometer analysis at a 595 nm wavelength. In more detail, 10 µL and 20 µL of sample diluted 1:10, and 50 µL of sample diluted 1:100 were analyzed.

µg BSA	Abs 595 (nm)
0	0
2	0,083
3	0,162
4	0,213
5	0,246
6	0,288
7	0,353
8	0,373
9	0,427
10	0,482

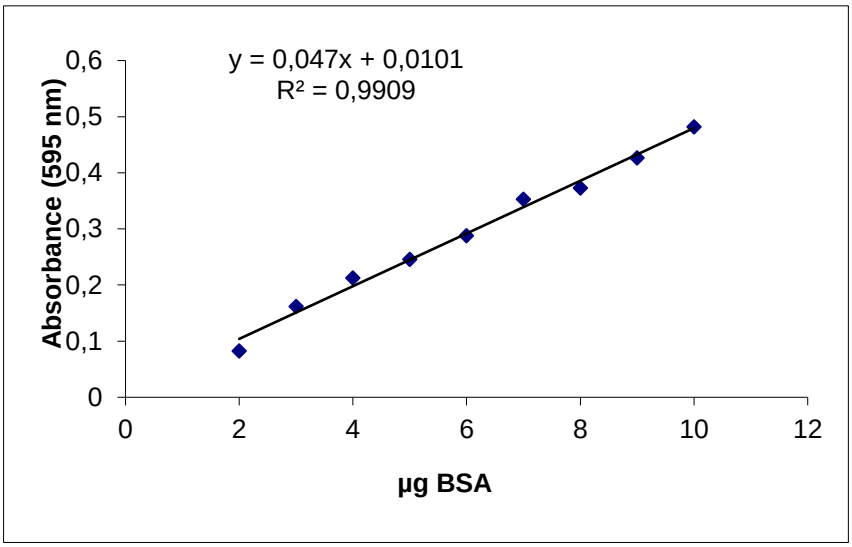


Figure 17. Straight calibration line to determine the protein concentration in kefir milk. For the construction of the line, increasing amounts of BSA were mixed with pure water and a fix amount of Bradford reagent (Coomassie). Each sample was measured for absorbance at 595 nm against a blank, containing only pure water mixed to Bradford reagent.

Sample dilution factor	Absorbance (Abs)	x value[a]	Concentration[b] (mg/mL)	Average value (mg/mL)	Standard deviation
1:10 (10 µL)	0.171	3.423	3.423	2.859	0.7816
1:10 (20 µL)	0.195	3.934	1.967		
1:100 (50 µL)	0.085	1.593	3.187		

Table 4. The values reported allowed the determination of proteins contained in the filtered milk kefir sample.

[a] x value=(Abs-0.0101)/0.047

[b] Concentration=(x value/µL added)*sample dilution factor

The samples diluted were analyzed by spectrophotometer in order to found the corresponding absorbance (Abs) values, as reported in **Table 4**. In order to calculate the x value corresponding to the protein concentration in each sample diluted, the Abs data were replaced to the y value of the straight-line equation y= 0.047x + 0.0101.

The protein concentration (x value) determined in each kefir sample diluted is divided by the quantity of kefir analyzed (mL) and then multiplied by the sample dilution factor. According to the aforementioned mathematical operations, the proteins concentration (mg / mL) is obtained.

Also, in order to obtain a representative value, the average value was determined (**Table 4**).

The protein concentration corresponds to 2.859 ± 0.7816 mg/mL.

4.3. Proteomic analysis

The presence of a wide post-translational modifications makes the milk proteome extremely complex. Post-translational modifications, including glycosylation, disulfide bond formation, phosphorylation, and proteolysis, can create a large number of different proteins. The 2DE of kefir proteins is shown in **Figure 18**. Each spot on the gel represents a single protein isoform contained in kefir milk. The protein isoforms are resolved from each other, as they migrate to different positions based on their size and pI, and are then visualized using a Coomassie Brilliant Blue.

This investigation intended to evaluate the peptide profile of kefir adopting LC/MS-MS liquid chromatography-mass spectrometric methods. The peptide profile should reveal polypeptides and bioactive components as the result of the proteolytic activity of the micro-organisms used as a starter culture. The fermentation process performed by micro-organisms improves the health-promoting effects of the fermented beverage due to the release of bioactive peptides.

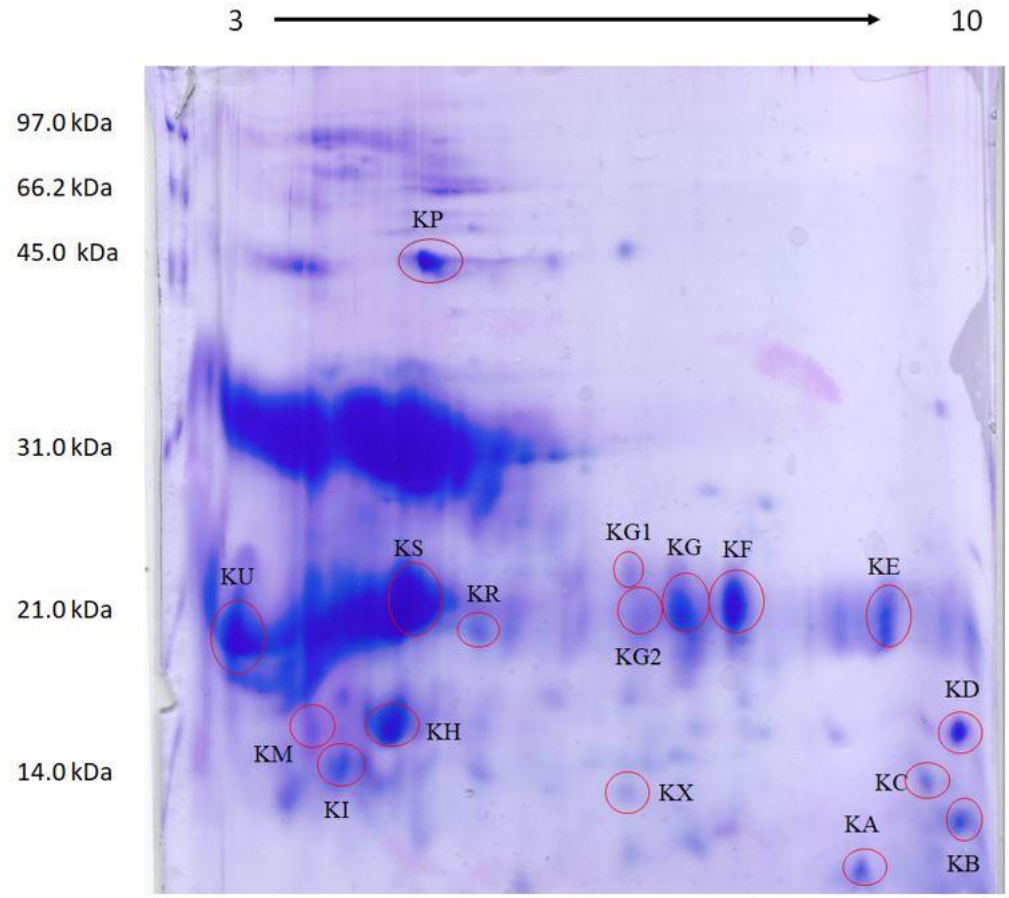


Figure 18. Two-dimensional electrophoresis gel of kefir sample performed at 3-10 pH range.

In **Figure 18**, the result of the two-dimensional electrophoresis performed at a 3-10 pH range is shown, and the spots identified by mass spectrometry are highlighted in red. In **Table 5**, the information about spots identification, pI, and molecular mass provided by PDQuest are detailed. There are some low molecular weight spots identified as follow: KA, KB, KC, KH, KI, KM, and KU. They are proteins derived from α–, and κ–caseins, and some of them hide specific amino acid sequences corresponding to bioactive peptides. It is known that milk proteins contain

bioactive peptides encrypted within the primary sequence, which can be released during gastrointestinal digestion by digestive enzymes or during the industrial processing.

4.3.2. Calculation of the normalized quantity

The quantification of micrograms for each spot shown in the gel was carried out as described in section 3.5.1.

The straight calibration line was obtained using a pure DHFR preparation as standard, which was loaded on a 15% polyacrylamide gel in different quantities in the range between 0.5-50 micrograms. After the de-staining step, the polyacrylamide gels were scanned and subjected to PDQuest analysis for the calculation of the normalized quantity for each electrophoretic band (corresponding to each protein concentration). Thus, two calibration lines were obtained. The first straight line, in the range 0.5-15 μg, and the second straight line, in the range 8-50 μg.

The amount (expressed in μg) contained in each spot was quantified by the straight calibration line as follow:

$y = 35.61x + 160.82$ $R^2 = 0.98$ (used for a range 0.5–15 μg).

The y-axis values correspond to the normalized quantities contained in each spot. These values were then replaced in the straight-line equation from which the microgram values of the protein spots were obtained. The values (expressed as μg) from each point are listed in **Table 5.**

SPOT	Normalized quantity ($x10^3$)	Quantity (µg)
KA	180	0.54
KB	182	0.594
KC	99	n.d.
KD	307.8	4.13
KE	788.2	17.62
KF	1082	25.87
KG	1519	38.14
KG1	281	3.37
KG2	579	11.74
KH	328	4.69
KI	478	8.91
KM	328	4.69
KP	427.3	7.48
KR	43	n.d.
KS	1431	35.4
KU	1306	32.2
KX	800	18.0

Table 5. PDQuest analysis of spots

The high amount of the proteins in the region of 20 and 30 kDa resulted in several limitations about their quantification. Therefore, a less quantity of sample should be analyzed to overcome this issue. In addition, a specific pH electrophoresis range 4-7could be suggested.

4.4. Mass-spectrometry results

In **Table 6** the identification of kefir milk proteins by LC-MS/MS followed by MASCOT software analysis is shown.

SPOT ID[a]	Protein name[b]	Score[c]	Mr (kDa)/pI[d]	Mr (kDa)/pI[e]	Sequence
KA	k-casein (*Bos taurus*)	35	18.96/5.93	8.2/9.0	FFSDKIAKYIPIQYVLSR
KB	k-casein (*Bos taurus*)	35	18.96/5.93	10.0/9.8	FFSDKIAKYIPIQYVLSR
KC	k-casein (*Bos taurus*)	35	18.96/5.93	11.8/9.6	FFSDKIAKYIPIQYVLSR
KD	Head protein (*Streptococcus virus 2972*)	76	12.7/7.85	14.0/9.7	AGTLVAGDGGSIFDDR
KE	Glycosylation-dependent cell adhesion molecule 1	44	17.15/ 6.22	19.2/8.9	LPLSILKEK

	(*Bos taurus*)				
KF	Glycosylation-dependent cell adhesion molecule 1 (*Bos taurus*)	29	17.15/ 6.22	21.3/7.6	NLENTVKETIK
KG	Glycosylation-dependent cell adhesion molecule 1 (*Bos taurus*)	37	17.15/ 6.22	20.3/7.2	SLFSHAFEVVK
KG1	Glycosylation-dependent cell adhesion molecule 1 (*Bos taurus*)	33	17.15/ 6.22	22.6/6.7	NLENTVKETIK
KG2	Glycosylation-dependent cell adhesion molecule 1 (*Bos taurus*)	36	17.15/ 6.22	19.7/6.8	SLFSHAFEVVK
KH	αs1-casein (*Bos taurus*)	43	22.96/4.90	14.0/4.7	FFVAPFPEVFGK
KI	αs1-casein (*Bos taurus*)	43	22.96/4.90	12.5/4.1	FFVAPFPEVFGK
KM	αs2-casein (*Bos taurus*)	24	24.33/8.35	14.4/3.9	NAVPITPTLNR
KP	Enolase (S. *thermophilus*)	93	47.0/4.67	45.3/4.8	LGANAILGVSIAVAR
KR	β-lactoglobulin (*Bos taurus*)	34	18.27/4.83	18.6/5.1	LIVTQTMKGLDIQKV
KS	β-lactoglobulin (*Bos taurus*)	126	18.27/4.83	19.8/4.8	LIVTQTMKGLDIQKV
KU	αs1-casein (*Bos Taurus*)	40	22.96/4.90	18.3/3.2	FFVAPFPEVFGK
KX	Olfactory receptor 7A17-like (*Bos taurus*)	31	34.8/8.04		VSSILGISSAQGKYK

Table 6. Spots identification

[a] Assigned spot ID as indicated in Figure 1.

[b] MASCOT results (SwissProt & NCBInr databases).

[c] MASCOT score reported.

[d] From SwissProt & NCBInr databases.

[e] Experimental values were calculated from the 2DE maps by the PDQuest software.

Peptides with biological activities, released during gastrointestinal digestion or food processing, play an essential role in metabolic regulation and modulation, suggesting their potential use as nutraceuticals and functional food ingredients for health promotion and disease risk reduction (Shahidi & Zhong, 2008).

It is assumed that the amount of bioactive peptides increases during the production of fermented milk products compared to raw milk and also, the composition of the peptide fraction changes due to the proteolytic action of microorganisms used as a starter.

According to LC-MS/MS analysis, many spots are identified. In more details, KA, KB and KC spots were identified as k-casein (*Bos taurus*), and in kefir are present as fragments of k-casein because of their low molecular weight (8.0÷11.8 kDa). The sequence obtained for the spots KA, KB and KC (YIPIQYVLSR) is contained in the casoxine-C, a bioactive peptide that acts mainly as an opioid antagonist, obtained from the digestion of k-casein.
This peptide has shown its activity against the suppressive effect of DAGO, a μ-opioid agonist evoking the contraction of the longitudinal muscle strips of the ileum (Takahashi et al., 1997). The same authors highlight similarity to the carboxy-terminal octapeptide of C3a, namely, C3a (70–77). This peptide derives from the cleavage of C3, after the activation of the complement resulting in the inflammatory response. Thanks to its activity similar to that of the C3a fragment, the casoxin-C is probably able to stimulate the release of histamine leading to a fast contraction of the intestinal smooth muscle and the release of prostaglandin E2 which exerts the anti-opioid activity of casoxin-C, resulting in the slow contraction. In addition, the casoxin-C seems to stimulate phagocytosis and therefore, it might enhance the host defense system against various infections (Takahashi et al., 1997).

The spots KH, KI, and KU, are fragments (12.5÷14.0 kDa) released from the α_{s1}-casein (*Bos taurus*) digestion. They report the sequence FFVAPFPEVFGK (**Table 6**), which is known as the angiotensin-I-converting enzyme (ACE) inhibitory peptide (FitzGerald et al., 2004). ACE is involved in blood pressure regulation by the production of the potent vasoconstrictor angiotensin II. Indeed, angiotensin II causes a reduction in urinary excretion of water and sodium, which plays an important role in long term stabilization of hypertension. Also, ACE acts in the degradation of bradykinin, a vasodilator. Therefore, inhibition of ACE results in a lowering of blood pressure and the enzyme represents an important therapeutic target in the treatment of hypertension (Amorim et al., 2019).

ACE inhibitory drugs are widely used in hypertensive patients despite several side effects after their administration are reported. The use of alternative natural ACE inhibitors such as peptides derived from caseins and whey proteins can be adopted to overcome the undesirable effects provided by the dosing of the synthetic drug (Ibrahim et al., 2017).

The spot KM is a peptide (14.4 kDa) derived from the α_{s2}-casein (*Bos taurus*), whereas the spots KE, KF, KG, KG1, and KG2 correspond to the Glycosylation-dependent cell adhesion molecule 1 (*Bos taurus*) namely lactophorin. It consists of 2 significant glycopeptides; 28 kDa (LP28) and 18 kDa (LP18). The first-one contains 5 partial phosphorylation sites (Ser29, Ser34, Ser38, Ser40, and Ser46), 3 O-glycosylation sites (Thr16, Thr60, and Thr86), and 1 Nglycosylation site (Asn77)

(Inagaki et al., 2012). It has been reported that lactophorin exists in several molecular forms determined by different glycosylation levels (Inagaki et al., 2010). Also, it is strongly expressed during lactation by the mammary epithelial cells. The expression of this protein is induced during pregnancy similarly to the hormonally induced milk proteins, and the protein can be typically found in the milk of the secreting mammary gland (Dowbenko et al., 1993). Inagaki and co-workers (Inagaki et al., 2010) theorized that different glycosylated forms of lactophorin are involved in mother-to-infant communication playing a role in functional differentiation and development. Besides, lactophorin exhibits potent inhibitory activity against human rotavirus (HRV**)**. HRV is one of the most important etiologic agents of severe gastroenteritis in infants and young children, and it was recognized as a significant cause of childhood diarrheal morbidity and mortality worldwide. Lactophorin exists in various molecular forms produced via post-translational modification and in more detail, LP16 has reported to have vigorous inhibitory activity against HRV replication as high-Mr glycoprotein fraction (F1) (Inagaki et al., 2012). Furthermore, in this study, at least five forms of lactophorin with similar molecular mass but different pI were detected.

Spot KR and KS were identified as β-lactoglobulin isoforms (*Bos taurus*). These peptides are inactive within the sequence of the precursor protein, but they can be released by in vivo or in vitro enzymatic proteolysis and become active. Hernández-Ledesma and co-authors reported that the release of fragment f(9–14) represented by GLDIQKV sequence was induced by the trypsin activity. Also, once released, these peptides have hypocholesterolemic activity (Hernández-Ledesma et al., 2008).

Lastly, spot KP has been identified as enolase from *Streptococcus thermophilus* widely used for the manufacture of yoghurt and cheese (Bolotin et al., 2004). Therefore, this strain could be a natural inhabitant of the kefir grain.

Chapter 5
CONCLUSION AND FUTURE PERSPECTIVE

Many studies reported the beneficial effects provided by kefir beverage that should be consumed regularly as part of the daily diet providing health benefits and reducing the risk of developing the diseases. The results obtained from this study can help to clarify the protein composition of kefir milk and also identify and characterize the bioactive peptides that could have a vital nutraceutical function and hence a positive impact on human health. Farnworth reported that kefir milk contains a large number of polypeptides and that the majority of bioactive peptides have molecular weights of ≤50 kDa (Farnworth, 2005). According to Farnworth statements, the results of the current investigation have reported a large number of polypeptides in commercial milk kefir and several peptides attributable to bioactive peptides. In particular, low molecular weight peptides deriving from the k-casein, α_{s1}-casein digestion, were found. These peptides may act by enhancing the host defence system against various infections and reducing blood pressure. Also, β-lactoglobulin isoforms reporting an essential role in the hypocholesterolemic activity and five isoforms of lactophorin with a different isoelectric point were identified. The results obtained can be considered as a useful preliminary study to perform future investigations such as the mapping of the glycosylation sites of the five lactophorin isoforms found by mass spectrometry**.**

The healthy properties hidden in milk peptides can offer strengths for interesting products development. Therefore, for the commercial production of novel bioactive peptides in large scale technologies, others analysis, including the ion-exchange chromatography, must be performed. This investigation may constitute the starting point to implement useful applications by transferring the health functional properties of bioactive peptides to create novel functional foods. However, it should be noted that through the two-dimensional electrophoresis technique, it has some limitations regarding the identification of proteins with Mr < 5.0 kDa, therefore, further analysis using other approaches will be necessary to identify the presence of milk-derived bioactive peptides.

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