DYNAMICS AND BIOINFORMATICS OF MICROBIAL SPOILAGE ECOLOGY OF KEFIR

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ABSTRACT

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In this study, it was aimed to characterize the microbiological properties, bacterial composition, and microbial stability of 5 different commercial milk kefir beverage products during refrigerated storage.

In order to determine the microbiological load and compositions, total mesophilic aerobic bacteria, yeast and molds, lactic acid bacteria, *Lactobacilli*, *Lactococci*, total coliforms, and *E.coli* were investigated by cultural conventional analysis for milk kefir beverages. According to the obtained data, microbiological and hygienic characteristics of the samples were found acceptable.

The bacterial load of the kefir beverage samples ranged between 7.086 and 8.794 $\log_{10}$ cfu.ml^{-1} for viable total aerobic mesophilic bacteria (TAMB), 6.792 and 8.382 og_{10} cfu.ml^{-1} for lactic bacteria (LAB), <10 and 6.322 $\log_{10}$ cfu.ml^{-1}for *Lactobacillus*, 5.857 and 8.146 $\log_{10}$ cfu.ml^{-1} *Lactococcus*, 5.176 and 7.218 $\log_{10}$ cfu.ml^{-1} for yeasts, negative for molds, coliform bacteria and negative for *E. coli*.

Principal component analysis (PCA) of the compounds separated the kefir beverages according to the storage time and kefir brands. Strong relationship were found between storage time and PC1 and between kefir brands and PC2.

To date, information on microbial properties, bacterial composition, and constancy of commercial kefir is scant, and to the best of our knowledge, this is the first research to contribute information on kefir beverages in microbial properties, bacterial composition, and their stability during refrigerated storage by evaluating Fourier Transform Infrared Spectroscopy (FTIR) spectra analysis and Bioinformatics besides cultural conventional analysis.

ABSTRACT

KEFİRİN MİKROBİYAL BOZULMA EKOLOJİSİNİN DİNAMİKLERİ VE BİYOİNFORMATİĞİ

Bu çalışmada, 5 farklı ticari süt bazlı kefir içecek ürününün buzdolabında depolama sırasında mikrobiyolojik özellikleri, bakteri bileşimi ve mikrobiyal stabilitesinin karakterize edilmesi amaçlanmıştır.

Mikrobiyolojik yük ve kompozisyonları belirlemek için süt kefir içecekleri için toplam mezofilik aerobik bakteri, maya ve küfler, laktik asit bakterileri, *Lactobacilli*, *Lactococci*, total koliformlar ve *E.coli* kültürel konvansiyonel analizlerle incelenmiştir. Elde edilen verilere göre örneklerin mikrobiyolojik ve hijyenik özellikleri kabul edilebilir bulunmuştur.

Kefir içecek örneklerinin bakteri yükü toplam canlı aerobik mezofilik bakteriler (TAMB) için 7.086 ile 8.794 $\log_{10}$ cfu.ml^{-1}, laktik bakteriler (LAB) için 6.792 ve 8.382 og_{10} kob.ml^{-1}, <10 ile 6.322 $\log_{10}$ arasında değişmektedir. *Lactobacillus* için cfu.ml^{-1}, 5.857 ve 8.146 $\log_{10}$ cfu.ml^{-1} *Lactococcus*, mayalar için 5.176 ve 7.218 $\log_{10}$ cfu.ml^{-1}, küfler, koliform bakteriler ve *E. coli* için negatiftir.

Bileşiklerin temel bileşen analizi (PCA), kefir içeceklerini saklama süresine ve kefir markalarına göre ayırmıştır. Depolama süresi ile PC1 arasında ve kefir markaları ile PC2 arasında güçlü bir ilişki bulunmuştur.

Bugüne kadar, ticari kefirin mikrobiyal özellikleri, bakteriyel bileşimi ve stabilitesi hakkındaki bilgi yetersizdir ve bildiğimiz kadarıyla, bu, kefir içeceklerinin mikrobiyal özellikleri, bakteriyel bileşimi ve buzdolabında depolama sırasındaki stabiliteleri hakkında Fourier Dönüşümü Kızılötesi Spektroskopisi (FTIR) spektrum analizi ve Biyoinformatik'i kültürel konvansiyonel analizi değerlendirerek bilgi sağlayan ilk araştırmadır.

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CHAPTER 1

INTRODUCTION

People were searching for how to protect the food long time and they found a way called fermentation, which is the oldest method that is known in the world. To protect food like milk from spoilage developed as fermented milk and this method was found in the Middle East and the Balkans, 10,000 years ago. Through time some progress developed like, people were using a small portion of the previous batch as a starter and putting this small portion of food into large-scale production (Litopoulou-Tzanetaki and Tzanetakis 2014). Found fermented milk products were classified into three groups as lactic, yeast-lactic and mold-lactic fermentation products.

As known worldwide kefir is generally made from cows' milk. Generally, kefir grains are added directly to milk as a starter culture. It is known that kefir is fermented differently from other milk products, which have grains to get fermented (Simova et al. 2002). The classic method of making kefir involves mixing kefir grains (2% – 10 % m/v) directly into pasteurized milk that has been cooled to 20–25°C. The beverage is incubated 24h at room temperature. Then, the grains are filtered, and the milk kefir is ready for consumption. If the milk kefir is fermented shortly the taste is sweeter but if it's fermented a long time the taste of the milk kefir is sour (Simova et al. 2002). Sequencing of 16S-rRNA is utilized for classification, identification and quantitation of all the microorganisms in microbiota. Classifying the microorganisms is done in genus and species level. The 16S-rRNA gene is highly conserved and by knowing this, in thousands of species the target gene is 16S-rRNA gene (Cox, Cookson, and Moffatt, 2013).

This study aimed to evaluate commercial kefir products in storage conditions, characterizing their microbial properties, bacterial composition, and their steadiness during refrigerated storage. To date, information on microbial properties, bacterial composition, and their stability of commercial kefir is scant, and to the best of our knowledge, this is the first preliminary research evaluating Fourier Transform Infrared Spectroscopy (FTIR) spectrum data and Bioinformatics besides cultural conventional analysis to provide on kefir beverages in microbial properties, bacterial composition, and their stability during refrigerated storage by.

CHAPTER 2

LITERATURE REVIEW

2.1. History of Kefir

The 21st century's most popular yogurt is called kefir, and it resembles a blend of thick, acidic, and slightly alcoholic, yellow and white dairy products (Enikeev 2012). Kefir, which predates written records, is a fermented milk drink emerging from the Caucasus. In ancient times, the nomadic sheep herders from the east realized that while they were traveling and carrying milk in their pouches, sometimes the milk turned in to a foamy beverage. These people called the beverage “kefir” that came from the word “keyif” and this word in Turkish means “pleasure (Turkmen 2017). There is no information about when the first kefir drink or kefir grains derived from. People were adding fresh milk while they were removing the fermented milk and it is known that kefir can be made from animal milk such as sheep, goat, cow, or vegetable milk such as soy milk.

2.2. Kefir Grains

Since kefir's starter is comprised of grains, which are a combination of various bacteria, yeasts, polysaccharides, and other microbial metabolic byproducts, in addition to milk protein curds, it differs from other fermented dairy beverages. Kefir grains are uneven in shape and has an uneven surface. These kefir grains in 2 cm in size. As shown in Figure 1 the grains look like cauliflower in shape and they have a gelatinous, elastic shape, a yellowish white appearance, and a distinct odor. When grains are put into the milk for fermentation, they develop, proliferate, and pass on their traits to subsequent generations of newly created grains and in the right conditions, kefir grains can continue to function for years. (Farnworth 2016).

Figure 1. Kefir Grains
(Source: Turkmen, 2017)

The kefir grains generally contain 90% water, whereas the dry bulk is composed of 57% carbohydrates, 33% proteins, and 6% ash. Kefir grains cause fermentation and there are group of microorganisms that are stick together by a polysaccharide matrix and this is known as kefiran and is made by *Lactobacillus kefiranofaciens*. A branching polysaccharide called kefiran is composed equally of galactose and glucose**.** The kefir grains consist of complex microbiological composition which are 83%-90% of lactic acid bacteria, 10%-17% yeasts, acetic acid bacteria and possibly mold which are all together on the matrix of the kefiran (Tamime et al. 2017; Sarkar 2007).

2.3. Probiotics as Kefir

Probiotics are foods that contain beneficial microorganisms for our wellbeing. When ingested, some microbes can be advantageous to one's health. The beneficial microorganism that is known as probiotics, can effect one's health in a good various ways like, aiding digestion, weight management and mental health. Although yogurt is the most well-known probiotic food in the Western diet, kefir is a far more effective source. Kefir grains are an example of probiotic symbiosis between yeast and bacteria. Kefir grains include approximately 61 strains of bacteria and yeast that makes the grains very rich and diverse source of probiotics, however diversity varies. Other fermented dairy products include no yeast and are created from fewer strains ("9 Evidence-Based Health Benefits of Kefir" n.d.).

2.4. Production of Milk Kefir

To make traditional kefir, pasteurized milk is added on kefir grains and stir and get it incubated for 24 hours at room temperature. After the fermentation is finished agitation of the kefir curd will make the kefir grains float because of the effect of carbon dioxide. The traditional kefir can be used after fermentation for future use in following kefir fermentations. Some researchers have argued that, to make traditional kefir, kefir grains must be used. However, industrial production of kefir is different from traditional kefir because it is not incubated with the grains. According to the same study, kefir made by incubating milk with kefir grains cannot be utilized as a starter culture for the following batches of kefir (Simova et al. 2002).

2.5. Storage of Kefir Grains

If you want to take a break of kefir you have to store it and there are several ways that you can store kefir. Firstly, you can store the kefir grains in milk and put in the fridge for 7 to 14 days and you can change the milk every 2 weeks. Secondly, you can store the kefir grains in water/sugar solution and put it in the fridge. This can stay up to 2 months in the fridge. Thirdly, you can dry them for few hours and put the dry grains in a jar and put it in the freezer and it can stay up to 6 months. Fourthly, you can wash the grains with fresh non chlorinated water and then leave them to dry few days on a paper towel until it gets completely dry. Then put powdered milk on the dried grains and put it in a zip bag for up to 6 months in the fridge ("How to Store Kefir Grains When You're Taking a Break from Kefir - KEFIRKO" n.d.).

2.6. Chemistry of Kefir Grains and Kefir Beverages

The structure of kefir differs due to the type, lipid content, the structure of grains and the creation methods of the milk. The typical structure of kefir has 89-90% moister, 0.2% lipid, 3% protein, 6% sugar, 0.7% ash and 1% lactic acid and alcohol (Sarkar 2007). It is reported that ethanol is present in kefir in different amounts in every product, like: 0.12-0.18% (Marshall and Cole 1985).

Traditional kefir's refreshing flavor is due to a combination of yeast and lactic acid fermentation. Still, package swelling can occur due to the yeast's generation of carbon dioxide. Some organizations employ starter cultures without yeast flora to prevent this problem. Due to the lack of alcohol fermentation and little to no carbon dioxide production, the flavor of kefir differs from that of true kefir. Researchers used a commercial kefir as a starter culture for 168 hours incubation, microbiological and biochemical parameters were determined. During the first 24 hours of incubation *Lactococcus* spp. were the richest strain but later, *Lactobacillus* spp. past *Lactococcus* spp. During the fermentation period, *Leuconostoc* strains were not detected and after 48 hours of incubation yeasts increased in number. This means that yeast populations were reported lower than other studies (García Fontán et al. 2006).

2.6.1. Kefir Flavor

Lactic acid is mostly found during kefir fermentation. There are flavor forming compounds in kefir that are carbonyl compounds, volatile organic acids and non-volatile acids that are secondary metabolites (Teixeira Magalhães et al. 2011). According to the species or strains present, the ratio and kind of flavor compounds produced by these microorganisms varies. This variety in the LAB composition may have a important impact on the quality of the last product (Mauriello et al. 2001).

Lactic acid, acetaldehyde, diacetyl, acetoin, acetone, ethanol, CO2, and acetic acid are some of the major and secondary end products produced by LAB. Lactic acid, in kefir, is a nonvolatile, odorless molecule that gives fermented foods their characteristic acidity. Lactic acid is produced when lactose is broken down by the homo-fermentative and hetero-fermentative LAB found in kefir grains (Ötles and Çağındı 2003).

Acetaldehyde is responsible for yogurt's distinctive "fresh fruit" fragrance. With concentrations ranging from 0.5 to 10 mg/L, this molecule is one of the main fragrance components detected in kefir (Güzel-Seydim et al. 2000).

Diacetyl is also a desirable component of many dairy products, and it is responsible for the buttery aroma of milk products at extremely low concentrations up to 5 mg/L (Güzel-Seydim et al. 2000).

Acetoin, at a concentration of 9 mg/L, has been found in high-quality kefir beverages. Acetoin is normally flavorless and odorless at amounts seen in cultured products, therefore it would have little taste value (Güzel-Seydim et al. 2000).

Acetone is a natural component of milk and cheese, and it is found in kefir beverages at amounts ranging from 0.6 to 4.91 mg/L. Acetone is thought to play a small role on the organoleptic properties of kefir, with acetone concentrations less than 1 mg/L unlikely to have a major effect on flavor. Acetone is thought to play a small role on the organoleptic properties of kefir, with acetone concentrations less than 1 mg/L unlikely to have a major effect on flavor (Aghlara et al. 2009).

Kefir ethanol concentrations have been observed to range from 0.01% to 2.5% (m/v), depending on the starter and kefir preparation process (Magalhães et al. 2011). Alcohol dehydrogenase, an enzyme found in both yeasts and LAB, converts acetaldehyde to ethanol and produces ethanol. Non-lactose-fermenting yeasts and lactose-fermenting yeasts can both be found in kefir. Lactose-fermenting yeasts lack sufficient alcohol dehydrogenase activity, resulting in a less yeast flavor in the final beverage than beverages made with non-lactose fermenting yeasts. Kefir's mild effervescence is due to carbon dioxide produced by alcoholic fermentation and heterofermentation (Liu, Chen, and Lin 2002).

Acetic acid is a short-chain volatile fatty acid present in kefir with amounts ranging from 200 to 850 mg/L (Garrote, Abraham, and De Antoni, 2001). Some other scientists did not observed acetic acid and in kefir beverages it was found in very low amounts and this did not influence the organoleptic nature of the beverage. Generally acetic acid has a flavor like vinegar but in kefir this is not principal flavor (Magalhães et al. 2011).

2.7. Microbiology of Kefir Grains and Kefir Beverages

Kefir grains are rich in microbial content and looks like cauliflower in structure. Lactic acid bacteria, acetic acid bacteria, and yeasts are the three types of microorganisms that present in symbiotic interaction in kefir grains. (Magalhães et al. 2010).

Kefir beverages microbial composition is related to the presence of kefir grains. Kefir grains microorganisms release to the milk and begin to multiply by using the nutrients that are inside the milk which are lactose that is used as a carbon and energy source. It is expected that kefir grains and beverages have similar composition. But, kefir beverage is not suitable to do inoculum to make a new batch so kefir grains are needed to produce traditional kefir. As a starter, kefir grains should be used instead of mixture of pure cultures. İt is reported that the integrity of grains are very important to get the

effervescent character, typical yeast flavor and the creamy texture of kefir (Simova et al. 2002).

2.7.1. Lactic Acid Bacteria (LAB)

The primary LAB population, which makes about 65% to 80% of the overall microbial population, can be homofermentative or heterofermentative. Kefir grains generally contain *lactobacilli*, *lactococci* and *Leuconostoc* sp. (Gao et al. 2013).

LAB has the possibility to ferment carbohydrates to make lactic acid and are generally used in the fermented food and beverage industry for their probiotic functions. They can spoil macromolecular components of food by destroying indigestible polysaccharides and changing taste components. On the other hand, they can also make exopolysaccharides (EPS), amines, bacteriocins, and short-chain fatty acids during metabolism. By breaking down the protein in food, LAB can create a range of tiny molecule peptides or free amino acids. In the kefir culture one of the main group of *lactobacilli* has a strong spoiling effect on milk protein in the beverage (Dallas et al. 2016).

In beverages the LAB population (especially the lactobacilli) is higher than the yeast population according to some researchers (Ertekin and Guzel-Seydim 2010). But some other scientist found more *Lactococci* than *Lactobacilli* (Rea et al. 1996). The second major group was the *Leuconostoc* species in Irish beverage kefir. *Leuconostoc* are found less in milk and are in a relationship with *Lactococci* (Rea et al. 1996).

2.7.2. Acetic Acid Bacteria (AAB)

In kefir grains, the microbial population of AAB is 20% (Magalhães et al. 2011). AAB were not present in some studies and were considered as contaminants (Angulo, Lope, and Lema 1993). Some scientist reported, AAB can stimulate the growth of other organisms because they can produce vitamin B12 (Rea et al. 1996). The consistency of kefir can be improved by using a starter that contains AAB and this means the presence of AAB can be important for good kefir consistency and is a quality product (“Technology of Kefir and Kumys | Semantic Scholar” n.d.).

AAB are also found in kefir beverage but it is not always found in kefir beverage and sometimes are conceived as contaminants (Angulo, Lope, and Lema 1993).

There are species of bacteria that were found in kefir grains and kefir beverages. If you need it to put in order lactic acid bacteria found in kefir grains and beverages are *Lb. acidophilus, Lb. crispatus, Lb. helveticus, Lb. kefiranofaciens, Lb. kefiri, Lb. otakiensis, Lc. lactis* and *Leu. mesenteroides.* Acetic asid bacteria found in kefir grains and beverages are *A. lovaniensis, A. pasteurianus* and *A. syzygii.* Yeasts that are found in kefir grains and beverages are, *Kl. marxianus* and *S. cerevisiae* (Gao and Li, 2016).

2.7.3. Yeasts

Yeasts that are found in kefir grains can be either lactose fermanting or non-lactose fermenting (Simova et al. 2002). Generally there few yeasts than LAB. But in some grains there more yeasts than LAB (Zajsek and Goršek 2010). Yeasts are also found in kefir beverages as lactose or non-lactose fermenting beverages (Miguel et al. 2010).

2.8. Infrared Spectroscopy (IR)

Infrared spectroscopy is a method that measures wavelength and intensity of infrared light absorption of a specimen. The infrared light is powerful enough to raise the energy level of molecular vibrations (Putzig et al. 1994). The IR light is being used for three different purposes and the wavelengths are different in every region such as, the far-IR region is used for inorganic molecules at wavelength is between 400 – 30 cm^{-1} .The mid-IR region is used for molecular finger print studies at the wavelength 1400 – 400 cm^{-1}. The near-IR region is used for food applications in the wavelength 4000 – 1400 cm^{-1}.

At the far-IR region inorganic compounds are interpreted. At the mid-IR region, the basic absorption bands provide the molecular fingerprint. This region especially needed for simple structural analysis and detection of natural material ingredients or additives by comparing the library. The essential bands in the mid-IR region are combined to create absorption bands in the near-IR region. The near-IR region is very useful for food applications, such as determining moisture, fat, and protein levels.

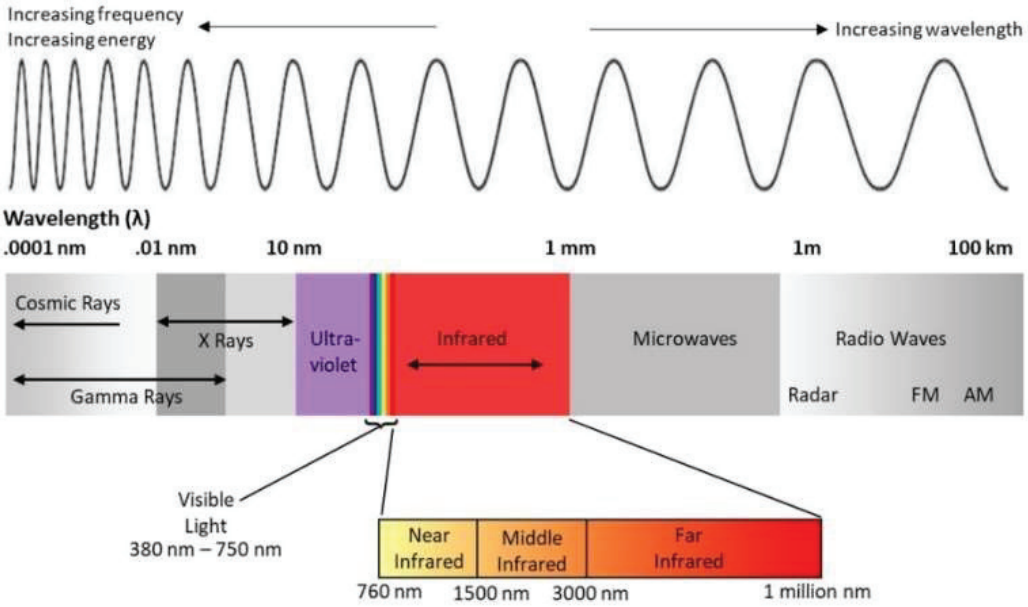


Figure 2. Electromagnetic Spectrum

(Source: "Near-Infrared (NIR) Light Sources for 3D Facial Recognition" n.d.)

2.8.1. Mid-IR Region

When mid-IR radiation is sent onto a sample, it will provide a spectral fingerprint to identify the sample's content. The sample receives mid-IR radiation, which is absorbed at various frequencies depending on the chemical makeup of the sample, to produce a mid-IR spectrum. As a result, a mid-IR spectrum's peaks and troughs strongly depend on the sample being examined. In the food and petrochemical industries, this analysis approach can be used to detect false or impure products. The overtone and combination bands produced with near-IR and visible light have lower intensity and more complicated spectra than the basic bands measured with mid-IR, which is advantageous for these and many other applications. ("Why Choose Mid-IR Spectroscopy? | Monospektra - Scientific Equipment and Industrial Solutions" n.d.).

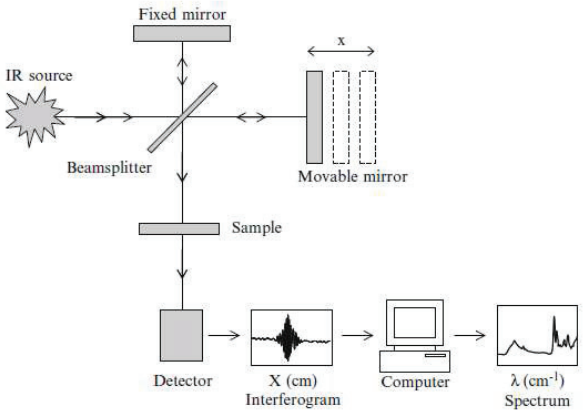


Figure 3. FTIR working principle

(Source: Ojeda and Dittrich, 2012)

2.9. Fourier Transform Infrared (FTIR) Spectroscopy

A sample is passed through by IR radiation during IR spectroscopy, which is a form of spectroscopy used in FTIR. The sample absorbs some of the IR radiation while transmitting some of it. The molecular absorption and transmission results in a spectrum, and this spectrum exhibits the sample's molecular fingerprint. (Table 3)

Molecular fingerprint of a sample means that every different molecular structure produces different infrared spectrum. A solid, liquid, or gas sample's IR absorption spectrum can be obtained using FTIR spectroscopy. High spectral resolution data throughout a broad spectrum range are simultaneously gathered by the FTIR spectrometer. Therefore, it is advantageous to simultaneously measure intensity in narrow wavelength ranges (Naumann, Helm, and Labischinski 1991).

The spectrum characteristics of cell constituents such fatty acids, membrane and intracellular proteins, polysaccharides, and nucleic acids are revealed by the strain-specific bacterial FTIR spectra (Mariey et al. 2001). The advantages of FTIR spectroscopy about microorganisms are, about the materials; it is fast and easy when compared with other methods, such as gun light screening and sequence analysis requires less time, low cost and only a small sample is required for the measurement. For the interpretation of the light series, multivariate statistical analysis is performed. It can be applied to microbiology to ascertain the make-up of bacteria and their cell components, taxonomic classification, the number of microorganisms present, process control, microbiological quality control, epidemiological research, and hygiene control. (Mouwen et al. 2005)

Table 1. Characteristics of IR bands.

Wavenumber (cm-1)	**Naumann,Helm, and Labischinski 1991**
3677.5	Hydroxyl (OH)
2988.12	v(C-H2) as (Fatty acids)
2927	v(C-H2) as (Fatty acids)
2344	Triple bonds (2500-1900 cm-1)
1700-1600	vC=O(carbonic acids and nucleic acids(1695))
	Amide-I from antiparallel B-sheets and B-turns of proteins (1685, 1675)
	Amide-I of α-helices of proteins (1655)
	Amide-I of B-sheets of proteins (1637)
1600-1500	Amide-II of proteins (1548)
	Tyrosine band (1515)
	Deformation heavy atoms (1500)
1394	v(C-O)s of COO-
	Amide-III of proteins
1250.8	v(P=O) as of PO2-
1066.35	C-O-C, C-O dominated by ring vibrations of carbohydrates C-O-P, P-O-P
1076.6	C-O-C, C-O dominated by ring vibrations of carbohydrates C-O-P, P-O-P
882.8	"Fingerprint" region

2.10. Attenuated Total Reflection (ATR)

As known, FTIR is well established laboratory-based technique. However, in order to generate high-quality spectra that can answer the users' inquiries, sample preparation can be tedious and time-consuming. ATR is a sampling technique that lets in light onto the sample to obtain high quality data of both liquid and solids. For FTIR Spectroscopy, ATR is one of the most commonly utilized sampling technologies. Due to the ubiquitous use of ATR it has the feasibility for the solid and liquid samples to be analyzed by making the measurement of virtually all substances easier. In ATR, the spectra can be measured without needing any sample preparation or dilution ("Attenuated Total Reflectance (ATR) | For FTIR Applications" n.d.).

When the sample meet a crystal, the light is sent into the crystal below a certain angle and this angle is known as the critical angle. The light passes through the crystal at an angle and collects on the other side (Figure 4). Information about the sample is revealed by an evanescent wave's contact with it. The inflection points of the light's propagation are where this standing wave known as the evanescent wave appears. It interacts with the sample and sends the chemical data to the detector along with it. The ability of ATR to give the user vibrational or chemical information about the target sample depends on this evanescent wave. This wave is controlled by a number of factors and enters the sample to a specific depth. Due of its connection to path length, penetration of depth is significant. Two factors influence the depth of penetration; ("Attenuated Total Reflectance (ATR) | For FTIR Applications" n.d.).

1. Wave number affects both penetration of depth and wave number. Throughout the entire spectrum that is being gathered, it is not constant. The lower your wavenumber, the greater the depth of penetration. Accordingly, when compared to the transmission experiment, peaks 400 wavenumbers lower will have a larger peak intensity than peaks at 4000 wavenumbers.
2. The difference between the refractive index of the crystal and the sample. A crystal like germanium with a higher refractive index will have a lower depth of penetration than something like diamond.

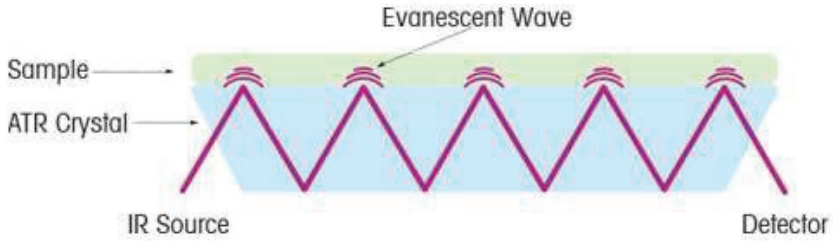


Figure 4. The principle of Attenuated Total Reflection ATR

(Source: "Attenuated Total Reflectance (ATR) | For FTIR Applications" n.d.)

2.11. Microbiome and Microbiota Studies

Single celled microorganisms are the first live micoroorganisms that were formed at least 3 billion years ago. When one says microorganism, firstly bacteria comes to ones mind but it is known that virus, fungi, alga, archaea and protozoa are also microorganisms. Some microorganisms can become dependant to multicellular organisms and they can live on or inside the host organism and these organisms are called microbiota. The microbiota that possesses the genetic material is called microbiome ("Microbiome" n.d.).

Microscopes and culture techniques have been the equipment of choice for scientists studying microbes in the laboratory for over a century. Researchers used microscopes to identify a microbe's shape and used culturing techniques to what microbes ate and what were the waste products they made. In these days it is known that the big majority of the Earth's microbiome is not able to be grown in a laboratory. In 1990s the gene sequencing technology emerged that read the microbial DNA. Scientists may now investigate and identify the "uncultured majority" of microorganisms in their natural settings. Sequencing a marker, that researchers can identify a microbe without sequencing its full genome, is a common technique. This technique shows the researchers that they can identify all the species in a short time that are present in a large number of samples. Gene sequencing technologies still have some limitations like they tell us which microbe is in the sample but doesn't always tell what they are doing. To learn the biological function, a study emerged called metagenomics. Metagenome means that the whole genome of every microbe in a sample that was taken from the habitat. 10,000 microbial genomes can be sequenced in a single experiment and when all the genes are analyzed in a taken sample researchers can learn what biological jobs these microbes do ("The

Science Behind The Microbiome | Kavli Foundation" n.d.). 16S ribosmal RNA (rRNA) gene is the preferred gene for the microbiota studies.

2.12. 16S rRNA

As known, ribosomes are found in all live cells and plays an important role in protein synthesis. Ribosomes contain two subunits, and both subunits contain proteins and ribosomal RNA (rRNA). Prokaryotes have a 70S ribosome, which is divided into two subunits: a small one known as the 30S subunit and a larger one known as the 50S subunit. While the 50S subunit has 23S and 5S rRNAs, the 30S subunit only possesses 16S rRNA. (Table 4) ("16S Ribosomal RNA Sequencing (Theory) : Microbiology Virtual Lab II : Biotechnology and Biomedical Engineering : Amrita Vishwa Vidyapeetham Virtual Lab" n.d.).

16S rRNA contain hypervariable regions that is useful to identify bacteria because that region is specific to each specie. Sequencing of 16S Ribosomal RNA is used for diversity identification in microorganisms to investigate phylogenetic studies.

Table 2. Ribosome of a Bacterium (Source: "Ribosome" n.d.)

Ribosome	Subunit	rRNAs
70S	50S	23S
		5S
	30S	16S

There are some of the benefits of employing ribosomal RNA in molecular techniques such as, there are ribosomes and rRNAs in all type of cells, in nature, RNA genes are remarkably conserved, and the sequencing approaches do not include microbial cell culture ("16S Ribosomal RNA Sequencing (Theory) : Microbiology Virtual Lab II : Biotechnology and Biomedical Engineering : Amrita Vishwa Vidyapeetham Virtual Lab" n.d.).

2.13. 16S rRNA Sequencing

16S rRNA sequencing is used for classification, identification and quantitation of all the microorganisms in a microbiota. Classification of the microorganisms are done in

the genus and species level. The 16S rRNA gene is form of all living creature's transcription mechanisms highly conserved component and therefore sequencing DNA samples that have thousands of different species are appropriate to be a target gene. Universal primers are designed to amplify the target conserved region of the 16S rRNA gene, enabling the gene to be amplified in numerous bacteria from a single sample. In figure 5, it is shown that the 16S rRNA gene has both conserved and variable sections. While the conserved region allows for universal amplification, sequencing the variable portions enables for differentiation across microorganisms like bacteria, archaea, and microbial eukarya. To identify a virus, metagenomics sequencing must be done because, in viruses there are no 16S marker gene (Cox, Cookson, and Moffatt 2013).

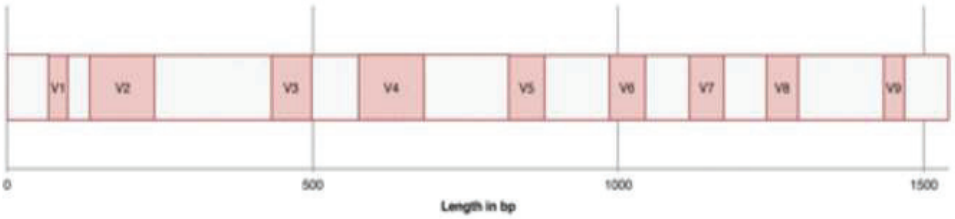


Figure 5. The E.coli 16S rRNA gene is approximately 1.5 kb in length, with nine variable regions that make it a good target for use as a phylogenetic marker gene

(Source: Cox, Cookson, and Moffatt, 2013).

2.14. 16s rRNA Next Generation Sequencing and Microbiota Composition Determination

In microbiota studies based on 16S rRNA gene the specific regions such as V1-V2 and V3-V4 are targeted by specific primers. For example, the forward primer Y1 (5'-TGGCTCAGGACGAACGCTGGCGGC-3'), the reverse primer Y2 (5'-CCTACTGCTGCCTCCCGTAGGAGT-3'), are used for V1-V2 region (Nalbantoglu et al. 2014). On the other hand, the forward primer Y3 (5'-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG-3') and the reverse primer Y4 (5'-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC-3') are generally used for the V3-V4 region (Castellanos-Rozo et al. 2020). For microbiota studies Next Generation Sequencing is usually performed by Illumina MiSeq, Illumina HiSeq, 454 (Roche) GS FLX+, 454 (Roche) GS Junior, SOLiD 5500xl, SOLİD

5500, Ion Torrent Ion Proton, Ion Torrent Ion PGM and Pacific Biosciences sequencer platforms (Song et al. 2016). The comparison of the sequencing platforms are like the following; the most commonly used sequencing platforms are Ion Torrent, SOLID, 454 (Roche), Illumina and Pacific Biosciences. Maximum read length ranges from 60 bp (SOLID) to 40 kbp (Pacific Bioscience). Run time ranges from a few hours to several days. The highest read accuracy is of 99.9 is reported for SOLID, 454 (Roche) and Illumina (Song et al. 2016).

After Sequencing is performed, the data in FASTQ format is obtained. The biological sequence is presented here together with the matching quality scores in text format. After obtaining the FASTQ files from the sequencer the quality of the reads is checked. There might be different reasons why the quality of the reads is bad. For instance, maybe good primer was not used, or the polymerase was expired or something else happened during the sequencing, the sequencing machine did some mistakes. So, there can be different reasons why the quality of the bases is not very good. The information is in the FASTQ files. To look and visualize the files there is a software called FASTQC. This software aims to do quality control checks on raw sequence data coming from the sequencer. Then QIIME2 (Bolyen et al. 2019) or Mothur (Schloss et al. 2009) is used to do quality trimming or data cleaning. After the data cleaning step taxonomic assignment of targeted 16S rRNA amplicon sequences is performed by QIIME2. Taxonomic assignment is the process of determining which taxa belong to the cleaned raw sequence data obtained. At the taxonomic determination stage, the experimentally obtained sequences are compared with the sequences of the taxa found in the databases, in the three most used reference databases which are GreenGenes (Mcdonald et al. 2012) Ribosomal Database Project (RDP) (Cole et al. 2008) and Silva (Quast et al. 2013).

After obtaining taxonomic assignments in microbiota studies, alpha and beta diversity index calculations are used to compare the samples with each other in terms of taxonomic content. Alpha diversity tries to determine what species are present in a particular environment. So, this measure of diversity is within one sample indicating how diverse one sample is. Beta diversity is how different are two communities. So, this diversity measurement is used for between sample comparisons. There are some common alpha diversity indices (“The Use and Types of Alpha-Diversity Metrics in Microbial NGS - CD Genomics” n.d.) which are,

1. Chao Index: There are two types of Chao Index which are Chao 1 and Chao 2. Chao 1 is an estimator based on abundance. Chao 2 is an estimator based on the incidence. Chao 1's index equation is given below:

$$S_1 = S_{obs} + \frac{F_1^2}{2F_2} \text{……...……..……..2.1}$$

S_{obs}= number of observed species
F1= Number of Singletons (are species that were seen once in the sample)
F2= Number of Doubletons (the number of species that are exactly seen twice in the sample)

2. Simpson Index: Takes into account that the species richness and evenness of an ecosystem and assigning a number to represent the biodiversity. Simpsons Reciprocal Index equation:

$$DI = \frac{N(N-1)}{\sum n(n-1)} \text{…….……..……..2.2}$$

N= Total number of organisms
n= Population of each individual species
D= Diversity Index (Higher means more diverse)

3. Shannon Index: The Shannon index is used in ecological literature as one of the most widely used diversity indices. This metric is based on the idea that it becomes harder to predict which letter will come next the more varied the letters are within a string of interest and the more uniformly their proportionate abundances are dispersed. The following equation is used to calculate it:

$$\text{Shannon Index (H)} = -\sum_{i=1}^{s} pi \ln pi \text{……2.3}$$

H= Value for Diversity
s= The total number of species in community
p= is the proportion (n/N) of individuals of one particular species found (n) divided by the total number of individuals found (N)

4. ACE Index: The Abundance-based coverage estimators (ACE) index, which uses an arbitrary abundance threshold to designate S abun as the number of abundant taxa and Srare as the number of rare taxa, is a diversity metric. The following equation is used to calculate it:

$$R = S_0 + a_0 \text{.....2.4}$$

.

Essentially, this equation inflates the number of rare taxa while also inflating the number of taxa using abundance 1.

5. Good's Coverage Index: Another alpha diversity estimator is Good's Coverage Index, which is calculated using the following equation:

$$1 - \frac{F_1}{N} \text{.....2.5}$$

.

N= The total number of individuals or the sum of abundances for all OTUs

F1=The number of singleton OTUs

CHAPTER 3.

MATERIALS AND METHODS

3.1. Materials

3.1.1. Kefir

5 different milk kefir commercial beverage samples were purchased. Milk kefir samples were used subsequently for microbial enumeration, FTIR analysis, and molecular microbial analysis.

Chemical and nutritional properties such as protein, fat, saturated fat, carbohydrate, sugar, salt, vitamin (B2, B12) and mineral (calcium, phosphor) content measured are given in Table 6.

As seen in table 6, Kefir 1 has the highest carbohydrate and protein content compared to all samples but has the lowest sugar content. Kefir 2 and Kefir 3 samples have almost the same nutritional and chemical properties. Kefir samples Kefir 1, Kefir 2 and Kefir 3 contain significantly more fat and saturated fat than the samples Kefir 4 and Kefir 5. Kefir 5 has the highest sugar content compared to all samples, and higher carbohydrate content in comparison with the Kefir 2, Kefir 3 and Kefir 4.

Table 3. Some nutritional and chemical properties of commercial milk kefir samples.

	Kefir Samples				
	Kefir 1	**Kefir 2**	**Kefir 3**	**Kefir 4**	**Kefir 5**
Energy and Nutrients	**For 100g**	**For 100 ml**	**For 100 ml**	**For 100 ml**	**100 ml**
Energy (kJ/kcal)	263/63	217/52	185/44	181/43	188/45
Fat (g)	3.2	3.2	2.3	2.5	1.7
Saturated Fat (g)	2.2	2.1	1.3	1.6	1.0
Carbohydrates (g)	5.2	3.2	3.2	2.4	4.7
Sugar (g)	2.3*	3.2	3.2	2.4	4.7*
Protein (g)	3.3	2.7	2.7	2.8	2.8
Salt (g)	0.2	0	0	0	0
Calcium (mg)	120	ND	ND	97	120
B12 (mg)	0.4	ND	ND	ND	ND
B2 (mg)	0.2	ND	ND	ND	ND
Phosphorus	106	ND	ND	ND	ND

* Sugar is due to the inherent factor of the product. It does not contain added sugar.

** Not determined.
*** (Kefir-1, KB), (Kefir-2, KE), (Kefir-3, KD), (Kefir-4, KC), (Kefir-5, KA)

3.2. Methods

3.2.1. Cultural Microbial Analysis

Commercial milk kefir samples of 5 different brands were microbiologically analyzed on the first day when opened and the seventh days of refrigeration storage according to the standard methods given in the following sections.

3.2.1.1. Total Bacteria, Coliform, and E. coli Count

Total aerobic mesophilic bacteria are count on plate count agar (PCA). According to aseptic conditions, 1 ml of the sample was taken and homogenized in 9 ml of sterile distilled water and decimal serial dilutions was done up to 10^{-7}. 0.1 ml of inoculum was inoculated onto the PCA medium and was spread and incubated at 30 °C for 2-3 days. Colonies that were count and were multiplied by the dilution rates and by doing this total live being was calculated of 1 ml milk kefir. Counts was done in three parallels and the results was given as mean ± standard deviation (ISO 1998, 2003).

To do total coliform count, MPN method (three tube method) was used. 1 ml of each dilution was inoculated into tubes containing Durham tube and 10 ml of Lauryl Sulfate Tryptose Broth (LSTB) medium and incubated at 35 ± 2°C for 48 hours. Tube which gas is observed after incubation was evaluated as a positive result. From LSTB tubes where the gas formation is observed, 10 mL Brillant Green Lactose Broth (BGLB) tubes with durham tube were inoculated with a loop and incubated at 35 ± 1°C for 48 hours. Tubes that gas is observed evaluated as a positive result for coliform bacteria. The number of coliforms determined by making numerical evaluation according to the MPN table and the results expressed as MPN/mL (Feng et al., 2002; ISO 2010).

3.2.1.2. Total Yeast Count

In total yeast count, plating was done by the spreading method on 1% oxytetracycline OGYE medium (pH 7.0 ± 0.2). 10 g of the sample was taken by aseptic conditions and homogenized in 90 ml of mycological sterile peptone water and a decimal

serial dilution up to 10^{-3} was prepared. 0.1 ml of inoculum was transferred to the medium for yeast count and was plated according to standard spread method and cultivated media were left for incubation for 5 days at 25°C. The yeast colonies were counted and multiplied by the dilution rates and the number of yeasts in 1 ml of kefir milk drink were calculated. Yeast count was made with three parallel and the results were given as mean standard deviation (ISO 1992; ISO, 2007).

3.2.1.3. Total Lactic Acid Bacteria, Lactobacilli and Lactococci Count

Man, Rogosa and Sharpe (MRS) Agar for *Lactobacillus and Bifidobacterium* species, M17 Agar for *Streptococcus and Lactococcus* species were used to count and isolate lactic acid bacteria from commercially purchased kefir samples. Cycloheximide (200 mg/L) sterilized by filtration was added to these media before microbiological planting to prevent mold and yeast growth. According to aseptic conditions, 10 g of kefir samples were taken and homogenized in 90 ml sterile % ($^{w}/_{v}$) peptone solution and a decimal serial dilution up to 10^{-6} was prepared. 1 ml of inoculum were transferred to MRS and *Laactobacilli* M17 agar medium for enumeration of lactic acid bacteria. According to bulk plate method planting was done and after the medium was solidified, it was left for incubation in an anaerobic jar with a gas pack (Anaerocult). MRS agar was incubated at 37°C and M17 Agar at 30°C (De Man et al. 1960; Van de Casteele, 2006). After the incubation period, colonies were counted and multiplied by the dilution rates and the number of lactic acid bacteria in 1 ml of kefir milk drink were calculated. Five samples were plated as parallel and these samples were purchased from the same batch production and the obtained results were given as average standard deviation.

3.2.2. Molecular Microbial Analysis

All sequencing and bioinformatics analyses were performed with service procurement. Briefly, the following steps were followed.

3.2.2.1. Bacterial Genomic DNA Extraction and 16S rRNA Library preparation

The EurX GeneMATRIX Tissue & Bacterial DNA Purification kit was used to isolate the samples' DNA according to the manufacturer's instructions.

The amount and quality of the isolated DNA were measured on the Victor3 fluorometer device using PicoGreen dye. An average of 50 ng/uL DNA was obtained from the samples.

The V3 and V4 regions of the 16S ribosomal RNA gene, which is the most frequently used variable region for species identification, (Klindworth et al. 2013) were targeted with the following primers Table 7. This primer pair amplified a region of approximately 460 bases. After cleaning the PCR products, Next Generation pre-sequencing libraries were created using the Nextera XT Index kit.

Table 4. Primers (Source: Klindworth et al. 2013)

Region	Sequence
16S Amplicon PCR Forward Primer	5' – TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG -3’
16S Amplicon PCR Reverse Primer	5' –GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC-3’

3.2.2.2. Next Generation Sequencing and Bioinformatic Analysis

The Illumina MiSeq device was used to sequence the libraries made from the samples. The reading images were processed and Real Time Analysis with the MiSeq Control Software v2.2 program bases were determined using the v1.18 program. Base reads were converted to FASTQ format with the bcl2fastq (v1.8.4) program. Short read results from 36 bases were excluded from the analysis for a clean data analysis.

Taxa identifications from phylum to species level and species diversity analyzes were made with the QIIME2 program (Bolyen et al. 2019). Within species the alpha diversity is calculated by Shannon, Simpson, and Chao indexes.

3.2.3. Fourier Transform-Infrared (FTIR) Spectroscopy Analysis

The commercially obtained kefir samples (day1, day2, day3, day7, day10) were evaluated by the FTIR spectroscopy GX Optica Perkin-Elmer supplied with an attenuated total reflectance (ATR) apparatus.

Before placing kefir sample on ZnSe crystal as a thin layer the crystal was cleaned by using dry paper with ethanol, and ultrapure water after each assessment and dried. First in FTIR-ATR spectrum ultra-pure water was used as a background. Secondly, as background the room air FTIR-ATR spectrum was used to evaluate instrumental conditions and H_2O interferences. In analysis, the spectra of the samples were taken between 4000 and 400 cm^{-1} wavelengths. Each sample was scanned 20 times at 4 cm^{-1} resolution at room temperature. Data were displayed and compared using Perkin Elmer Spectrum (version 10.4.3) software after acquiring the spectra. Statistical programs were used to evaluate obtained absorbance values.

3.2.4. Statistical Analysis

Three separate series of trials were conducted. On the first, second, third, seventh, and tenth days that the samples were kept in the refrigerator, each assay was performed twice. SPSS 16.0 was used to statistically analyze the outcomes (SPSS Inc., Chicago, IL). ANOVA was used to assess the significance of variance differences at a level of $P<0.05$. Means and standard deviations were calculated and the Duncan's multiple range test was employed to differentiate means of the treatments.

3.2.5. Principal Component Analysis (PCA)

Using R 3.5.0 and the stats (v3.6.2) package, principal component analysis (PCA) was carried out. The 'prcomp' function was used to examine baseline corrected, centered, and scaled FTIR data. To illustrate the differences and similarities across samples, spectral data were used. Principal component (PC) scatter plots in two dimensions (2D) were used to display the findings.

CHAPTER 4

RESULTS AND DISCUSSION

4.1. Cultural Microbial Analysis

Commercial kefir drinks were analyzed for microbiological attributes in accordance with the recommendations of *Codex Alimentarius* for fermented milk products (Codex Stan 243-2003). Average total aerobic mesophilic bacteria, lactic acid bacteria, *Lactobacilli*, *Lactococci*, yeast, coliforms and *E. coli* counts of five different commercial kefir beverage samples were enumerated and results were given as Table 8.

For five milk kefir samples mould, coliforms and *E. coli* were not determined. As can be seen from Table 8, the bacterial content of the kefir beverage samples differed from 7.086 to 8.794 $\log_{10}$ cfu.ml^{-1} for viable total aerobic mesophilic bacteria (TAMB), 6.792 and 8.382 $\log_{10}$ cfu.ml^{-1} for lactic bacteria (LAB), <10 and 6.322 $\log_{10}$ cfu.ml^{-1} for *Lactobacillus*, 5.857 and 8.146 $\log_{10}$ cfu.ml^{-1} *Lactococcus*, 5.176 and 7.218 $\log_{10}$ cfu.ml^{-1} for yeasts.

Samples had a number of total microorganisms of at least 10^7 colony-forming units cfu.ml^{-1} and a total yeast number not less than 10^4 cfu.ml^{-1}.

Lactic acid bacteria counts of milk kefir samples were among 6.792 $\log_{10}$ cfu.ml^{-1} and 8.382 $\log_{10}$ cfu.ml^{-1}. At day 1 and at day 7 the highest LAB counts were enumerated for the sample K1 7.839 $\log_{10}$ cfu.ml^{-1} and 8.382 $\log_{10}$ cfu.ml^{-1}, respectively.

Contradictorily, the highest yeast count was counted for the K2 kefir sample at the 1st day of storage (6.833 $\log_{10}$ cfu.ml^{-1}) and K4 kefir sample at the 7th days of storage (7.218 $\log_{10}$ cfu.ml^{-1}).

All kefir brands contain lactic kefir culture contents and kefir yeast and contain at least 1.0x10^6 cfu.ml^{-1} viable microorganisms. Although brand Kefir 4 (KC) has claim on the packaging that contains *Bifidobacterium* and *Lactobacillus acidophilus* at least 1.0x10^6 cfu.ml^{-1} no *Lactobacilli* were detected. This result also confirmed by the sequencing data that is no *Bifidobacterium* was detected in Kefir 4 (KC). Similarly, sequencing data showed that K1 (KB), K3 (KD) and K4 (KC) kefir samples were not including *Lactobacillus acidophilus.*

Table 5. TAM, LAB, yeast, Lactobacilli, Lactococci (cfu.ml^{-1}), coliforms and E. coli (MPN/ml^{-1}) counts of commercial milk kefir samples.

Sample Code		TAM count (cfu.ml^{-1})	LAB count (cfu.ml^{-1})	*Lactobacilli* count (cfu.ml^{-1})	*Lactococci* count (cfu.ml^{-1})	Yeasts count (cfu.ml^{-1})	Mould count (cfu.ml^{-1})	Coliform count (MPN.ml^{-1})	*E. coli* count (MPN.ml^{-1})
K1	Day1	2.85×10^7 (7.455±0.15)	6.9×10^7 (7.839±0.45)	1.40×10^5 (5.146±0.20)	1.1×10^7 (7.041±0.15)	2.5×10^6 (6.398±0.24)	Absent	Absent	Absent
	Day7	6.22×10^8 (8.794±0.30)	2.41×10^8 (8.382±0.06)	5.15×10^5 (5.712±0.70)	1.4×10^8 (8.146±0.20)	1.8×10^6 (6.255±0.61)	Absent	Absent	Absent
K2	Day1	1.22×10^7 (7.086±0.02)	6.2×10^6 (6.792±0.03)	1.0×10^3 (3.00±0.80)	1.25×10^7 (7.097±0.00)	6.8×10^6 (6.833±0.83)	Absent	Absent	Absent
	Day7	8×10^7 (7.903±0.13)	3.35×10^7 (7.525±0.29)	1.85×10^4 (4.267±0.02)	2.15×10^6 (6.332±0.05)	1.1×10^7 (7.041±0.84)	Absent	Absent	Absent
K3	Day1	4.4×10^7 (7.643±0.02)	3.96×10^7 (7.598±0.01)	3.1×10^4 (4.491±0.05)	6.5×10^7 (7.812±0.10)	1.5×10^5 (5.176±0.0)	Absent	Absent	Absent
	Day7	1.5×10^8 (8.176±0.01)	8.5×10^7 (7.929±0.01)	2.5×10^4 (4.398±0.70)	7.2×10^5 (5.857±0.05)	2.5×10^6 (6.398±0.29)	Absent	Absent	Absent
K4	Day1	9×10^7 (7.954±0.00)	5.9×10^7 (7.77±0.05)	<10	6×10^7 (7.845±0.002)	4.1×10^6 (5.613±0.35)	Absent	Absent	Absent
	Day7	2.5×10^8 (8.398±0.04)	1.1×10^8 (8.041±0.04)	<10	8.5×10^7 (7.9±0.01)	1.65×10^7 (7.218±0.02)	Absent	Absent	Absent
K5	Day1	6.5×10^7 (7.813±0.21)	1.11×10^7 (7.045±0.62)	2.1×10^6 (6.322±0.12)	3.6×10^7 (7.556±0.80)	9.6×10^5 (5.982±0.19)	Absent	Absent	Absent
	Day7	6.85×10^7 (7.836±0.01)	8.5×10^6 (6.929±0.64)	1.8×10^4 (4.255±0.04)	4.45×10^7 (7.648±1.10)	2.5×10^6 (6.398±0.82)	Absent	Absent	Absent

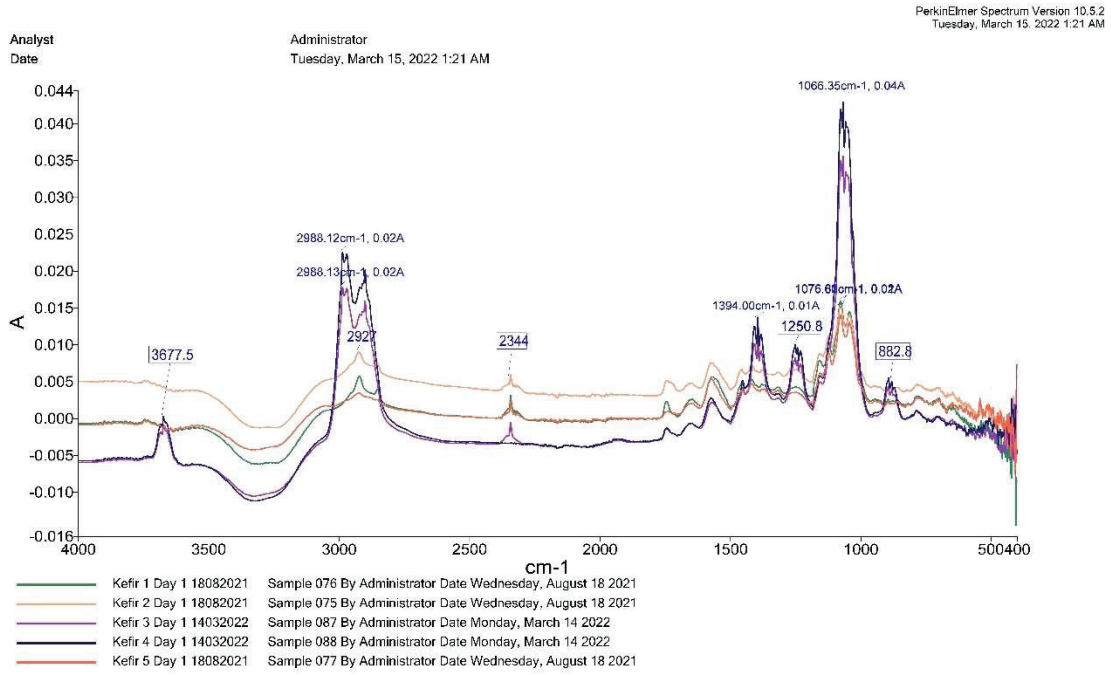
4.2. Comparison of Kefir samples with FTIR

The commercially obtained kefir samples were analyzed by the FTIR spectroscopy and spectra obtained for the 1st, 2nd, 3rd and 7th days while ultra-pure water (Figures 6-14) used as a background and for the 1st, 2nd, 3rd and 10th days the room air (Figures 15-18) were used as a background are given in Figures 15-18. The FTIR spectrum of a commercial kefir sample confirms the presence of carbohydrates, proteins, amino acids, fatty acids as well as organic acids.

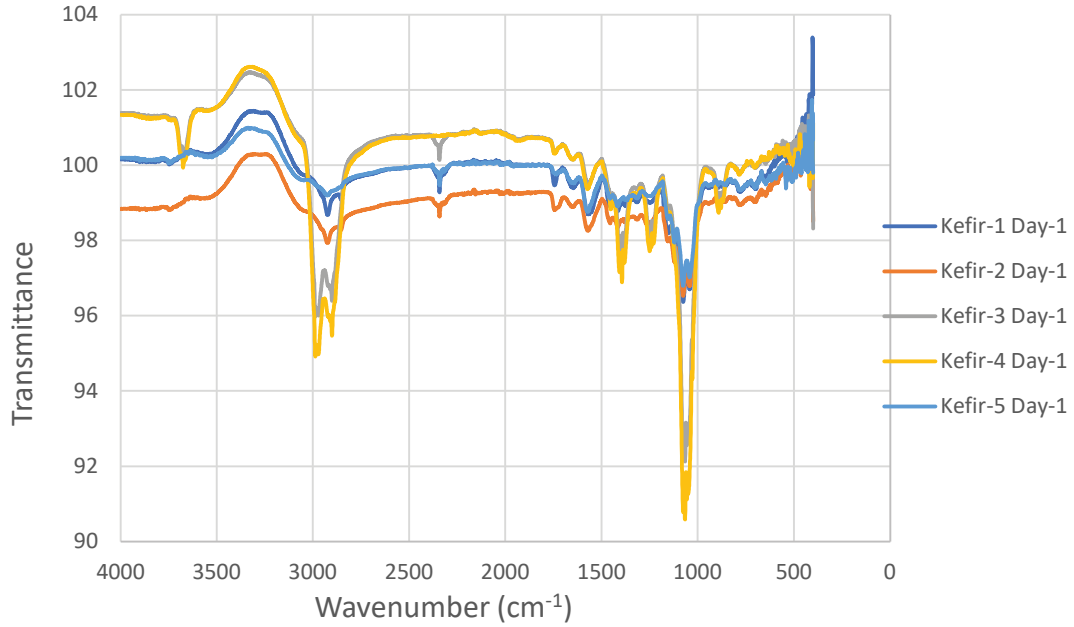
FTIR spectra obtained for kefir samples (1st, 2nd, 3rd and 7th) (Figure 6-14) have a broad peak at 2922-2988 cm^{-1} that can be ascribed to hydroxyl (-OH) groups stretching in associated with carbohydrate structures or hydrogen (-H) bonding stretching vibration of the complex carbohydrates such as polysaccharides. The peak between 3800 and 3200 cm^{-1} was assigned to the intramolecular -OH or intermolecular -H bonding stretching vibration of the polysaccharide whereas the weak absorption near 2924-2925.8 cm^{-1} was related with the both symmetrical and asymmetrical C–H stretching modes of aliphatic -CH_2. The peaks at 2915.49 cm^{-1} are associated with C-H bending in fatty acids, 1639 cm^{-1} fit to the carbonyl (C=O) stretching or amino (N-H) and methylidyne radical (C-H) bending vibration of the milk proteins (2924-2925.8 cm^{-1}). The region at 2920 and 2850 cm^{-1} may be due to the anti-symmetric and symmetric stretching of CH_2 groups from the fatty milk components. The comparatively strong peak in the region of 1700–1550 cm^{-1} was characteristic of polysaccharides and attributed to C-O stretching, indicating the presence of this functional group in the EPS (Singh et al. 2011). The peaks at 1548 and 1336 cm^{-1}, designated the typical absorption of -COOH or carboxylate ($RCOO^-$) groups similar to the polysaccharide produced by *B. animalis* RH and *B. licheniformis* (Shang et al. 2013; Singh et al. 2011). The existence of strong absorbance between 1450–850 cm^{-1} associated with the fingerprint region. Also, strong absorption band at 1161 and 1070 cm^{-1} validated the characteristics of the EPS obtained and proposed that the monosaccharide consisted in it had a pyranose ring. The additional peak at 836 cm^{-1} showed the presence of α-glycosidic linkages of the samples (Ye et al. 2009). Around 891 cm^{-1} absorption peak was found designating the presence of β-glycosidic linkage in the EPS (Coimbra et al. 2002). Similarly, EPS was produced by *L. kefiranofaciens*, *L. plantarum*, *S. thermophilus*, and by kefir grain contain β-glycosidic linkages (Kooiman 1968).

4.2.1. Comparison of Kefir Samples on Day-1

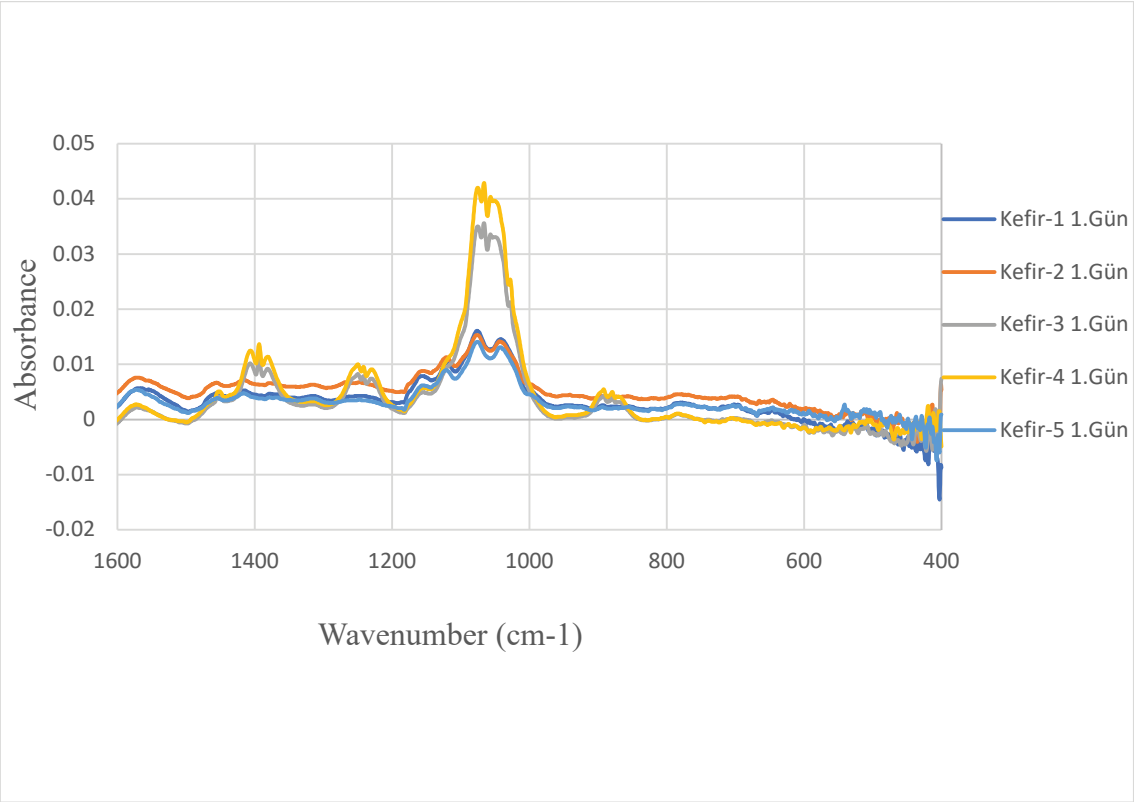
Figure 6a, 6b and 6c show the absorbance, transmittance and fingerprint spectra of the milk kefir samples on day-1, respectively.



(a)



(b)



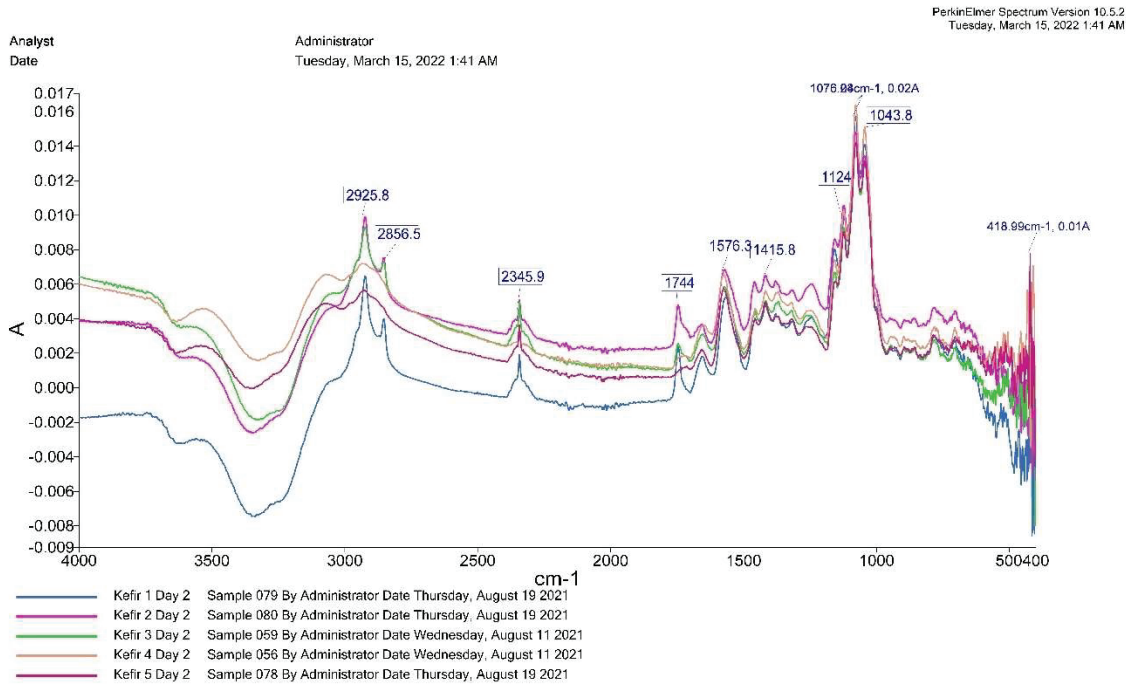
(c) Fingerprint Spectra

Figure 6. Comparison of Kefir samples on Day-1

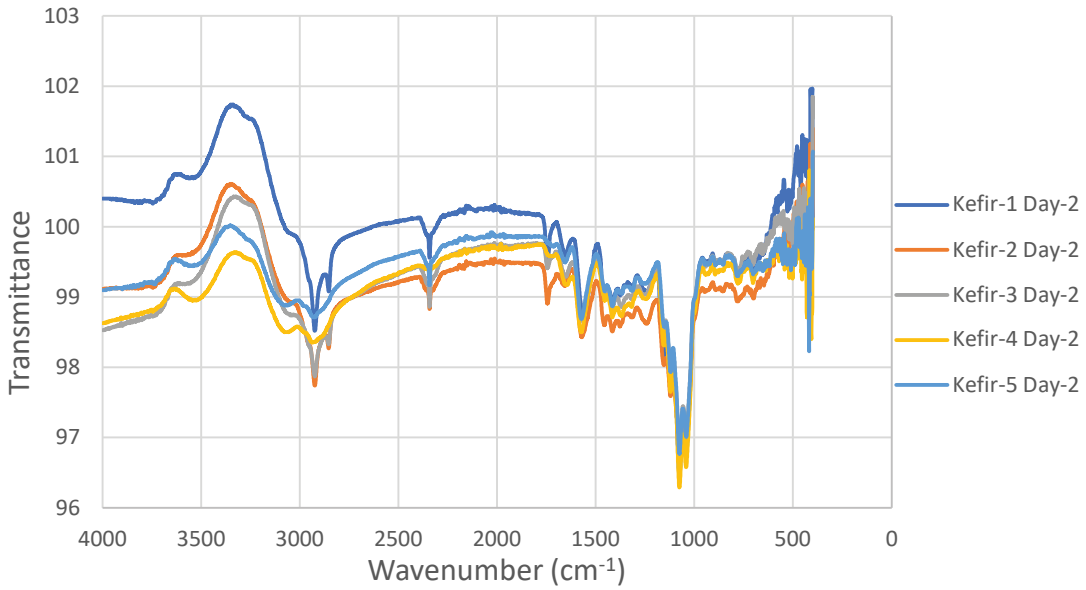
Variations in the bands assigned to polysaccharides (850–1450 cm^{-1}) revealed differences in the moieties of the carbohydrates of microbial cells in all five samples. Burgain et al. (2015) showed that a decrease in pH leads to physico-chemical changes in the external layer of peptidoglycans from the cell wall. The changes in the spectral fingerprints of polysaccharides were more pronounced in the older biofilms.

4.2.2. Comparison of Kefir Samples on Day-2

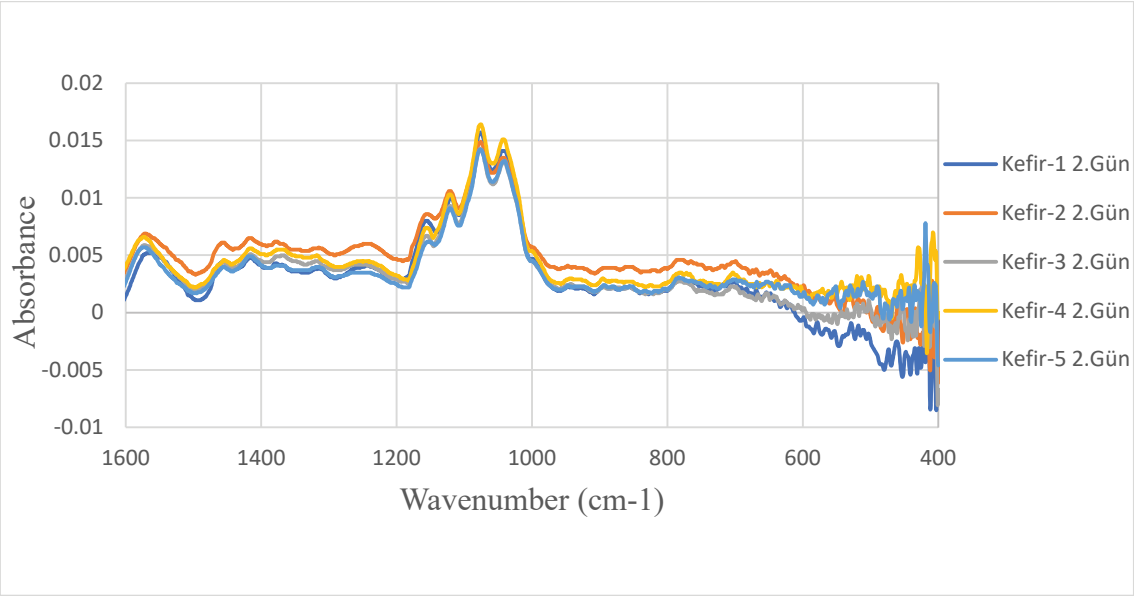
Figure 7a, 7b and 7c show the absorbance, transmittance and fingerprint spectra of the milk kefir samples on day-2, respectively.


PerkinElmer Spectrum Version 10.5.2
Tuesday, March 15, 2022 1:41 AM
Analyst
Administrator
Date
Tuesday, March 15, 2022 1:41 AM
2925.8
2856.5
2345.9
1744
1576.3
1415.8
1124
1043.8
418.99cm-1, 0.01A
A
cm-1
Kefir 1 Day 2 Sample 079 By Administrator Date Thursday, August 19 2021
Kefir 2 Day 2 Sample 080 By Administrator Date Thursday, August 19 2021
Kefir 3 Day 2 Sample 059 By Administrator Date Wednesday, August 11 2021
Kefir 4 Day 2 Sample 056 By Administrator Date Wednesday, August 11 2021
Kefir 5 Day 2 Sample 078 By Administrator Date Thursday, August 19 2021

(a)


Transmittance
Wavenumber (cm-1)
Kefir-1 Day-2
Kefir-2 Day-2
Kefir-3 Day-2
Kefir-4 Day-2
Kefir-5 Day-2

(b)



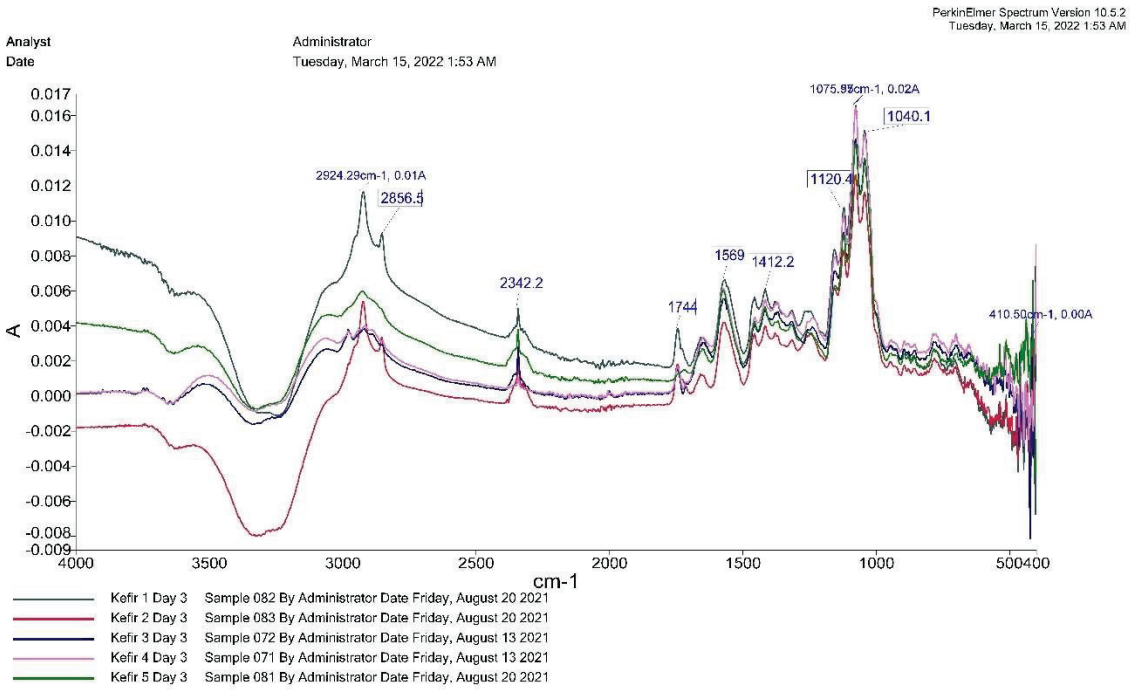
(c) Fingerprint Spectra

Figure 7. Comparison of Kefir Samples on Day-2

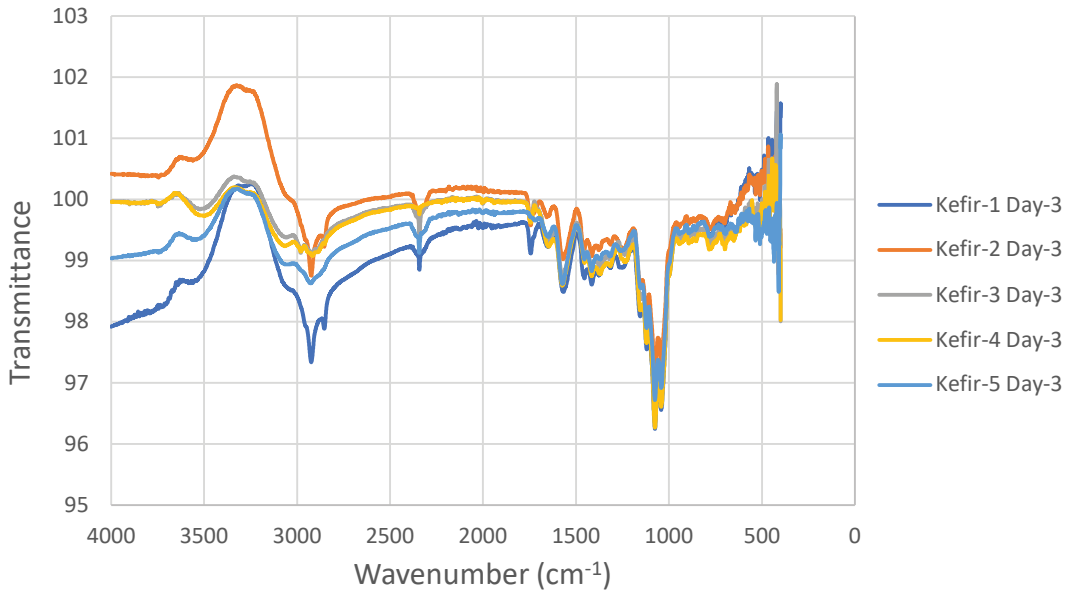
FTIR spectra are strain specific, and they reveal the characteristic features of all cellular components, such as fatty acids, membrane proteins, intracellular proteins, polysaccharides and nucleic acids. According to Naumann et al. (2001), for identification purposes, five spectral regions in IR spectra can be distinguished. They are called spectral 'windows': W1 (3000–2800 cm^{-1}) is the fatty acid region; W2 (1700–1500 cm^{-1}) contains the amide I and II bands of proteins and peptides; W3 (1500–1200 cm^{-1}) is a mixed region of fatty acid bending vibrations, proteins, and phosphate-carrying compounds; W4 (1200–900 cm^{-1}) contains absorption bands of the carbohydrates in microbial cell walls; W5 (900–700 cm^{-1}) is the 'fingerprint region' that contains weak but very unique absorbances that are characteristic of specific bacteria (Naumann et al., 1991).

4.2.3. Comparison of Kefir Samples on Day-3

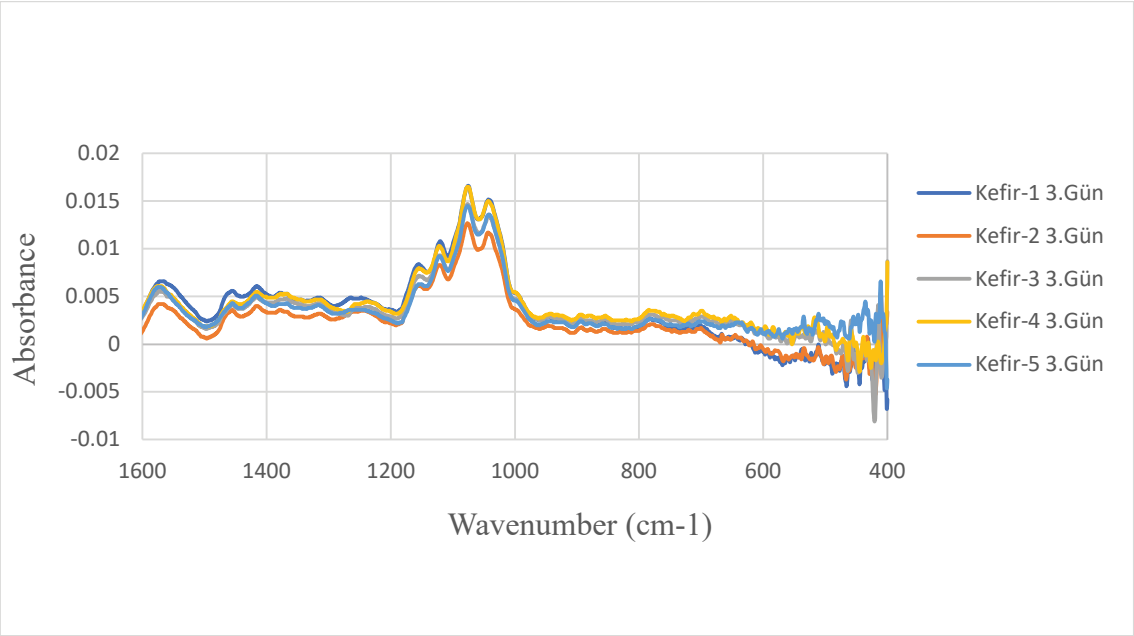
Figure 8a, 8b and 8c show the absorbance, transmittance and fingerprint spectra of the milk kefir samples on day-3, respectively.


PerkinElmer Spectrum Version 10.5.2
Tuesday, March 15, 2022 1:53 AM
Analyst
Administrator
Date
Tuesday, March 15, 2022 1:53 AM
2924.29cm-1, 0.01A
2856.5
2342.2
1744
1569
1412.2
1120.4
1075.93cm-1, 0.02A
1040.1
410.50cm-1, 0.00A
A
cm-1
Kefir 1 Day 3 Sample 082 By Administrator Date Friday, August 20 2021
Kefir 2 Day 3 Sample 083 By Administrator Date Friday, August 20 2021
Kefir 3 Day 3 Sample 072 By Administrator Date Friday, August 13 2021
Kefir 4 Day 3 Sample 071 By Administrator Date Friday, August 13 2021
Kefir 5 Day 3 Sample 081 By Administrator Date Friday, August 20 2021

(a)


Transmittance
Wavenumber (cm-1)
Kefir-1 Day-3
Kefir-2 Day-3
Kefir-3 Day-3
Kefir-4 Day-3
Kefir-5 Day-3

(b)

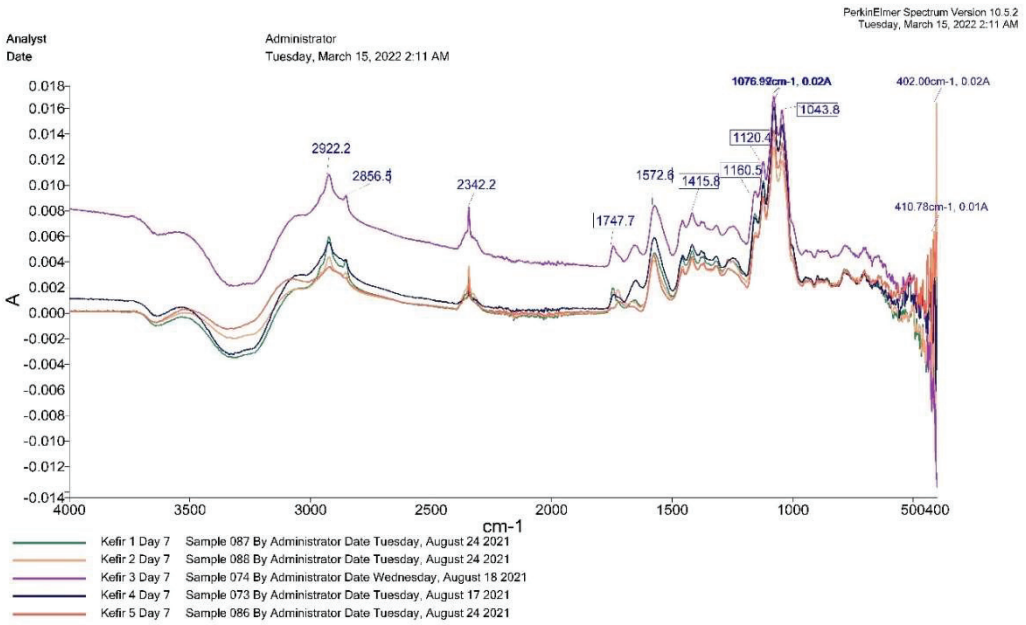


(c) Fingerprint Spectra

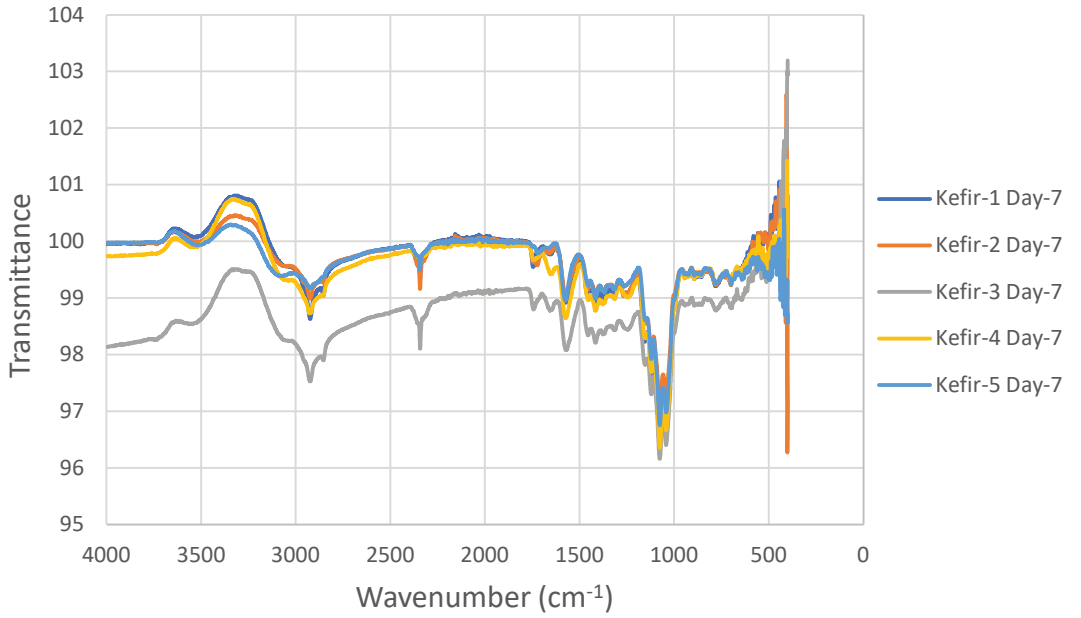
Figure 8. Comparison of Kefir Samples on Day-3

4.2.4. Comparison of Kefir Samples on Day-7

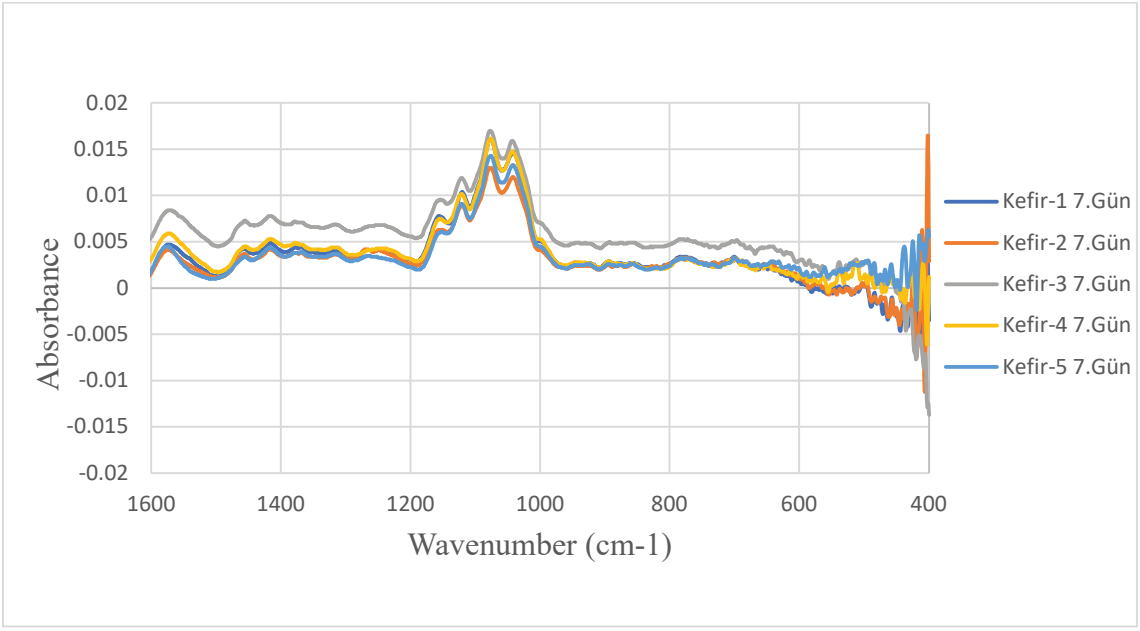
Figure 9a, 9b and 9c show the absorbance, transmittance and fingerprint spectra of the milk kefir samples on day-7, respectively.



(a)



(b)



(c) Fingerprint Spectra

Figure 9. Comparison of Kefir Samples on Day-7

4.2.5. Comparison of Kefir-1 with Days

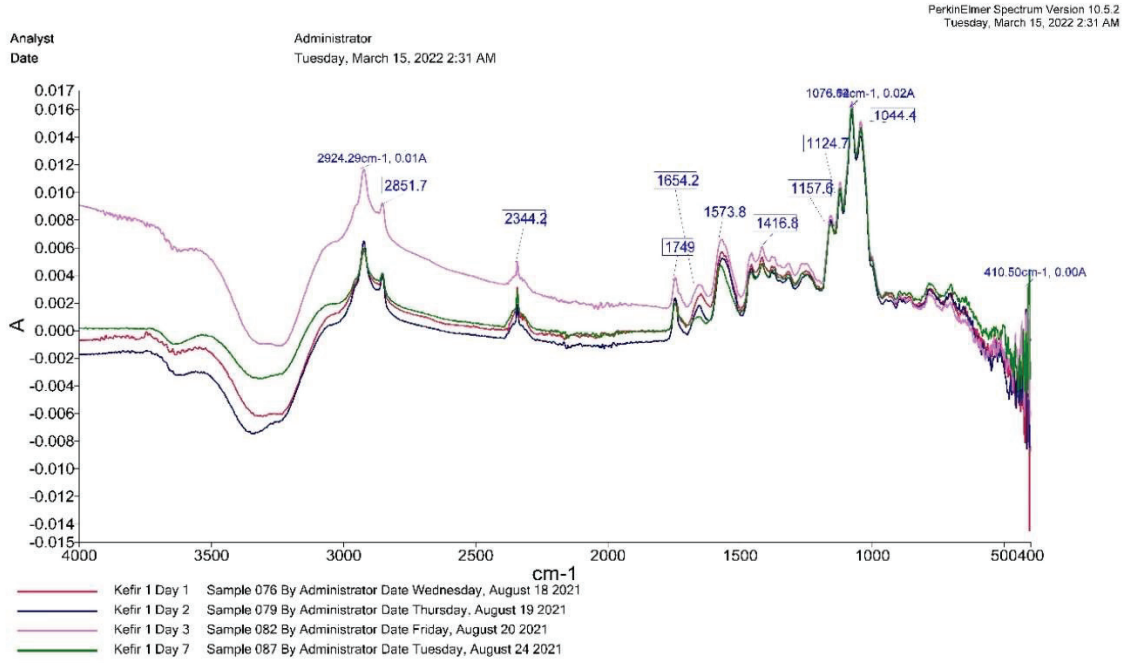


Figure 10. Comparison of Kefir-1 with Days

4.2.6. Comparison of Kefir-2 with Days

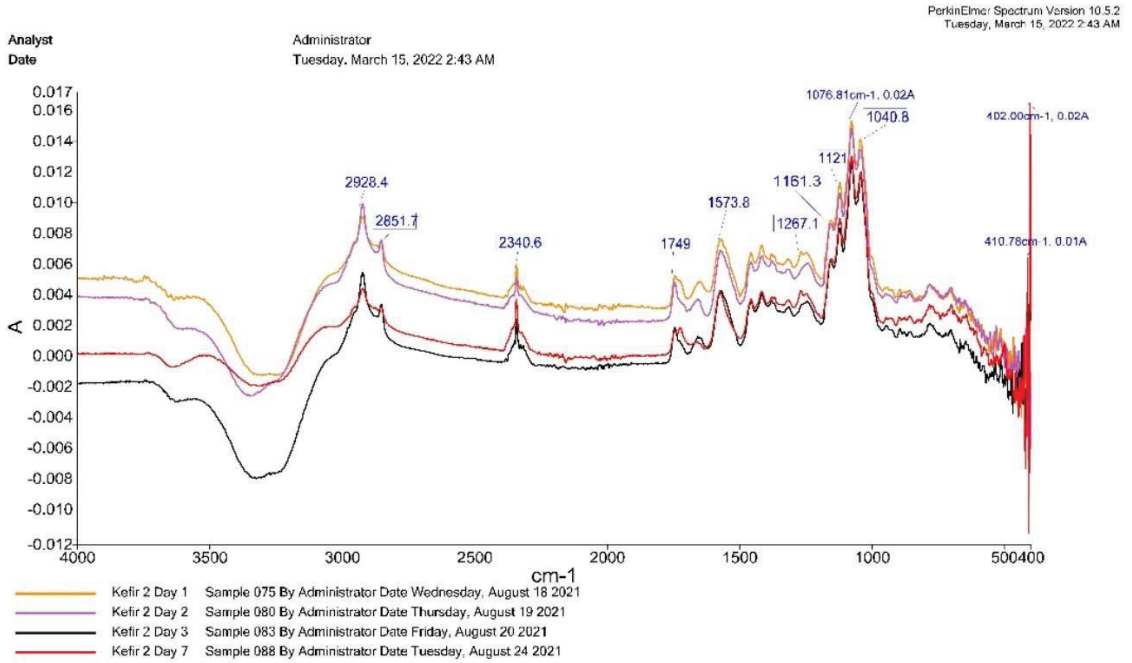


Figure 11. Comparison of Kefir-2 with Days

4.2.7. Comparison of Kefir-3 with Days

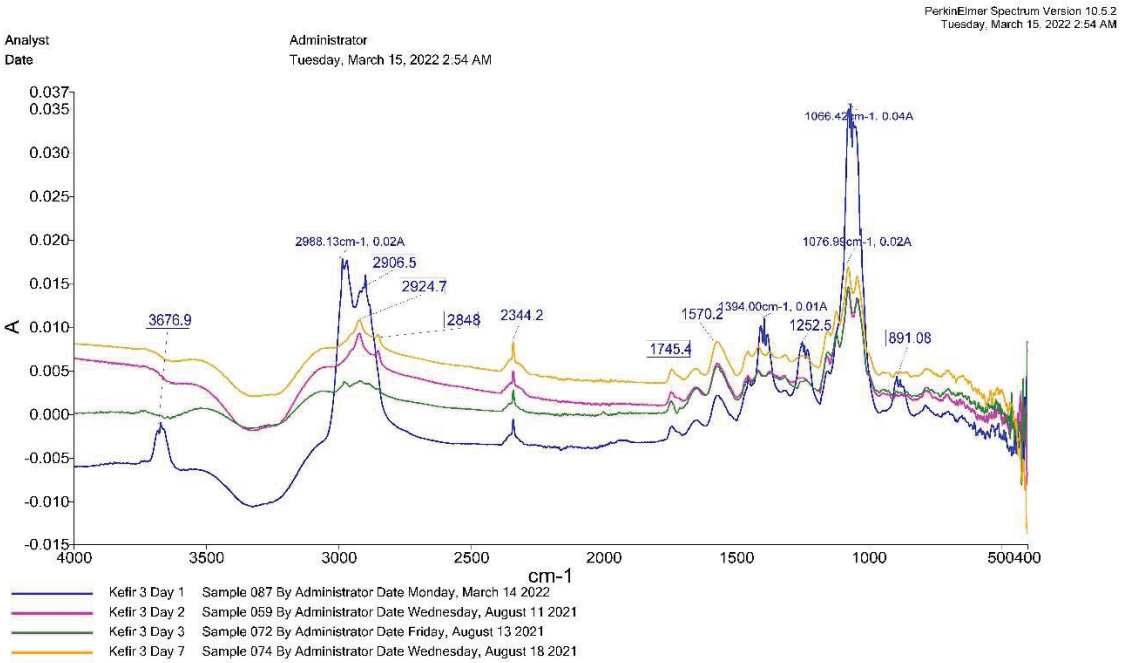


Figure 12. Comparison of Kefir-3 with Days

4.2.8. Comparison of Kefir-4 with Days

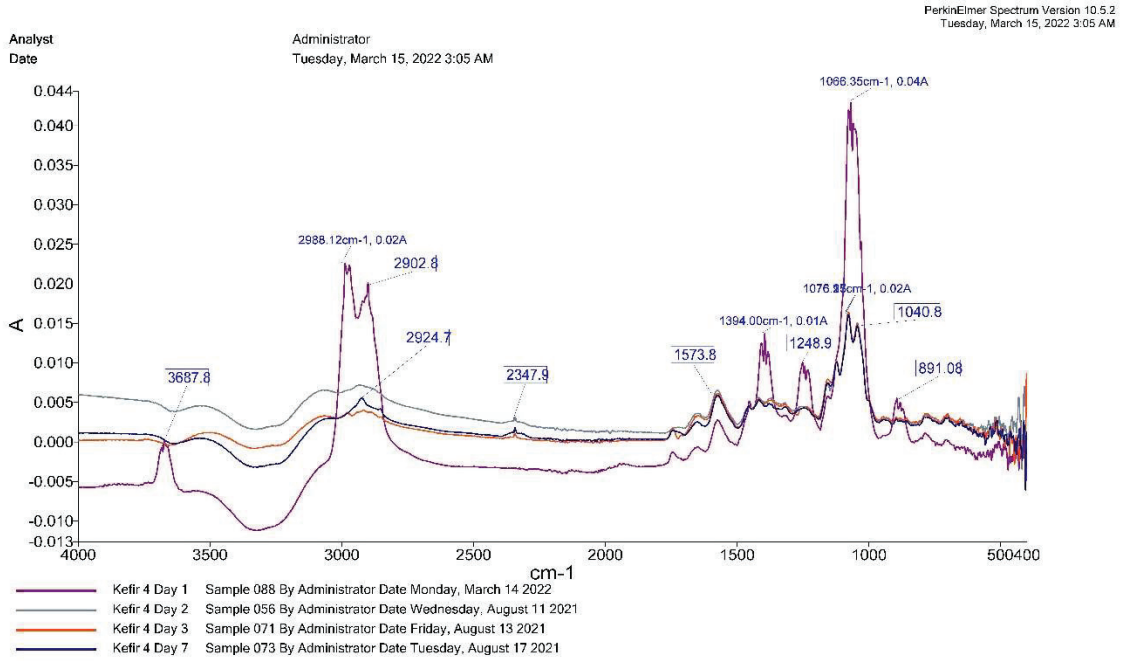


Figure 13. Comparison of Kefir-4 with Days

4.2.9. Comparison of Kefir-5 with Days

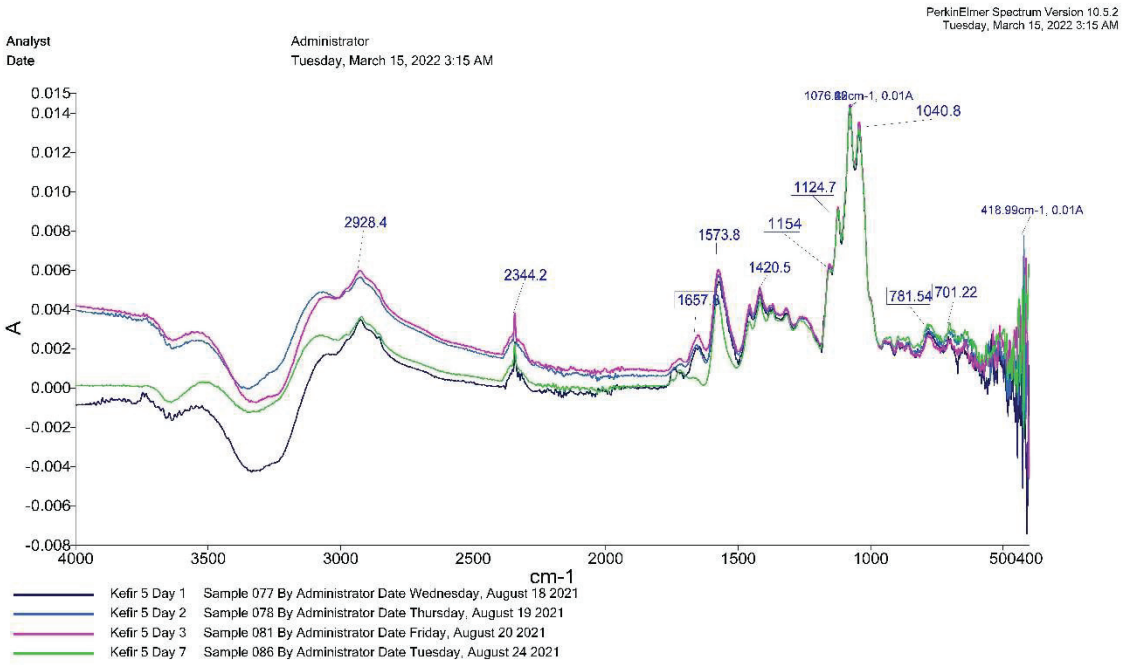


Figure 14. Comparison of Kefir-5 with Days

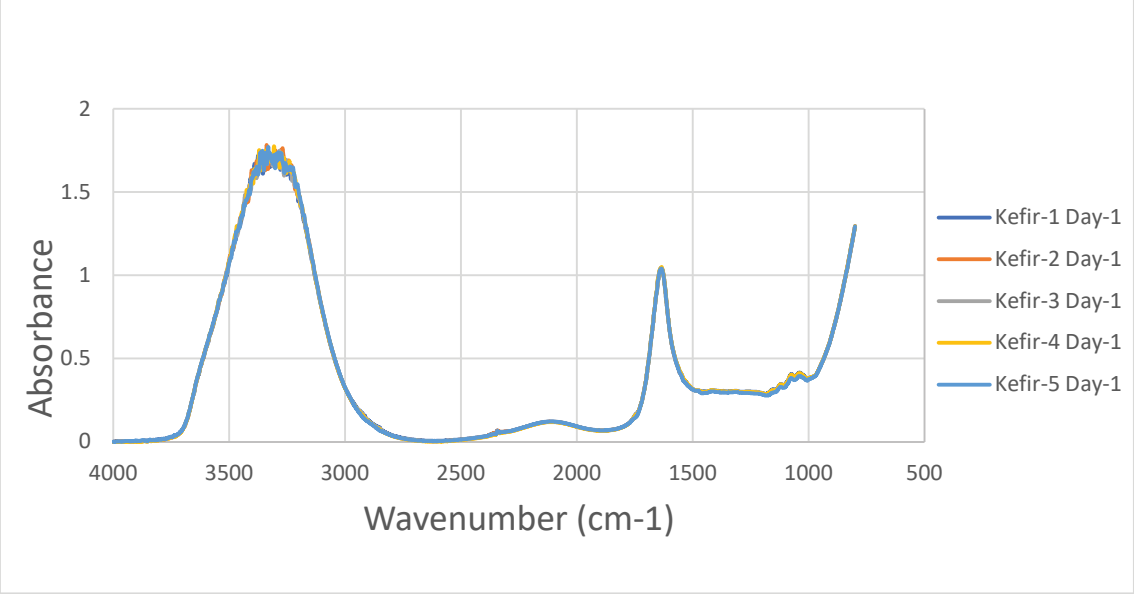


Figure 15. Comparison of Kefir samples on Day-1 (background as air)

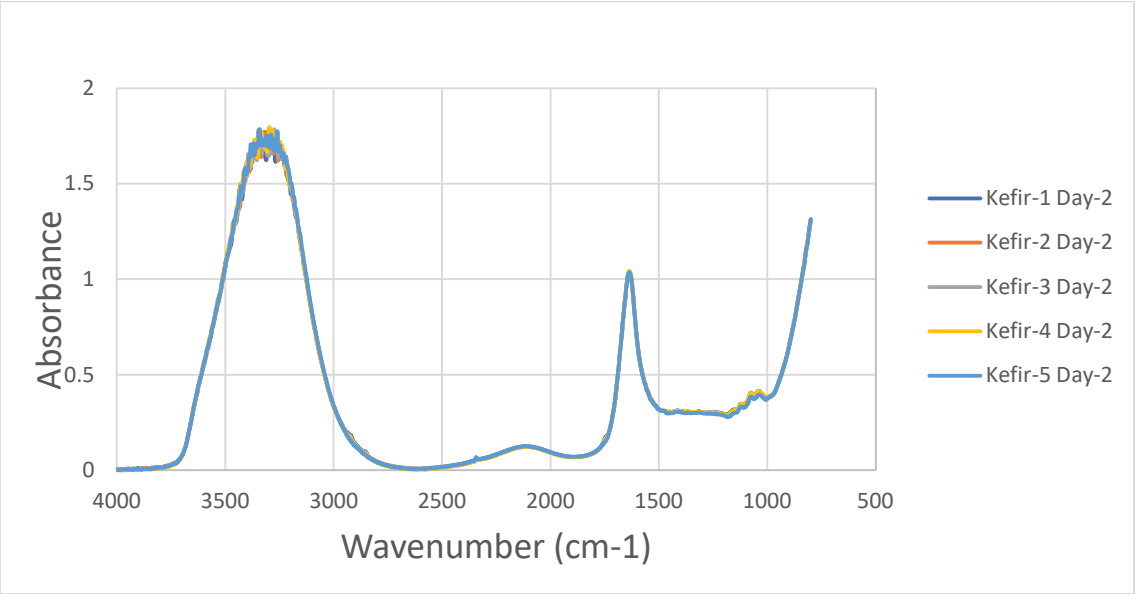


Figure 16. Comparison of Kefir samples on Day-2 (background as air)

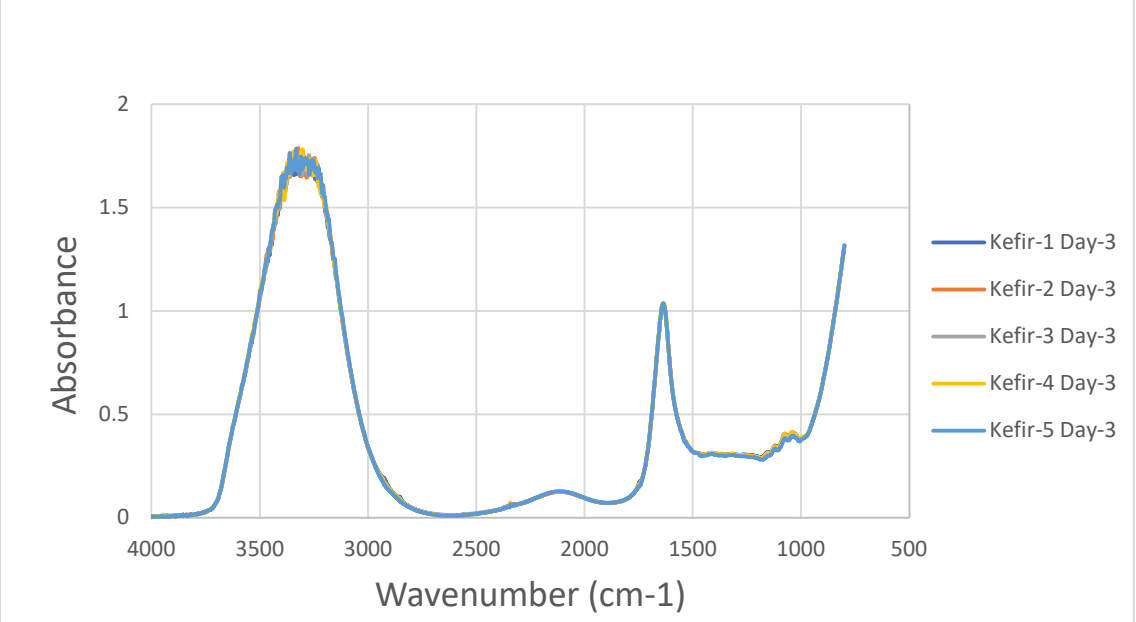


Figure 17. Comparison of Kefir samples on Day-3(background as air)

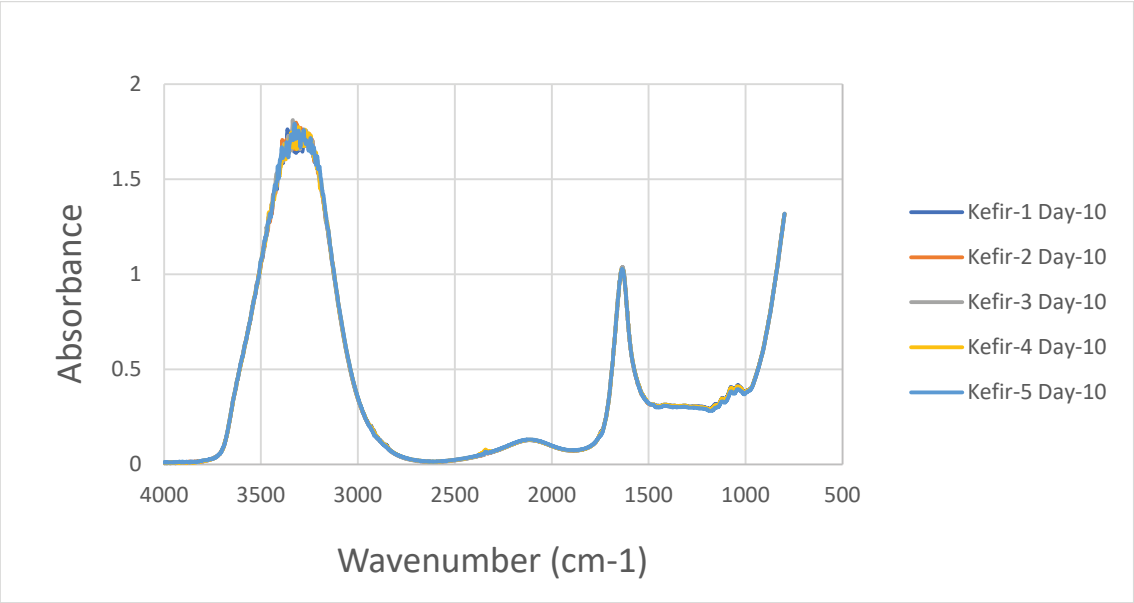


Figure 18. Comparison of Kefir samples on Day-10 (background as air)

4.2.10. PCA Analysis

Figure 19-21 and 22-24 show the PCA analysis for cropped data and PCA analysis for whole data of the milk kefir samples, respectively. Figure 25 shows the PCA anaylsis for FTIR background as air. Figure 26 shows the PCA anlaysis of the finger print region of all kefir samples.

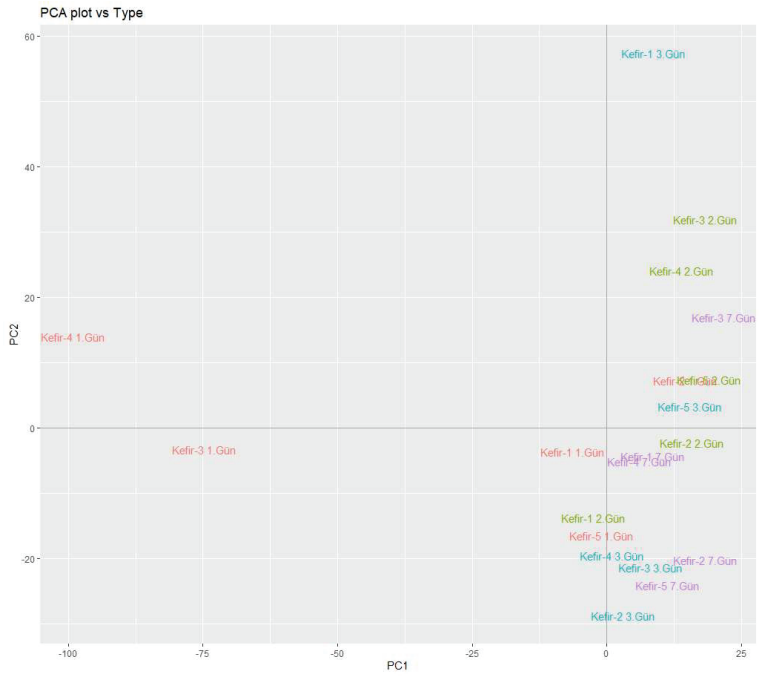


Figure 19. PCA analysis for cropped data PC1 vs PC2

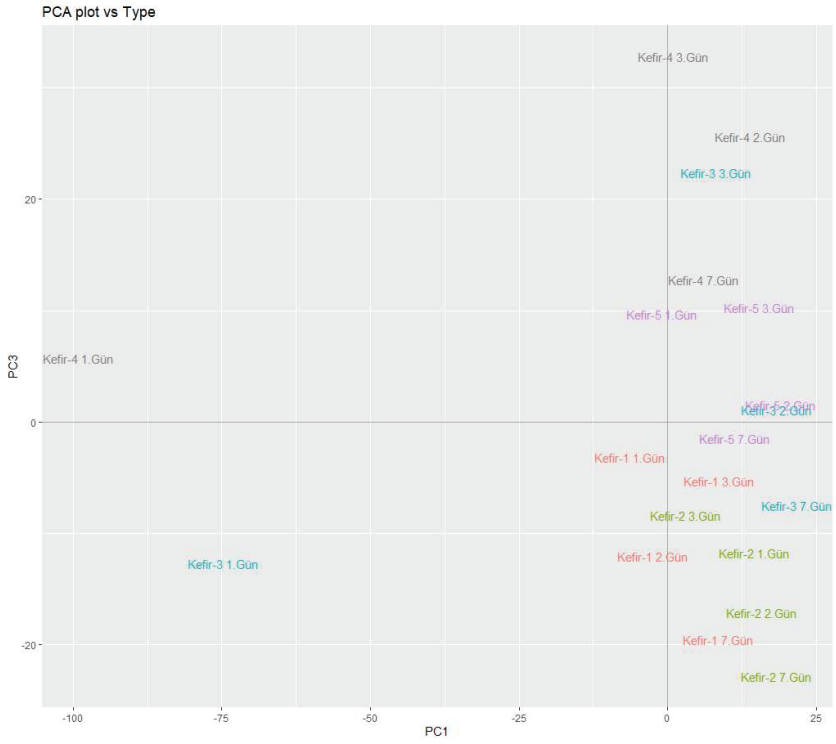


Figure 20. PCA analysis for cropped data PC1 vs PC3

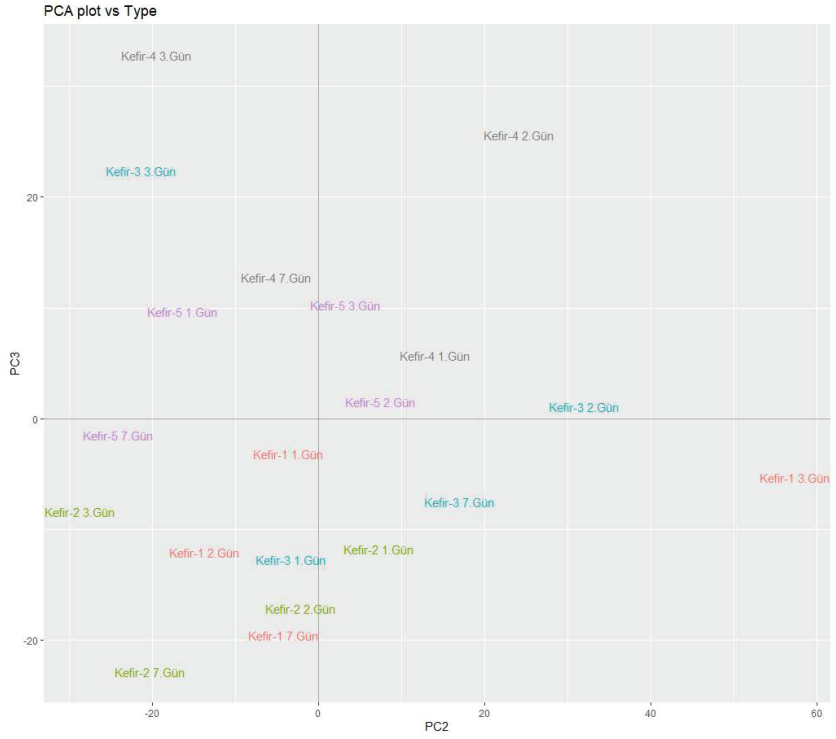


Figure 21. PCA analysis for cropped data PC2 vs PC3

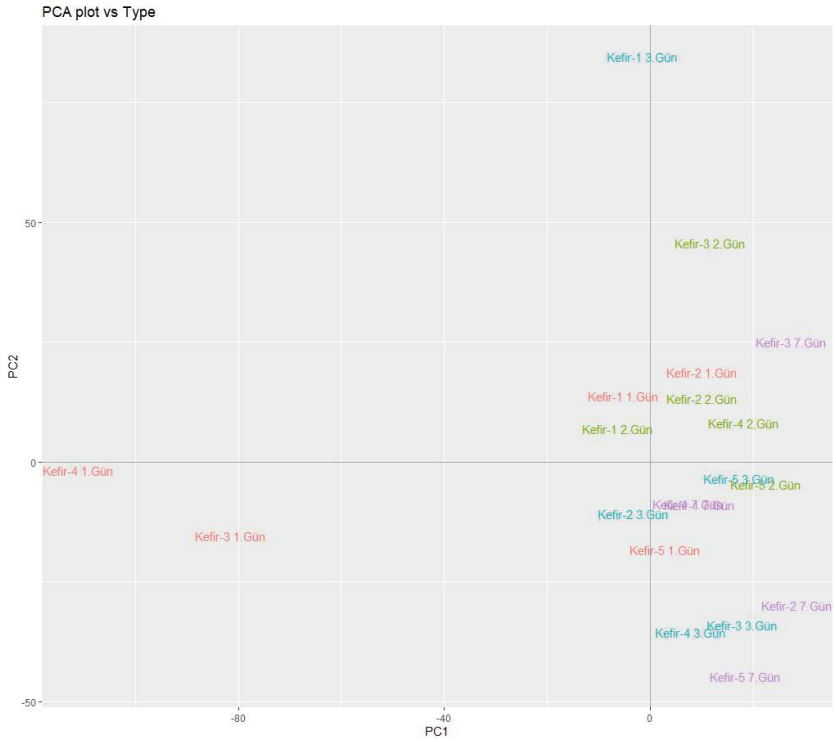


Figure 22. PCA analysis for whole data PC1 vs PC2

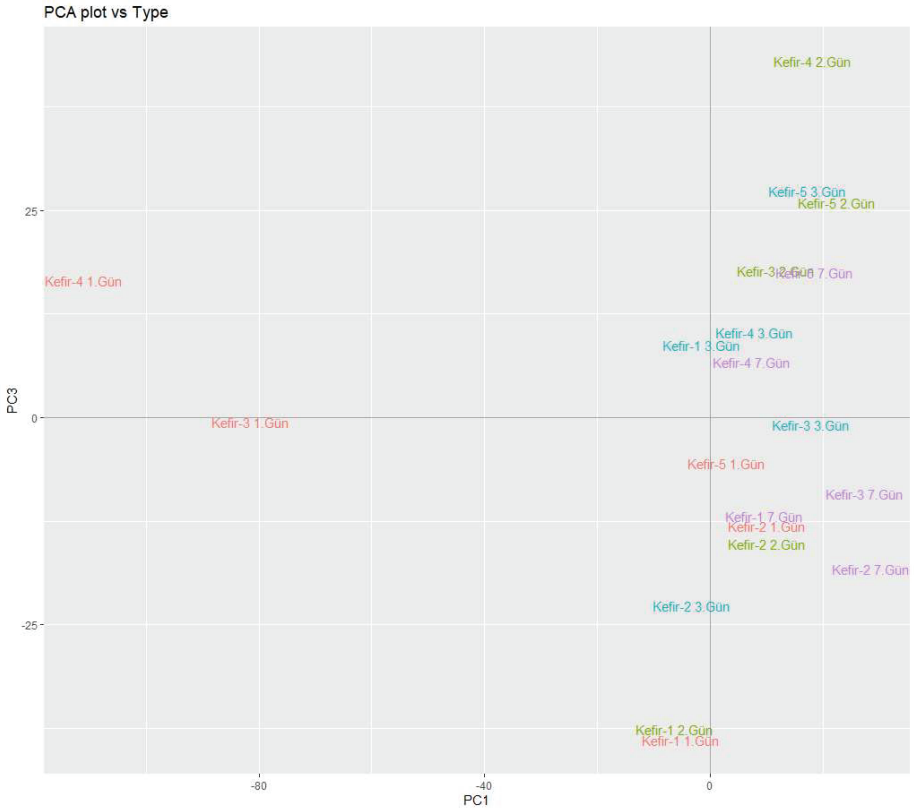


Figure 23. PCA analysis for whole data PC1 vs PC3

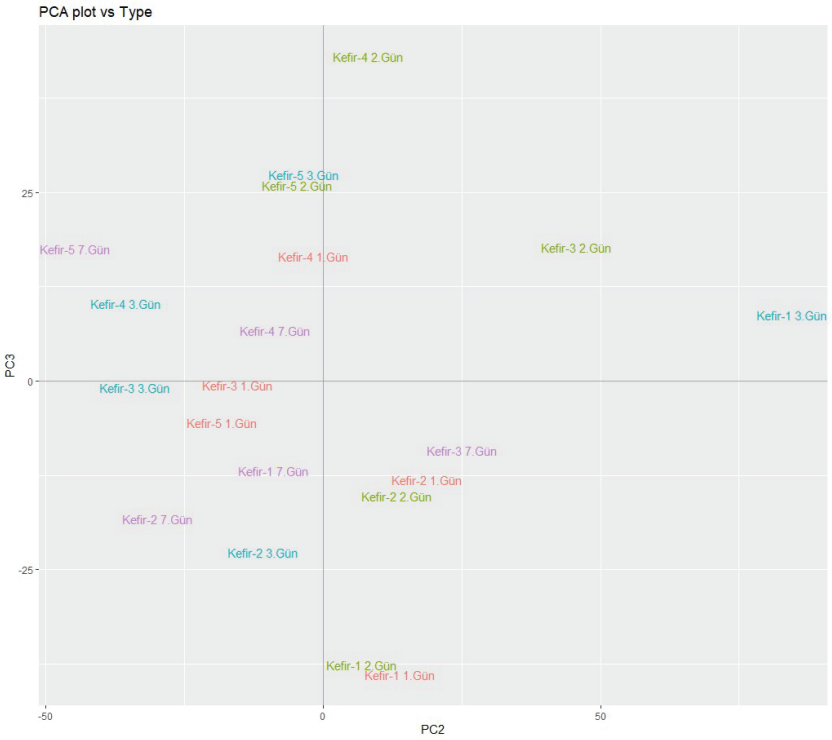


Figure 24. PCA analysis for whole data PC2 vs PC3

Figure 25. PCA Analysis (FTIR background as air)

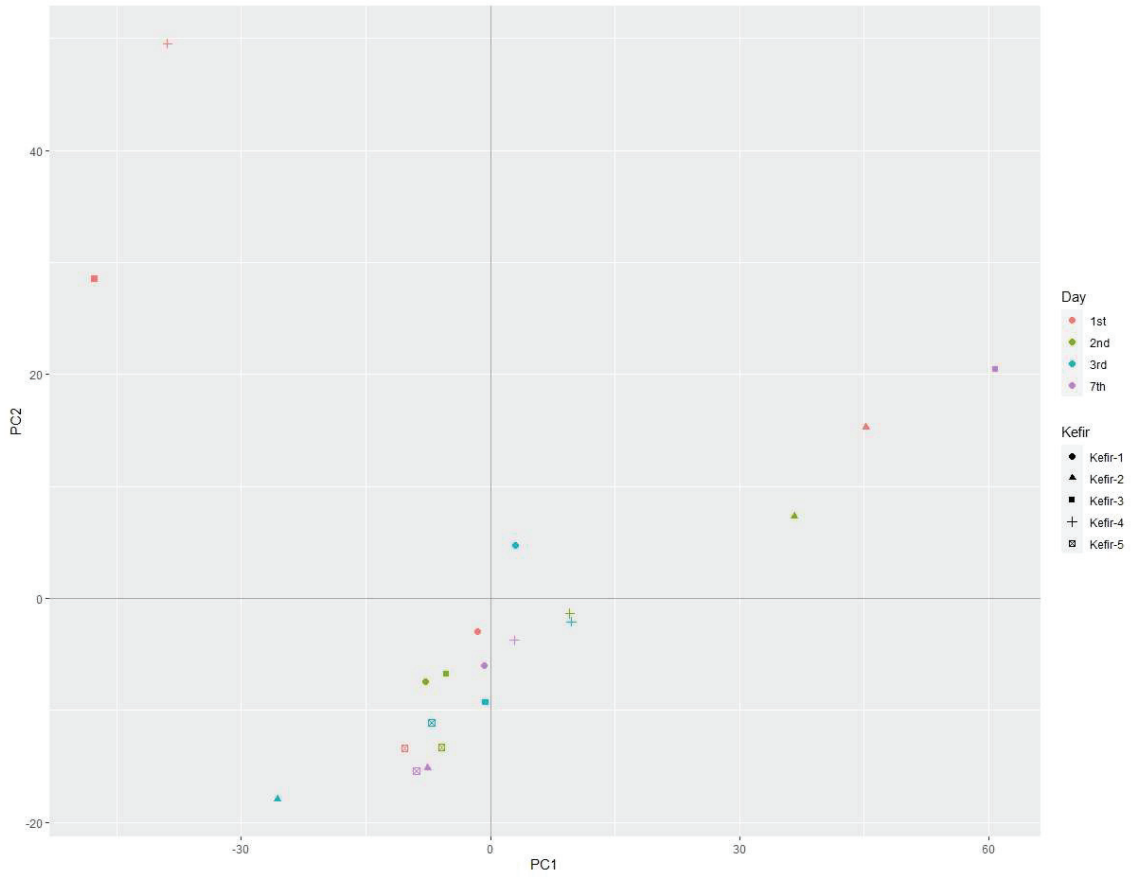


Figure 26. PCA Analysis for the fingerprint region (1600-600 cm-1)

In the cropped data PC-1 and PC-2, PCA plot, generally the samples are scattered at PC1 between 0-25. But exceptions such as kefir-3 day-1 and kefir-4 day-1 are scattered through -100 and -75 at the PC1. Some of the kefir samples that overlap are like each other such as kefir-2 day1 and kefir-5 day-2, kefir-1 day-7, and kefir-4 day-7. The samples are better scattered throughout PC-2 than PC-1.

In the cropped data PC-1 and PC-3, PCA plot, generally the samples are scattered in PC-3 broadly, on the other hand, the samples are also scattered throughout PC-1 between 0-25 except kefir-4 day-1 and kefir-3 day-1 are scattered between -100 and -75 at PC-1. Kefir-3 and kefir-5 on the second day are similar to each other because they are overlapped.

In the cropped data PC-2 and PC-3, PCA plot, the samples are scattered broadly. But at PC-3 it is better seen than PC-2 that the samples are scattered throughout the plot. Samples that are close to each other are similar in composition.

In the whole data PC-1 and PC-2, at PC-2 the samples are scattered more broadly than at PC-1. At PC-1 the samples are scattered between 0-25. There are overlapped samples that are closely related to each other on the PCA plot.

In the whole data PC-1 and PC-3, at PC-3 the samples are scattered more broadly than at PC-1. At PC-1 the samples are gathered between 0-25 but there are some exceptions that kefir-1 day-2 and kefir-1 day 2 are between -40 and 0. There are more closely related samples in this PCA plot

In the whole data PC-2 and PC-3, the samples are scattered more broadly through PC-3, than PC-2. In this PCA plot, the samples are not that closely related to each other but there are exceptions such as kefir-5 day-3 and kefir-5 day-2 they are near to each other means that they are similar in composition.

At figure 25, the samples are scattered equally at PC-1 and PC-2. Kefir-2 day-3 and kefir-3 day-2 are similar to each other. Kefir-3 day-3 and kefir-2 day-10 are close to each other means that they are similar to each other.

4.3. Comparison of Taxonomic Diversity in Kefir Samples

4.3.1. Phylum level taxonomic diversity among kefir samples

The most abundant phylum in all kefir samples was Firmicutes ranging from 66.55% in KA to 90.34% in KC. (Firmicutes: KA: 66.55% KB: 74.24% KC: 90.34% KD: 82.68% KE: 85.1 %) (Figures 21-25).

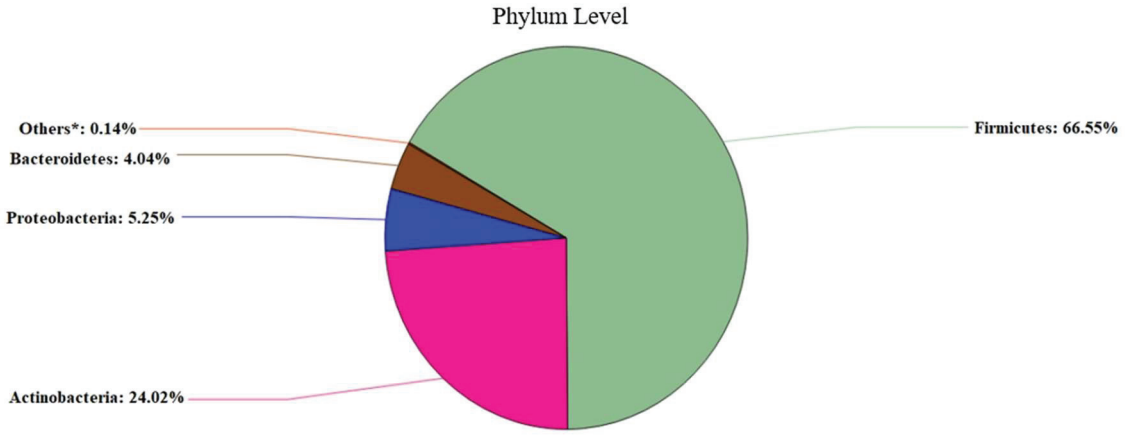


Figure 26. Phylum level taxonomic composition of sample KA

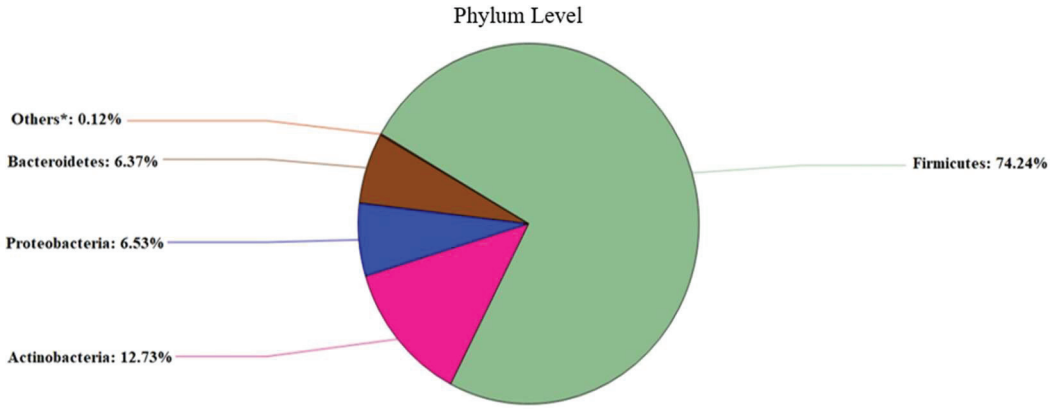


Figure 27. Phylum level taxonomic composition of sample KB

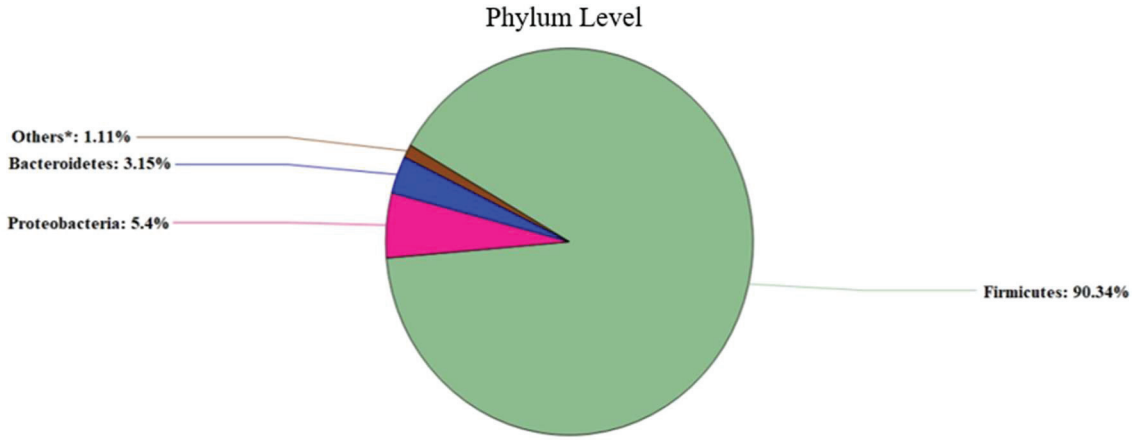


Figure 28. Phylum level taxonomic composition of sample KC

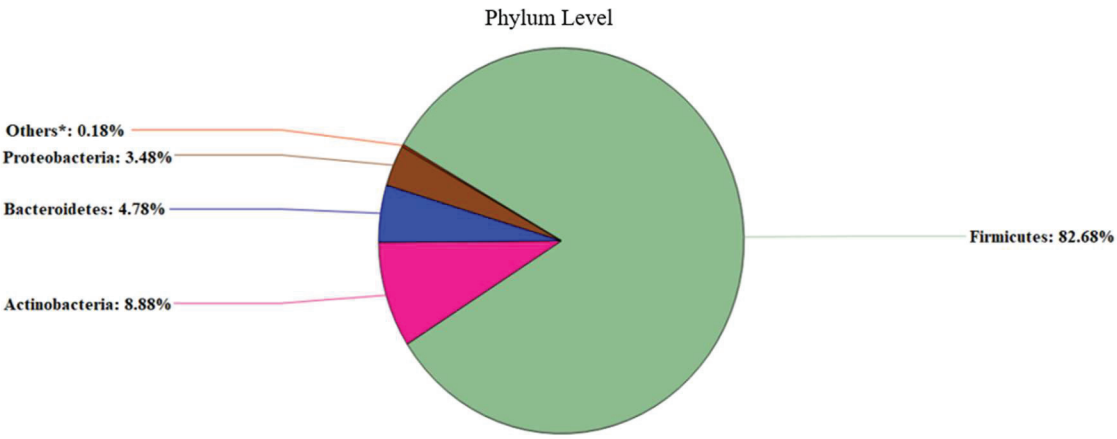


Figure 29. Phylum level taxonomic composition of sample KD

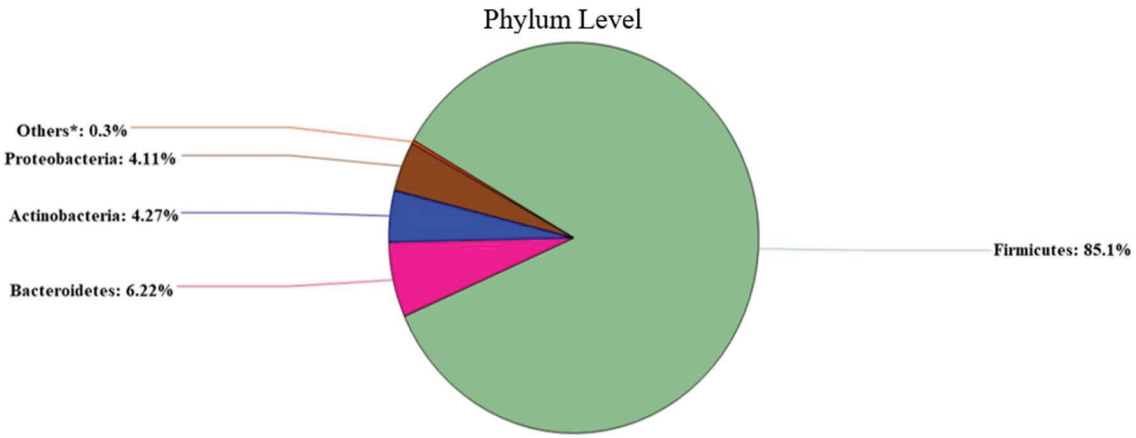


Figure 30. Phylum level taxonomic composition of sample KE

4.3.2. Class level taxonomic diversity among kefir samples

The most abundant class in all kefir samples was Bacilli ranging from 65.3% in KA to 89.57% in KC. (Bacilli: KA: 65.3%, KB: 73.2%, KC: 89.57%, KD: 80.89%, KE: 82.35%) (Figures 26-30).

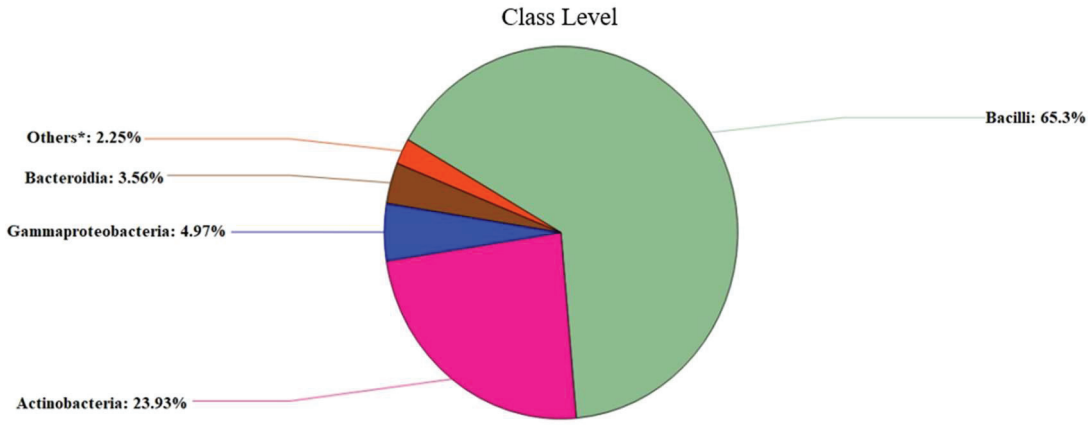


Figure 31. Class level taxonomic composition of sample KA

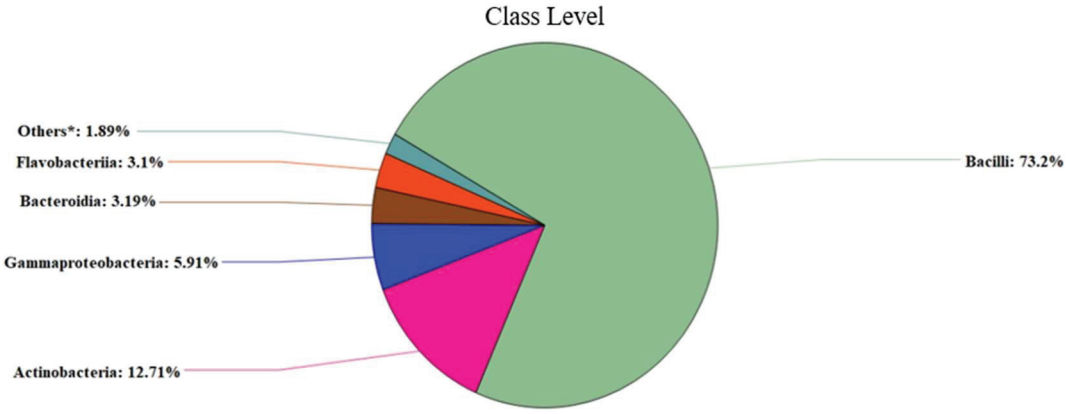


Figure 32. Class level taxonomic composition of sample KB

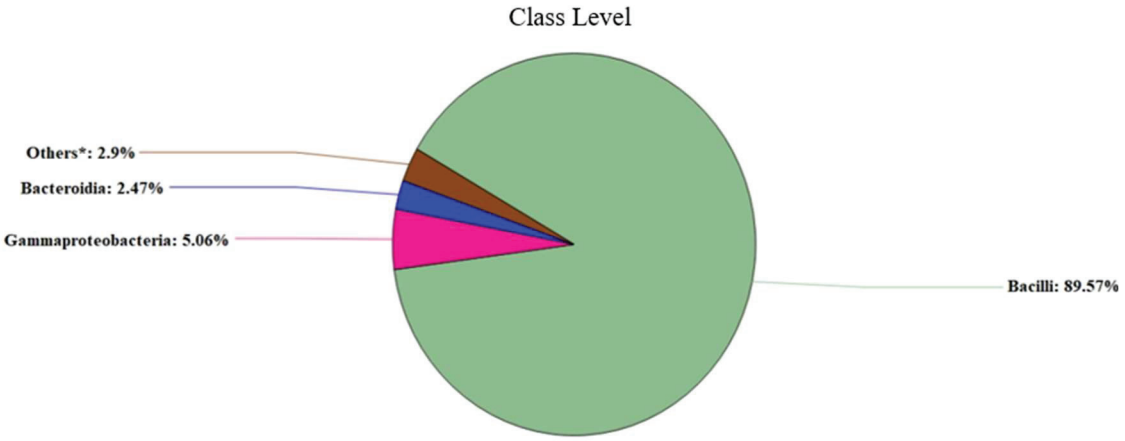


Figure 33. Class level taxonomic composition of sample KC

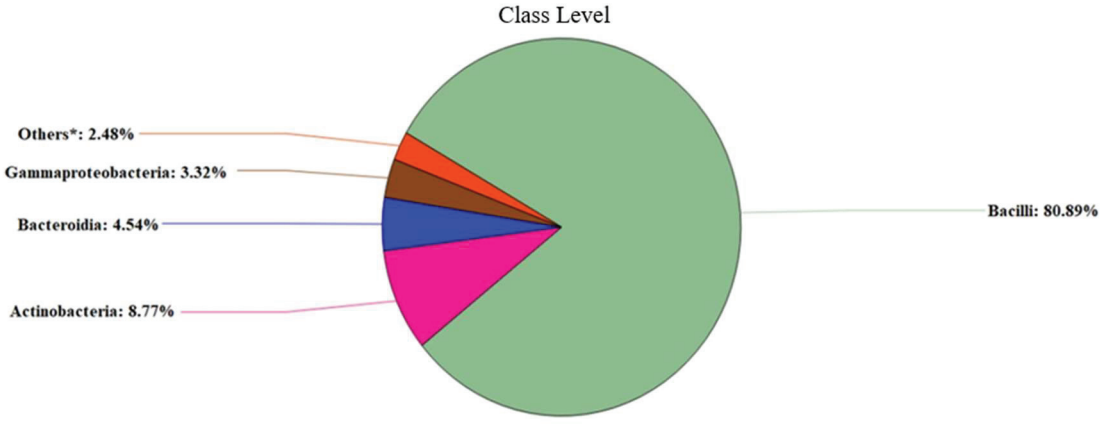


Figure 34. Class level taxonomic composition of sample KD

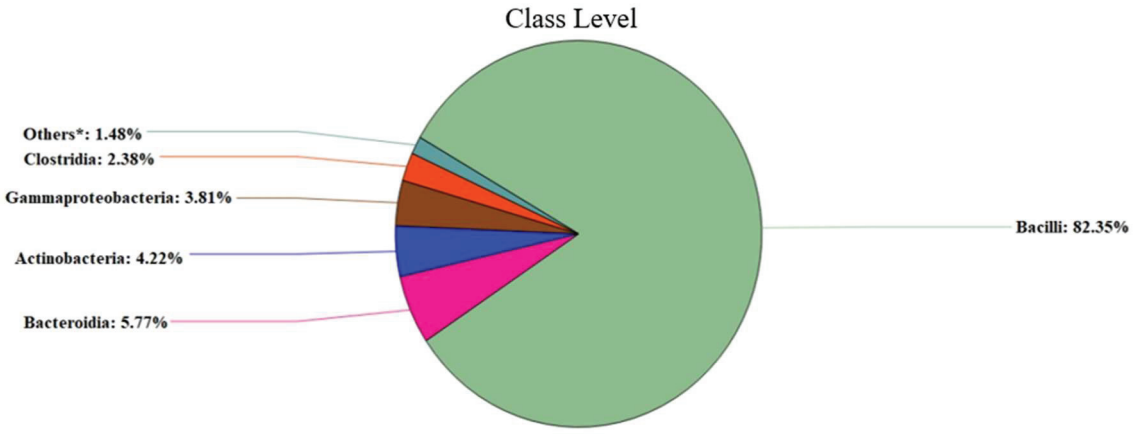


Figure 35. Class level taxonomic composition of sample KE

4.3.3. Order level taxonomic diversity among kefir samples

The most abundant order in all kefir samples was Lactobacillales ranging from 65.21% in KA to 89.46% in KC. (Lactobacillales: KA: 65.21%, KB: 73.1%, KC: 89.46%, KD: 80.73%, KE: 82.26%) (Figures 31-35).

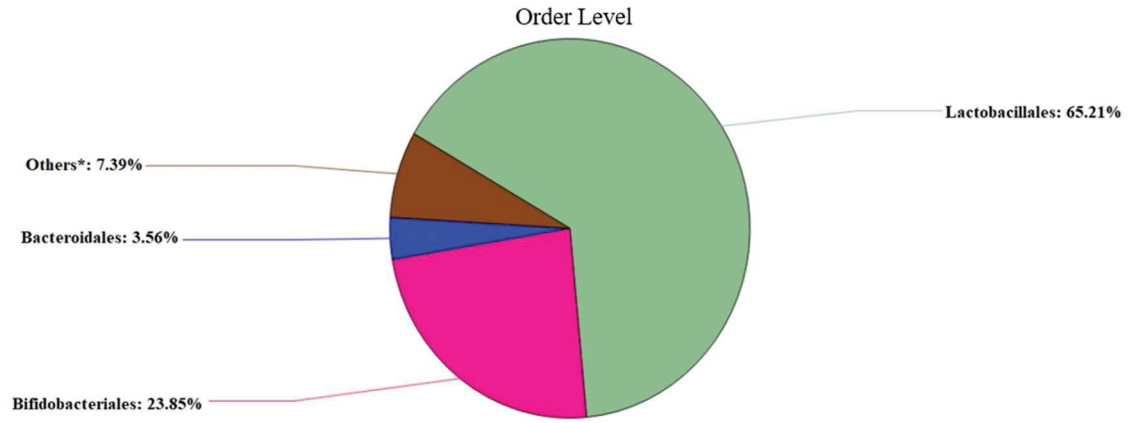


Figure 36. Order level taxonomic composition of sample KA

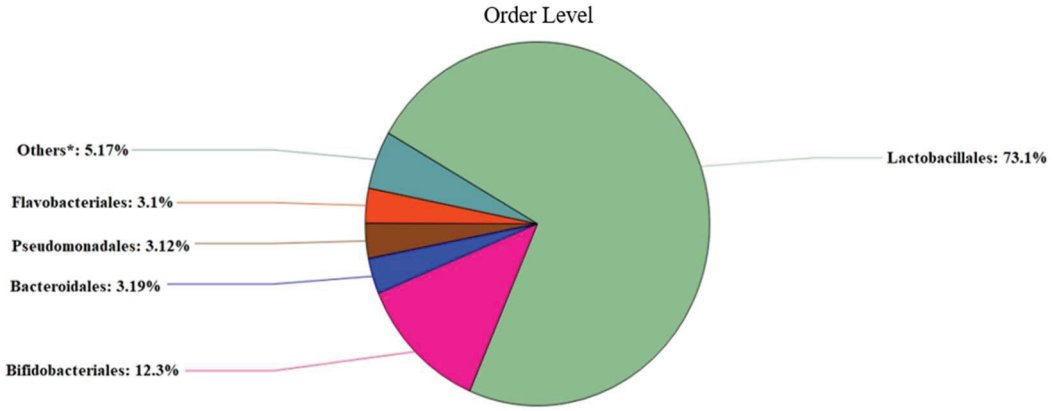


Figure 37. Order level taxonomic composition of sample KB

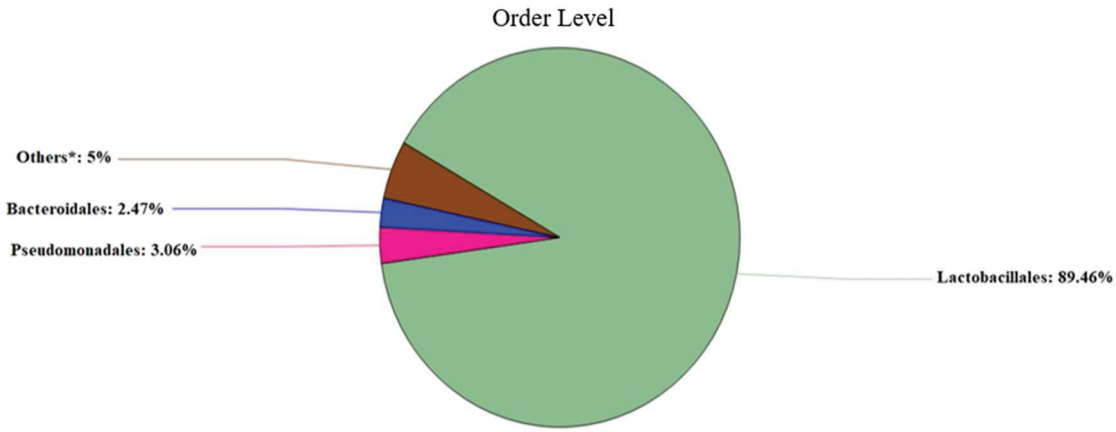


Figure 38. Order level taxonomic composition of sample KC

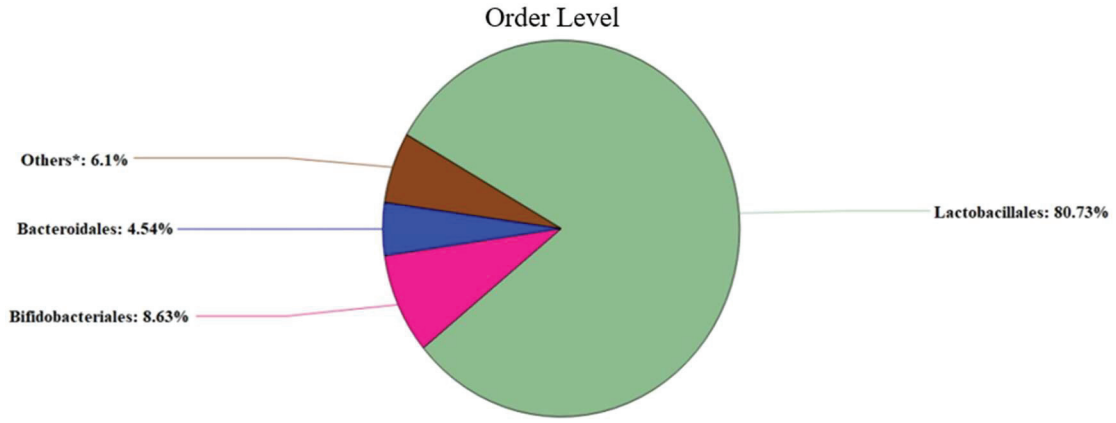


Figure 39. Order level taxonomic composition of sample KD

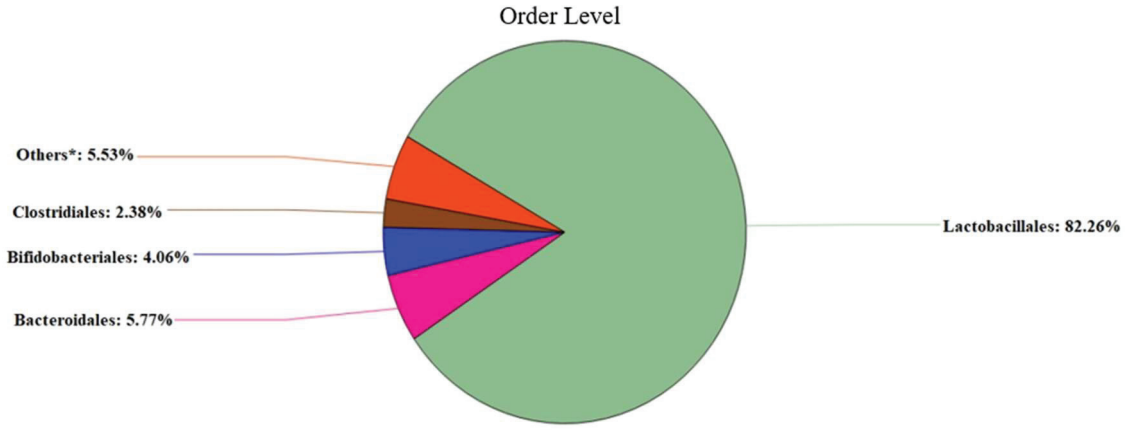


Figure 40. Order level taxonomic composition of sample KE

4.3.4. Family level taxonomic diversity among kefir samples

As given in Table 9 the most abundant family in all kefir samples was *Streptococcaceae* ranging from 54.33% in KA to 87.26% in KC (*Streptococcaceae:* KA: 54.33%, KB: 67.29%, KC: 87.26%, KD: 77.49%, KE: 75.39%). The second most abundant family in all kefir samples was *Bifidobacteriaceae* ranging from 4.06% in KE to 23.85% in KA. The third most abundant family in all kefir samples was *Lactobacillaceae* ranging from 2.14% in KC to 8.53% in KA. The forth most abundant family was *Leuconostocaceae* ranging from 2.31% in KA to 4.51% in KE (Figures 36-40).

Table 6. Comparison of dominant bacteria family between kefir samples

Family Level	**KA**	**KB**	**KC**	**KD**	**KE**
Streptococcaceae	54.33%	67.29%	87.26%	77.49%	75.39%
Bifidobacteriaceae	23.85%	12.30%	NP	8.63%	4.06%
Lactobacillaceae	8.53%	NP	2.14%	2.17%	2.34%
Leuconostocaceae	2.31%	3.99%	NP	NP	4.51%
Flavobacteriaceae	NP	3.10%	NP	NP	NP
Moraxellaceae	NP	2.91%	2.67%	NP	NP
Bacteroidaceae	NP	NP	NP	2.18%	2.49%
Prevotellaceae	NP	NP	NP	NP	2.39%
Others*	10.99%	10.41%	7.93%	9.53%	8.82%

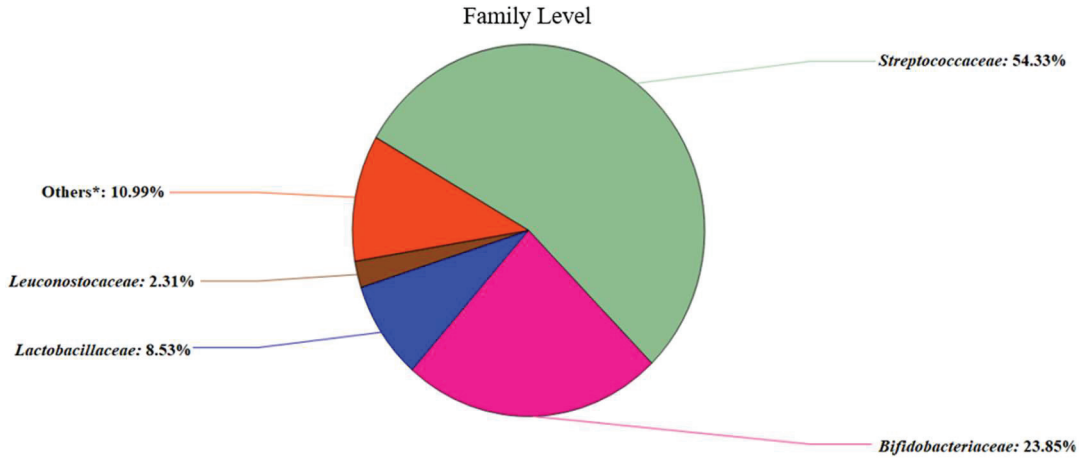


Figure 41. Family level taxonomic composition of sample KA

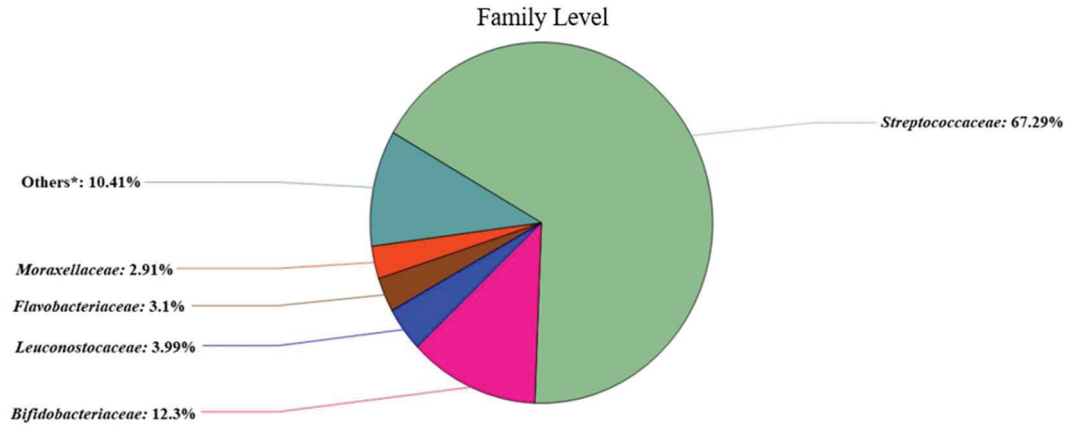


Figure 42. Family level taxonomic composition of sample KB

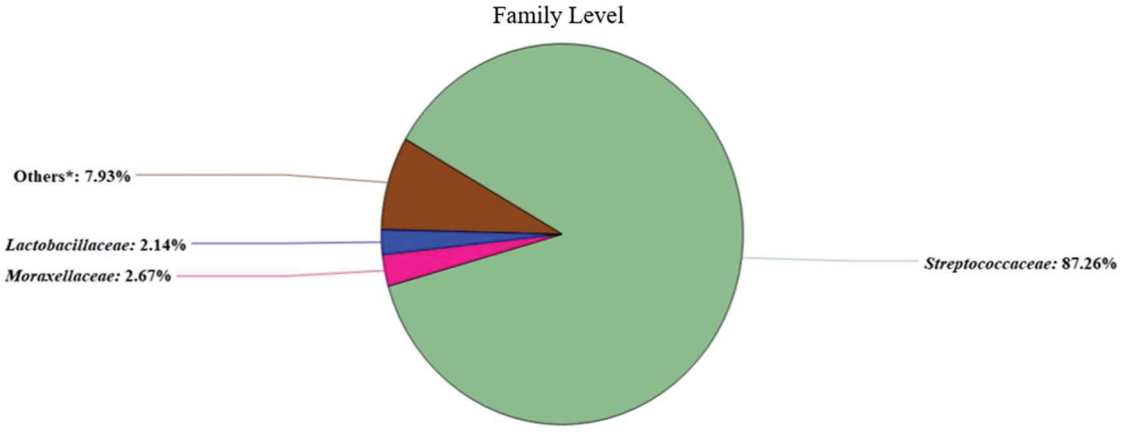


Figure 43. Family level taxonomic composition of sample KC

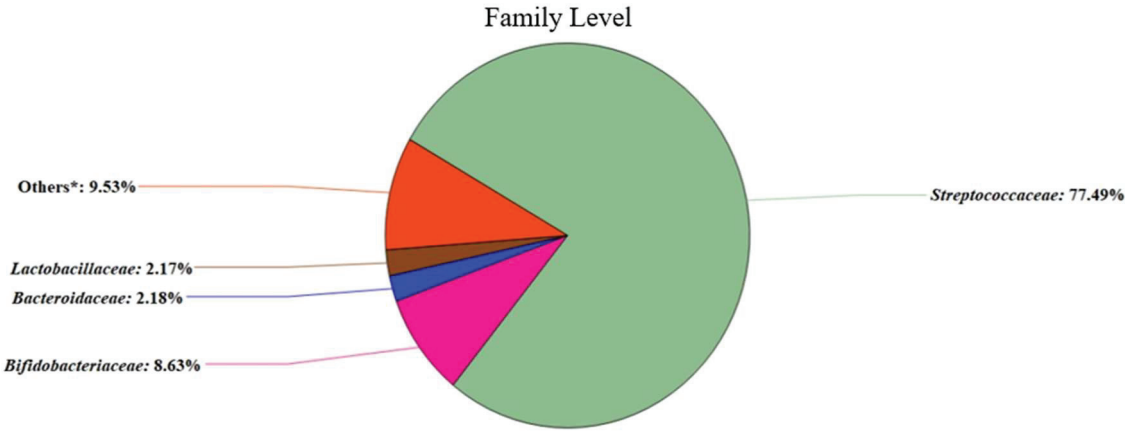


Figure 44. Family level taxonomic composition of sample KD

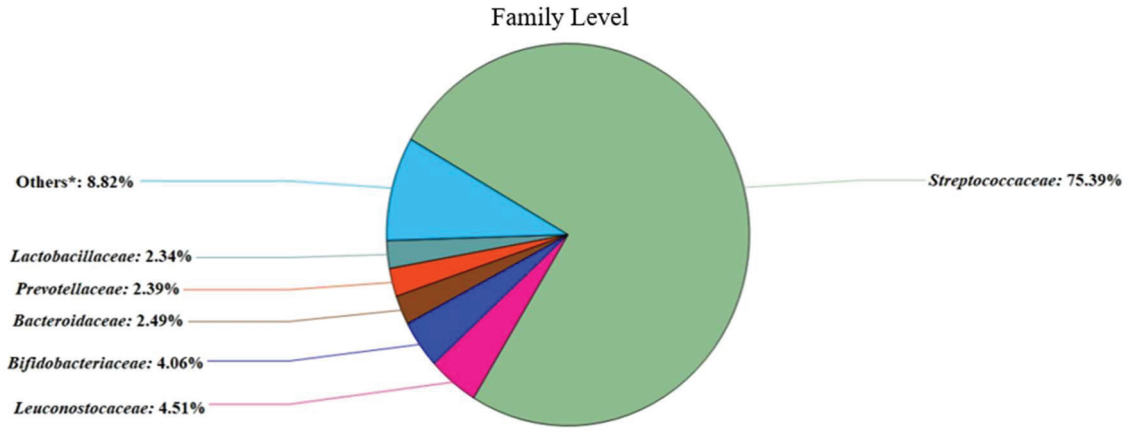


Figure 45. Family level taxonomic composition of sample KE

4.3.5. Genus level taxonomic diversity among kefir samples

Genus level taxonomic diversity among kefir samples are indicated in Table 10. Most abundant genus found in the beverages was *Streptococcus* ranging from 39.34% in KA to 73.19% in KC. (*Streptococcus:* KA: 39.34%, KB: 54.3%, KC: 73.19%, KD: 40.43%, KE: 48.9%) (Figures 41-45). Although the second most abundant genus in all kefir samples was *Bifidobacterium* ranging from 4.06% in KE to 23.85% in KA, it was not detected in sample KC. The third most abundant genus in all kefir samples was *Lactococcus* ranging from 12.98% in KB to 37.06% in KD. The fourth most abundant genus in all kefir samples was *Lactobacillus* ranging from 2.14% in KC to 8.53% in KA. (Table 10). Genus *Lactobacillus* were not found in the sample KB, genera

Chryseobacterium and *Prevotella* were only found in the samples KB and KE, respectively.

Table 7. Comparison of dominant bacteria genus between kefir samples

Genus	**KA**	**KB**	**KC**	**KD**	**KE**
Streptococcus	39.34%	54.30%	73.19%	40.43%	48.90%
Bifidobacterium	23.85%	12.30%	NP	8.63%	4.06%
Lactococcus	14.99%	12.98%	14.06%	37.06%	26.49%
Lactobacillus	8.53%	NP	2.14%	2.17%	2.33%
Leuconostoc	2.31%	3.99%	NP	NP	4.51%
Chryseobacterium	NP	3.02%	NP	NP	NP
Bacteroides	NP	NP	NP	2.18%	2.48%
Prevotella	NP	NP	NP	NP	2.35%
Others*	10.99%	13.40%	10.60%	9.53%	8.88%

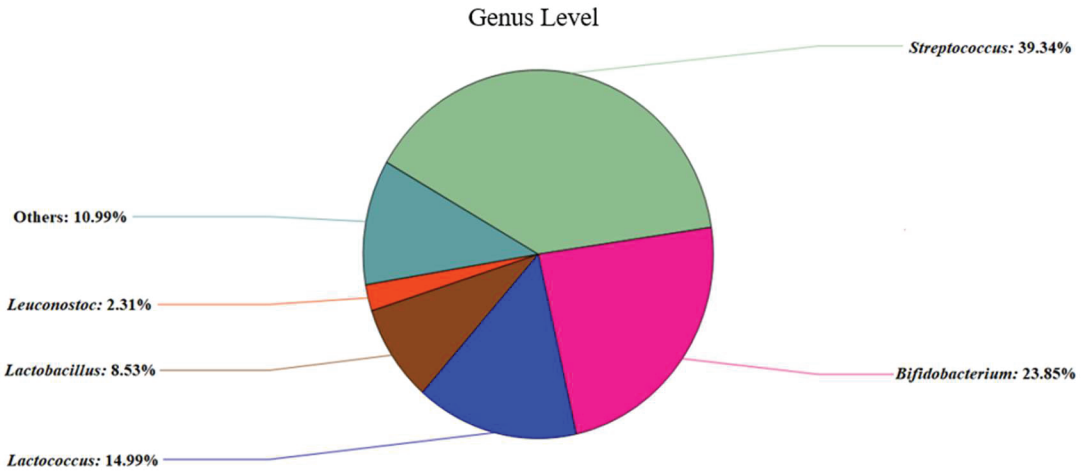


Figure 46 Genus level taxonomic composition of sample KA

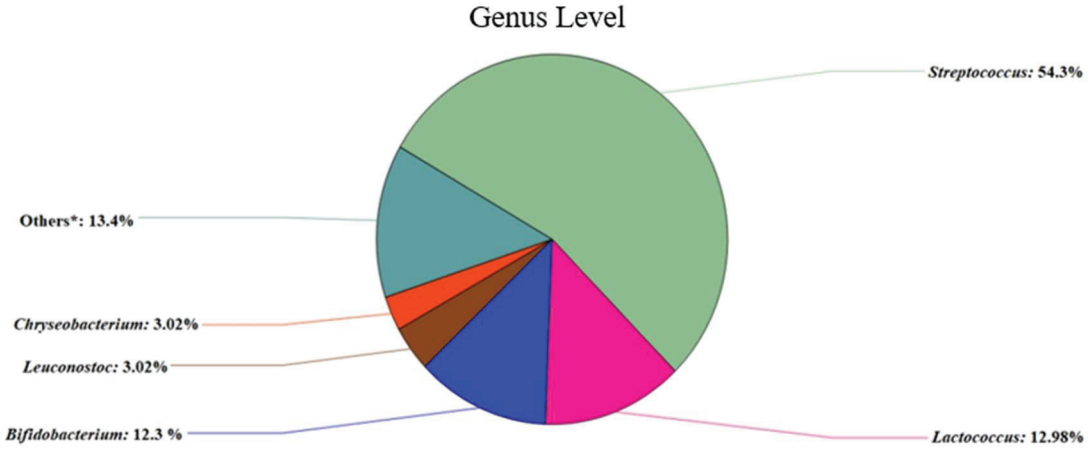


Figure 47. Genus level taxonomic composition of sample KB

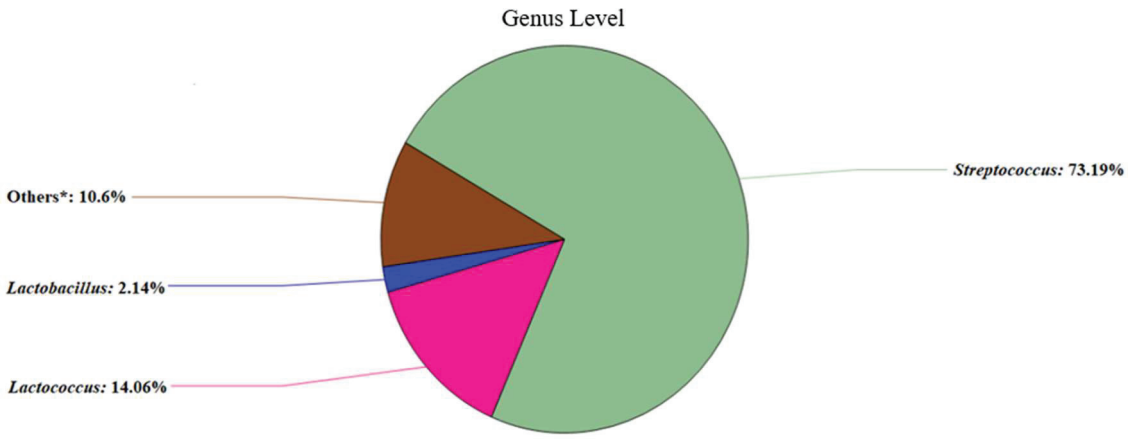


Figure 48. Genus level taxonomic composition of sample KC

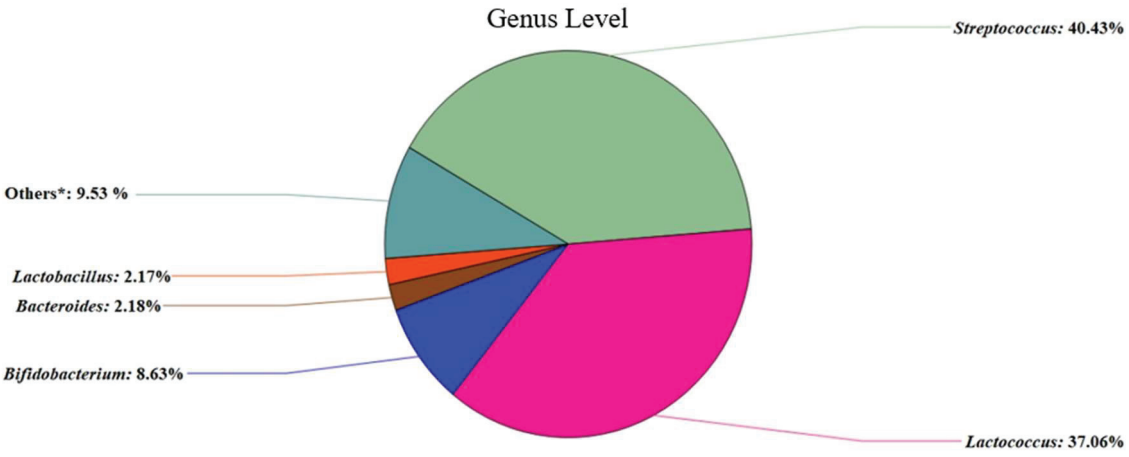


Figure 49. Genus level taxonomic composition of sample KD

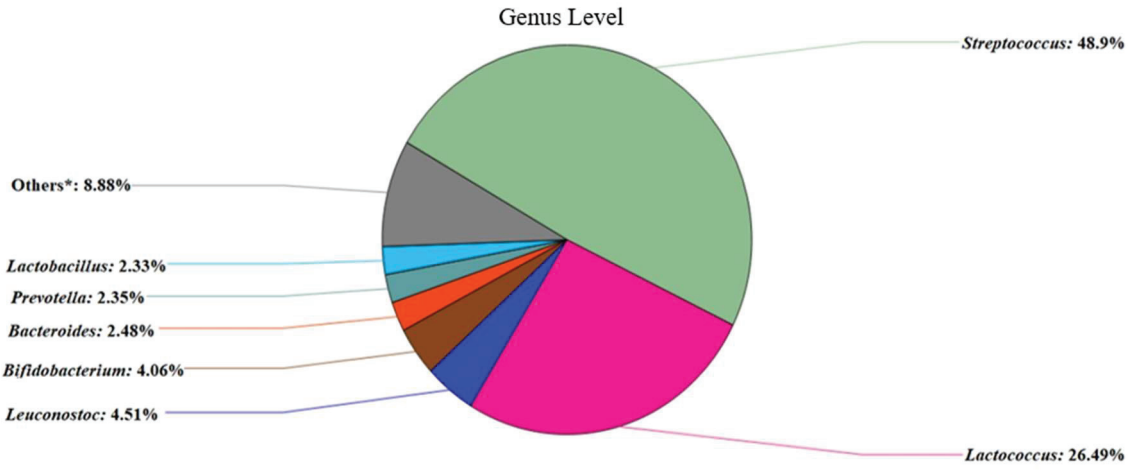


Figure 50. Genus level taxonomic composition of sample KE

4.3.6. Species- level taxonomic diversity among kefir samples

The most abundant species in all kefir samples were *Streptococcus thermophilus* ranging from 39.22% in KA to 73.14% in KC. (*Streptococcus thermophilus*: KA: 39.22%, KB: 54.12%, KC: 73.14%, KD: 40.19%, KE: 48.86%). The second most abundant species in all kefir samples was *Lactococcus lactis* ranging from 11.91% in KB to 36.85% in KD. The third most abundant species in all kefir samples was *Bifidobacterium animalis* ranging from 4.01% in KE to 23.79% in KA, however this species were not found in sample KC. The fourth most abundant species in all kefir samples was *Lactobacillus acidophilus* were found only in KA and KE at 2.11% and 8.33% levels, respectively. *Prevotella copri* was found only in sample KE (Table 11).

Table 8. Comparison of dominant bacteria species between kefir samples

Species	**KA**	**KB**	**KC**	**KD**	**KE**
Streptococcus thermophilus	39.22%	54.12%	73.14%	40.39%	48.86%
Bifidobacterium animalis	23.79%	12.20%	NP	8.51%	4.01%
Lactococcus lactis	14.41%	11.91%	14.00%	36.85%	26.28%
Lactobacillus acidophilus	8.33%	NP	NP	NP	2.11%
Leuconostoc pseudomesenteroides	2.26%	3.91%	NP	NP	4.36%
Prevotella copri	NP	NP	NP	NP	2.29%
Others	11.98%	17.86%	12.86%	14.26%	12.09%

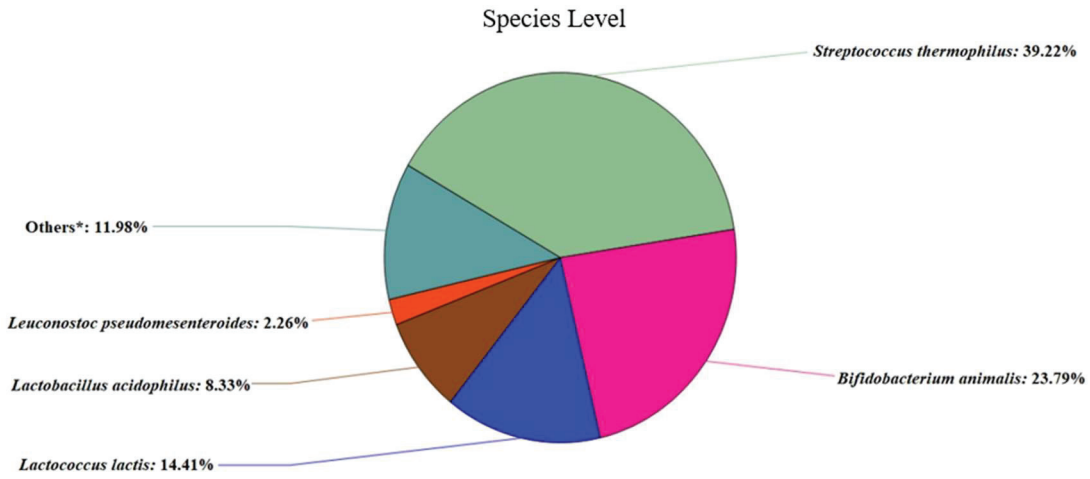


Figure 51. Species level taxonomic composition of sample KA

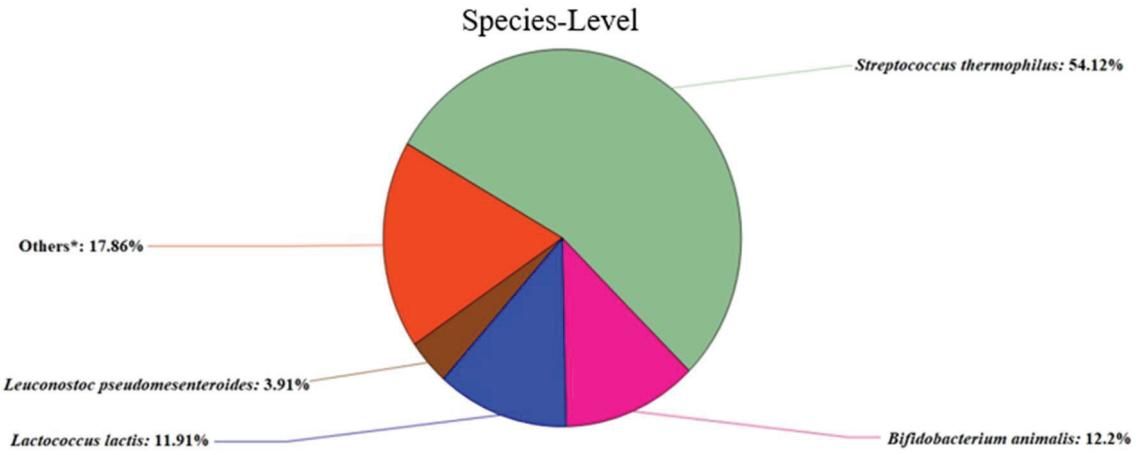


Figure 52. Species level taxonomic composition of sample KB

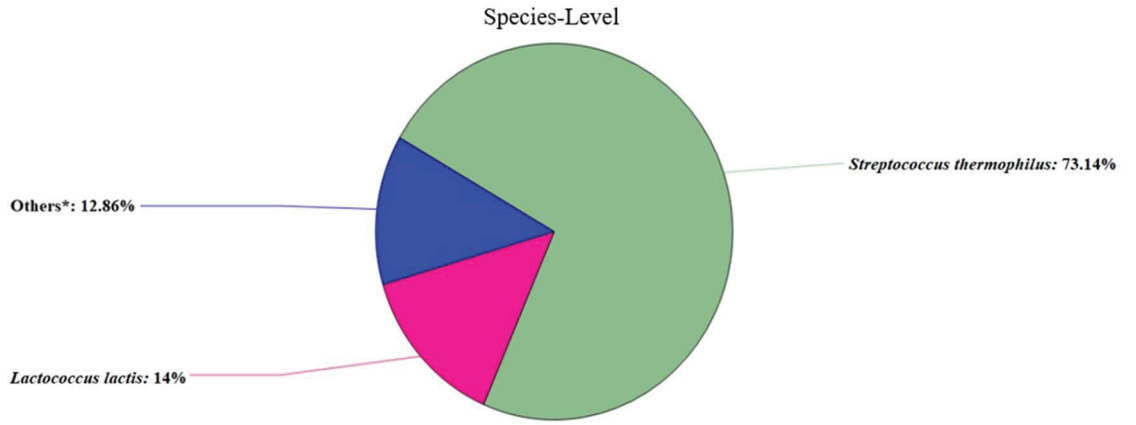


Figure 53. Species level taxonomic composition of sample KC

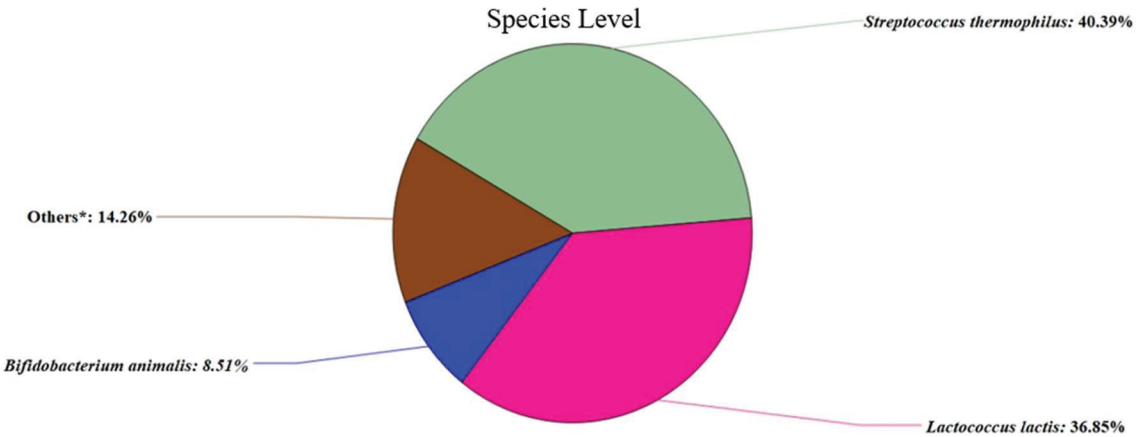


Figure 54. Species level taxonomic composition of sample KD

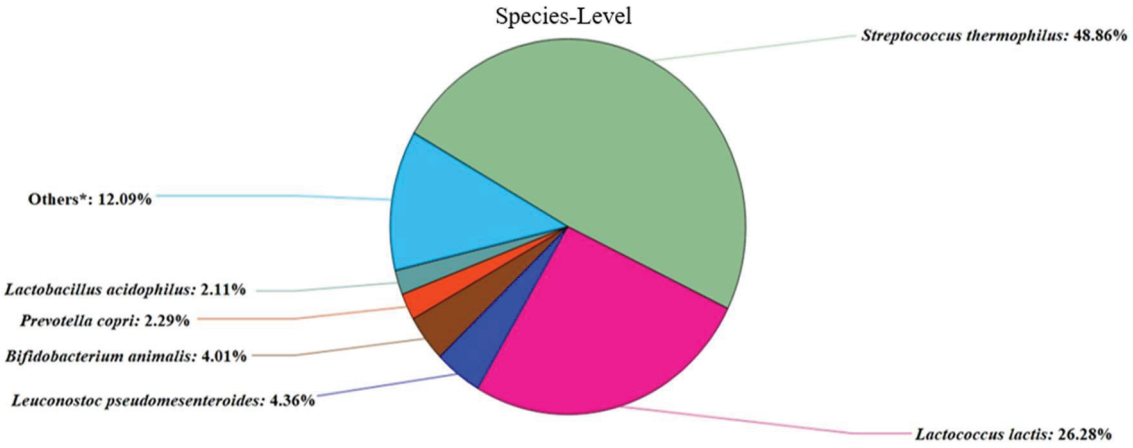


Figure 55. Species-level taxonomic composition of sample KE

Based on a phylogenetic analysis showing the relatedness of a kefir beverage based on species level taxonomic diversity KA and KB samples, KE and KD samples were more closely related to each other (Figure 51). KC sample was the most different sample compared to the other kefir beverage samples. These analyses suggest that the species level taxonomic diversity is similar in KA and KB samples, and KD and KE samples.

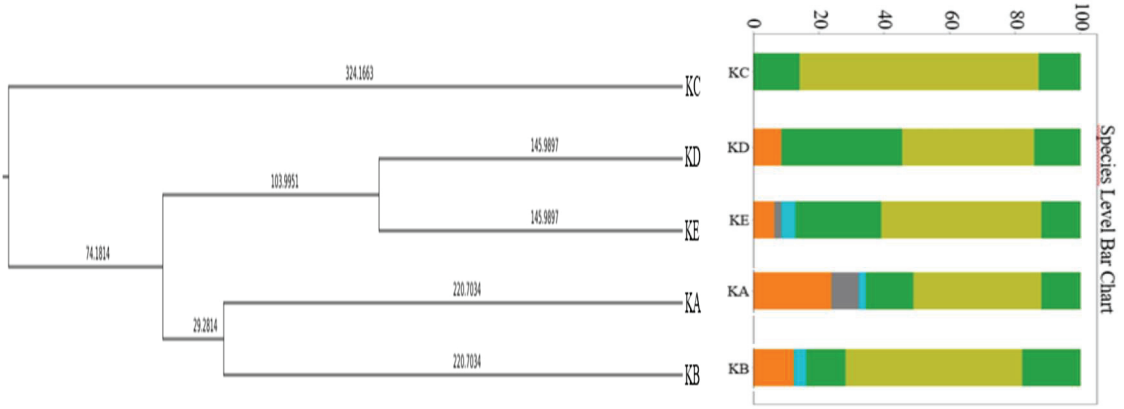


Figure 56 Phylogenetic analysis showing the relatedness of kefir samples based on the species level taxonomic diversity

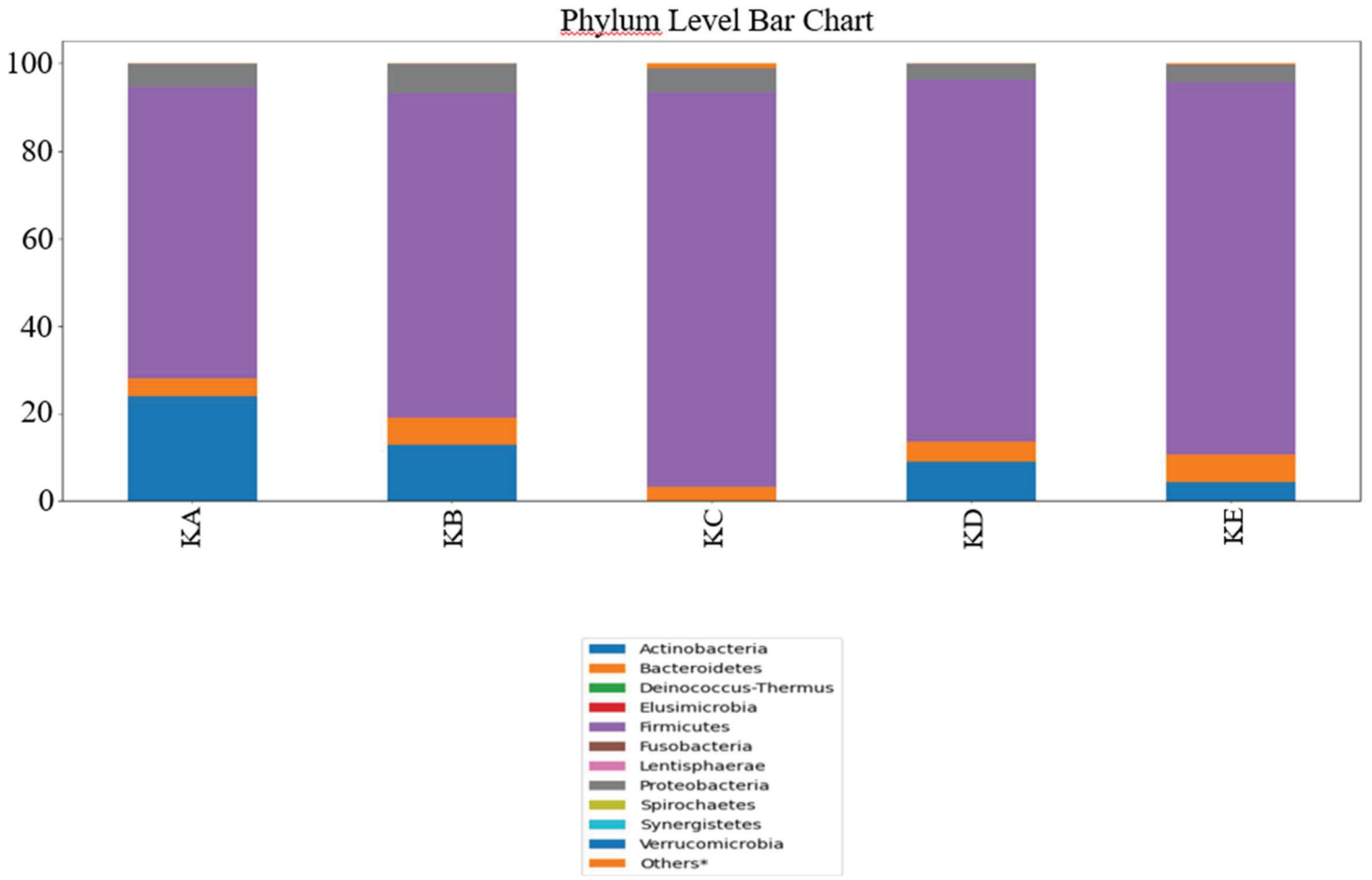


Figure 57. Phylum level taxonomic comparison of kefir samples

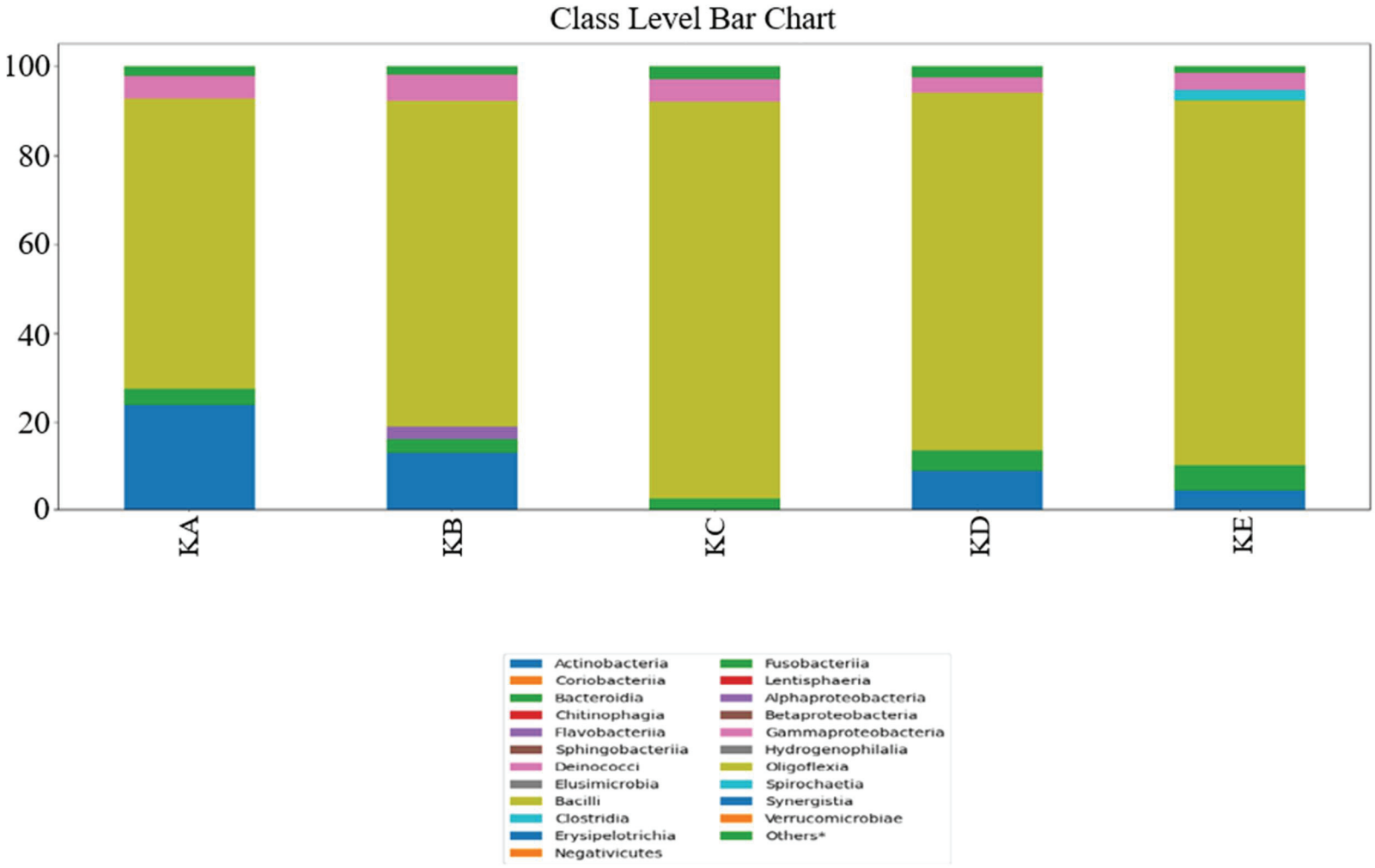


Figure 58. Class level taxonomic comparison of kefir samples

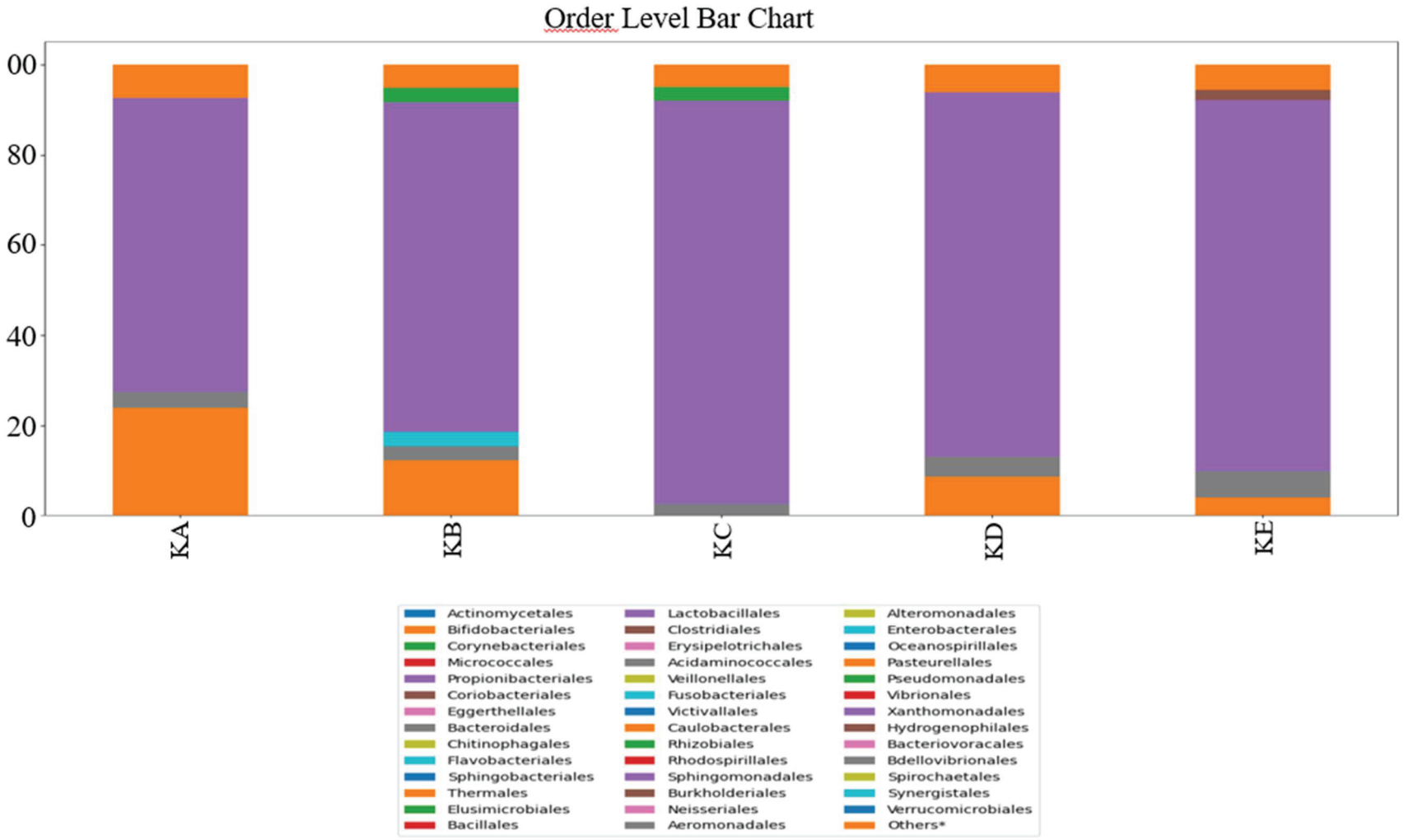


Figure 59. Order level taxonomic comparison of kefir samples

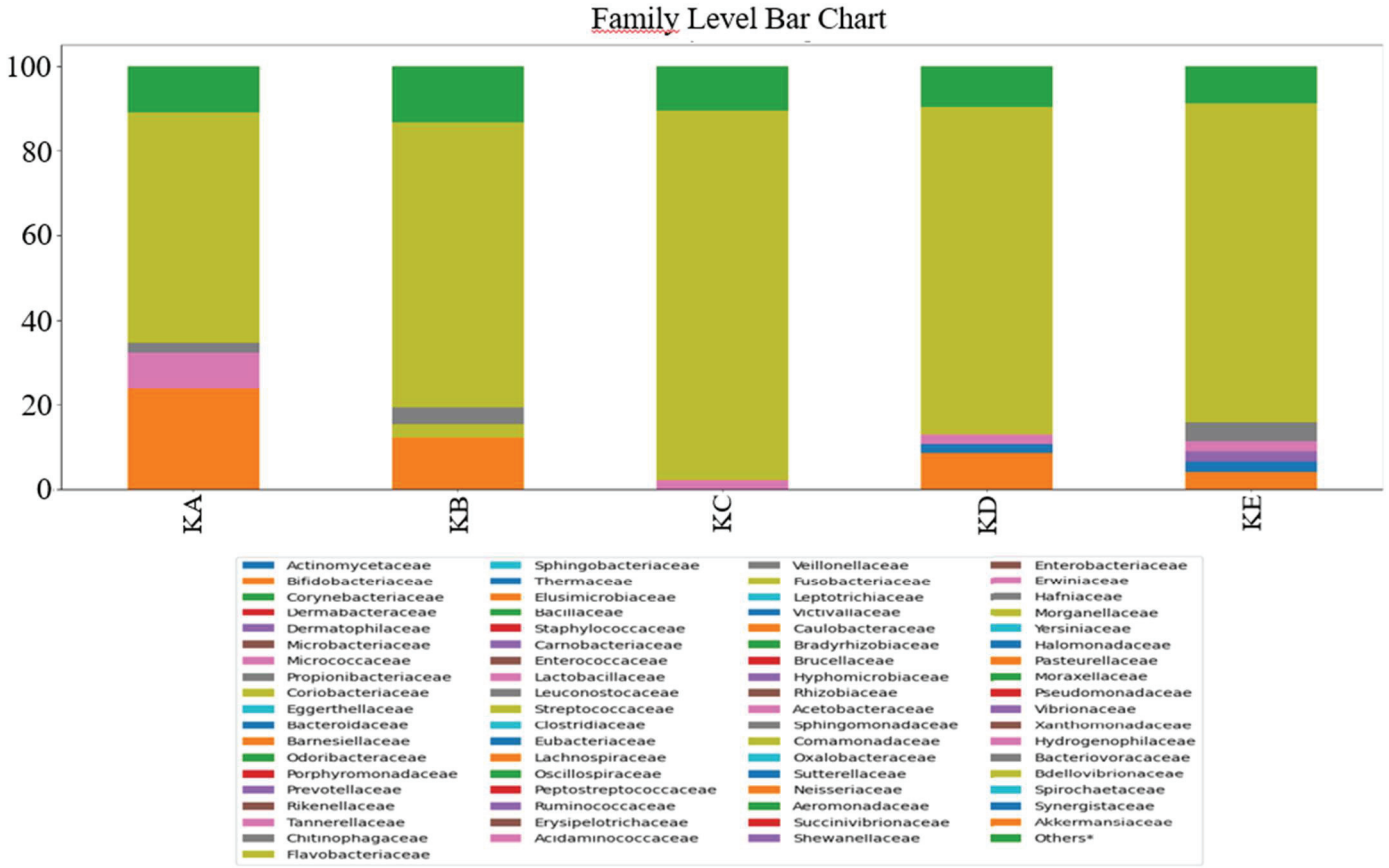


Figure 60. Family level taxonomic comparison of kefir samples

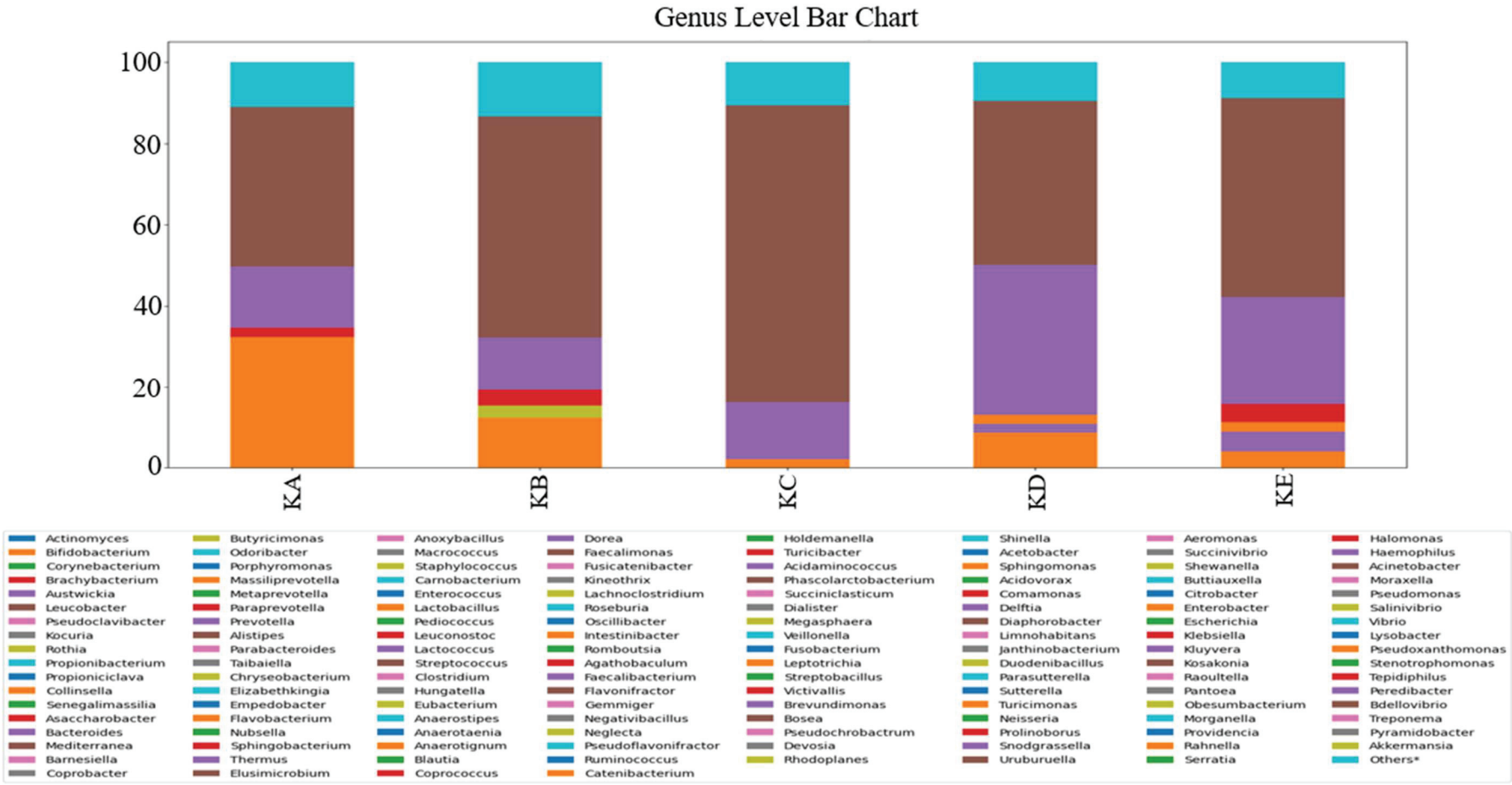


Figure 61. Genus level taxonomic comparison of kefir sample

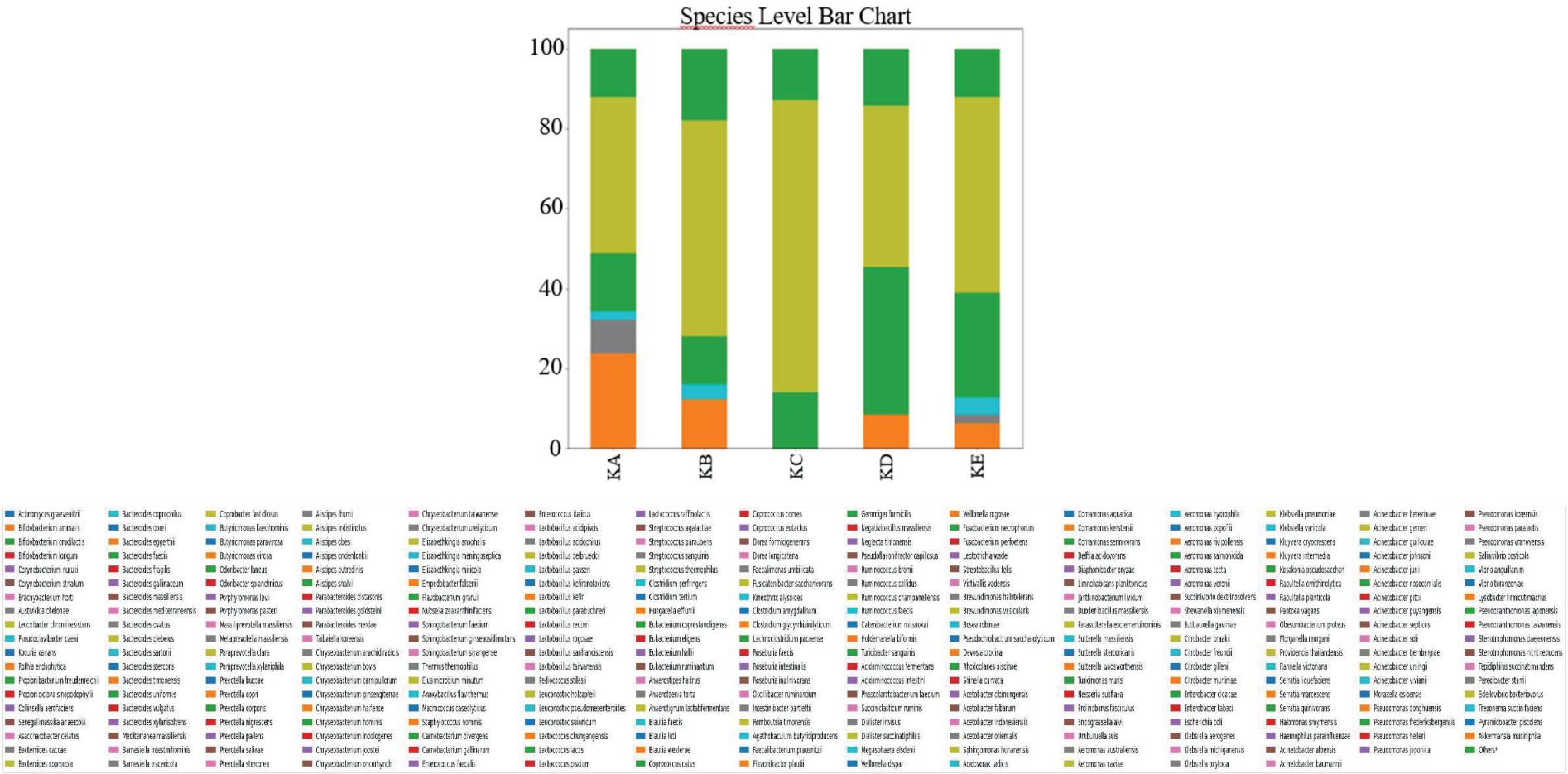


Figure 62. Species level taxonomic comparison of kefir samples

4.4. Comparison of Alpha Diversity in Kefir Sample

4.4.1. Shannon Index

The highest level of shannon diversity index was observed in KA sample ranging between 2.5 and 3.0. The lowest shannon diversity index was observed in KC sample ranging between 1.4 and 1.8.

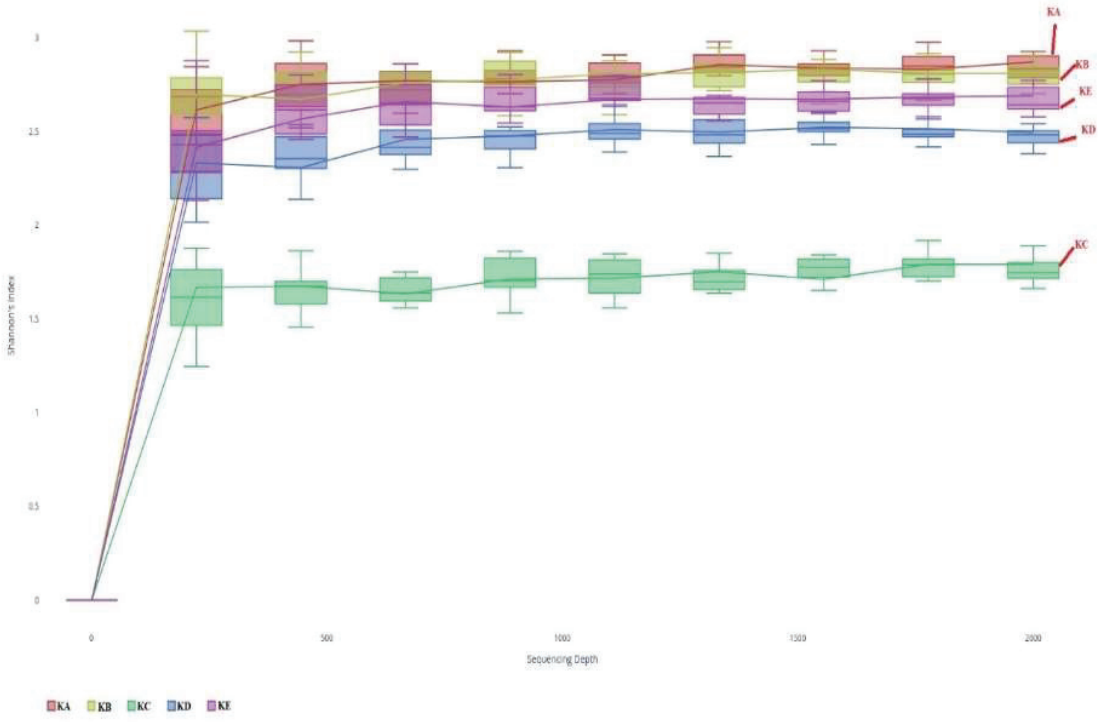


Figure 63. Shannon Index

4.4.2. Simpson Index

The highest level of simpson diversity index was observed in KA sample with an average value of 0.72. The lowest simpson diversity index was observed in KC sample with an average value of 0.4. The simpson index values of KB, KD and KE was very similar to each other with average values of 0.65.

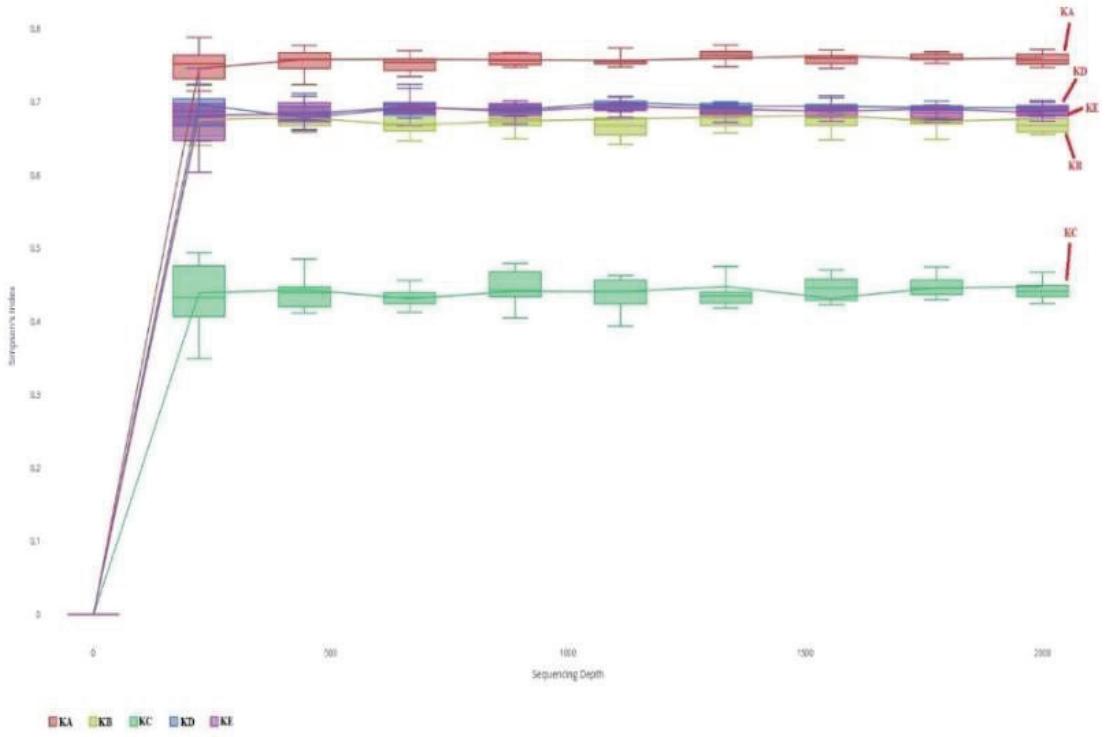


Figure 64. Simpson Index

4.4.3. Chaol Index

The chao1 diversity index values of KA, KB, KD and KE was very similar to each other with values around 100, the lowest chao1 diversity index value was observed in KC sample with an average value around 60.

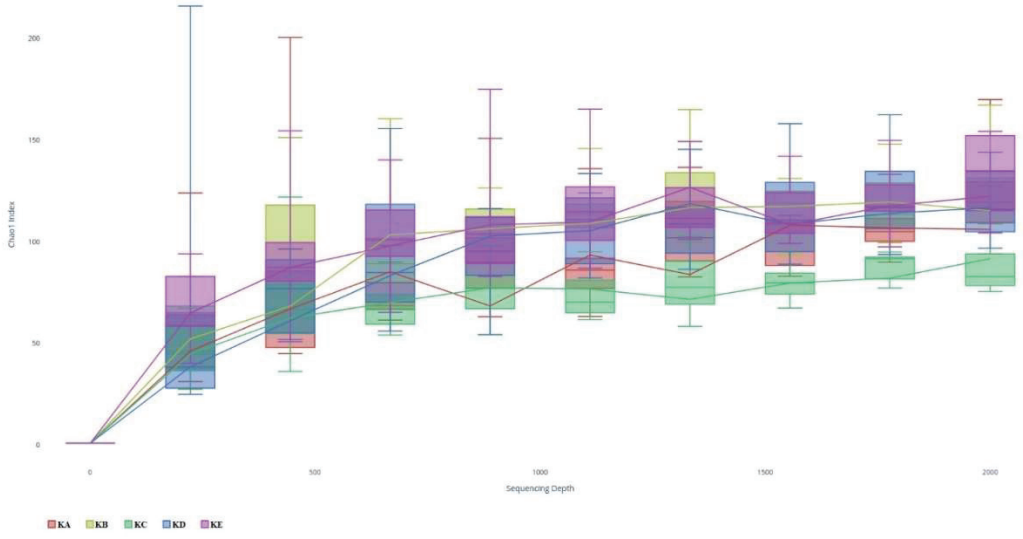


Figure 65. Chao1 Index

4.5. Comparison of microbial diversity estimates based on classical microbiological and sequence-based microbiota approaches among kefir samples

In KA, on day 1 and day-7 of the microbiological count total LAB count was 0.17% and 0.11 respectively, but in NGS total LAB count was 65.17%. The microbiological *Lactobacilli* count on day-1 and day-7 was 0.03% and 2.62% respectively. The NGS-*Lactobacilli* count was 8.53%. The microbiological *Lactococci* count on day-1 and day-7 was 0.57% and 0.64% respectively. The NGS-*Lactococci* count was 14.99%.

In KB the microbiological count on day-1 and day-7 the total LAB count was 2.3% and 0.38% respectively. The NGS total LAB count was 71.27%. The microbiological *Lactobacilli* count on day-1 and day-7 was 0.003% and 8.33% respectively. The NGS-*Lactobacilli* count was not present. The microbiological *Lactococci* count on day-1 and day-7 was 0.33% and 0.16% respectively. The NGS-*Lactococci* count was 12.98%.

In KC, the microbiological count on day-1 and day-7 the total LAB count was 0.66% and 0.44% respectively. The NGS total LAB count was 89.39%. The microbiological *Lactobacilli* count on day-1 and day-7 was lower than 10, means that it is not present. The NGS-*Lactobacilli* count was 2.14%. The microbiological *Lactococci* count on day-1 and day-7 was 0.66% and 0.30% respectively. The NGS-*Lactococci* count was 14.06%.

In KD, the microbiological count on day-1 and day-7 the total LAB count was, 0.90% and 0.45%. The NGS total LAB count was 79.66%. The microbiological *Lactobacilli* count on day-1 and day-7 was 0.00075% and 15.000% respectively. The NGS-*Lactobacilli* count was 2.17%. The microbiological *Lactococci* count on day-1 and day-7 was 1.75% and 0.0035% respectively. The NGS-*Lactococci* count was 37.06%.

In KE, the microbiological count on day-1 and day-7 the total LAB count was, 0.6% and 0.375% respectively. The NGS total LAB count was 82.23%. The microbiological *Lactobacilli* count on day-1 and day-7 was 0.00008% and 0.00025% respectively. The NGS-*Lactobacilli* count was 2.33%. The microbiological *Lactococci* count on day-1 and day-7 was 1.024% and 0.025% respectively. The NGS-*Lactococci* count was 26.49%.

Table 9. Comparison of kefir samples according to microbiological count and Next Generation Sequencing (NGS)

Sample	**Time**	**LAB (cfu.mL^{-1})**		***Lactobacilli* (cfu.mL^{-1})**		***Lactococci* (cfu.mL^{-1})**		**NGS-LAB**	**NGS-*Lactobacilli***	**NGS-*Lactococci***
Kefir-A	**Day-1**	1.11×10^7	0.17%	2.1×10^6	0.03%	3.6×10^7	0.57%	65.17%	8.53%	14.99%
	Day-7	8.5×10^6	0.11%	1.8×10^4	2.62%	4.45×10^7	0.64%			
Kefir-B	**Day-1**	6.9×10^7	2.3%	1.40×10^5	0.003%	1.1×10^7	0.33%	71.27%	NP	12.98%
	Day-7	2.41×10^8	0.38%	5.15×10^5	8.33%	1.4×10^8	0.16%			
Kefir-C	**Day-1**	5.9×10^7	0.66%	<10	0%	6×10^7	0.66%	89.39%	2.14%	14.06%
	Day-7	1.1×10^8	0.44%	<10	0%	8.5×10^7	0.30%			
Kefir-D	**Day-1**	3.96×10^7	0.90%	3.1×10^4	0.00075%	6.5×10^7	1.75%	79.66%	2.17%	37.06%
	Day-7	8.5×10^7	0.45%	2.5×10^4	15.000%	7.2×10^5	0.0035%			
Kefir-E	**Day-1**	6.2×10^6	0.6%	1.0×10^3	0.00008%	1.25×10^7	1.024%	82.23%	2.33%	26.49%
	Day-7	3.35×10^7	0.375%	1.85×10^4	0.00025%	2.15×10^6	0.025%			

4.6. Comparison of FTIR fingerprint region data with sequenece based microbial diversity among Kefir samples

Both homofermentative and heterofermentative lactic acid bacteria (LAB), *Lactobacillaceae* family (*Lactobacillus* and *Leuconostoc* genera) and *Streptococcaceae* family (*Lactococcus* and *Streptococcus* genera), acetic acid bacteria *Acetobacteraceae* family (*Acetobacter* genera) and yeasts *Saccharomycetaceae* family (*Kluyveromyces* and *Saccharomyces* genera) form the microorganisms usually found in kefir grains (Bengoa et al. 2019; Garrote et al. 2001; Leite et al. 2013; Pogačić et al. 2013; Zheng et al. 2020).

Lactic acid bacteria group, which are fermentative, produce relatively large amounts of lactic acid from carbohydrates. Mainly, *Streptococcus thermophilus* and the species of *Lactococcus, Leuconostoc, Pediococcus, and Lactobacillus* genera are included in the group.

Spherical or ovoid (1 μm) shaped *Streptococcus* species are nonmotile, facultative anaerobes; mesophiles arranged in pairs or chains and can grow at 50°C. *Str. thermophilus* is used in dairy fermentation as well as in milk kefir beverage production; can be present in raw milk.

Facultative anaerobe, nonmotile, ovoid elongated *Lactococcus* cells (0.5-1.0 μm) having arrangement as pairs or short chains are mesophiles, however can grow at 10°C and produce lactic acid. *Lactococcus* species are used to produce many foods mainly fermented dairy foods by bioprocessing. *Lactococcus lactis* subsp. *lactis* and *L. lactis* subsp. *cremoris* present in raw milk and plants and, some strains have potential as food biopreservatives since they produce bacteriocins with a relatively wide host range against Gram-positive bacteria.

Although some are very long but others are coccobacilli rod-shaped *Lactobacillus* cells appear in single or in small and large chains and also differ extensively in shape and size. They are facultative anaerobes; most species are nonmotile; mesophiles (but some are psychrotrophs); can be homo- or heterolactic fermentors. Found in plant sources, milk, meat, and feces. Many of them such as *Lactobacillus delbrueckii* subsp. *bulgaricus*, *Lab. helveticus* and *Lab. plantarum* are used in food bioprocessing. Some species such as *Lab. acidophilus, Lab. reuteri* and *Lab. casei* subsp. *casei* are used as probiotics. Some species like *Lab. sake* and *Lab. curvatus* can grow in products stored at refrigerated temperature while several strains produce bacteriocins having a wide spectrum can be used as food biopreservatives.

Facultative anaerobe, nonmotile *Leuconostoc* cells are spherical or lenticular and occur in pairs or chains. These heterofermentative LAB found in plants, meat, and milk are mesophiles but some species and strains can grow at or below 3°C. Psychrotrophic strains are associated with spoilage (gas formation) of vacuum-packaged refrigerated foods while some are used in food fermentation. *Leuconostoc mesenteroides* subsp. *mesenteroides, Leu. carnosum, Leu. lactis, Leu. mesenteroides* subsp. *dextranicum* produces dextran while growing in sucrose. Several strains produce bacteriocins, some with a wide spectrum against Gram-positive bacteria, and these have potential as food biopreservatives.

Lactobacillus and *Leuconostoc* can grow in both the presence and absence of oxygen and during metabolism of nutrients species from these genera can produce gas ($C0_2$. H_2. H_2S). Some *Lactobacillus* and *Leuconostoc* species are psychrotrophs that can grow at refrigerated temperature (≤ 5°C). Several species from *Lactobacillus, Lactococcus,* and *Streptococcus* are tolerant of an acidic environment i.e. aciduric that can survive at low pH (< 4.0). Species or strains of *Lactobacillus*, *Leuconostoc* and *Lactococcus* are able to produce slime as they synthesize polysaccharides.

Acetic acid bacteria such as *Acetobacter aceti* produce acetic acid and have high resistance to acetic acid released into the fermentative medium.

Activity of kefir grains is provided by conservation of the bacteria and yeast proportion attained by continuous fermentation patterns that lead to their biomass proliferate. Temperature, pH, washing of the grains, renewal of milk, and the presence of nutrients influence this increase (Bourrie et al 2016; Garrote et al. 1998; 2001; Guzel-Seydim et al. 2011; Simova et al. 2002; Rattray and O'Connell 2011).

Kefir drink microbiota is distinct from that of the kefir grains (Simova et al. 2002). The physicochemical properties and microbial content of kefir drink is affected by the type of the milk used, grain to milk ratio, temperature and time of fermentation cycle and storage conditions (Hecer et al. 2019; Gul et al. 2013; Magra et al. 2012; Vieira et al. 2015; Wszolek et al. 2001).

Traditional kefir drink typically uses cow's milk as substrate (Gul et al. 2013; Nielsen 2014). Whole, semi-skimmed, or skimmed milk can be utilized however a kefir produced by skimmed milk have significantly lower nutritional quality (Irigoyen et al. 2005; Rea et al. 1996; Vieira et al. 2015). Grain to milk ratio, typically altering between 2% and 10% (w/v), affects the kefir microbial content, and higher rates of grain inoculum enhance lactic acid quantities, allocating sharper pH decline (Garrote et al. 2000; Wszolek

et al. 2001; Irigoyen et al. 2005). The viscosity is also influenced, since higher ratios of kefir grain inoculum produce a more acidic, but less viscous, kefir (Wszolek et al. 2001; Irigoyen et al. 2005). Lactose content is the predominant nutritional component affected by the amount of grain inoculum and smaller ratios of inoculation results in kefir with higher lactose levels (Irigoyen et al. 2005; Leite et al. 2013).

Distinctive kefir fermentation takes place between 20 and 25°C for almost 24 h, with pH ranges between 4.2 and 4.6 (Garrote et al., 2000; Wszolek et al., 2001; Zajšek, and Goršek, 2010). The chemical composition of kefir changes during fermentation mainly due to lactose transformation to lactic acid by homofermentative LAB causing the pH to decrease and acidity to increment, followed by the surplus hydrolyzation into glucose and galactose by the enzymatic activity of β-galactosidase available in the grains (Barukčić et al. 2017; De Vrese et al. 1992; Leite et al. 2013). Additionally heterofermentative LAB transform glucose into lactic acid, ethanol and CO_2, the former being the most predominant organic acid after fermentation, and in this environment proteins are broken down into peptides (Guzel Seydim et al. 2000; Irigoyen et al. 2005; Magra et al. 2012; Otles and Cagindi 2003). Lactic acid produced in kefir has antimicrobial effect and since it behaves as a natural preservative, enables the homemade product to have a lower risk of contaminants (Bengoa et al. 2019; Garrote et al, 2000; Walsh et al. 2016).

At least 2.8% protein, less than 10% fat, and at least 0.6% lactic acid account for the chemical constitution of kefir drink reflects its nutritional value and is the recommended quality standards for kefir are (Codex, 2003). Following grain separation kefir drink can be consumed immediately or preserved in refrigerator (Farnworth 2005; Garrote et al. 1998; Otles and Cagindi 2003). Characteristics of kefir drink as well as fermented milk products must be asserted during storage. Howbeit, since continuous metabolic activities of surplus kefir microbiota may take place, the composition of refrigerated kefir may be influenced during storage (Garrote et al. 1997; Grønnevik et al. 2011; Magra et al. 2012; Irigoyen et al. 2005). Generally, kefir drink can retain a shelf life of 3–12 days (Garrote et al. 1998). It was reported that during refrigerated (4°C) storage viscosity of kefir drink declined unexpectedly with time, while total fat, lactose, dry matter, and pH remain constant until 14 days of storage and lactic acid slightly increases after 7 days storage (Guzel-Seydim et al. 2000; Magra et al. 2012; Irigoyen et al. 2005; Vieira et al. 2015). The lipolytic activity in milk fat by LAB is limited yet it can still confer to the production of free fatty acids (Kim and Liu 2002)

When we compare the FTIR finger print regions of kefir samples, the spectrums of Kefir1 (KB) and Kefir5 (KA) samples are similar to each other, while the spectrums of Kefir3 (KD) and kefir 4 (KC) samples are similar. Although the entire FTIR fingerprint region of kefir2 (KE) sample is not similar to other kefir samples, kefir 1 kefir 2 and kefir 5 781.54 and 701.22 cm1 give the same peaks. When we compare the FTIR finger print data (Figure 6-9.) with the sequence-based microbial diversity prediction data (Figure 56), Kefir 1 and Kefir5 are similar. Kefir 4 and kefir 3 are similar to each other.

CHAPTER 5

CONCLUSIONS

For five milk kefir samples mould, coliforms and *E. coli* were not determined. As can be seen from Table 8, the bacterial content of the kefir beverage samples varied from 7.086 to 8.794 $\log_{10}$ cfu.ml^{-1} for viable total aerobic mesophilic bacteria (TAMB), 6.792 and 8.382 $\log_{10}$ cfu.ml^{-1} for LAB, <10 and 6.322 $\log_{10}$ cfu.ml^{-1} for *Lactobacillus*, 5.857 and 8.146 $\log_{10}$ cfu.ml^{-1} *Lactococcus*, 5.176 and 7.218 $\log_{10}$ cfu.ml^{-1} for yeasts. Since in all of the samples LAB numbers were found and in any of the samples coliforms and *E. coli* were not detected, according to microbiological analysis results of the milk kefir samples, it can be concluded that the microbiological criteria and safety of the samples are proper for the legislation. By FTIR analysis a correlation can be made according to storage duration. When storage duration increases PC1 value increases and different brands scatter through PC2.

All kefir brands contain lactic kefir culture contents and kefir yeast and contain at least 1.0x10^6 cfu.ml^{-1} viable microorganisms. Although brand Kefir 4 (KC) has claim on the packaging that contains *Bifidobacterium* and *L. acidophilus* at least 1.0x10^6 cfu.ml^{-1}no *Lactobacilli* were detected. This result also confirmed by the sequencing data that is no *Bifidobacterium* was detected in Kefir 4 (KC). Similarly, sequencing data showed that K1 (KB), K3 (KD) and K4 (KC) kefir samples were not including *L. acidophilus.* The most abundant species in all kefir samples were *Str.thermophilus* ranging from 39.22% in KA to 73.14% in KC. (*Str. thermophilus*: KA: 39.22%, KB: 54.12%, KC: 73.14%, KD: 40.19%, KE: 48.86%). The second most abundant species in all kefir samples was *Lactococcus lactis* ranging from 11.91% in KB to 36.85% in KD. The third most abundant species in all kefir samples was *Bif. animalis* ranging from 4.01% in KE to 23.79% in KA, however this species were not found in sample KC. The 4th most abundant species in all kefir samples was *L. acidophilus* were found only in KA and KE at 2.11% and 8.33% levels, respectively. *Prevotella copri* was found only in sample KE.

Based on a phylogenetic analysis showing the relatedness of a kefir beverage based on species level taxonomic diversity KA and KB samples, KE and KD samples were more closely related to each other. KC sample was the most different sample

compared to the other kefir beverage samples. These analyses suggest that the species level taxonomic diversity is similar in KA and KB samples, and KD and KE samples.

REFERENCES

"16S Ribosomal RNA Sequencing (Theory) : Microbiology Virtual Lab II : Biotechnology and Biomedical Engineering : Amrita Vishwa Vidyapeetham Virtual Lab." n.d. Accessed April 24, 2022. https://vlab.amrita.edu/?sub=3&brch=76&sim=1421&cnt=1.

"9 Evidence-Based Health Benefits of Kefir." n.d. Accessed April 24, 2022. https://www.healthline.com/nutrition/9-health-benefits-of-kefir.

Aghlara, Arezou, Shuhaimi Mustafa, Yazid A. Manap, and Rosfarizan Mohamad. 2009. "Characterization of Headspace Volatile Flavor Compounds Formed during Kefir Production: Application of Solid Phase Microextraction." *International Journal of Food Properties* 12 (4): 808–18. https://doi.org/10.1080/10942910802073189.

Angulo, Luis, Eliseo Lope, and Cesar Lema. 1993. "Microflora Present in Kefir Grains of the Galician Region (North-West of Spain)." *Journal of Dairy Research* 60 (2): 263–67. https://doi.org/10.1017/S002202990002759X.

"Attenuated Total Reflectance (ATR) | For FTIR Applications." n.d. Accessed April 24, 2022.https://www.mt.com/us/en/home/products/L1_AutochemProducts/ReactIR/attenuated-total-reflectance-atr.html.

Barukčić, I.; Gracin, L.; Jambrak, A.R.; Božanić, R. 2017. "Comparison of chemical, rheological and sensory properties of kefir produced by kefir grains and commercial kefir starter." *Mljekarstvo* 67, 169–176. doi:10.15567/mljekarstvo.2017.0301.

Bengoa, A.A.; Iraporda, C.; Garrote, G.L.; Abraham, A.G. 2019. "Kefir micro-organisms: Their role in grain assembly and health properties of fermented milk." J. Appl. Microbiol., 126, 686–700. doi:10.1111/jam.14107.

Bolyen, Evan, Jai Ram Rideout, Matthew R Dillon, Nicholas A Bokulich, Christian C Abnet, Gabriel A Al-Ghalith, Harriet Alexander, et al. 2019. "Reproducible, Interactive, Scalable and Extensible Microbiome Data Science Using QIIME 2." *Nature Biotechnology* 37 (8): 852–57. https://doi.org/10.1038/s41587-019-0209-9.

Bourrie, B.C.T.; Willing, B.P.; Cotter, P.D. 2016. "The Microbiota and Health Promoting Characteristics of the Fermented Beverage Kefir." *Frontiers in Microbiology.* 7, 647. doi:10.3389/fmicb.2016.00647.

C. P. Miguel, Maria Gabriela da, Patrícia G Cardoso, Karina T Magalhães, and Rosane F Schwan. 2011. "Profile of microbial communities present in Tibico (Sugary Kefir) grains from different Brazilian States." *World Journal of Microbiology and Biotechnology* 27 (8): 1875–84. https://doi.org/10.1007/s11274-010-0646-6.

Casteele, S.V., Vanheuverzwijn, T., Ruyssen, T., Assche, V.P., Swings, J., Huys, G., 2006. "Evaluation of culture media for selective enumeration of probiotic srains of

Lactobacilli and *Bifidobacteria* in combination with yoghurt or cheese starters", *International Dairy Journal* 16, 1470-1476.

Castellanos-Rozo, José, Rubén Pérez Pulido, Mª José Grande, Rosario Lucas, and Antonio Gálvez. 2020. "Analysis of the bacterial diversity of Paipa cheese (a traditional raw cow's milk cheese from Colombia) by high-throughput sequencing." *Microorganisms* 8 (2): 218. https://doi.org/10.3390/microorganisms8020218.

Codex Alimentarius International Food Standards. Codex Standard for Fermented Milks (Codex Stan CXS 243-2003) Codex Alimentarius; FAO: Rome, Italy; ISBN 5856420187. Available online: http://www.fao.org/fao-who-codexalimentarius/codex-texts/list-standards/en/ (accessed on 12 January 2021).

Coimbra, M. A., Gonçalves, F., Barros, A. S., & Delgadillo, I. 2002. "Fourier transform infrared spectroscopy and chemometric analysis of white wine polysaccharide extracts." *Journal of Agricultural and Food Chemistry*, 50, 3405–3411.

Cole, J R, Wang Q, Cardenas E, Fish J, Chai B, Farris R J, Kulam-Syed-Mohideen A S, et al. 2008. "The Ribosomal Database Project: Improved Alignments and New Tools for RRNA Analysis." *Nucleic Acids Research* 37: 141–45. https://doi.org/10.1093/nar/gkn879.

Cox, Michael J., William O.C.M. Cookson, and Miriam F. Moffatt. 2013. "Sequencing the Human Microbiome in Health and Disease." *Human Molecular Genetics* 22 (R1): 88–94. https://doi.org/10.1093/hmg/ddt398.

Dallas, D., Citerne, F., Tian, T., Silva, V., Kalanetra, K., Frese, S., et al. 2016. "Peptidomic analysis reveals proteolytic activity of kefir microorganisms on bovine milk proteins." *Food Chemistry* 197, 273–284. doi: 10.1016/j.foodchem.2015. 10.116

De Man, J.C., Rogosa, M., Sharpe, M.E. 1960. "A medium for the cultivation of *Lactobacilli*." *Journal of Applied Bacteriology* 23:130–136.

De Vrese, M.; Keller, B.; Barth, C.A. 1992. "Enhancement of intestinal hydrolysis of lactose by microbial β-galactosidase (EC 3.2.1.23) of kefir." *British Journal of Nutrition* 67, 67–75. doi:10.1079/bjn19920009.

Enikeev, Ruslan. 2012. "Development of a new method for determination of exopolysaccharide quantity in fermented milk products and its application in technology of kefir production." *Food Chemistry* 134 (4): 2437–41. https://doi.org/https://doi.org/10.1016/j.foodchem.2012.04.050.

Ertekin, B., and Guzel-Seydim Z.B. 2010. "Effect of Fat Replacers on Kefir Quality." *Journal of the Science of Food and Agriculture* 90 (4): 543–48. https://doi.org/10.1002/jsfa.3855.

Feng, P., Weagant, S. D., Grant M. A., Burkhardt, W. 2002. Chapter 4. "Enumeration of *Escherichia coli* and the Coliform Bacteria," In Food and Drug Administration

(FDA), Bacteriological Analytical Manual Online, 8th Edition, Silver Spring, Berlin, 1998.
Farnworth, E.R. 2016. “Kefir : From Folklore to Regulatory Approval” 4179 (February). https://doi.org/10.1300/J133v01n04.

Farnworth, E.R. 2005. Kefir—A complex probiotic. Food Science and Technology Bulletin. *Functional Foods* 2, 1–17, doi:10.1616/1476-2137.13938.

Gao, Jie, Fengying Gu, Jie He, Jianzhong Xiao, Qihe Chen, Hui Ruan, and Guoqing He. 2013. “Metagenome analysis of bacterial diversity in Tibetan kefir grains.” *European Food Research and Technology* 236 (3): 549–56. https://doi.org/10.1007/s00217-013-1912-2.

Gao, Xin, and Bo Li. 2016. “Chemical and microbiological characteristics of kefir grains and their fermented dairy products: A review.” *Cogent Food and Agriculture* 2 (1). https://doi.org/10.1080/23311932.2016.1272152.

Garbers, I-m., T J Britz, and R C Witthuhn. 2004. “PCR-Based Denaturing gradient gel electrophoretic typification and identification of the microbial consortium present in kefir grains.” *World Journal of Microbiology and Biotechnology* 20 (7): 687–93. https://doi.org/10.1007/s11274-004-2624-3.

García Fontán, María C, Sidonia Martínez, Inmaculada Franco, and Javier Carballo. 2006. “Microbiological and chemical changes during the manufacture of kefir made from cows’ Milk, Using a commercial starter culture.” *International Dairy Journal* 16 (7): 762–67. https://doi.org/https://doi.org/10.1016/j.idairyj.2005.07.004.

Garofalo, C., Osimani A., Milanović V., Aquilanti L., De Filippis F., Stellato G., Di Mauro S., et al. 2015. “Bacteria and yeast microbiota in milk kefir grains from different Italian regions.” *Food Microbiology* 49 (1): 123–33. https://doi.org/10.1016/J.FM.2015.01.017.

Garrote, G.; Abraham, A.; De Antoni, G. 1997. Preservation of Kefir Grains, a Comparative Study. LWT, 30, 77–84, doi:10.1006/fstl.1996.0135.

Garrote, G.L.; Abraham, A.G.; De Antoni, G.L. 1998. "Characteristics of kefir prepared with different grain [ratio] milk ratios." *Journal of Dairy Research* 65, 149–154, doi:10.1017/s0022029997002677.

Garrote, G.L., Abraham, A.G. and De Antoni, G.L. 2001. “Chemical and microbiological characterisation of kefir grains.” *Journal of Dairy Research* 68 (4): 639–52. https://doi.org/DOI: 10.1017/S0022029901005210.

Garrote, G.L.; Abraham, A.G.; De Antoni, G.L. 2000. I"nhibitory power of kefir: The role of organic acids." *Journal of Food Protection* 63, 364–369, doi:10.4315/0362-028x-63.3.364.

Grønnevik, H.; Falstad, M.; Narvhus, J.A. "Microbiological and chemical properties of Norwegian kefir during storage." *International Dairy Journal* 2011, 21, 601–606, doi:10.1016/j.idairyj.2011.01.001.

Gul, O.; Mortas, M.; Atalar, I.; Dervisoglu, M.; Kahyaoglu, T. "Manufacture and characterization of kefir made from cow and buffalo milk, using kefir grain and starter culture." *Journal of Dairy Science* 2015, 98, 1517–1525, doi:10.3168/jds.2014-8755.

Gulitz, A., Stadie J., Wenning M., Ehrmann M. A, and Vogel R.F. 2011. "The microbial diversity of water kefir." *International Journal of Food Microbiology* 151 (3): 284–88. https://doi.org/https://doi.org/10.1016/j.ijfoodmicro.2011.09.016.

Guzel-Seydim, Z.; Kok-Tas, T.; Ertekin-Filiz, B.; Seydim, A.C. 2011. "Effect of different growth conditions on biomass increase in kefir grains." *Journal of Dairy Science* 94, 1239–1242, doi:10.3168/jds.2010-3349.

Guzel-Seydim, Z.; Seydim, A.C.; Greene, A.K. "Organic acids and volatile flavor components evolved during refrigerated storage of kefir." *Journal of Dairy Science* 2000, 83, 275–277, doi:10.3168/jds. s0022-0302(00)74874-0.

Güzel-Seydim, Z.B., Seydim, A.C. Greene, A.K., Bodine A. B. 2000. "Determination of organic acids and volatile flavor substances in kefir during fermentation." *Journal of Food Composition and Analysis* 13 (1): 35–43. https://doi.org/10.1006/jfca.1999.0842.

Hamet, Fernanda M., Londero A., Medrano M., Vercammen E., Koenraad Van Hoorde, Graciela L Garrote, Huys G., Vandamme P., and Abraham A. G. 2013. "Application of culture-dependent and culture-Independent methods for the identification of *Lactobacillus kefiranofaciens* in mcrobial consortia pesent in kefir grains." *Food Microbiology* 36 (2): 327–34. https://doi.org/https://doi.org/10.1016/j.fm.2013.06.022.

Hecer, C.; Ulusoy, B.; Kaynarca, D. 2019. "Effect of different fermentation conditions on composition of kefir microbiota." I*nternational Food Research Journal* 26, 401–409.

"How to Store Kefir Grains When You're Taking a Break from Kefir - KEFIRKO." n.d. Accessed April 24, 2022. https://kefirko.com/how-to/how-to-store-kefir-grains-when-taking-break-from-kefir/?v=ebe021079e5a.

Hsieh, Hsin-Hui, Sheng-Yao Wang, Tzu-Li Chen, Yen-Lin Huang, and Ming-Ju Chen. 2012. "Effects of Cow's and Goat's Milk as Fermentation Media on the Microbial Ecology of Sugary Kefir Grains." *International Journal of Food Microbiology* 157 (1): 73–81. https://doi.org/https://doi.org/10.1016/j.ijfoodmicro.2012.04.014.

Huang, Ying, Fei Wu, Xiaojun Wang, Yujie Sui, Longfei Yang, and Jinfeng Wang. 2013. "Characterization of *Lactobacillus plantarum* Lp27 isolated from Tibetan kefir grains: A potential probiotic bacterium with cholesterol-lowering effects." *Journal of Dairy Science* 96 (5): 2816–25.

https://doi.org/https://doi.org/10.3168/jds.2012-6371.

Irigoyen, A.,"" Arana, I., Castiella, M., Torre, P., Ibáñez, F. 2005. "Microbiological, physicochemical, and sensory characteristics of kefir during storage." *Food Chemistry* 90, 613–620, doi: 10.1016/j.foodchem.2004.04.021.

ISO, 1992. International Organization for Standardization. ISO 6611:1992. "Milk and milk products - Enumeration of yeast and moulds - Colony count technique at 25°C." Genewa, Switzerland: International Organisation Standardisation.

ISO, 1998. International organization for standardization. ISO 15214:1998. "Microbiology of food and animal feeding stuffs-Horizontal method for the enumeration of mesophilic lactic acid bacteria-colony count technique at 30 °C." Genewa, Switzerland: International Organisation Standardisation.

ISO, 2003. International Organization for Standardization. ISO 4833:2003. "Microbiology of food and animal feeding stuffs - Horizontal method for the enumeration of microorganisms-Colony-count technique at 30°C." Genewa, Switzerland: International Organisation Standardisation.

ISO, 2007. International organization for standardization. PN-ISO 6611:2007. "Milk and milk product. Determination of the number of yeast and/or mold colony forming units. Plate method at 25°C." Genewa, Switzerland: International Organisation Standardisation.

ISO, 2010. International organization for standardization. ISO 4831:2010. "Microbiology of food and animal feeding stuffs-Horizontal method for the detection and enumeration of coliforms-Most probable number technique." Genewa, Switzerland: International Organisation Standardisation.

Kim, Y.J.; Liu, R.H. 2002. "Increase of conjugated Linoleic acid content in milk by fermentation with lactic acid bacteria." *Journal of Food Science* 67, 1731–1737, doi:10.1111/j.1365-2621.2002.tb08714.x.

Klindworth, A., Elmar Pruesse, Schweer T., Peplies J., Quast C., Horn M., and Glöckner F.O. 2013. “Evaluation of general 16S ribosomal RNA gene PCR primers for classical and next-generation sequencing-based diversity sudies.” *Nucleic Acids Research* 41 (1). https://doi.org/10.1093/NAR/GKS808.

Kooiman, K. 1968."The chemical structure of kefiran, the water-soluble polysaccharide of the kefir grain." *Carbohydrate Research* 7, 200–211.

Latorre-García, Lorena, Lucas del Castillo-Agudo, and Julio Polaina. 2007. “Taxonomical classification of yeasts Isolated from kefirbased on the sequence of their ribosomal RNA genes.” *World Journal of Microbiology and Biotechnology* 23 (6): 785–91. https://doi.org/10.1007/s11274-006-9298-y.

Leite, A. M.O., Mayo, B. C. Rachid,T.C.C. Peixoto, R. S. Silva, J. T. Paschoalin, V. M.F. and Delgado S. 2012. “Assessment of the microbial diversity of Brazilian Kefir Grains by PCR-DGGE and pyrosequencing analysis.” *Food Microbiology* 31

(2): 215–21. https://doi.org/10.1016/J.FM.2012.03.011.

Leite, A.M.O.; Leite, D.C.A.; Del Aguila, E.M.; Alvares, T.S.; Peixoto, R.S.; Miguel, M.A.L.; Silva, J.T.; Paschoalin, V.M.F. "Microbiological and chemical characteristics of Brazilian kefir during fermentation and storage processes." *Journal of Dairy Science* 2013, 96, 4149–4159, doi:10.3168/jds.2012-6263.

Litopoulou-Tzanetaki, E., and N. Tzanetakis. 2014. "*Fermented Milks: Range of Products*. *Encyclopedia of Food Microbiology: Second Edition*. Second Edition. Vol. 1. Elsevier. https://doi.org/10.1016/B978-0-12-384730-0.00120-8.

Liu, J-R., Chen M-J., Lin C-W. 2002. "Characterization of polysaccharide and volatile compounds produced by kefir grains grown in soymilk." *Journal of Food Science* 67 (1): 104–108. https://doi.org/https://doi.org/10.1111/j.1365-2621.2002.tb11367.x.

Magalhães, Karina Teixeira, Maria Alcina Pereira, Ana Nicolau, Giuliano Dragone, Lucília Domingues, José António Teixeira, João Batista de Almeida Silva, and Rosane Freitas Schwan. 2010. "Production of Fermented Cheese Whey-Based Beverage Using Kefir Grains as Starter Culture: Evaluation of Morphological and Microbial Variations." *Bioresource Technology* 101 (22): 8843–50. https://doi.org/10.1016/j.biortech.2010.06.083.

Magra, T.I.; Antoniou, K.D.; Psomas, E.I. "Effect of milk fat, kefir grain inoculum and storage time on the flow properties and microbiological characteristics of kefir." *J. Texture Stud*. 2012, 43, 299–308, doi:10.1111/j.1745-4603.2011.00343. x.

Mariey, L., Signolle J. P., Amiel C., and Travert J. 2001. "Discrimination, Classification, identification of microorganisms using FTIR spectroscopy and chemometrics." *Vibrational Spectroscopy* 26 (2): 151–59. https://doi.org/10.1016/S0924-2031(01)00113-8.

Marshall, V.M., Cole W.M. 1985. "Methods for making kefir and fermented milks based on kefir." *Journal of Dairy Research* 52 (3): 451–56. https://doi.org/10.1017/S0022029900024353.

Mauriello, G., Moio L., Moschetti G., Piombino P., Addeo F., and Coppola S. 2001. "Characterization of lactic acid bacteria strains on the basis of neutral volatile compounds produced in whey." *Journal of Applied Microbiology* 90 (6): 928–42. https://doi.org/10.1046/j.1365-2672.2001.01327.x.

Mcdonald, D., Price M.N., Goodrich J., Nawrocki E.P., Desantis T.Z., Probst A., Andersen G.L, R Knight., and Hugenholtz P. 2012. "An improved greengenes taxonomy with explicit ranks for ecological and evolutionary analyses of bacteria and archaea." *The ISME Journal* 6: 610–18. https://doi.org/10.1038/ismej.2011.139.

"Microbiome." n.d. Accessed April 24, 2022. https://www.niehs.nih.gov/health/topics/science/microbiome/index.cfm.

Miguel, M., Cardoso P., Lago L., and Schwan R.. 2010. “Diversity of bacteria pesent in milk kefir grains using culture-dependent and culture-Independent methods.” *Food Research International* 43 (June): 1523–28. https://doi.org/10.1016/j.foodres.2010.04.031.

Mouwen, D J M, Weijtens M J B M, Capita R, Alonso-Calleja C, and Prieto M. 2005. “Discrimination of Enterobacterial repetitive intergenic consensus PCR types of *Campylobacter coli* and *Campylobacter jejuni* by Fourier transform infrared spectroscopy.” *Applied and Environmental Microbiology* 71 (8): 4318–24. https://doi.org/10.1128/AEM.71.8.4318-4324.2005.

Nalbantoglu, U., Cakar A., Dogan H., Abaci N., Ustek D., Sayood K., and Can H. 2014. “Metagenomic analysis of the microbial community in kefir grains.” *Food Microbiology* 41: 42–51. https://doi.org/10.1016/j.fm.2014.01.014.

Naumann, D., Helm D., and Labischinski H. 1991. “Microbiological characterizations by FT-IR spectroscopy.” *Nature* 351 (6321): 81–82. https://doi.org/10.1038/351081a0.

Nejati, F.; Capitain, C.C.; Krause, J.L.; Kang, G.-U.; Riedel, R.; Chang, H.-D.; Kurreck, J.; Junne, S.; Weller, P.; Neubauer, P. 2022. "Traditional grain-based vs. commercial milk kefirs, How different are they?" *Applied Sciences 12*, 3838. https://doi.org/10.3390/app12083838

“Near-Infrared (NIR) Light Sources for 3D Facial Recognition.” n.d. Accessed April 24, 2022. https://www.azooptics.com/Article.aspx?ArticleID=1666.

Nielsen, B., Gürakan, G.C., Ünlü, G. "Kefir: a multifaceted fermented dairy product." *Probiotics Antimicrobial Proteins* 2014, 6, 123–135, doi:10.1007/s12602-014-9168-0.

Ojeda, J.J, and M Dittrich. 2012. “Fourier Transform Infrared Spectroscopy for Molecular.” *Microbial Systems Biology: Methods and Protocols, Methods in Molecular Biology,* 881: 279–306. https://doi.org/10.1007/978-1-61779-827-6.

Ötles, S., and O. Çağındı. 2003. “Kefir: a probiotic dairy-composition, nutritional and therapeutic aspects.” *Pakistan Journal of Nutrition*. 2, 54–59, doi:10.3923/pjn.2003.54.59.

Pogačić, T.; Šinko, S.; Zamberlin, S.; Samaržija, D. "Microbiota of Kefir Grains." *Mljekarstvo* 2013, 63, 3–14.

Putzig, C.L, Leugers M.A., Mckelvy M.L., Gary E M., Nyquist R. A, Papenfuss R.R, Yurga L. 1994. “Infrared Spectroscopy.” *Analytical Chemistry* 66. https://pubs.acs.org/sharingguidelines.

Quast, C., Pruesse E., Yilmaz P., Gerken J., Schweer T., Yarza P., Peplies J., and F. Glöckner O. 2013. “The SILVA Ribosomal RNA gene database project: improved data processing and web-based tools.” *Nucleic Acids Research* 41 (D1): D590–96. https://doi.org/10.1093/nar/gks1219.

Rattray, F.P.; O'Connell, M.J. 2011. "Fermented Milks—Kefir. In Encyclopedia of Dairy Sciences" Fuquay, J.W., Fox, P.F., McSweeney, P.L.H., Eds.; Academic Press Inc.: London, UK, pp. 518–524.

Rea, M.C.; Lennartsson, T.; Dillon, P.; Drinan, F.D.; Reville, W.J.; Heapes, M.; Cogan, T.M. 1996. "Irish kefir-like grains : their structure, microbial composition and fermentation kinetics." *Journal of Applied Bacteriology* 81 (1): 83–94. https://doi.org/10.1111/j.1365-2672.1996.tb03286.x.

"Ribosome." n.d. Accessed April 24, 2022. https://www.genome.gov/genetics-glossary/Ribosome.

Sarkar, S. 2007. "Potential of kefir as a dietetic beverage - A Review." *British Food Journal* 109 (4): 280–90. https://doi.org/10.1108/00070700710736534.

Schloss, .P.D, Westcott S.L., Ryabin T., Hall J.R., Hartmann M., Hollister E.B., Lesniewski R.A., et al. 2009. "Introducing Mthur: Open-Source, platform-independent, community-supported Software for describing and comparing microbial communities." *Applied and Environmental Microbiology* 75 (23): 7537–41. https://doi.org/10.1128/AEM.01541-09.

Schoevers, A. Britz, T.J. 2003. Influence of different culturing conditions on kefir grain increase. *International Journal of Dairy Technology*, 56, 183–187, doi:10.1046/j.1471-0307.2003.00104.x.

Simova, E., Beshkova, D., Angelov, A., Hristozova, T., Frengova, G. and Spasov, Z. 2002. "Lactic acid bacteria and yeasts in kefir grains and kefir made from them", *Journal of Industrial Microbiology and Biotechnology* 28 (1): 1–6. https://doi.org/10.1038/sj.jim.7000186.

Shang, N., Xu, R. H., Li, P. L. 2013." Structure characterization of an exopolysaccharide produced by *Bifidobacterium animalis* RH." *Carbohydrate Polymers*, 91, 128–134.

Singh, R. P., Shukla, M. K., Mishra, A., Kumari, P., Reddy, C. R. K., Jha, B. 2011. "Isolation and characterization of exopolysaccharides from seaweed associated bacteria *Bacillus licheniformis*." *Carbohydrate Polymers*, 84, 1019–1026.

Song, Hae Jung, Jun Mo Lee, Louis Graf, Mina Rho, Huan Qiu, Debashish Bhattacharya, and Hwan Su Yoon. 2016. "A Novice's guide to analyzing NGS-derived organelle and Metagenome Data." *Algae* 31 (2): 137–54. https://doi.org/10.4490/algae.2016.31.6.5.

Tamime, A. Y., M. Saarela, M. Wszolek, H. Ghoddousi, D. M. Linares, and N. P. Shah. 2017. *Production and Maintaining Viability of Probiotic Micro-Organisms in Dairy Products*. *Probiotic Dairy Products: Second Edition*. https://doi.org/10.1002/9781119214137.ch4.

Taş, Tuğba Kök, F. Yeşim Ekinci, and Zeynep B. Guzel-Seydim. 2012. "Identification of Microbial Flora in Kefir Grains Produced in Turkey Using PCR." *International*

Journal of Dairy Technology 65 (1): 126–31. https://doi.org/10.1111/j.1471-0307.2011.00733.x.

"Technology of Kefir and Kumys | Semantic Scholar." n.d. Accessed April 24, 2022. https://www.semanticscholar.org/paper/Technology-of-kefir-and-kumys-Koroleva/696adaa06d8e91e97ec0474720350afd2e11f7f4.

Teixeira Magalhães, Karina, Gilberto Vinícius de Melo Pereira, Cássia Roberta Campos, Giuliano Dragone, and Rosane Freitas Schwan. 2011. "Brazilian Kefir: Structure, Microbial Communities and Chemical Composition." *Brazilian Journal of Microbiology* 42: 693–702. http://www.ncbi.nlm.nih.

"The Science Behind The Microbiome | Kavli Foundation." n.d. Accessed April 24, 2022. https://kavlifoundation.org/news/science-behind-microbiome.

"The Use and Types of Alpha-Diversity Metrics in Microbial NGS - CD Genomics." n.d. Accessed February 21, 2022. https://www.cd-genomics.com/microbioseq/the-use-and-types-of-alpha-diversity-metrics-in-microbial-ngs.html.

Turkmen, Nazli. 2017. "Chapter 29 - Kefir as a Functional Dairy Product." In *Dairy in Human Health and Disease Across the Lifespan*, edited by Ronald Ross Watson, Robert J Collier, and Victor R Preedy, 373–83. Academic Press. https://doi.org/https://doi.org/10.1016/B978-0-12-809868-4.00029-7.

Van de Casteele S., Vanheuverzwijn T., Ruyssen T., Van Assche P., Swings J., Huys G., 2006. Evaluation of culture media for selective enumeration of probiotic strains of lactobacilli and bifidobacteria in combination with yoghurt or cheese starters. Int. Dairy J. 16, 1470–1476, https://doi.org/10.1016/j.idairyj.2005.12.002

Vardjan, T, P Mohar Lorbeg, I Rogelj, and A Čanžek Majhenič. 2013. "Characterization and Stability of Lactobacilli and Yeast Microbiota in Kefir Grains." *Journal of Dairy Science* 96 (5): 2729–36. https://doi.org/10.3168/jds.2012-5829.

Vieira, C.P.; Álvares, T.S.; Gomes, L.S.; Torres, A.G.; Paschoalin, V.M.F.; Conte-Junior, C.A. Kefir Grains Change Fatty Acid Profile of Milk during Fermentation and Storage. PLoS ONE 2015, 10, e0139910, doi: 10.1371/journal.pone.0139910.

Walsh, A.M.; Crispie, F.; Kilcawley, K.; O'Sullivan, O.; O'Sullivan, M.G.; Claesson, M.J.; Cotter, P.D. Microbial Succession and Flavor Production in the Fermented Dairy Beverage Kefir. mSystems 2016, 1, e00052-16, doi:10.1128/msystems.00052-16.

Wang, S Y, H C Chen, J R Liu, Y C Lin, and M J Chen. 2008. "Identification of Yeasts and Evaluation of Their Distribution in Taiwanese Kefir and Viili Starters." *Journal of Dairy Science* 91 (10): 3798–3805. https://doi.org/10.3168/jds.2007-0468.

"Why Choose Mid-IR Spectroscopy? | Monospektra - Scientific Equipment and Industrial Solutions." n.d. Accessed April 24, 2022.

https://www.monospektra.com/why-choose-mid-ir-spectroscopy/.

Witthuhn, R Corli, Tersia Schoeman, and Trevor J Britz. 2004. “Isolation and Characterization of the Microbial Population of Different South African Kefir Grains.” *International Journal of Dairy Technology* 57 (1): 33–37. https://doi.org/https://doi.org/10.1111/j.1471-0307.2004.00126.x.

Wszolek, M.; Tamime, A.; Muir, D.; Barclay, M. Properties of Kefir made in Scotland and Poland using Bovine, Caprine and Ovine Milk with Different Starter Cultures. LWT 2001, 34, 251–261, doi:10.1006/fstl.2001.0773.

Ye, L. B., Zhang, J.-S., Yang, Y., Zhou, S., Liu, Y.-F., Tang, Q.-J., et al. 2009. Structural characterisation of a heteropolysaccharide by NMR spectra. *Food Chemistry*, 112, 962–966.

Zajšek, K.; Goršek, A. Modelling of batch kefir fermentation kinetics for ethanol production by mixed natural microflora. Food Bioprod. Process. 2010, 88, 55–60, doi: 10.1016/j.fbp.2009.09.002.

ZAJŠEK, KATJA, and A Goršek. 2010. “Effect of Natural Starter Culture Activity on Ethanol Content in Fermented Dairy Products.” *International Journal of Dairy Technology* 63 (February): 113–18. https://doi.org/10.1111/j.1471-0307.2009.00549.x.

Zheng, J.; Wittouck, S.; Salvetti, E.; Franz, C.M.A.P.; Harris, H.M.B.; Mattarelli, P.; O’Toole, P.W.; Pot, B.; Vandamme, P.; Walter, J.; et al. A taxonomic note on the genus Lactobacillus: Description of 23 novel genera, emended description of the genus Lactobacillus Beijerinck 1901, and union of Lactobacillaceae and Leuconostocaceae. Int. J. Syst. Evol. Microbiol. 2020, 70, 2782–2858, doi:10.1099/ijsem.0.004107.

Zhou, Jianzhong, Xiaoli Liu, Hanhu Jiang, and Mingsheng Dong. 2009. “Analysis of the Microflora in Tibetan Kefir Grains Using Denaturing Gradient Gel Electrophoresis.” *Food Microbiology* 26 (8): 770–75. https://doi.org/https://doi.org/10.1016/j.fm.2009.04.009.

APPENDIX

Table 1. Properties given on the package label of milk kefir brand Kefir -1 sample

KEFIR-1				
Energy and Nutrients	**For 100g**	**For 100g RI***	**For 1 Portion (250 ml)**	**For 1 Portion RI*%**
Energy (kJ/kcal)	263/63	3%	656/157	8%
Fat (g)	3.2	5%	8	11%
Saturated Fat (g)	2.2	5%	5.5	11%
Carbohydrates (g)	5.2	2%	13	5%
Sugar (g)	2.3	3%	5.8	6%
Protein (g)	3.3	7%	8.3	17%
Salt (g)	0.2	3%	0.5	8%
Calcium (mg)	120	15%	300	38%
B12 (mg)	0.4	16%	1	40%
B2 (mg)	0.2	16%	0.6	39%
Phosphorus	106	15%	265	38%

* Values refer to the average adult's Reference Intake (RI) level (8400 kJ/2000 kcal)
Sugar is due to the inherent factor of the product. It does not contain added sugar.

Table 2 Properties given on the package label of milk kefir brand Kefir-2 sample

KEFIR-2				
Energy and Nutrients	**For 100ml**	**For 100 ml*RI%**	**For 1 Portion (250 ml)**	**For 1 Portion*RI%**
Energy (kJ/kcal)	217/52	3%	548/130	7%
Fat (g)	3.2	5%	8	11%
Saturated Fat (g)	2.1	11%	5.3	26%
Carbohydrate (g)	3.2	1%	8	3%
Sugar (g)	3.2	4%	8	9%
Protein (g)	2.7	5%	6.7	14%
Salt (g)	0	0%	0	0%

* Values refer to the average adult's Reference Intake (RI) level (8400 kJ/2000 kcal)

Table 3 Properties given on the package label of milk kefir brand Kefir-3 sample

KEFIR-3	
Energy and Nutrients	**For 100ml**
Energy (kj/kcal)	217/52
Fat (g)	3.2
Saturated Fat (g)	2.1
Carbohydrate (g)	3.2
Sugar (g)	3.2
Protein (g)	2.7
Salt (g)	0

Table 4 Properties given on the package label of milk kefir brand Kefir-4 sample

KEFİR-4				
Energy and Nutrients	**For 100 ml**	**For 100g RI***	**For 1 Portion (260 ml)**	**For 1 Portion RI***
Energy (kj/kcal)	181/43	2%	470/113	6%
Fat (g)	2.5	4%	6.5	9%
Saturated Fat (g)	1.6	8%	4.2	21%
Carbohydrate (g)	2.4	1%	6.2	2%
Sugars (g)	2.4	3%	6.2	7%
Protein (g)	2.8	6%	7.3	15%
Salt (g)	0	0%	0	0%
Calsium (mg)	-	-	252	32%

* Values refer to the average adult's Reference Intake (RI) level (8400 kJ/2000 kcal)

Table 5 Properties given on the package label of milk kefir brand Kefir-5 sample

KEFİR-5		
Energy and Nutrients	**For 100 ml**	**For 100 ml *RI%**
Energy (kJ/kcal)	216/52	3%
Fat (g)	2.4	3%
Saturated Fat (g)	1.5	8%
Carbohydrate (g)	4.7	2%
Sugar (g)	4.7	5%
Protein (g)	2.8	6%
Salt (g)	0	0%
Minerals		**NRV%
Kalsiyum (mg)	120	15%

* Values refer to the average adult's Reference Intake (RI) level (8400 kJ/2000 kcal)
** NRV Nutrition Reference Value. Sugar is due to the inherent factor of the product. It does not contain added sugar.