Development of a sheep's milk kefir using species isolated from kefir products

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Abstract

The aim of the research described in this thesis was to develop a good tasting kefir using sheep's milk. Kefir is a refreshing and effervescent milk beverage fermented with bacteria and yeast. Different combinations of bacteria, yeast and treatment result in different qualities of Kefir. An optimal kefir has a pleasant slightly sour flavour and is slightly effervescent. It is drunk chilled.

To arrive at preferred or optimal kefir the following steps occurred:

- Yeast and bacteria were isolated using MRS, M17, and DRGB agar from five commercial and three homemade kefir products. 54 isolates were identified using 16s rDNA PCR for bacteria, and 26s rDNA for yeast.
    - The commercial yeasts were*: Saccharomyces cerevisiae,* and *Torulaspora delbrueckii,* the commercial bacteria were: *Lactococcus lactis* subsp*. lactis, Leuconostoc mesenteroides, Lactobacillus plantarum, Lactococcus lactis* subsp*. cremoris,* and *Leuconostoc pseudomesenteroides,* and *Leuconostoc pseudomesenteroides.*
    - The homemade yeast were: *Kluyveromyces marxianus, Kazachstania unispora, Pichia membranifaciens,* and *Clavispora lusitaniae,* and the homemade bacteria were: *Lactobacillus delbrueckii, Lactobacillus kefiranofaciens,* and *Lactobacillus kefiri.*
    - *Streptococcus thermophiles* was the only isolate found in both homemade and commercial kefir.

One isolate of each species identified was used to form a starter culture and grown in gamma sterilized sheep's milk, allowing the assessment of the growth characteristics required for kefir. The characteristics assessed were; cell counts, pH, textural properties, effervescence, and the levels of lactose, glucose, galactose, ethanol, lactic acid, acetic acid, and diacetyl using HPLC. The results from these tests were used to determine the optimum mix of species for a sheep's milk kefir.

Four different mixes of 5-6 isolates were chosen based on the results of the individual isolates and grown in sheep's milk and tested for the same characteristics as the pure isolates as well as taste tested. This optimised kefir was made by inoculating $1x10^6$cuf/mL of each isolate to sheep's milk, sealed in the final container and fermented at 30°C for 24 hours. After cooling to 4°C the final product has a refreshing sour taste and effervescence, with a pH of 4.6, and a cell count above $3x10^9$cfu/mL which decreases to above $9x10^8$cfu/mL after five weeks, which is over 10^6cfu/mL required for labelling purposes.

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1. Introduction

Spring Sheep Dairy is a sheep dairy company with a sheep dairy farm in the Central Plateau of the North Island, New Zealand. Their products currently include infant formula, calcium chews, and luxury ice cream, and they are looking to investigate the potential of a kefir made with sheep's milk. Kefir is a fermented milk beverage considered to have a range of health benefits.

1.1 Hypothesis

The microflora of bovine kefir can be isolated and used as a starter for a satisfactory sheep's milk kefir.

1.2 Research question

How will the microflora isolated from bovine kefir grow in sheep's milk as a kefir beverage?

1.3 Aim

To develop a kefir which will:

1. be made of sheep's milk,
2. have probiotic potential,
3. have an alcohol content lower than 0.5%,
4. have an acceptable taste,
5. be effervescent, and
6. have an acceptable texture.

1.4 Objectives

To develop a kefir using sheep's milk, the following objectives will be completed, and discussed, in this thesis:

1. bacteria and yeasts will be isolated from kefir products and identified,
2. from the identified species, starter cultures will be prepared and grown in sterilized sheep's milk to assess the growth characteristics,
3. the isolates will then be selected based on a number of criteria to be used as a mixed kefir culture, and finally
4. a sheep's milk kefir will be developed using the selected starter cultures.

2. Literature review

2.1 What is kefir?

Kefir is a yeast-lactic fermented milk beverage: it is refreshing, slightly sour with a thick consistency and light effervescence. It is fermented by 'grains' - clumps of bacteria and yeast - which are reused from batch to batch. Kefir originated in the Caucasus mountain region in Russia (Guzel-Seydim, Kok-Tas, & Greene, 2010) and is a popular drink in Russia, both homemade and commercial varieties. As a yeast fermented product it contains a small amount of alcohol, from 0.02% to 1.5% (Mainville & Farnworth, 2008). The lower alcohol products tend to be commercial variety's which have reduced the yeast fermentation, resulting in a trade-off between undesirable package swelling and desirable effervescence due to the carbon dioxide production by yeast. Thus lower alcohol products can have a deleterious effect on the sensory properties in comparison to traditional kefir, especially for the formation of carbon dioxide bubbles (Guzel-Seydim, Kok-Tas, & Greene, 2010). Kefir can be produced with many products: cow, sheep, goat, buffalo and mare milk, soy milk, coconut water, and fruit juices, with cow milk being the most common version (Hui, Meunier-Goddik, Josephsen, Nip, & Stanfield, 2005). Kefir is considered to have a huge range of health benefits due to the live micro-organisms present and the products of fermentation. Health benefits claimed include: improved gut health, lactose intolerance control, improved immune system, and potential anti-carcinogenic activity (Yerlikaya, Kensenkas, & Ozer, 2013). Due to these health properties kefir is becoming increasingly popular worldwide, with the CEO of PepsiCo Europe Zein Abdalla, declaring it "one of the fastest-growing beverage categories" (Pepsico, 2012).

2.2 How is traditional kefir produced?

According to Mainville and Farnworth (2008) the traditional method of making kefir is to mix the grains with milk and allow to propagate at room temperature for around 24 hours, the grain is then removed and added to the next batch, and the resultant kefir is stored at 4°C until it is consumed. This method is not appropriate on a large scale for a number of reasons.

- The safety issues of transferring the grain from one batch to another and the potential for one contaminated batch to ruin and shut down all successive batches.
- The lack of control over the species in the grains may result in the production of undesirable attributes.
- Reduced control over the final product and lack of uniformity.

However the grain is integral to the organoleptic properties of kefir, which makes large scale production difficult.

2.3 What is a kefir grain?

A kefir grain is a clump of different yeast and bacteria in symbiosis held within a complex construct of polysaccharides and proteins. As shown in Figure 1, they resemble small cauliflower heads: white and lumpy, with an elastic texture, between 3mm and 30mm in size (de Oliveira Leite, et al., 2013). The major bacterial genera present in the kefir grain (and the resulting kefir) are; *Lactobacillus, Lactococcus, Leuconostoc,* and *Acetobacter* (Guzel-Seydim, Kok-Tas, & Greene, 2010). The yeasts are often from the genera *Candida* and *Saccharomyces* and other non-lactose and lactose fermenting yeast (Mainville & Farnworth, 2008). The grains are difficult to obtain commercially; they are generally acquired by the propagation of current grains, so grains tend to be passed from household to household (Mainville & Farnworth, 2008). This produces difficulties in identifying the micro-organisms required for kefir, as new bacteria and yeast can be incorporated into the grain resulting in a huge variety of micro-organisms found in kefir grains throughout the world. An example is *Acetobacter* (acetic acid bacteria) which is one of the four main types of bacteria found in kefir, however some studies do not find *Acetobacter*, indicating that it could potentially be a contaminant (Mainville & Farnworth, 2008). Other contaminants such as members of the *Enterobacteriaceae* and *Clostridiaceae* families, and *Pseudomonas* species and pathogenic fungi *Dipodascus capitus* and *Trichosporon coremiiforme* have been found from gene sequencing kefir grains (de Oliveira Leite, et al., 2013). However the label 'contaminant' is also very location specific, with some countries viewing certain kefir attributes developed from these 'contaminated' kefir grains as desirable, while other countries do not (Law, 1997). This reinforces the need for proper micro-organism control in the commercial production of kefir.

Figure 1 Photograph taken of milk kefir grains

The grain itself is very important for the microbiological composition of the kefir, as the yeast and bacteria exist in symbiosis – meaning some species are reliant on others for growth and survival. There are examples of bacteria that when isolated from the grain and added as pure cultures struggle or cannot grow in milk, or exhibited enhanced growth when another species is added (Hui, Meunier-Goddik, Josephsen, Nip, & Stanfield, 2005). This is further seen under an electron microscope, where the bacteria surround the yeast in close proximity, indicating potential interactions (Mainville & Farnworth, 2008). This symbiosis exhibited in the kefir grain is also thought to enhance the drinks stability regardless of milk quality variations (Mainville & Farnworth, 2008). Clumps of microbes within a gel structure can be seen in Figure 2 and Figure 3 (water kefir grains were used instead of milk based on their translucency). This complex microbiology and structure of the kefir grain is why, unlike yoghurt and similar fermented products, kefir cannot be used to produce more kefir by inoculating milk with the original product, and why it is so difficult to reproduce on commercial large scale (Mainville & Farnworth, 2008).

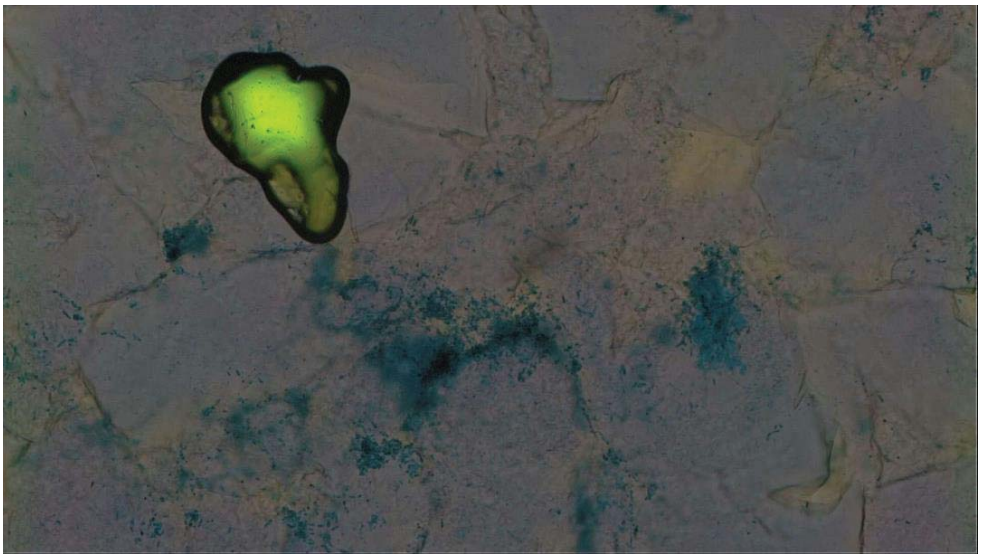

Figure 2 Water kefir grain stained with crystal violet at 10x magnification, the green is an air bubble and the dark blue dots microbes

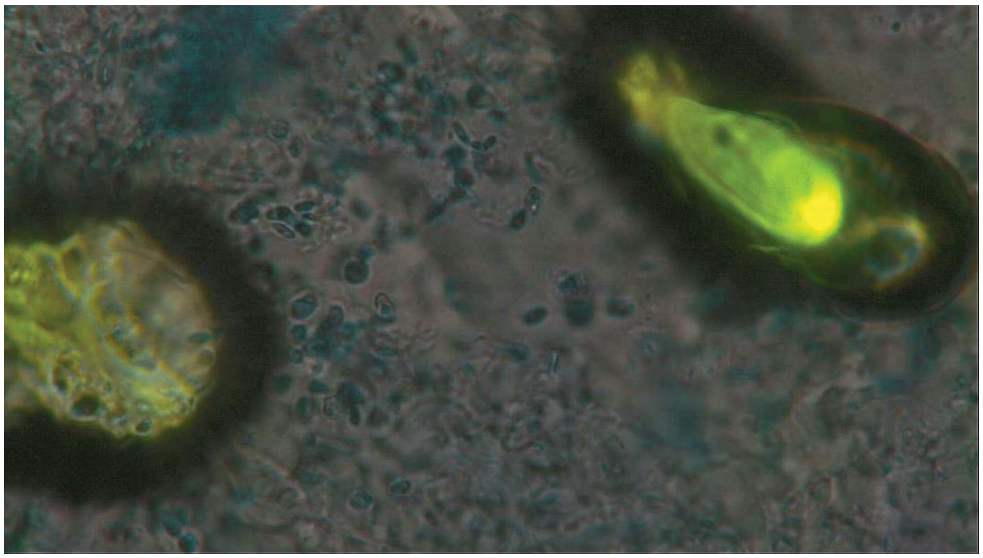

Figure 3 Water kefir grain stained with crystal violet at 40x magnification, the green is an air bubble and the yeast discernible in dark blue

Kefir grains are traditionally produced at room temperature, this has resulted in the grains adapting to the growth conditions of the environment – hot countries or regions vs cold countries or regions and other environmental factors – through their propagation (Miguel M. G., Cardoso, Lago, & Schwan, 2010). This means that different countries and regions have different microbial content in their kefir grains and the resulting kefir, thus affecting the taste and texture of the kefir (Miguel M. G., Cardoso, Lago, & Schwan, 2010).

2.4 Alternative options to kefir grains

While kefir grains are considered vital for the production of kefir, there are various methods for 'grain free' kefir for use on a commercial scale. However, while most of these methods do not accurately reproduce the organoleptic properties of traditional kefir, they are either 'close enough' or sold to new markets (such as North America which have little prior knowledge on the properties of original kefir) (Mainville & Farnworth, 2008). Examples of this being that some commercial products labelled 'kefir' do not contain any yeast at all.

Commercial production of kefir commonly involves using freeze dried lactic acid and yeast cultures as a starter (Guzel-Seydim, Kok-Tas, & Greene, 2010). This is more controllable than other 'grain-free' methods where a grain is still used as an initial inoculum for a 'mother culture' to create a smaller quantity of kefir that is then strained and added to the main batch (Mainville & Farnworth, 2008). However using the freeze dried method the selection of micro-organisms to use is very important, with a range of different species being recommended from different

sources. The selection of species is also difficult due to the huge variation of species in a kefir grain, with one study finding over 120 different strains of *Lactobacillus* (Takizawa, et al., 1998).

2.5 Methods of production

Most kefir is produced by adding the kefir grain or starter to pasteurised and homogenised milk at a rate of around 2-3% grain/culture and leaving to ferment at 25°C for 24 hours, when the pH drops to around 4.4-4.6, then stored under refrigeration (to 4°C) (Guzel-Seydim, Kok-Tas, & Greene, 2010). However other sources provide a range of fermentation temperatures from 18-25°C, and different methods including slow cooling and maturing steps, where after a set pH is reached the kefir is slowly cooled to 8-10°C before being left to mature for 15-20 hours to allow flavour and aroma to develop (Mainville & Farnworth, 2008).

One issue in the large scale manufacture of kefir is the yeast; it creates alcohol and carbon dioxide which results in the bulging of the packages (when laminated paper board is used) (Mainville & Farnworth, 2008). A method of production to help alleviate this problem is to do 2-step fermentation. This is when only bacteria are used to inoculate the milk initially, and when a certain pH is achieved (around 4.4-4.6) the kefir is cooled and yeast is added but it is stored at 4°C to prevent further fermentation (Law, 1997). However this method does not produce the effervescent character or yeasty aroma expected of a kefir product.

A similar method that produces carbon dioxide and effervescence is to add the yeast in the secondary fermentation step, sometimes with a small amount of sugar that the yeast can ferment (Law, 1997). This may also produce ethanol, but controlling the amount of sugar added will limit the alcohol production. This method is also reported to be shelf stable at 5°C for 42 days, using the bacteria specified (Law, 1997).

An important factor in the processing of kefir is agitation and the fermentation container. One method is to inoculate the pasteurised milk in the final packaging, and leave to ferment in the bottle (Mainville & Farnworth, 2008; Law, 1997). This is sometimes referred to as the 'set' method, as the milk can solidify as a soft curd. However this pours as a lumpy liquid, and is considered to not be acceptable for most consumers (Mainville & Farnworth, 2008). The more recent and popular method is the "stirred' method, where the milk is inoculated in a large container and left to ferment and coagulate with agitation, before being slowly cooled in the same container (Mainville & Farnworth, 2008). This method is considered to produce a more acceptable kefir: however there are a range of different variables that affect the organoleptic properties (Mainville & Farnworth, 2008; Law, 1997).

2.6 Yoghurt manufacture comparison

In comparison to kefir manufacture, yoghurt manufacture was investigated, including the methods as described by Watson Dairy Consulting (2017).

The first step in yoghurt manufacture is increasing the solids-not-fat (SNF). This is done to produce a smoother, more consistent texture with a better mouth-feel. This is either done by adding extra skim milk powder to the milk, or removing the water using evaporation, ultrafiltration or reverse osmosis.

There are two main methods of yoghurt production: stirred and set. Set yoghurt is often considered more premium, and tends to contain exclusively milk and culture. The milk is incubated and cooled in the final purchase pottle, resulting in gel-like yoghurt. Stirred yoghurt is made in large scale vats, where the milk is inoculated, incubated and cooled while being stirred. The stirring results in a smoother creamier type yoghurt, fruit and other flavours are often included for taste, and stabilisers and setting agents such as gelatine to produce a consistent product.

The milk is heated prior to fermentation to denature the proteins, improving the texture and viscosity. It is normally heated to around 90-95°C for 5-10 minutes (high temperature short hold HTSH), or 80-85°C for 30 minutes (low temperature holding LTH). The temperature/time chosen is often dependent on the heating process used: plate heat exchangers use HTSH and vessel heating uses LHT. After this initial heat treatment, the milk is cooled to 40-45°C (picked to match the optimum growing temperature of the starter) and the starter culture is added, usually *Latobacillus delbrueckii* sub. *bulgaricus* and *Streptococcus thermophiles*. If the yoghurt is set yoghurt, the final purchase pottles are filled with the inoculated milk before being incubated and then cooled to refrigeration temperature. For stirred yoghurt, the inoculated milk is incubated in a large vessel while being stirred before being cooled and used to fill the final purchase pottles.

2.7 Sensory attributes

There are a number of different sensory attributes that can be ascribed to kefir.

Cais-Sokolinska, Dankow, and Pikul (2008), used the temperatures of 23°C and 26°C for two different grain cultures incubated for 16-18 hours – until reaching pH 4.6. The curd was then cooled to 20°C and bottled before further cooling to 6°C. The sensory qualities Cais-Sokolinska, Dankow, and Pikul looked for were: bitter taste, yeasty taste, fermented taste, sour taste, fermented odour, sour odour, viscosity, astringency, serum separation, and overall acceptability. The attributes correlated with an increased acceptability were: yeasty taste, fermented taste,

fermented odour, viscosity, and astringency, with; serum separation, sour odour and bitter taste being negatively correlated.

Sady, Domagala, Grega, and Najgebauer-Lejko, (2007) found that the kefirs they tested with the highest rheological parameters got the lowest sensory scores for consistency, so the tasters did not like viscous kefir. This was thought to be due to the fact traditional kefir is refreshing, and the thick consistency is not considered refreshing.

Muir, Tamime, and Wszolek, (1999) conducted a sensory test using a trained panel of Scottish consumers to assess 'traditional' and 'modified' kefir, as well as buttermilk and yogurt. The attributes Muir, Tamime, and Wszolek used in the analysis were split into 3 groups: odour (fruity, sweet, acid/sour, creamy/milky, and musty), flavour (intensity, persistence, salty, fruit, sweet, sour/acid, creamy/milky, bitter, and other), and mouth-feel (viscosity, slimy, chalky/floury, gummy/sticky, serum separation, mouth coating, and melt-in-the-mouth). The regression co-efficient for the partial least squares model were positive (increased acceptability) for: flavour- fruity, sweet, creamy- odour- fruity, sweet, creamy- mouth-feel- viscous, melt-in-the-mouth, chalky, and mouth coating. Muir, Tamime, and Wszolek concluded that the Western European market preferred the 'modified kefir' which were made from defined micro-organisms rather than a kefir grain.

2.8 Variables effecting kefir production

The methods of manufacturing kefir can have an effect on its final taste and texture, as well as its shelf life. These can be seen in Figure 4 below.

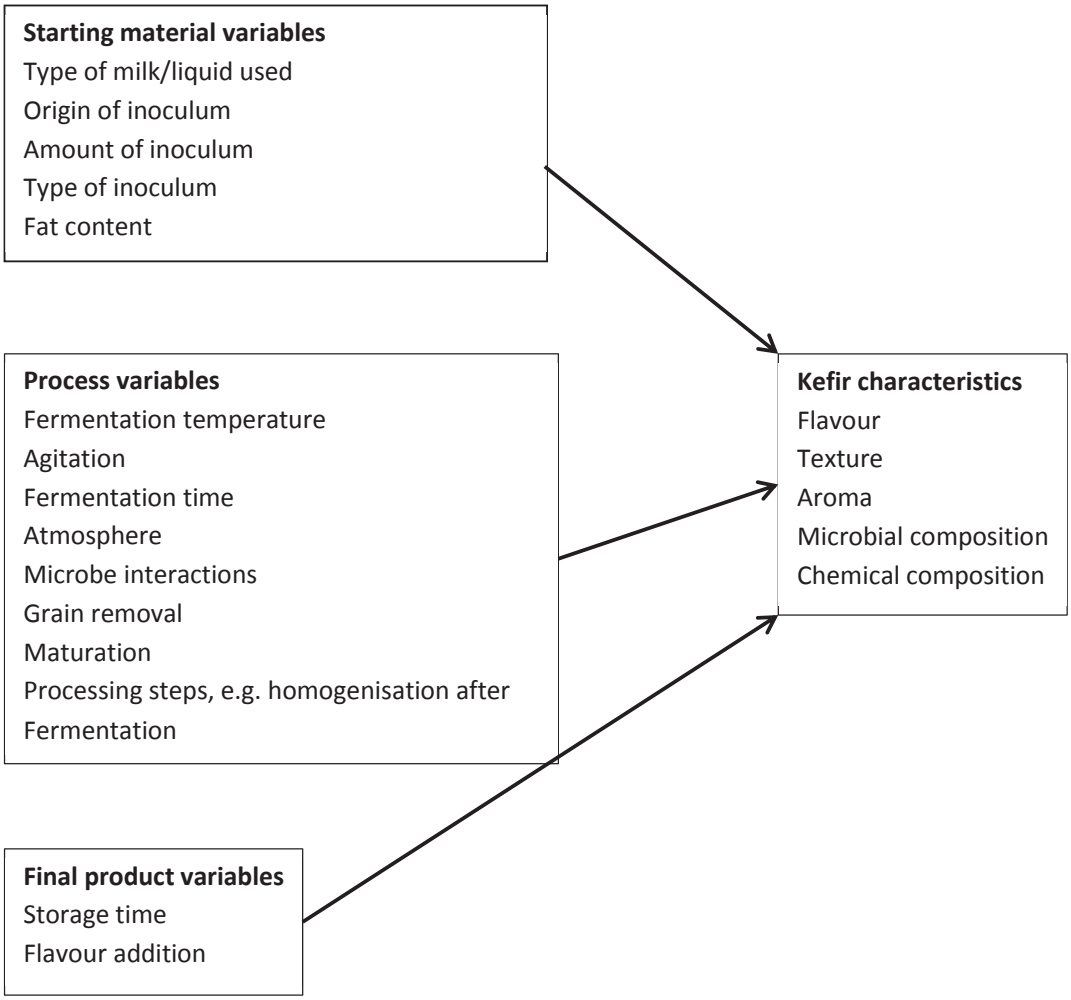

Figure 4 Variables affecting the final characteristics of kefir (Mainville & Farnworth, 2008).

2.8.1 Fermentation time and temperature

The fermentation temperature has an effect on how the species grow and what compounds they produce. Most species will have an optimal temperature for growth, where the cell count will increase the fastest. In suboptimal temperature ranges, some species will metabolize different compounds, which in the case of kefir can produce undesirable flavour characteristics. The time of fermentation affects the cell count, it should include the lag and log phase of micro-organism growth, but if fermentation is continued the cells may begin to die off. Increased fermentation time can also lead to splitting of the milk due to the lowered pH, as shown in Figure 5, and an increase of strong flavours (often 'cheesy' flavours) which can be undesirable in kefir. However

too short a fermentation time and the starter may not have had the time to grow sufficiently to reach a high cell count and produce the required compounds to change the product from milk into kefir, such as lactic acid to lower the pH.

Figure 5 splitting of milk after extended fermentation with kefir grains

2.8.2 Amount of inoculum

The amount of inoculum added to the kefir has an effect on the final product. The ratio of grain to milk has an effect on the pH of the kefir, with an increase in grain resulting in a lower pH, the optimum grain to milk ratio is between 1:30 and 1:50 (Rattray & O'Connel, 2011). Irigoyen, Arana, Castiella, Torre, and Ibanez, (2004) Compared an inoculation of 1% kefir grains and 5% kefir grains in milk, and 5% grains had a higher acceptability based on a tasting panel. They found that the higher grain inoculation resulted in a lower pH, more viscous product with higher yeast, and acetic acid cell counts with less lactose.

2.8.3 Sheep's milk composition

The composition of the media used to grow bacteria will have an effect on how fast it grows and to what cell count. It will also affect the by-products of fermentation, which are often the major flavour contributors in fermented products.

According to Raynal-Ljutovac, Lagriffoul, Paccard, Guillet, and Chilliard, (2008) the average total solids of sheep's milk is 18.1%, making the total water 81.9%. In skim milk the average total

solids without fat in sheep's milk is 11.28%. This gives the percentage of skim milk powder at 13.8% with 87.2% water.

Cataldi, Angelotti, and Bianoco, (2003) examined the monosaccharide and disaccharide composition of a range of milks, including sheep's milk, using chromatography. The sugars in sheep's milk were determined to be; lactose 4100±0.1mg/100mL, glucose 0.32±0.05mg/100mL, and galactose 0.31±0.02mg/100mL.

Yaman, Elmali, and Kamber, (2010) compared the growth and storage of kefir made from cow, sheep, and goat's milk. Table 1 and Table 2 below show the change in microbial levels and pH over fermentation and storage for cow and sheep's milk (Goat's milk had similar results to cow, and have not been included). It can be seen that sheep's milk had a higher final lactobacillus cell count of 8.79cfu/mL verse 7.57cfu/mL after 21 hours fermentation, and sheep's milk continued to have a higher cell count over the storage period. Yaman, Elmali, and Kamber thought that the higher levels of *Lactobacillus* in sheep's milk was due to the fact it is a better medium for the bacterial growth, as it contains higher levels of essential vitamins for the growth of *Lactobacillus* spp. strains, such as: pantothenic acid, folic acid, riboflavin, and niacin.

Table 1 (Yaman, Elmali, & Kamber, 2010) Changes in pH and cfu counts for Lactobacillus, yeast, and Lactococcus + Leuconostoc over 21 hour fermentation at 25°C

	pH		Log cfu/mL Lactobacillus		Log cfu/mL yeast		Log cfu/mL Lactococcus + Leuconostoc	
Time (h)	cow	sheep	cow	sheep	cow	sheep	cow	sheep
0	7.02	7.03	4.11	4.30	3.60	3.30	4.36	4.14
3	6.62	6.82	5.04	5.47	4.39	5.17	4.78	5.77
6	6.36	6.63	6.08	6.41	4.90	5.34	6.08	5.95
9	6.04	6.29	6.90	7.38	4.60	5.15	6.60	7.28
12	5.70	5.26	7.25	9.54	5.20	5.36	7.28	8.61
15	4.81	5.26	8.69	9.68	5.30	5.38	8.69	9.78
18	4.56	5.06	8.78	8.69	5.67	5.47	8.95	9.45
21	4.54	4.52	7.57	8.79	5.72	5.86	9.28	9.32

Table 2 (Yaman, Elmali, & Kamber, 2010) Changes in pH and cfu counts for Lactobacillus, yeast, and Lactococcus + Leuconostoc over 7 days of storage at 4°C

	pH		Log cfu/mL Lactobacillus		Log cfu/mL yeast		Log cfu/mL Lactococcus + Leuconostoc	
Day	cow	sheep	cow	sheep	cow	sheep	cow	sheep
1	4.41	4.49	7.48	8.84	5.81	5.84	9.68	9.00
2	4.28	4.42	7.41	8.15	5.90	5.60	9.55	9.11
3	4.24	4.37	7.30	7.76	5.50	5.58	9.56	8.95
4	4.22	4.34	7.47	7.47	5.48	5.47	8.60	8.30
5	4.12	4.31	7.17	7.23	5.60	5.47	7.60	8.20
6	4.08	4.3	7.04	7.28	5.47	5.47	7.30	8.00
7	4.02	4.3	7.00	7.23	5.47	5.44	7.25	8.00

2.8.4 Atmospheric conditions

Different atmospheric conditions may have an effect on the kefir: the micro-organism population make-up, and organoleptic properties. This is due to the requirements of different organisms. Some are aerobic and require oxygen to grow, some are anaerobic and require an absence of oxygen to grow, and others are facultative and can grow in either absence or presence of oxygen. Therefore in a kefir system the atmospheric conditions can affect the microbes' growth and therefore the flavour and texture. In in bottle fermentation (where the fermentation is sealed from external atmosphere) the atmosphere can change from aerobic to anaerobic as the microbes utilize oxygen and/or produce carbon dioxide. However in uncovered fermentation there can be an aerobic and anaerobic zone, as the top layer is exposed to oxygen; whereas the liquid underneath is not. Stirred fermentation is aerobic, unless the atmosphere is altered, as air is continuously being incorporated into the milk.

Kok-Tas, Seydim, Ozer, and Guzel-Seydim, (2012) compared normal atmospheric conditions with 10% CO_2 at 25°C till pH 4.6 (22hr), for both a grain and starter culture. The 10% CO_2 had no significant effect on the growth of yeast, and there was no effect on the overall *Lactobacillus* species and *Lactococcus* species. However the probiotic bacteria *Lactobacillus acidophilus* and *Bifidobacterium* species are anaerobic, so a statistically significant increase was seen under 10%CO_2 conditions (Kok Tas, Ekinci, & Guzel-Seydim, 2012).

2.9 Exopolysaccharides

Exopolysaccharides and polysaccharides that are excreted out of the cell by a number of bacteria can aid in the textural properties of fermented milks, such as making it thicker. They can also be slimy and ropey, which can be detrimental or beneficial based on the final products desirable characteristics.

A number of *Lactobacilli* produce exopolysaccharides which are beneficial in kefir for textural and stabilising properties. *Lactobacillus kefiranofaciens* and *Lactobacillus kefiri* produce the exopolysaccharide kefiran. Rimada and Abraham, (2001) found that the production of polysaccharides from kefir grains increased with increasing temperature and higher grain ratio. The time taken to reach pH4 decreased under the same parameters. It was also noted that as the temperature of fermentation increased above 37°C the grain weight decreased, Rimada and Abraham assumed this to be from the polysaccharide forming the grain being dissolved into the liquid due to the increasing temperature.

Rimada and Abraham, (2006) found that in milk gels supplemented with kefiran, a pre-heat treatment (similar to that used in yoghurt manufacture) increased the viscosity of the kefiran gel. This may mean that the kefir produced with skim milk powder will be thicker due to the heating during spray drying, or that pre-heating could be used to make a thicker kefir.

2.10 Microbial interactions

Kefir is considered to be a good example of microbial interactions, as not all the species found in kefir have the ability to ferment lactose, the only sugar present in milk. This means that the nutrients required for these species to grow need to be provided somehow – by other microbes.

Garrote, Abraham, and De Antomi, (2010) explain the five different types of interactions microbes can have when growing in the same system: competition, mutualism, parasitism, amensalism, and commensalism. These interactions either benefit, have an adverse effect, or no effect on the individual species in the system. For a system of two species, mutualism (symbiosis) is when both species benefit from growing in the same system, an example is when one species produces a bi-product that the other species needs for growth, and that species in turn produced a bi-product which aids the growth of the original species. Competition is when the species compete for the same resources; so both species are limited in growth by each other. Parasitism arises when one of the species benefits and the other is adversely affected. Amensalism occurs when one species has a detrimental effect on the other but is not effected itself, an example of this is acid and alcohol production limiting the growth of a species.

Commensalism occurs when one species is not affected and the other is benefited, it is similar to mutualism, however the second species does not produce anything that benefits the first.

2.11 Species make-up of milk kefir

As well as the effects of manufacturing on the final product, the selection of the culture has a considerable effect. As shown in Figure 4, the starting variables include the type, origin and amount of inoculum used. As the aim of this thesis is to isolate and identify species from kefirs, and then utilize them in the development of a sheep's milk kefir, it is important to look at the literature to assess what species are common to kefir.

2.11.1 Microflora found in kefir

A number of studies have identified the species present in milk kefirs. The identified species have been tabulated in Table 3, as well as the species stated to be present by a number of commercially available kefirs and kefir starters. From this the most commonly identified species can be determined, and the most commonly used in commercial kefirs. This gives an indication of what kind of bacteria will be found which will allow better isolation techniques. It will also aid in the selection of isolates to be used in the development of the final kefir.

Table 3 Micro-organisms identified in kefir in various studies, and advertised on various commercial kefir products, the micro-organism names have been used as stated in the reference. The final column is the total number of references for the species; Reasearch, Commercial.

Micro-organism	Research paper	Commercial product	
Lactobacillus casei	Tibet (Zhou, Xiaoli, Jiang, & Dong, 2009), Turkey (Kesmen & Kacmaz, 2011), Brazil (Zanirati, et al., 2014), Turkey (Nalbantoglu, et al., 2014)	(Lifeway kefir, 2016) (Clover Stornetta Farms Inc, 2015) (Evolve Kefir, N.D), (Wallaby Yogurt Company, 2016), (Body Ecology, 2016)	4,5
Lactobacillus plantaum	Brazil (Miguel M. G., Cardoso, Lago, & Schwan, 2010), Tibet (Gao, et al., 2015), Turkey (Nalbantoglu, et al., 2014)	(Lifeway kefir, 2016)	3,1
Lactobacillus kefyr		(The Kefir Company, 2011)	0,1
Lactobacillus delbrueckii	Turkey (Nalbantoglu, et al., 2014)		1,0
Lactobacillus delbrueckii subsp. *bulgaricus*		(Clover Stornetta Farms Inc, 2015), (Wallaby Yogurt Company, 2016)	0,2
Lactobacillus delbrueckii subsp. *lactis*		(Wallaby Yogurt Company, 2016)	0,1
Lactobacillus acidophil		(Clover Stornetta Farms Inc, 2015) (Evolve Kefir, N.D)	0,2
Lactobacillus acidophilus	Turkey (Kesmen & Kacmaz, 2011), Ireland (Dobson,	(Wallaby Yogurt Company, 2016), (Mad Millie, 2016),	4,2

	O'Sullivan, Cotter, Ross, & Hill, 2011), Turkey (Nalbantoglu, et al., 2014), Turkey (Kok Tas, Ekinci, & Guzel-Seydim, 2012)	(Lifeway kefir, 2016)	
Lactobacillus rhamnosus	Turkey (Nalbantoglu, et al., 2014)	(Evolve Kefir, N.D), (Wallaby Yogurt Company, 2016), (Lifeway kefir, 2016)	1,2
Lactobacillus helveticus	Tibet (Zhou, Xiaoli, Jiang, & Dong, 2009), Turkey (Kesmen & Kacmaz, 2011), Brazil (Miguel M. G., Cardoso, Lago, & Schwan, 2010), Ireland (Dobson, O'Sullivan, Cotter, Ross, & Hill, 2011), Turkey (Unsal, 2008), Turkey (Nalbantoglu, et al., 2014), Turkey (Kok Tas, Ekinci, & Guzel-Seydim, 2012)		7,0
Lactobacillus kefiranofaciens	Tibet (Zhou, Xiaoli, Jiang, & Dong, 2009), Turkey (Kesmen & Kacmaz, 2011), Belgium (Korsak, et al., 2014), Brazil (Leite, et al., 2012), Ireland (Dobson, O'Sullivan, Cotter, Ross, & Hill, 2011), Taiwan (Chen, Wang, & Chen, 2008), Tibet (Gao, et al., 2015), Brazil (Leite A. M., et al., 2013), Brazil (Zanirati, et al., 2014), Turkey (Nalbantoglu, et al., 2014), Italy (Garofalo, et al., 2015)		11,0
Lactobacillus kefiranofaciens subsp. *kefiranofaciens*	Brazil (Leite, et al., 2012), Turkey (Unsal, 2008), Turkey (Kok Tas, Ekinci, & Guzel-Seydim, 2012)		3,0
Lactobacillus kefiranofaciens subsp. *kefirgranum*	Brazil (Leite, et al., 2012), Ireland (Dobson, O'Sullivan, Cotter, Ross, & Hill, 2011), Slovenia (Vardjan, Lorbeg, Rogelj, & Majhenic, 2013), Turkey (Kok Tas, Ekinci, & Guzel-Seydim, 2012)		4,0
Lactobacillus kefiri	Tibet (Zhou, Xiaoli, Jiang, & Dong, 2009), Turkey (Kesmen & Kacmaz, 2011), Brazil (Miguel M. G., Cardoso, Lago, & Schwan, 2010), Taiwan (Chen, Wang, & Chen, 2008), Belgium (Korsak, et al., 2014), Brazil (Leite, et al., 2012), Ireland (Dobson, O'Sullivan, Cotter, Ross, & Hill, 2011), Tibet (Gao, et al., 2015), Slovenia (Vardjan, Lorbeg, Rogelj, & Majhenic, 2013), Brazil (Leite A. M., et al., 2013), Brazil (Zanirati, et al., 2014), Turkey		13,0

	(Nalbantoglu, et al., 2014), Italy (Garofalo, et al., 2015)		
Lactobacillus lactis	Turkey (Kesmen & Kacmaz, 2011)	(Lifeway kefir, 2016)	1,0
Lactobacillus kefir	Turkey (Kesmen & Kacmaz, 2011), Brazil (Miguel M. G., Cardoso, Lago, & Schwan, 2010)		2,0
Lactobacillus raffinolactis	Turkey (Kesmen & Kacmaz, 2011)		1,0
Lactobacillus parakefir	Brazil (Miguel M. G., Cardoso, Lago, & Schwan, 2010), Slovenia (Vardjan, Lorbeg, Rogelj, & Majhenic, 2013), Brazil (Leite A. M., et al., 2013), Turkey (Nalbantoglu, et al., 2014)		4,0
Lactobacillus paracasei	Brazil (Miguel M. G., Cardoso, Lago, & Schwan, 2010), Tibet (Gao, et al., 2015), Turkey (Nalbantoglu, et al., 2014)		3,0
Lactobacillus paracasei subsp. *paracasei*		(Wallaby Yogurt Company, 2016)	0,1
Lactobacillus parakefiri	Belgium (Korsak, et al., 2014), Brazil (Leite, et al., 2012), Ireland (Dobson, O'Sullivan, Cotter, Ross, & Hill, 2011)		3,0
Lactobacillus satsumensis	Brazil (Miguel M. G., Cardoso, Lago, & Schwan, 2010), Brazil (Zanirati, et al., 2014)		2,0
Lactobacillus uvarum	Brazil (Miguel M. G., Cardoso, Lago, & Schwan, 2010)		1,0
Lactobacillus mali	Brazil (Zanirati, et al., 2014)		1,0
Lactobacillus buchneri	Brazil (Leite, et al., 2012), Turkey (Nalbantoglu, et al., 2014)		2,0
Lactobacillus parabuchneri	Brazil (Leite, et al., 2012), Ireland (Dobson, O'Sullivan, Cotter, Ross, & Hill, 2011), Turkey (Nalbantoglu, et al., 2014)		3,0
Lactobacillus amilovorus	Brazil (Leite, et al., 2012)		1,0
Lactobacillus amylovorus	Turkey (Nalbantoglu, et al., 2014)		1,0
Lactobacillus crispatus	Brazil (Leite, et al., 2012), Turkey (Nalbantoglu, et al., 2014), Italy (Garofalo, et al., 2015), Turkey (Kok Tas, Ekinci, & Guzel-Seydim, 2012)		4,0
Lactobacillus brevis	Turkey (Nalbantoglu, et al., 2014)		1,0
Lactobacillus pentosus	Turkey (Nalbantoglu, et al., 2014)		1,0
Lactobacillus salivarius	Turkey (Nalbantoglu, et al., 2014)		1,0
Lactobacillus sunkii	Turkey (Nalbantoglu, et al., 2014)		1,0
Lactobacillus johnsonii	Turkey (Nalbantoglu, et al., 2014)		1,0
Lactobacillus gasseri	Turkey (Nalbantoglu, et al., 2014)		1,0
Lactobacillus rossiae	Turkey (Nalbantoglu, et al., 2014)		1,0
Lactobacillus sakei	Turkey (Nalbantoglu, et al., 2014)		1,0
Lactobacillus reuteri	Turkey (Nalbantoglu, et al., 2014)	(Lifeway kefir, 2016)	1,1
Lactobacillus gallinarum	Turkey (Nalbantoglu, et al., 2014)		1,0

Lactobacillus otakiensis	Turkey (Nalbantoglu, et al., 2014)		1,0
Lactobacillus kalixensis	Turkey (Nalbantoglu, et al., 2014)		1,0
Lactobacillus rapi	Turkey (Nalbantoglu, et al., 2014)		1,0
Lactobacillus diolovorans	Turkey (Nalbantoglu, et al., 2014)		1,0
Lactobacillus parafarraginis	Turkey (Nalbantoglu, et al., 2014)		1,0
Lactobacillus intestinalis	Italy (Garofalo, et al., 2015)		1,0
Lactococcus lactis	Tibet (Zhou, Xiaoli, Jiang, & Dong, 2009), Turkey (Kesmen & Kacmaz, 2011), Belgium (Korsak, et al., 2014), Brazil (Leite, et al., 2012), Ireland (Dobson, O'Sullivan, Cotter, Ross, & Hill, 2011), Taiwan (Chen, Wang, & Chen, 2008), Brazil (Zanirati, et al., 2014), Turkey (Nalbantoglu, et al., 2014), , Italy (Garofalo, et al., 2015)		9,0
Lactococcus lactis subsp. *cremoris*	Brazil (Leite, et al., 2012), Brazil (Leite A. M., et al., 2013)	(The Kefir Company, 2011) (Evolve Kefir, N.D), (Wallaby Yogurt Company, 2016), (Mad Millie, 2016)	2,4
Lactococcus lactis subsp. *lactis*	Turkey (Unsal, 2008), Brazil (Leite A. M., et al., 2013)	(The Kefir Company, 2011) (Evolve Kefir, N.D), (Wallaby Yogurt Company, 2016), (Mad Millie, 2016)	2,4
Lactococcus lactis subsp. *lactis* biovar. *diacetylactis*		(Wallaby Yogurt Company, 2016), (Mad Millie, 2016)	0,2
Lactococcus diolivorans	Turkey (Kesmen & Kacmaz, 2011), Turkey (Nalbantoglu, et al., 2014)		2,0
Lactococcus garvieae	Ireland (Dobson, O'Sullivan, Cotter, Ross, & Hill, 2011), Turkey (Nalbantoglu, et al., 2014)		2,0
Leuconostoc cremoris		(Lifeway kefir, 2016), (Body Ecology, 2016)	0,2
Leuconostoc mesenteroides subsp. *cremoris*		(The Kefir Company, 2011) (Evolve Kefir, N.D), (Wallaby Yogurt Company, 2016)	0,3
Leuconostoc mesenteroides	Tibet (Zhou, Xiaoli, Jiang, & Dong, 2009), Turkey (Kesmen & Kacmaz, 2011), Belgium (Korsak, et al., 2014), Taiwan (Chen, Wang, & Chen, 2008), Turkey (Unsal, 2008), Brazil (Leite A. M., et al., 2013), Brazil (Zanirati, et al., 2014), Turkey (Nalbantoglu, et al., 2014)		8,0
Leuconostoc pseudomesenteroides	Turkey (Kesmen & Kacmaz, 2011)		1,0
Streptococcus		(Lifeway kefir, 2016)	0,1

diacetylactis			
Streptococcus thermophiles	Turkey (Kesmen & Kacmaz, 2011), Tibet (Gao, et al., 2015), Italy (Garofalo, et al., 2015), Turkey (Kok Tas, Ekinci, & Guzel-Seydim, 2012)	(Evolve Kefir, N.D), (Clover Stornetta Farms Inc, 2015), (Wallaby Yogurt Company, 2016), (Mad Millie, 2016)	4,4
Oenococcus oeni	Brazil (Zanirati, et al., 2014), Turkey (Nalbantoglu, et al., 2014)		2,0
Pediococcus claussenii	Turkey (Nalbantoglu, et al., 2014)		
Pediococcus damnosus	Turkey (Nalbantoglu, et al., 2014)		
Pediococcus halophilus	Turkey (Nalbantoglu, et al., 2014)		
Pediococcus pentosaceus	Turkey (Nalbantoglu, et al., 2014)		
Pediococcus lolii	Turkey (Nalbantoglu, et al., 2014)		
Tetragenococcus halophilus	Turkey (Nalbantoglu, et al., 2014)		
Acetobacter syzyggi	Turkey (Kesmen & Kacmaz, 2011), Brazil (Miguel M. G., Cardoso, Lago, & Schwan, 2010), Turkey (Unsal, 2008)		3,0
Acetobacter orientalis	Belgium (Korsak, et al., 2014), Italy (Garofalo, et al., 2015)		2,0
Acetobacter lovaniensis	Belgium (Korsak, et al., 2014), Turkey (Unsal, 2008), Brazil (Leite A. M., et al., 2013), Italy (Garofalo, et al., 2015)		4,0
Acetobacter fabarum	Italy (Garofalo, et al., 2015)		1,0
Gluconobacter japonicus	Brazil (Miguel M. G., Cardoso, Lago, & Schwan, 2010)		1,0
Gluconobacter frateurii	Belgium (Korsak, et al., 2014)		1,0
Gluconobacter cerinus	Belgium (Korsak, et al., 2014)		1,0
Propionobacterium freudenreichii subsp. *shermanii*		(Wallaby Yogurt Company, 2016)	0,1
Bifidobacterium bifidum	Turkey (Kok Tas, Ekinci, & Guzel-Seydim, 2012)		1,0
Bifidobacterium lactis		(Evolve Kefir, N.D), (Wallaby Yogurt Company, 2016), (Lifeway kefir, 2016)	0,3
Bifidobacterium sp.	Turkey (Kesmen & Kacmaz, 2011)	(Mad Millie, 2016)	1,1
Bifidobacterium breve	Ireland (Dobson, O'Sullivan, Cotter, Ross, & Hill, 2011)	(Lifeway kefir, 2016)	1,1
Bifidobacterium choerinum	Ireland (Dobson, O'Sullivan, Cotter, Ross, & Hill, 2011)		1,0
Bifidobacterium longum	Ireland (Dobson, O'Sullivan, Cotter, Ross, & Hill, 2011)	(Lifeway kefir, 2016)	1,1
Bifidobacterium pseudolongum	Ireland (Dobson, O'Sullivan, Cotter, Ross, & Hill, 2011)		1,0
Likely contaminants			
Pseudomonas sp.	Tibet (Zhou, Xiaoli, Jiang, & Dong, 2009), Turkey (Kesmen & Kacmaz, 2011), (Chen, Wang, &		2,0

	Chen, 2008)		
Peanibacillus sp.	Turkey (Kesmen & Kacmaz, 2011)		1,0
Enterococcus faecium	Turkey (Unsal, 2008)		1,0
Enterobacter amnigenus	Belgium (Korsak, et al., 2014)		1,0
Enterobacter sp. LH-CAB10	Belgium (Korsak, et al., 2014)		1,0
Escherichia coli	(Chen, Wang, & Chen, 2008)		1,0
Yeasts			
Saccharomyces unisporus	Argentina (Diosma, Romanin, Rey-Burusco, Londero, & Garrote, 2013), Spain (Latorre-Garcia, del Castillo-Agudo, & Polaina, 2007)	(The Kefir Company, 2011)	2,1
Saccharomyces cerevisiae	Tibet (Zhou, Xiaoli, Jiang, & Dong, 2009), Brazil (Leite, et al., 2012), Tibet (Gao, et al., 2015), Argentina (Diosma, Romanin, Rey-Burusco, Londero, & Garrote, 2013), Brazil (Leite A. M., et al., 2013), (Marsh, O'Sullivan, Hill, Ross, & Cotter, 2013), Italy (Garofalo, et al., 2015), (Miguel M. G., Cardoso, Magalhaes-Guedes, & Schwan, 2013)		8,0
Saccharomyces turicensis	Taiwan (Wang S. Y., Chen, Liu, Lin, & Chen, 2008)		1,0
Saccharomyces humaticus	Spain (Latorre-Garcia, del Castillo-Agudo, & Polaina, 2007)		1,0
Saccharomyces exiguus	Spain (Latorre-Garcia, del Castillo-Agudo, & Polaina, 2007)		1,0
Saccharomyces boulardii		(Body Ecology, 2016)	0,1
Saccharomyces florentinus		(Lifeway kefir, 2016)	0,1
Saccharomyces bayanus	(Miguel M. G., Cardoso, Magalhaes-Guedes, & Schwan, 2013)		1,0
Klyveromyces marxianus var. *marxianus*		(The Kefir Company, 2011)	0,1
Klyveromyces marxianus	Tibet (Zhou, Xiaoli, Jiang, & Dong, 2009), Belgium (Korsak, et al., 2014), Argentina (Diosma, Romanin, Rey-Burusco, Londero, & Garrote, 2013), Slovenia (Vardjan, Lorbeg, Rogelj, & Majhenic, 2013), (Marsh, O'Sullivan, Hill, Ross, & Cotter, 2013), Taiwan (Wang S. Y., Chen, Liu, Lin, & Chen, 2008), Turkey (Kok Tas, Ekinci, & Guzel-Seydim, 2012), (Miguel M. G., Cardoso,		8,0

	Magalhaes-Guedes, & Schwan, 2013)		
Kluyveromyces lactis	Spain (Latorre-Garcia, del Castillo-Agudo, & Polaina, 2007)		1,0
Issatchenkia occidentalis	Argentina (Diosma, Romanin, Rey-Burusco, Londero, & Garrote, 2013)		1,0
Issatchenkia orientalis	Spain (Latorre-Garcia, del Castillo-Agudo, & Polaina, 2007)		1,0
Kazachstania unispora	Tibet (Zhou, Xiaoli, Jiang, & Dong, 2009), Brazil (Leite, et al., 2012), Tibet (Gao, et al., 2015), (Marsh, O'Sullivan, Hill, Ross, & Cotter, 2013), Italy (Garofalo, et al., 2015)		5,0
Kazachstania exigua	Tibet (Zhou, Xiaoli, Jiang, & Dong, 2009), Belgium (Korsak, et al., 2014), Slovenia (Vardjan, Lorbeg, Rogelj, & Majhenic, 2013), , Italy (Garofalo, et al., 2015)		4,0
Kazachstania barnetti	(Marsh, O'Sullivan, Hill, Ross, & Cotter, 2013)		1,0
Kazachstania turicensis	Italy (Garofalo, et al., 2015)		1,0
Pichia kudriavzevii	(Marsh, O'Sullivan, Hill, Ross, & Cotter, 2013)		1,0
Pichia occidentalis	(Marsh, O'Sullivan, Hill, Ross, & Cotter, 2013)		1,0
Pichia fermentans	(Marsh, O'Sullivan, Hill, Ross, & Cotter, 2013), Taiwan (Wang S. Y., Chen, Liu, Lin, & Chen, 2008), (Miguel M. G., Cardoso, Magalhaes-Guedes, & Schwan, 2013)		3,0
Pichia membranifaciens	(Miguel M. G., Cardoso, Magalhaes-Guedes, & Schwan, 2013)		1,0
Pichia guilliermondi	(Miguel M. G., Cardoso, Magalhaes-Guedes, & Schwan, 2013)		1,0
Pichia anomala	(Miguel M. G., Cardoso, Magalhaes-Guedes, & Schwan, 2013)		1,0
Candida colliculosa		(Mad Millie, 2016)	0,1
Candida valdiviana	(Miguel M. G., Cardoso, Magalhaes-Guedes, & Schwan, 2013)		1,0
Candida parapsilosis	(Miguel M. G., Cardoso, Magalhaes-Guedes, & Schwan, 2013)		1,0
Rhodosporidium kratochvilovae	Slovenia (Vardjan, Lorbeg, Rogelj, & Majhenic, 2013)		1,0
Naumovozyma sp.	Belgium (Korsak, et al., 2014)		1,0

Naumovozyma castelli	(Marsh, O'Sullivan, Hill, Ross, & Cotter, 2013)		1,0
Zygosaccharomyces lentus	(Marsh, O'Sullivan, Hill, Ross, & Cotter, 2013)		1,0
Zygosaccharomyces mellis	(Miguel M. G., Cardoso, Magalhaes-Guedes, & Schwan, 2013)		1,0
Likely contaminants			
Davidiella tassiana	(Marsh, O'Sullivan, Hill, Ross, & Cotter, 2013)		1,0
Penicillium sp *vega347*	(Marsh, O'Sullivan, Hill, Ross, & Cotter, 2013)		1,0
Dekkera anomala	(Marsh, O'Sullivan, Hill, Ross, & Cotter, 2013), Italy (Garofalo, et al., 2015), , (Miguel M. G., Cardoso, Magalhaes-Guedes, & Schwan, 2013)		3,0
Dekkera bruxellensis	(Marsh, O'Sullivan, Hill, Ross, & Cotter, 2013)		1,0
Hanseniaspora guilliermondii	Italy (Garofalo, et al., 2015)		1,0
Eurotium amstelodami	(Marsh, O'Sullivan, Hill, Ross, & Cotter, 2013)		1,0
Wallemia sebi	(Marsh, O'Sullivan, Hill, Ross, & Cotter, 2013)		1,0
Microdochium nivale	(Marsh, O'Sullivan, Hill, Ross, & Cotter, 2013)		1,0
Cryptococcus sp. *vega039*	(Marsh, O'Sullivan, Hill, Ross, & Cotter, 2013)		1,0
Teatosphaeria knoxdaviesii	(Marsh, O'Sullivan, Hill, Ross, & Cotter, 2013)		1,0
Cyberlindnera jadinii	(Marsh, O'Sullivan, Hill, Ross, & Cotter, 2013)		1,0
Malassezia pachydermatis	(Marsh, O'Sullivan, Hill, Ross, & Cotter, 2013)		1,0
Heterobasidion annosum	(Marsh, O'Sullivan, Hill, Ross, & Cotter, 2013)		1,0
Paziza campestris	(Marsh, O'Sullivan, Hill, Ross, & Cotter, 2013)		1,0
Ganoderma lucidum	(Marsh, O'Sullivan, Hill, Ross, & Cotter, 2013)		1,0
Dioszegia hungarica	(Marsh, O'Sullivan, Hill, Ross, & Cotter, 2013)		1,0
Dipodascus capitatus	Spain (Latorre-Garcia, del Castillo-Agudo, & Polaina, 2007)		1,0
Trichosporon coremiiforme	Spain (Latorre-Garcia, del Castillo-Agudo, & Polaina, 2007)		1,0

Table 3 above shows the species found in a number of studies assessing the species present in kefirs, and as advertised on commercial kefirs. There are a total of 85 different bacterial species

identified, and 49 different yeast species. This is a very large variation in species with not one being present in all kefirs. This shows that kefir can be a very diverse product and there is not a set requirement for species, as well as the variation in product qualities this would likely produce. Table 4 below shows the most common species of yeast and bacteria found in kefir.

Table 4 The microbes with the highest number of references

Species	**Instances**		
Bacteria	**literature**	**commercial**	**total**
Lactococcus lactis (all subsp)	13	6	19
Lactobacillus kefiranofaciens (all subsp)	18	0	18
Lactobacillus kefiri	13	0	13
Leuconostoc mesenteroides (all subsp)	8	3	11
Lactobacillus casei	4	4	8
Lactobacillus helveticus	7	0	7
Lactobacillus delbrueckii (all subsp)	1	3	4
Streptococcus thermophiles	4	3	7
Lactobacillus acidophilus	4	1	5
Lactobacillus parakefir	4	0	4
Lactobacillus paracasei (all subsp)	3	1	4
Lactobacillus plantarum	3	1	4
Lactobacillus crispatus	4	0	4
Acetobacter lovaniensis	4	0	4
Yeast			
Saccharomyces unisporus/Kazachstania unispora	7	1	8
Klyveromyces marxianus (all subsp)	7	1	8
Saccharomyces cerevisiae	7	0	7
Saccharomyces exiguus/Kazachstania exigua	5	0	5

It is interesting to note the variation in species from species advertised on the commercial kefirs to the species found in tested kefirs. 9 different commercial kefirs were assessed, and only 5 contained yeast (Table 3 and Appendix 6.4). Yeast gives kefir its distinctive effervescence, and is included in the definition of kefir as a 'yeast-lactic' fermentation. This indicates that a number of products that market themselves as kefir are in fact more in a nature of a drinking yoghurt. Species commonly associated with yoghurt are also included in a number of the commercial

kefirs, such as *Lactococcus lactis, Lactobacillus delbrueckii,* and *Streptococcus thermophiles.* Whereas species highly prevalent in kefir, such as *Lactobacillus kefiranofaciens* and Lactobacillus *kefiri* (Table 4) are not in any of the commercial kefirs, even though kefir is their predominant environment and lends it name to these two species.

Marsh, O'Sullivan, Hill, Ross, and Cotter, (2013) tested 25 different kefirs for bacteria and yeast, using ITS PCR for the yeast identification, they found a total of 23 different yeast species, with at least 15 being considered environmental contaminants due to their commonly associated environments. This is a much larger range of fungi than any of the other studies looked at, however it is also for a greater number of kefir samples. It is interesting that only 9 species were identified in the grains, with 14 identified exclusively in the kefir milk. Some of the fungi identified only in the milk could have been caused by environmental contamination and from mishandling grains, especially as the grains were propagated over a 4 month period. However it should be noted that it is difficult to determine whether a species is a contaminant or not due to the high variation of species in kefir as well as environmental species which may be naturally in kefir as they are ubiquitous. Contamination is also subjective, an example being acetobacter which some consumers consider to be a contaminant and others view as a requirement (Mainville & Farnworth, 2008).

The fungi *Heterobasidion annosum, Paziza campestris, Teratosphaeria knowdaviesii,* and *Diozegia hungarica* were all found in only one kefir milk sample each, and they were reported to all be considered as environmental fungi. This indicates that they are all likely to be contaminants. *Davidella tassiana* fungus is a cause of cladosporium rot in dying plants and other organic matter and is likely to be a contaminant of milk kefir (Kassemeyer & Berkelmann-Lohnertz, 2009). *Pichia kudriavzevii* is commonly found in agricultural products, and often on humans and animals and is not considered to be a common spoilage yeast*,* however it is a common clinical isolate and therefore a likely contaminant in kefir (Kurtzman, Fell, & Boekhout, 2011). *Pichia fermentans* is a yeast commonly associated with wine production, as it is an environmental yeast and often found on human skin, leaves, soil, and fruit (especially grapes) therefore it is a likely contaminant in kefir having been picked up from the environment (UCDavis, 2014).

Dekkera bruxellensis is very similar to *Saccharomyces cerevisiae* and is a major contaminant in biofuel and wine production and is a likely contaminant (Borneman, Zeppel, Chambers, & Curtin, 2014). *Zygosaccharomyces lentus* is a food spoilage yeast which can grow in low pH foods, and is therefore a contaminant (Steels, James, Robberts, & Stratford, 1999). *Eurotium amstelodami* is a common food spoilage fungi often found in baked goods, and is a likely contaminant (Abellana,

Magri, Sanchis, & Ramos, 1999). *Wallemia sebi* is a fungus commonly found in agricultural environments, is a potential causative of 'farmers lung disease' and has been detected as a slow growing spoilage fungi in a range of food products and is a likely contaminant in kefir milk (Zeng, Westermark, Rasmuson-Lestander, & Wang, 2004). *Microdochium nivale* is an agricultural fungus and is the cause of 'pink snow mould' on grass which can kill the grass, and is a likely contaminant (PennState College of Agricultural Sciences, 2016). *Malasszeia pachydermatis* is a pathogenic yeast causing skin infections in dogs and humans, and is therefore a likely contaminant in kefir milk (Morris, O'Shea, Shofer, & Rankin, 2005). *Ganoderma lucidum* is a mushroom forming fungi which is used in herbal medicine and is therefore a likely contaminant (Wachtel-Galor, Yuen, Buswell, & Benzie, 2011).

Dekkera anomala is considered a food spoilage yeast, particularly in the beverage industry, and is therefore considered a contaminant in kefir milk (Gray, Rawsthorne, Dirks, & Phister, 2011). However, it was also found by Miguel M. G., Cardoso, Magalhaes-Guedes, & Schwan, (2013), who considered it to be a contaminant. Garofalo, et al., (2015) also found *Dekkera anomala* as a major (high percentage incidence) in all of the 6 kefir grains analysed. This indicates that it may not be a contaminant in the kefir, but rather undetected due to limits in the technology, and the slow growth of *Dekkera anomala* preventing its isolation on media or from the kefir milk samples. This limit of technology may apply to a number of the assumed contaminants, and some of them may be naturally found in kefir grains, as there is no definitive method for identifying contaminants in kefir due to the extensive range of species identified and the geographical variations.

The quantity of contaminant yeast found is interesting, Marsh, O'Sullivan, Hill, Ross, & Cotter, (2013) was the only study looked at which used culture-independent ITS sequencing on the direct kefir and kefir grains. Latorre-Garcia, del Castillo-Agudo, and Polaina, (2007), Miguel M. G., Cardoso, Magalhaes-Guedes, and Schwan, (2013), Diosma, Romanin, Rey-Burusco, Londero, and Garrote, (2013), and Magalhaes, Pereira, Campos, Dragone, and Schwan, (2011) also used ITS sequencing, however after using culture-dependent isolation. Zhou, Xiaoli, Jiang, and Dong, (2009), Korsak, et al., (2014), Leite, et al., (2012), Gao, et al., (2015), Vardjan, Lorbeg, Rogelj, and Majhenic, (2013), Leite A. M., et al., (2013), and Garofalo, et al., (2015) all used culture-independent 26s PCR-DGGE analysis, and did not find a similar range of contaminants as Marsh, O'Sullivan, Hill, Ross, and Cotter, (2013). This could be due to the quantity of kefirs assessed in each study, however with the large quantity of studies the total quantity of kefirs assessed would be greater than the 25 assessed by Marsh, O'Sullivan, Hill, Ross, and Cotter, (2013). This implies that ITS sequencing is better at identifying low frequency species than 26s PCR.

In almost all of the studies assessed, which compared both culture-dependent and culture-independent methods, there was a discrepancy between the species found with each method (Kesmen & Kacmaz, 2011; Miguel M. G., Cardoso, Lago, & Schwan, 2010; Chen, Wang, & Chen, 2008; Unsal, 2008; Gao, et al., 2015; Vardjan, Lorbeg, Rogelj, & Majhenic, 2013; Leite A. M., et al., 2013; Zanirati, et al., 2014).This discrepancy was thought to be due to the difficulty in obtaining high quality DNA from the grains and kefir, template competition affecting the detection of rarer bacteria for the culture-independent techniques, the low cell counts of these un-isolated species, and the specific growth requirements of different species not being met by media for the culture-dependent techniques. The method used for the identification also had an effect on the results. The general conclusions from these studies were that for an accurate description of the species in kefir, both culture dependent and independent techniques should be utilised.

Nalbantoglu, et al., (2014) compared whole genome shotgun (WGS) metagenomics with 16s pyrosequencing, and determined that WGS provided a higher resolution of the bacteria community. Leite, et al., (2012) compared PCR-DGGE and pyrosequencing. Pyrosequencing found more species of bacteria in the kefir, as was hypothesised, as it allows the identification of bacteria present in low levels, which is often unobserved using other techniques.

While it is useful to know that multiple techniques are required for accurate assessment of the ecology of kefir, this thesis is focused on the potential of utilising kefir species in a product, so only culturable species are of interest.

Table 5 shows the media used for the isolation of species from kefir by a number of studies. There were a range of media used, but MRS is the dominant medium, being used in all the studies excluding those looking exclusively for yeast. A number of studies also included various antibacterial or antifungal agents to allow for more selective growth.

Table 5 Media used for isolation of microbes from kefir from various studies

Reference	Media used for isolation
(Kesmen & Kacmaz, 2011)	MRS agar + 200ppm cyclohexamide M17 agar + 200ppm cyclohexamide
(Miguel M. G., Cardoso, Lago, & Schwan, 2010)	MRS agar + 4mL nystatin
(Chen, Wang, & Chen, 2008)	MRS agar + 200ppm cyclohexamide M17 agar + 0.5% lactose + 200ppm cyclohexamide
(Unsal, 2008)	MRS agar
(Magalhaes, Pereira, Campos, Dragone, & Schwan, 2011)	Edwards modified medium Nutrient agar LUSM medium MRS medium M17 medium 135 medium(acetic acid bacteria) YEPG medium (Yeast)
(Gao, et al., 2012)	MRS agar AC agar (acetic acid bacteria) YDP agar (yeasts)
(Gao, et al., 2015)	MRS medium Elliker medium Potato dextrose agar (yeasts)
(Witthuhn, Schoeman, & Britz, 2005)	MRS agar +3 g 100 mL^{-1} ethanol + 0.5g/100 mL cycloheximide potassium carboxymethyl cellulose agar + 10mL/mL TCC KCA + 30µg/L vancomycin Acetobacter peroxydans medium yeast extract lactate medium +0.002% v/v naladixic acid malt extract agar (yeasts) yeast extract chloramphenicol agar (yeasts)
(Diosma, Romanin, Rey-Burusco, Londero, & Garrote, 2013)	yeast extract glucose chloramphenicol agar (yeasts)
(Vardjan, Lorbeg, Rogelj, & Majhenic, 2013)	MRS agar KPL agar KDM agar
(Leite A. M., et al., 2013)	MRS agar + 200mg/kg cyclohexamide M17 agar +0.5% glucose +200mgmg/kg cyclohexamide Yeast extract glucose chloramphenicol agar (yeast)
(Zanirati, et al., 2014)	MRS agar in lactic whey +200ppm cyclohexamide
(Latorre-Garcia, del Castillo-Agudo, & Polaina, 2007)	YDP agar (yeasts) Sabouraud agar (yeasts) LS agar +0.1% sodium tetrazolium (yeasts)
(Miguel M. G., Cardoso, Magalhaes-Guedes, & Schwan, 2013)	Malt extract yeast glucose peptone extract + 100mg/mL chloramphenicol + 50mg/L chlortetracycline
(Irigoyen, Arana, Castiella, Torre, & Ibanez, 2004)	MRS agar + 200mg/L cyclohexamide M17 agar + 200mg/L cyclohexamide OGYE + 1% oxytetracycline

A number of the studies looked at the variation in the species present in the kefir grain, and the resultant kefir milk, as well as the change in species frequency over subsequent inoculations with kefir milk (as opposed to re-using grains). Korsak, et al., (2014) compared the ecology of kefir grains and the resulting kefirs, from both 5% and 10% grain ratio. The grains they used were from Belgium: two from individuals (A, B), two from the Ministry of Agriculture (C, D), and one from a supermarket (E). The bacteria Korsak, et al., found can be seen in Figure 6. The figure displays the relative abundance of reads, giving an indication of the percentage each bacterium makes up of the kefir grains and resulting kefir. *Lactobacillus kefiranofaciens* is clearly the most common bacteria in all the samples, excluding the supermarket sample (E) where *Lactococcus lactis* is the most prevalent bacteria; however *Lactobacillus kefiranofaciens* is still present. This study was slightly unusual as, while many studies recommend the final pH of a traditional kefir to be 4.6 with an inoculation ratio of 1-5%, the pH achieved after 24 hours at 25°C was between 5.14 and 5.27 at the 5% inoculation rate for all samples except the supermarket sample which had a pH of 4.57. The lower pH of the supermarket kefir (E) is likely due to the higher presence of lactic acid forming *Lactococcus lactis,* which is often found in kefir.

It is also interesting to note that the relative abundance of bacteria changes from grain to kefir in Figure 6, as seen particularly in the case of *Acetobacter orientalis* and *Gluconobacter frateurii/cerinus.* This serves as an example of why kefir cannot be used to successfully re-inoculate milk to create more kefir, as the distribution of bacteria has changed. *Enterobacter amnigenus* and *Enterobacter* sp. LH-CAB10 are likely to be contaminants, especially *Enterobacter amnigenus* which is pathogenic (Stock & Wiedemann, 2002). These two contaminant species are only present in the kefir and grains from individuals, so they have likely been incorporated by mishandling of the grains and kefir production. Dobson, O'Sullivan, Cotter, Ross, and Hill, (2011) Also compared the kefir grain to kefir milk, and again found the kefir grain to be over 80% *Lactobacillaceae* with the resulting milk containing over 60% *Streptococcaceae.* This variation of grain to milk re-iterates the importance of the grain in the production of kefir. It is important to consider the difficulty of blending the grains into a smooth liquid appropriate for use in the DNA extraction techniques due to their very rubbery texture. So the distributions may be more indicative of the surface of the kefir grains, while the interiors may be more hidden if the grains remained lumpy in the blending step. However it would be expected that the surface microflora to be more similar to the kefir milk than the internal grain microflora as the milk is in direct contact with the surface of the grain.

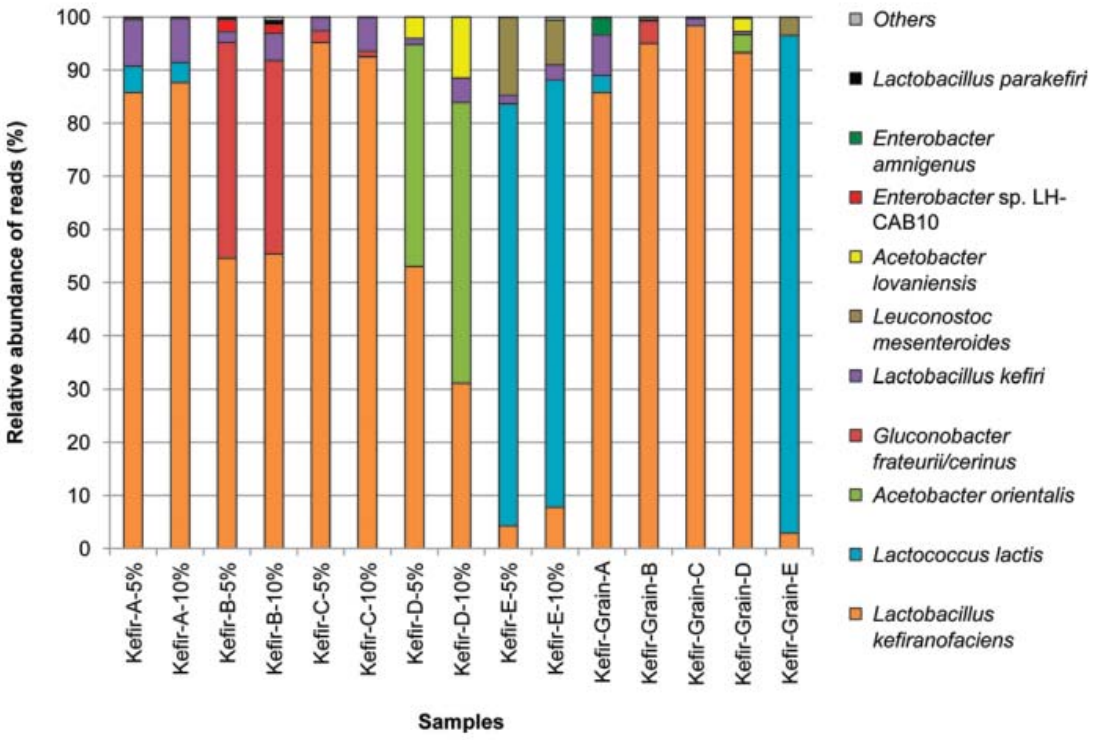

Figure 6 "Relative abundance of bacterial species in samples of kefir and grains. Bar chart represents the relative abundance of bacterial species (in relation to the number of reads) detected by 16S rDNA metagenetics for the 10 samples of kefir (with 5 and 10% inoculation rates) and 5 samples of grains (A-E)" (Korsak, et al., 2014)

Gao, et al., (2015) investigated the microbial diversity and stability over primary cultivation and sub cultivation in Tibetan kefir using both culture-independent and culture-dependent methods. The identified microbes and their relative frequency over the fermentation time and the various sub cultivations can be seen in Figure 7. It is interesting to note the instability of the kefir over various sub cultivations, supporting the theory that kefir liquid cannot be used to cultivate milk, and the grains are required to produce kefir. The inclusion of new bacteria and yeast species in the sub cultivations (not present in the primary fermented product), *Lactobacillus helveticus, Microbacterium testaceum,* and *Meyerozyma guilliermondii* is unexplained in the study (they are not included in Table 3 due to not being present in the initial kefir). However it could be a result of the PCR-DGGE method used to identify the microbes not being sensitive enough to pick up the bacteria and yeast in very small quantities in the initial kefir, but as they were grown in sub cultivations the quantity of the cells increased and so could be detected.

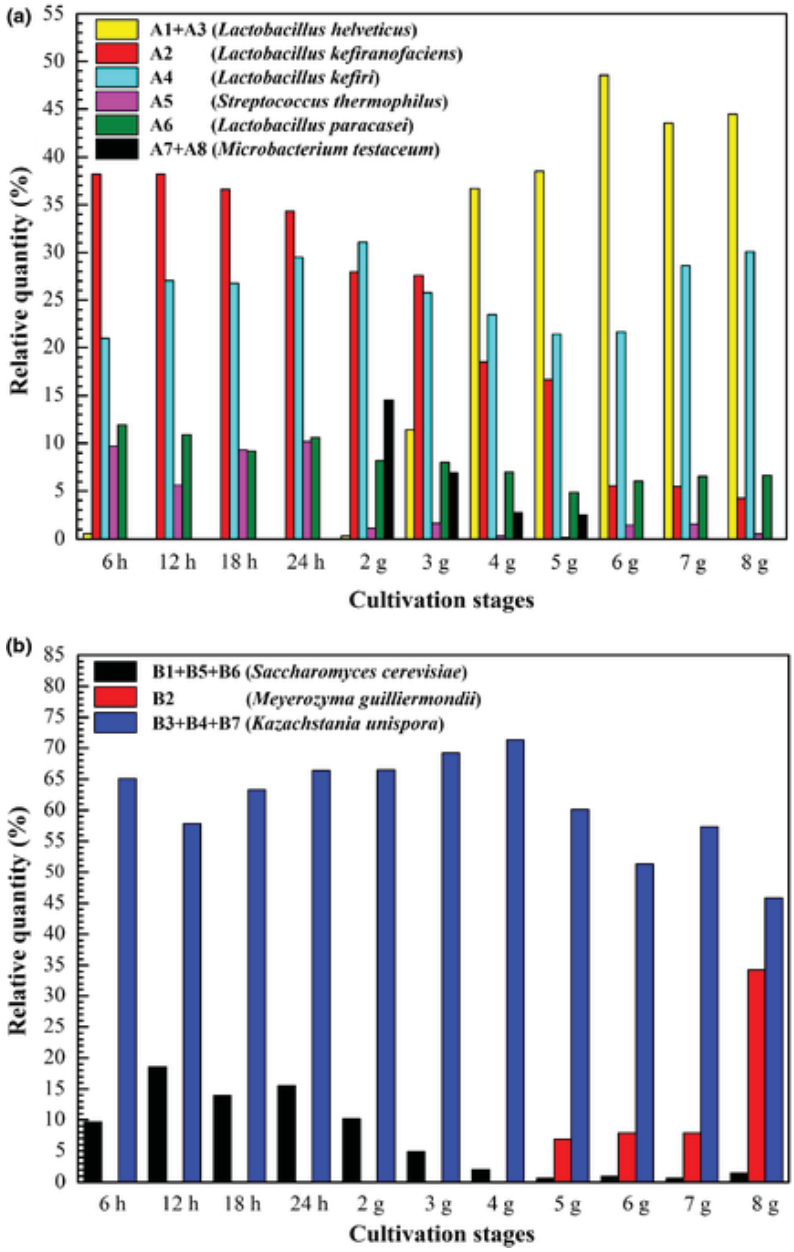

Figure 7 "The relative quantities of different predominant bacterial species (a) and yeast species (b) in the Tibetan kefir at different cultivation stages, A1–A8 represent the bacterial species of sites A1–A8 in Fig. a, B1–B7 represent the yeast species of sites B1–B7 in Fig. b, h represents the abbreviation for hour, g represents the abbreviation for subcultivation generation." (Gao, et al., 2015)

Marsh, O'Sullivan, Hill, Ross, and Cotter, (2013) identified the species present in 25 kefir grains and their subsequent kefir milks. The relative abundance of bacteria can be seen in Figure 8 and of yeast in Figure 9. Shown in Figure 8, the genus *Leuconostoc* was detected in only 4 grains, but all of the resultant kefir milks, and, between levels of ~1-20% relative abundance, which is a considerable increase. However this increase is not as large as *Lactococcus,* shown in red in Figure 8 which visibly increases in abundance, for example sample IR2, in which the grain has

~3% and the resultant kefir has ~93%. *Lactobacillus* relative abundance decreased from grain to kefir considerably, for example sample UK3 in Figure 8 shows the relative abundance drop from ~99% in the grain to ~3% in the kefir milk. This change from the grains to milk is likely to be the result of the bacteria's ability to grow in milk, and which species grow better in the other conditions such as temperature (room temperature in the this study). This would allow the fastest growing bacteria to outcompete the others, and result in higher abundance. Another potential reason for the difference in the grain verse milk species abundance could be the purpose the species are used for in the kefir grain. For example *Lactobacillus kefiranofaciens* and *Lactobacillus kefiri* are some of the bacteria responsible of producing kefiran, the exopolysaccharide which is considered to be potentially responsible for the grain structure (Mainville & Farnworth, 2008; Vos, et al., 2009). This could imply that the *Lactobacillus* mostly grow within the grain expanding it, rather than being released into the milk. However these bacteria, and the exopolysaccharide they produce, are also considered to be responsible for some of the textural aspects of kefir i.e. its increased thickness and 'slimy texture' (Vos, et al., 2009).

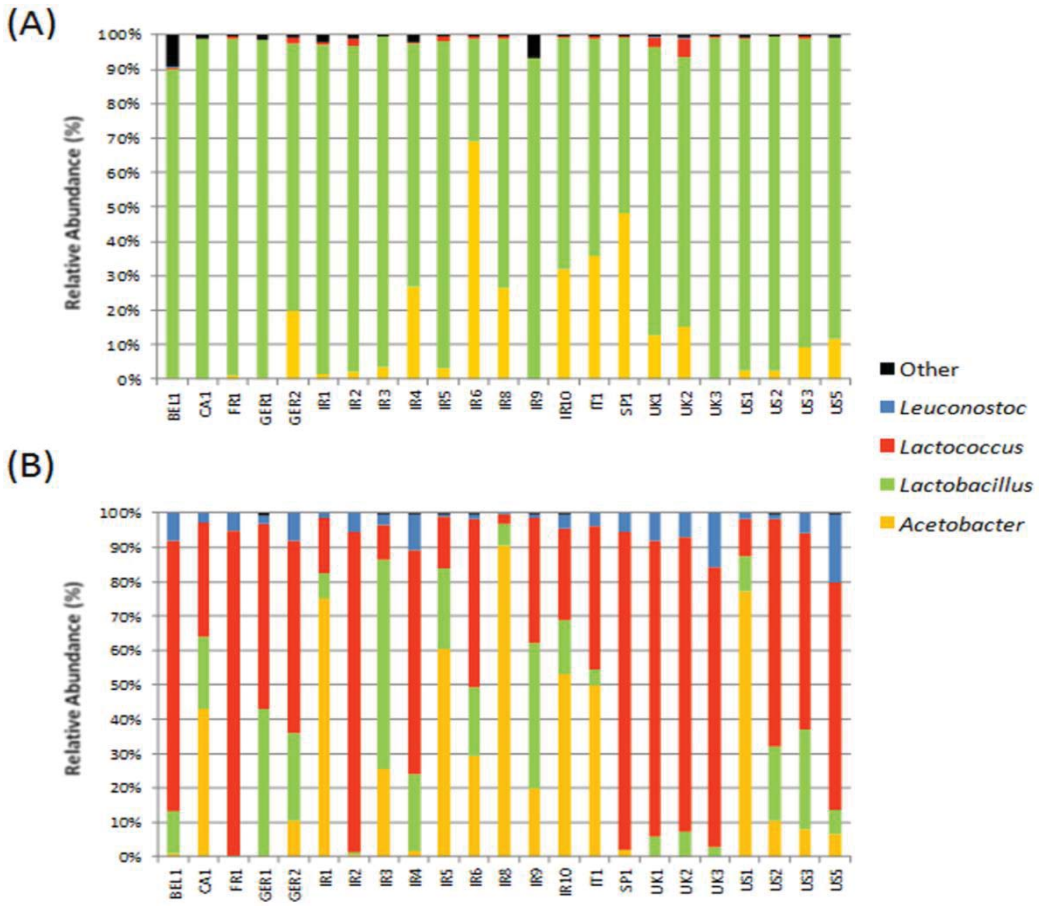

Figure 8 "16S phylogenetic composition of the bacterial component of the kefir grain (A) and kefir fermented milk (B) at genus level." (Marsh, O'Sullivan, Hill, Ross, & Cotter, 2013)

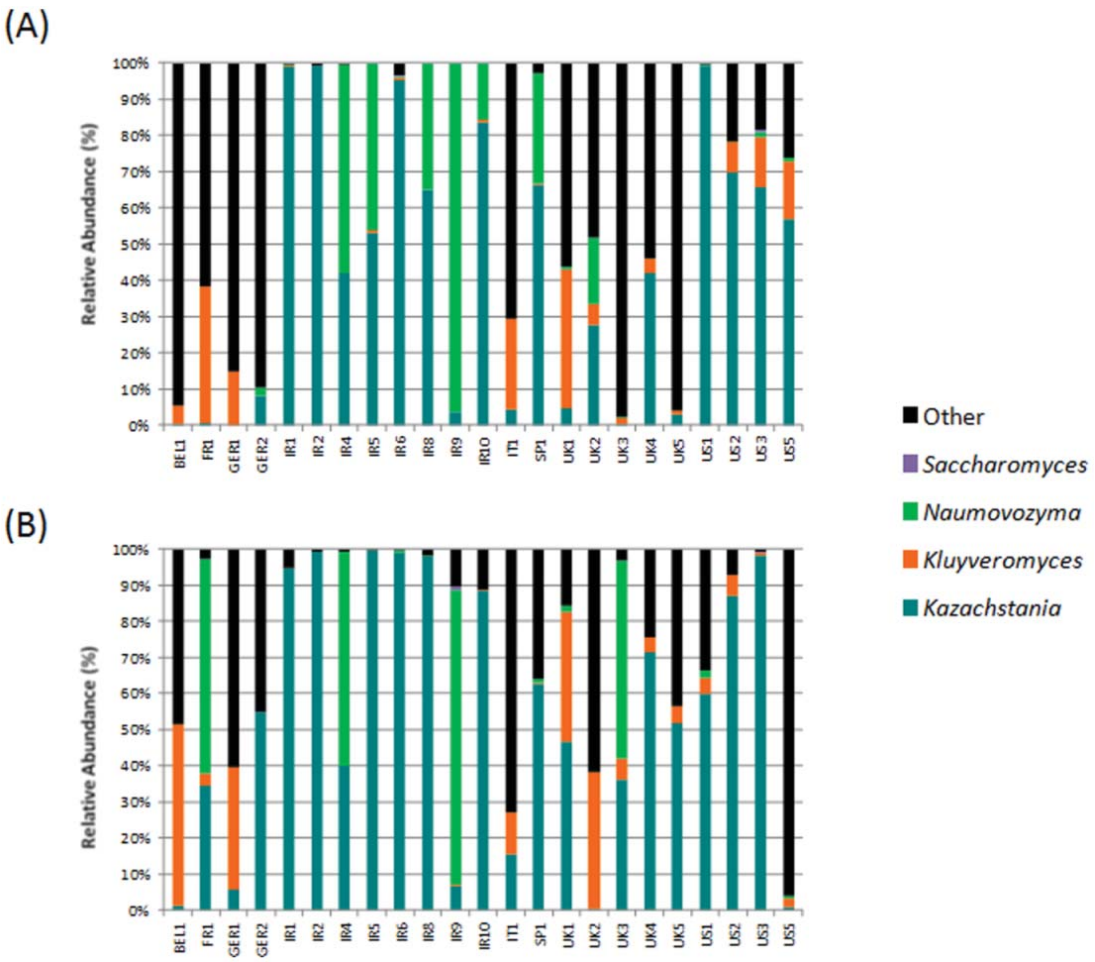

Figure 9 "ITS phylogenetic composition of the fungal component of the kefir grain (A) and kefir fermented milk (B) at genus level." (Marsh, O'Sullivan, Hill, Ross, & Cotter, 2013)

Figure 9, Figure 8, Figure 6, and Table 6 show the diversity of species present in kefir grains. This diversity shows that there is no common species that form kefir, and that classifying it is very difficult. This is also supported by the over 130 different species previously identified in kefir (Table 3). This also implies that different kefirs may have considerably different textural and flavour properties, and what may be considered a defective flavour by some consumers may be a required flavour by others. An example of this is acetobacter which some markets consider a contaminant and others do not (Mainville & Farnworth, 2008).

Table 6 Distribution frequency of the cultural microbial of 4 different kefir grains (Gao, Fengying, Abdella, Ruan, & He, 2012).

Sample	Species identified	% abundance
TK-ZJUJ01	*Kluyveromyces marxianus*	3
	Saccharomyces cerevisiae	7
	Bacillus subtilis	24
	Lactococcus lactis	67
TK-ZJUJ02	*Kluyveromyces marxianus*	10
	Kazachstania unispora	33
	Pichia kudriavzevii	3
	Lactobacillus kefiri	54
TK-ZJUJ04	*Pichia guilliermondii*	2
	Kazachstania unispora	71
	Lactobacillus plantarum	21
	Acetobacter fabarum	6
TK-ZJUJ03	*Kluyveromyces marxianus*	1.7
	Kazachstania unispora	2.3
	Kazachstania exugia	13
	Lactococcus lactis	23.5
	Lactobacillus plantarum	23.5
	Leuconostoc lactis	23.5

2.12 Regulatory requirements of kefir

CODEX (2010) declare kefir to be a fermented milk with a 'starter culture prepared from kefir grains, *lactobacillus kefiri,* species of the genera *Leuconostoc, Lactococcus* and *Acetobacter* growing in a strong specific relationship. Kefir grains constitute both lactose fermenting yeasts (*Kluyveromyces marxianus*) and non-lactose fermenting yeasts (*Saccharomyces unisporus, Saccharomyces cerevisiae* and *Saccharomyces exiguus*)'. However from the literature reviewed it is clear that this is not an accurate representation of kefir, an example being the limited presence of *Lactobacillus kefiri* in the literature kefirs, and absence in the commercial kefirs. There is no regulatory standard for kefir in New Zealand, and therefore in this thesis it was decided not to restrict the product development to the CODEX description, but rather what is

expected of a kefir, being a yeast-lactic fermentation with a low pH and refreshing effervescence.

Of the nine commercial kefirs assessed (Table 3 and Appendix 6.4) only five were reported to contain yeast. Again this does not adhere to the definition given by CODEX. This implies that either the commercial products should be considered a drinking yoghurt as opposed to a kefir, or that CODEX is not an accurate description.

CODEX (2010) also state the minimum sum of micro-organisms in the starter culture to be 10^7cfu/g, however the Australia New Zealand Food Standards Code Standard 2.5.3 (2015) declare the required starter to be 10^6cfu/g. CODEX also state that for yoghurt, when a claim is made on the labelling in relation to a specific micro-organism, the required cell count of that organism is over 10^6cfu/g. There is no similar requirement for kefir, but it was decided that this value should be considered a requirement for any micro-organisms, as it enables them to be used on the label.

It is also required for kefir to contain less than 0.5% ethanol. It is compulsory for foods containing over 0.5% ethanol to declare it on the label according to Australia New Zealand Food Standards Code Standard 2.7.1 (2015). It could have negative effects on the marketing of kefir if it was required to declare an ethanol content.

2.13 Proteolysis

A key mechanism by which the microflora affects the physical structure of the curd and the presence of desirable or undesirable flavour or aroma compounds is proteolysis (Beal & Helinck, 2014). Proteolysis is when the proteolytic system of bacteria degrades proteins. In milk, this tends to be casein which is broken down into peptides and free amino acids. Lactic acid bacteria often require an external source of amino acids and peptides, which are often not readily available in (bovine) milk (Fernandez-Espla, Maria, Garault, Monnet, & Rul, 2000). This means the bacteria are reliant on the proteolysis of the casein to produce these amino acids and peptides. Cell envelope proteinases are a key enzyme in this process, as they break down the casein into oligosaccharides which can then be transported into the bacteria and further broken down (Fernandez-Espla, Maria, Garault, Monnet, & Rul, 2000). While proteolysis is important for the flavour development of fermented milks, it was decided that the time required for analysis the proteolysis was out of the scope and timeframe of this study.

3. Methods

3.1 Isolation from kefir

3.1.1 Stock culture isolation

13 glass vials containing freeze dried micro-organisms isolated from homemade kefir were received from Fonterra. They were all dated 1983, except one from 2003. They were labelled; K1, K2, K10, K12, K13, K14, K15 2003, K15 1983, K16, K17, K18, KY1, and KY2. KY1 and KY2 were assumed to be yeasts and tested accordingly (they were subsequently identified as yeast so this assumption was correct).

The vials were aseptically opened and a small amount (under 200µL) of Merck brain heart infusion (BHI) broth was added to the vial to rehydrate and loosen the bacteria. A drop was then added to or streaked on the media below.

Media used;

- Oxoid milk plate count agar (MPCA)
- Oxoid brain heart infusion (BHI) broth
- Difco M17 broth with 5% added sterile filtered 10% lactose solution
- Merck DRBC agar (KY1 and KY2 only)
- Merck Saburoud broth (SAB) +2% glucose (KY1 and KY2 only).

These were then left to grow at 30°C for 4 days, and broth or colonies streaked out on;

- Difco M17 agar with 5% added sterile filtered 10% lactose solution and 10% glucose solution (M17 L+G)
- Acumedia MRS agar
- Merck BHI agar
- Merck Rogosa agar, in a anaerobic jar containing a gas pack (K1 –K18 only)
- Merck Saburoud agar (KY1 and KY2 only).

The most successful agar for each isolate was then used for further streaking and incubation then storage on slants at 4°C.

3.1.2 Isolation from kefir

For the isolation of species from mixed micro-organism kefir both freeze dried kefir starters and liquid kefirs were used. The aim was to visually determine different colonies (species) and isolate them till pure.

For the freeze dried starters, 0.1g was rehydrated in 5mL peptone water before being streaked onto each plate. The liquid kefirs were streaked directly. The media used for isolation were;

- M17 L+G agar
- M17 L+G agar, +0.05% cyclohexamide (sterile filtered)
- MRS agar, in a anaerobic jar containing a gas pack
- MRS agar
- DRBC agar

The plates were incubated at 30°C for 48 hours, or longer if little differentiation between colonies was observed.

Distinct colonies were re-streaked on the same agar until pure. They were then streaked onto both M17 L+G and MRS agar and visually identical isolates were discarded. When pure they were observed under a microscope to determine whether yeast or bacteria, and stored on agar slants at 4°C.

3.2 PCR for sequencing for bacterial (16s rDNA) and yeast (26srDNA) identification

The isolates were initially grown in MRS and Difco M17 L+G broth for 3 days, and the broths with the most growth were used for PCR and sequencing. The isolates were then prepared according to the method below before being sent to the Massey University Genome Service for sequencing. The centrifuge was set at 10,000G.

1. 25µL Master mix, 20µL RNAse free water, 1µL of 10µM each primers (Y1 and Y2 for bacteria and NL1 and NL4 for yeast), and 3µL of a culture were mixed in a 0.2mL Eppendorf tube.
2. The samples were then placed in the "PCR machine" which cycled through the required temperatures. For yeasts; initial denaturation at 95°C for 15 min, followed by 35 cycles of 95°C for 20s, 55°C for 40s, and 72°C for 45s, followed by a final elongation step at 72°C for 5 min, and then cooled to 4°C (Neubeck, et al., 2015). For bacteria; 94°C for 5 minutes, followed by 94°C for 45s, 60°C for 45s, and 72°C for 1 min for 30 cycles, and then 72°C for 5 min, and then cooled to 4°C (Young, Downer, & Eardly, 1991).
3. 10µL of each sample were then run though a 2% agarose gel to determine if DNA was present and compared to a Invitrogen E-Gel low range quantitative DNA ladder, as shown in Figure 10 for bacteria and Figure 11 for yeast.

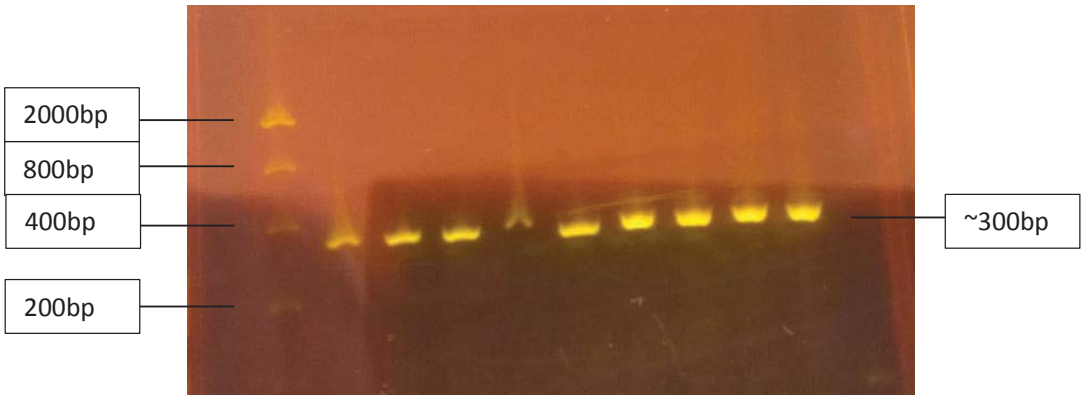

Figure 10 Gel electrophoresis results for a selection of bacteria, left to right; Ladder, K1, K2, K10, K13, K14, K15, K16, K17, K18, negative

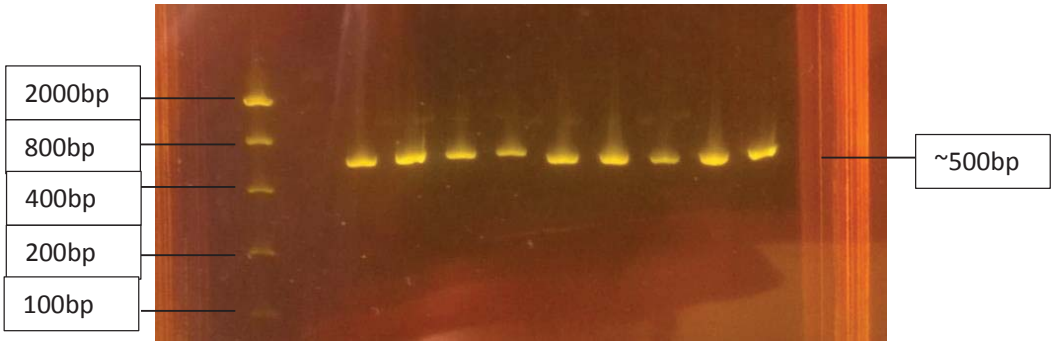

Figure 11 Gel electrophoresis results for a selection of yeast, left to right; Ladder, negative, H1, H2, H3, H4, KY1, KY2, DW, DPW, DRP

4. A zymo research DNA clean and concentrate kit was used, and the methods followed as according to kit instructions.
5. 1µL of each sample was then pipetted onto the Titertek Berthold Colibri microvolume spectrometer and the readouts for ng/ µL DNA and the A260/A280 (purity) were recorded, with an acceptable range being below 2.20 for the A260/A280 ratio, and above 20 ng/ µL for DNA concentration.
6. Each sample selected for sequencing was given two consecutive numeric labels, one for each primer. 0.2mL Eppendorf tubes were labelled with each sample number.
7. 0.5µL of each PCR product in 0.2mL Eppendorf tubes was made up to 16µL using RNAse free water.
8. 4µL of 1pM of each primer were added to their required tubes (one primer in one of the samples tubes, and the other primer in the other sample tube).
9. The samples were then taken to the Massey University Genome Service for sequencing. An example of the resulting data can be seen in Figure 10 and Figure 11. The nucleotide base code was then submitted into BLAST, where the sequence was compared to the BLAST database and the highest likelihood bacteria was chosen.

3.3 Sub species characterisation

3.3.1 Lactococcus strains

For the biochemical characterisation of the *Lactococcus lactis* isolates, storage slopes were used to take sterile loop samples to inoculate the following media:

- M17 L+G agar, +4%NaCl
- Arginine decarboxylase test
- Autoclaved 9mL phenol red broth in Durham tubes + 10% filter sterilized:
    - Lactose
    - Maltose
    - Sucrose
    - Ribose
    - Glucose (positive control)

The inoculated media were incubated at 30°C for 3 days before being assessed for growth or colour change (acid production).

3.3.2 Streptococcus determination

In order to screen these isolates for polysaccharide production, pre-prepared mitis-Salivarius agar was streaked with *Streptococcus* isolates from the storage slopes using a sterile loop.

The plates were then incubated at 30°C for 3 days to determine the presence of globular colonies, an indication of *Streptococcus salivarius*.

3.4 Impedance

To determine the growth curves of the isolates and provide a rapid method of estimation bacterial counts, impedance measurements were used with the following media:

- 10mL M17 L+G broth in 25mL bottles
- 10mL MRS broth in 25mL bottles
- 10mL M17 L+G broth in Bactrac tubes
- 10mL MRS broth in Bactrac tubes
- M17 L+G agar
- MRS agar
- 9mL peptone in 10mL bottles

Each isolate from storage slopes was used to inoculate a 25mL bottle of M17 L+G and MRS. This was incubated at 30°C for 24 hours.

0.1mL of each incubated broth was added to a Bactrac tube of the corresponding media and placed in the Bactrac, which measured the M and E values (media impedance and electrode capacitance respectively) every 20 minutes at 30°C for 3 days, after an initial 1.5 hour warm up. The values of E and M were then plotted against time.

1mL of each incubated broth was used for serial dilutions, with the final 3 being plated in duplicate with the media's corresponding agar. The plates were then incubated for 3 days at 30°C before the colonies were counted. This provided data to calibrate the impedance growth curved for viable bacteria (colony forming unit equivalents) and was used in subsequent experiments to estimate microbial levels in samples.

3.5 Optical density

Optical density was used as an alternative measure of growth and estimate of bacterial numbers.

Media:

- 1L bottle MRS containing stirrer bar
- 1L bottle M17 L+G containing stirrer bar
- MRS agar
- M17 L+G agar
- RO water
- 11mL MRS in 25mL bottle
- 11mL M17 L+G in 25mL bottle
- 9mL peptone water in 10mL bottles.

10mL of MRS (isolate HB3, *Lactobacillus kefiri,* aerobically, and isolate RST, *Lactobacillus kefiranofaciens,* anaerobically) and M17 (isolate DDP, *Pichia membranifaciens,* aerobically) were inoculated from the storage slopes and incubated at 30°C until visual growth was observed.

1mL was then removed and used for serial 10-fold dilutions, and the last 3 dilutions were plated in duplicate with the agar corresponding to the media used.

20mL of the 1L bottles of un-inoculated media were aseptically removed into a sterile bottle, to provide blanks for the optical density measurements.

10mL of the incubated media were then added to the 1L bottle, containing a stirrer bar, of the required media. This was incubated at 30°C.

An important feature of optical density measurements is the wavelength of light used, and it needs to be consistent. Suzuki, et al. (2013) used optical density to assess the growth of *Bifidobacterium longum* in MRS broth at a wavelength of 550nm. Their measurements range from 0 to 1.2 over 12 hours. Pak, et al. (2013) used optical density to assess the growth of *Lactobacillus bulgaricus* in MRS broth. However instead of measuring the density in MRS broth, 1mL of the sample was centrifuged for 2 minutes at 16,000G, and the pellet was suspended in 1mL phosphate buffered saline solution. The optical density was measured at a wavelength of 600nm. The optical density increased from 0 - 3.25 over 24 hours. Homayouni, Azizi, Ehsani, Yarmand, and Razavi, (2008) measured the optical density of *Lactobacillus casei* and *Bifidobacterium lactis* growing in MRS broth at a wavelength of 525.2nm. These studies show a range of wavelengths used between 525nm and 600nm, so optical density was measured using a spectrophotometer with a wavelength sweep, with 600nm and 525nm being recorded.

Readings were taken at the initial inoculation time, and then after 16 hours of incubation at 9:30am readings were taken, followed by readings at 1:30pm, and 5:30pm. This was repeated every day with both a RO water blank and media blank until the OD levelled out or the test was stopped. Prior to each reading the incubated media was stirred on a stirring plate until the cell mass appeared equally dispersed in the media.

Optical density begins to become non-representative of the bacterial growth at approximately OD≤0.4. This means that after this optical density is reached, the sample needs to be diluted and the optical density calculated from the dilution factor (Widdel, 2010). So the samples and media were diluted with RO water if they exceeded 0.4 and the results subsequently multiplied by the dilution factor.

Plate counts were also done at 9:30am and 5:30pm each day to monitor the viable cells.

3.6 Starter culture preparation

Media:

- Peptone water
- 9mL peptone in 10mL bottles
- MRS agar
- M17 L+G agar.

1mL from 1L bottles containing incubated media (as described in sections 3.4 Impedanceand 3.5 Optical density) was used for serial dilutions, with the final 3 dilutions plated in duplicate using the agar for each isolate as prescribed in Table 11. These plates were then incubated at 30°C for 3 days before the colony count was recorded.

Half of the 1L incubated media was added to autoclaved 500mL centrifuge tubes and centrifuged at 5000RPM (~3500G) for 10 minutes, the clarified media was poured off leaving the sedimented cells. The rest of the incubated broth was then added and this was repeated.

The sedimented cells were then mixed with peptone water to wash them, before being re-centrifuged and the peptone poured out. This was then repeated.

20mL of autoclaved 10% skim sheep's milk was added to the concentrated bacteria/yeast and mixed well, before 1mL aliquots were put in 1.5mL Eppendorf tubes. The tubes were then frozen at -80°C. After freezing, 1mL of each sample was defrosted at room temperature before being used for serial dilution plate counts to determine the cell count of the starter to be used for further use.

3.7 Inoculation ratio of kefir grains

The cell counts in milk using traditional kefir grains were used to determine the inoculation ratio of traditional kefir.

Media:

- M17 L+G agar
- 9mL peptone water in 10mL bottles
- store brought cow's milk
- kefir grains.

Kefir grains were removed from kefir and added to cow's milk at a ratio of 5% grains. This was briefly shaken, before 1mL milk was removed and used for serial dilutions and plate counts.

The plates were then incubated at 30°C for 3 days before the colonies were counted.

3.8 Growth in sheep's milk

Gamma sterilized whole sheep's milk powder was aseptically reconstituted at 18% total solids with autoclaved water and 80g were added to sterilized 100mL duram bottles.

1 x 10^6 cfu of each isolate as prepared in method 3.6 was added to a bottle of milk in duplicate and incubated at 30°C for 24 hours and then cooled at 4°C for 2 hours. The incubated milk was then separated into autoclaved 5mL Eppendorf tubes and stored at 4°C for further testing.

3.9 HPLC

In order to detect sugars, organic acids, and alcohol in the kefir, HPLC using the Aminex HPX-87H column was used. This has been used for the detection of compounds in kefir and other milk products in a number of studies (Fiorda, et al., 2016; Zeppa, Conterno, & Gerbi, 2001; Pirisino, 1983; Leite A. M., et al., 2013).

For each run done on the HPLC, standards of known concentration were put through at the beginning and end of the runs. These were used to calculate the amounts in each sample, so any variation in runs would be accounted for by the standards.

The standards used were:

- Lactose 5%
- Glucose 5%
- Galactose 10%
- Lactic acid 10%
- Acetic acid 10%
- Diacetyl 10%
- Ethanol 10%.

The samples were prepared by:

1. adding 1.5mL of sample to a 2mL Eppendorf tube
2. pipetting 0.15mL 1M HCl into sample and shaking
3. centrifuging at 14,000G for 10minutes (or until separated)
4. filter the supernate through 0.22µm PTFE filters into HPLC bottles with inserts.

The HPLC was then run at 65°C using the mobile phase 3mM H_2SO_4 at a flow rate of 0.6mL/min. The results were analysed using Shimadzu LabSolutions software.

3.10 Gas production

Each isolate was grown in sheep's milk to determine the gas production. This was done by capturing the gas produced. A system was set up where 20g of inoculated gamma sterilized reconstituted sheep's milk (18%) was sealed with a bung with piping connected to an inverted

10mL measuring cylinder filled with water in a beaker containing water to measure gas production in mL, the set-up is shown in Figure 12. This was left to incubate at 30°C, with the gas production being assessed at 24 and 48 hours.

CO_2 production can also be measured by the change in weight of the system; as the CO_2 is released as a gas. This was done by van Breda, Jolly, & van Wyk (2013). So the weight of the sheep's milk was also measured before and after incubation.

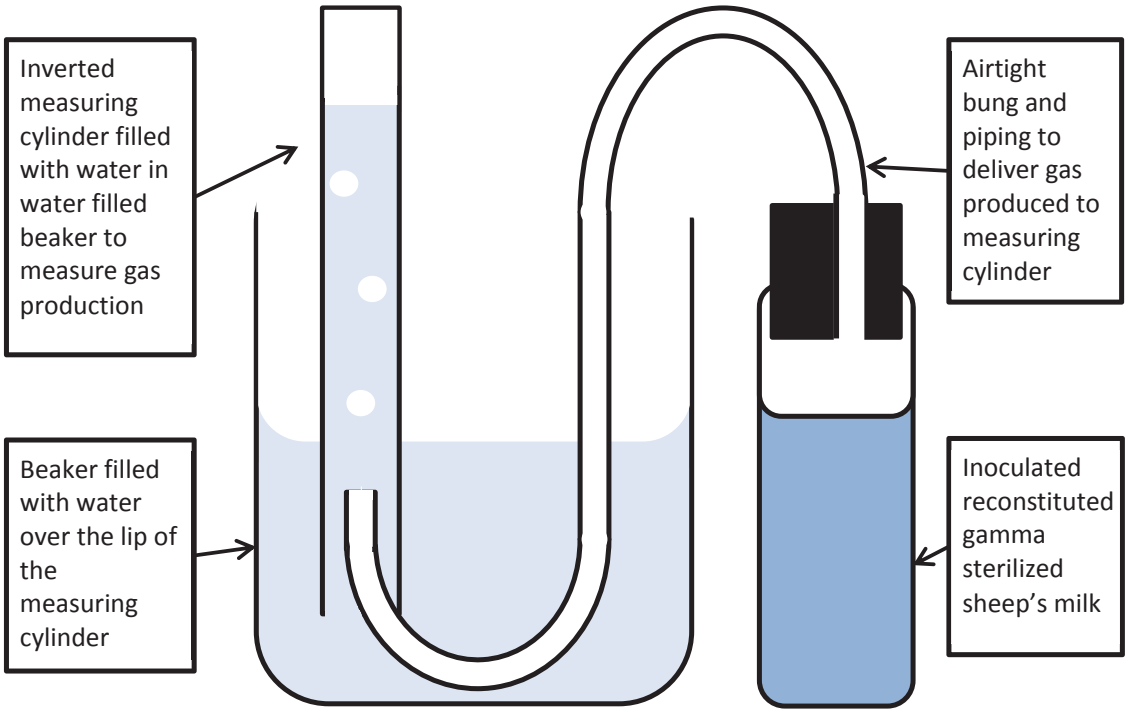

Figure 12 Diagram of the set up for measuring gas production of cultures

3.11 Syneresis

To determine syneresis; a 4cm internal diameter buchner funnel was set up attached to a vacuum. 2 Whatman number 1 filter papers were placed on the base of the funnel (2 were used due to 1 often breaking due to the large holes in the funnel base). 10g of sample was placed on the filter paper and a vacuum was pulled for 2 minutes. A set-up can be seen in Figure 13. Versions of this method have been used in a number of studies (Paucean, Socaciu, Vodnar, & Mudura, 2010; Sahan, Yasar, & Hayaloglu, 2008; Wu, Hulbert, & Mount, 2000).

The weight of the flask prior to filtration was measured, and the weight of the flask after filtration (containing the released whey) was measured, these were then used to determine the amount of weight lost from the 10g added.

Figure 13 Set-up of vacuum filtration unit to measure syneresis

3.12 Rheology

It was decided to use a quick and easy method to gauge an approximate value for viscosity that can be compared across all kefirs. A fast method was required due to the quantity of fermented milks that were to be tested, as well as restrictions in transferring fermented out of the microbiology laboratory. Concentric circles were placed 5mm apart up to 50mm, as shown in Figure 14, this was laminated and 5mL of each sample was pipetted into the centre of the circles. A photograph was taken of each sample as well as a recording identifying the circle the kefir spread to. No timing was used, as after being placed on the laminated paper, there was no further spread over time. The laminated paper was also wiped with ethanol and allowed to dry after each use to prevent interactions between the remaining surface fat and the kefir. This also allowed for a visual record as a photograph was taken of each sample tested.

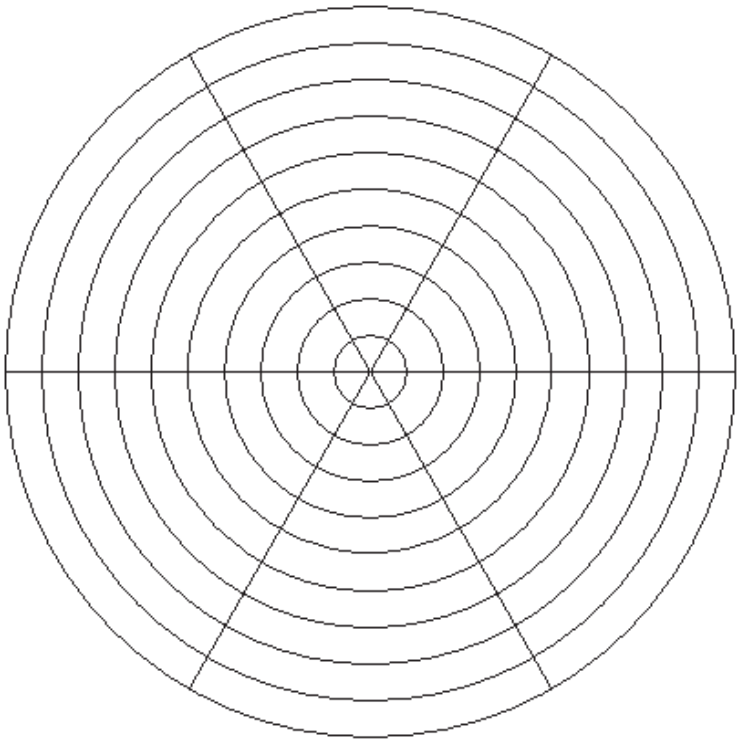

Figure 14 Circles placed 5mm apart to allow for an estimation of viscosity

3.13 Kefir fermentation

To prepare a kefir representative of a final product, and enable the starter cultures with the best potential to produce the product based on preliminary experiments, the following procedure was followed:

1. Whole sheep's milk powder was reconstituted in water at 18% total solids 82% water
2. The required amount of starter culture to achieve approximately 10^6cells/mL milk of each isolate was added
3. This was then fermented at 30°C for 24 hours, before being cooled to 4°C.

3.14 Statistics

Technical and biological duplicates were done for plate counts, these four results were used to plot error bars of standard deviation around the mean.

Biological duplicate samples were done for HPLC, and the range was plotted as error bars around the mean.

4. Results and discussion

4.1 Isolation

4.1.1 Homemade kefir

4.1.1.1 Freeze dried vials

Thirteen freeze dried vials of micro-organisms isolated from kefir in 1983 were gifted to the project. Method 3.1.1 Stock culture isolation was used to rehydrate and grow each isolate.

Unfortunately the control for the M17 broth was contaminated, so the rest of the inoculated M17 broths were discarded. None of the bacterial isolates grew well on the MPCA, either as white pin-prick colonies, not at all, or very few colonies. The growth can be seen in Table 7. Both of the yeasts, KY1 and KY2, grew well on all media.

The bacteria appeared to grow better on Difco M17 + 5% Lactose agar in comparison to Mereck M17 agar, but were still struggling. It was decided to streak from the initial BHI onto Brain Heart agar, as there was growth in the initial BHI broths and it was thought that the bacteria would grow better through sub-culturing on the same medium. However as can be seen in Table 7, the bacteria did not grow well on BH agar. Most of the bacteria were growing on the Difco M17 + L agar; however 3 of the isolates only had a few colonies growing. It was decided that the addition of 5% glucose as well as 5% lactose to the M17 medium would encourage growth as the bacteria may not all be able to ferment lactose successfully.

It was observed that K1 and K13 had both small and large colonies on the agar. A large and small colony from each was selected and streaked out to determine if a contaminant was present. On successive cultures it was observed that colony size variation was a continued occurrence, therefore it was decided that there was no contaminants present.

Table 7 Growth of bacterial isolates on various media. Y; some growth was observed, N; no growth was observed, W; very weak growth, L; low number of colonies present.

Isolate	MPCA	BHI broth	Mereck M17 broth	Difco M17 broth + 0.5% lactose	Mereck M17 agar	Difco M17 agar + 0.5% lactose	Rogosa agar, anaerobic conditions	Brain Heart agar	Difco M17 agar + 0.5% lactose and glucose	MRS agar
K1	Y	Y	Y	Y	Y	L	N	L	Y	Y
K2	Y	Y	Y	Y	L	L	N	L	Y	Y
K10	Y	Y	Y	Y	Y	L	N	Y	Y	y
K12	N	N	N	N	N	N	N	N	N	N
K13	Y	Y	Y	Y	Y	Y	N	N	Y	Y
K14	Y	Y	Y	Y	Y	Y	N	N	Y	Y
K15 03	N	N	N	N	N	N	N	N	N	N
K15 83	L	N	W	W	N	Y	Y	N	Y	Y
K16	Y	Y	Y	Y	N	Y	L	W	Y	Y
K17	L	W	W	W	N	Y	Y	N	Y	Y
K18	Y	Y	Y	Y	N	Y	Y	W	L	Y

Based on Table 7, it was decided that the most successful agar was Difco M17 with added lactose and glucose, and it was decided to use this as the main media with MRS which was also successful. It was also noted that K12 and K15 03 did not grow, and it was assumed these isolates did not survive and attempts to grow them were discontinued, K15 83 was labelled as K15 from then on. 4 of the isolates grew in anaerobic conditions; K15 83, K16 (low colonies), K17, and K18.

The isolates K15 83, K16, K17, and K18 were sub-cultured back onto anaerobic Rogosa agar to help improve their growth, and they all successfully grew however were still weak. It was determined that they grew better on Rogosa under anaerobic conditions than on Difco M17 with added lactose and glucose in aerobic conditions, so it was determined these isolates prefer anaerobic conditions. The isolates were also grown on Difco M17 L+G in anaerobic conditions to compare agars, and there was very similar growth on both these agar.

All of the bacterial isolates were grown on MRS agar, as this is selective for lactobacilli, to assess whether there was an increase of growth on this agar. Most of the isolates grew on MRS, however the anaerobic isolates K15-K18 grew better than Difco M17 L+G and better or similar to

Rogosa, so it was decided to continue the growth of these isolates on MRS agar. Whereas the aerobic isolates K1-K14 were more successful on Difco M17 L+G.

4.1.1.2 Kefir from household

Two different kefirs were received from various households in Palmerston North to isolate the microorganisms present.

8 different yeasts were isolated from the two homemade kefirs, and 5 bacteria in total. Initially more isolates were identified however these were all streaked on M17 L+G and MRS agar and visually identical isolates were removed.

The first kefir received was isolated using method 3.1.2 Isolation from kefir, however without the addition of cyclohexamide in MRS, and DRBC agar plates were also used. All but two of the 19 isolates found were yeast, as kefir normally contains between 1-3 yeasts, it was assumed that there were many identical species within the isolates. On DRBC agar there were four distinct colony formations, so these were taken as the only isolates from the first homemade kefir. For the second kefir this was also done.

This large yeast yield was also an issue due to the yeast colonies being larger than the bacterial colonies which might result in them in being obscured on the agar and not isolated. Because of this, it was decided to add anti-fungal agents to some of the agar to assist in the isolation of bacteria by supressing the yeasts growth. Witthuhn, Schoeman, and Britz, (2005) used 0.5g/100mL (0.5%) cyclohexamide in MRS agar in the isolation of bacteria from kefir; therefore it was decided to use cyclohexamide as the anti-fungal agent. However according to the Sigma-Aldrich (2016) product sheet, 3.5mL of 0.1% cyclohexamide solution in 500mL (0.0007%) is sufficient. This is a considerable variation in the concentration, and it was decided to use 0.1% sterile filtered cyclohexamide solution, added to the agar to make a final concentration of 0.005% (this was also limited by the quantity available and its ability to dissolve at a maximum 2%).

4.1.2 Commercial kefir

A number of commercial kefirs were used as a source of microbes for isolation. This was done due to the limitation of homemade kefirs available, and to find microbes that may not be commonly in homemade kefirs but may still be of use for the development of a kefir.

The micro-organisms of 5 different commercially available kefirs and kefir starters were isolated using method 3.1.2 Isolation from kefir. The kefirs and kefir starters used were:

- The Kefir Company Coconut Kefir (coconut water based kefir),
- Mad Millie Kefir sachet (freeze dried starter),
- Body Ecology Kefir sachet (freeze dried starter),
- Nature's Goodness Kefir sachet (freeze dried starter), and
- The Collective Kefir (cow's milk based kefir).

Four of the selected kefirs provided information on what species the kefir contained. Nature's goodness and the collective kefir did not. The species for each of the four kefirs are discussed below.

Mad Millie's freeze dried starter: *Lactococcus lactis* subsp. *Lactis, Lactococcus lactis* subsp. c*remoris, Lactococcus lactis* subsp. *Lactis biovar*. *diacetylactis, Leuconostoc, Lactobacillus acidophilus, Bifidobacterium* species*, Streptococcus thermophiles,* and *Candidia colliculosa* (Mad Millie, 2016). There was one yeast isolate found, as expected from the given species, and this is expected to be identified as *Candidia colliculosa.* There were 8 bacterial isolates, which may or may not indicate duplicates as the label does not state species for *Bifidobacterium* or *Leuconostoc.*

Body ecology freeze dried starter; *Lactococcus lactis, Lactococcus cremoris, Lactococcus diacetylactis, Leuconostoc cremoris, Lactobacillus plantarum, Lactobacillus casei,* and *Saccharomyces boulardii* (Body Ecology, 2016).7 bacteria were isolated, so some duplicates are expected as there are only 6 bacterial species in the starter. There was also no yeasts isolates from this starter.

The Kefir Company's coconut water kefir; *Lactococcus lactis* subsp. *Lactis, Lactococcus lactis* subsp. c*remoris, Lactococcus lactis* subsp. *diacetylactis, Leuconostoc mesenteroides* subsp. *cremoris, Lactobacillus kefyr, Klyveromyces marxianus* var. *marxianus,* and *Saccharomyces unisporus* (The Kefir Company, 2011). Interestingly The Kefir Company claims to use the 'Body Ecology Kefir starter' however they have listed different species to Body Ecology on their website. Also as the sugar content of coconut water is mostly sucrose (depending on the coconut varietal) this may affect the growth of some of the micro-organisms (Prades, Dornier, Diop, & Pain, 2012). There were 3 yeast isolates and 4 bacterial isolates from The Kefir Company's coconut water kefir.

There was five isolates found in the Collective Kefir, and one yeast isolate. Nature's Goodness failed to grow on the media, so it was inoculated in UHT milk and incubated at 30°C and this was streaked out, with one isolate being found.

4.2 Identification

The 57 isolates were then identified using PCR (method 3.2 PCR for sequencing for bacterial (16s rDNA) and yeast (26srDNA) identification). The results from BLAST (National Center for Biotechnology Information, N.D) and Seqmatch (Michigan State University, 2014) are shown in Table 8 below.

Table 8 PCR results run through BLAST and seqmatch for each sample

	Sample			BLAST top results	Seqmatch result
Freeze dried vials	1	K1	A.Y1	*Streptococcus thermophiles* 99%	*Streptococcus salivarius* 98.3%
			B.Y2	*Streptococcus thermophiles* 99%	*Streptococcus salivarius* 97%
	2	K2	A.Y1	*Streptococcus thermophiles* 99%	*Streptococcus salivarius* 98.9%
			B.Y2	*Streptococcus thermophiles* 99%	*Streptococcus salivarius* 96.5%
	3	K10	A.Y1	*Streptococcus thermophiles* 99%	*Streptococcus salivarius* 97.3%
			B.Y2		
	4	K13	A.Y1	*Streptococcus thermophiles* 99%	*Streptococcus salivarius* 98.7%
			B.Y2	Uncultured bacterium clone 100% *Streptococcus thermophiles* 99%	*Streptococcus salivarius* 96.9%
	5	K14	A.Y1	Uncultured bacterium clone 100% *Streptococcus thermophiles* 99%	*Streptococcus salivarius* 98.7%
			B.Y2	Uncultured bacterium clone 100% *Streptococcus thermophiles* 99%	*Streptococcus salivarius* 96.6%
	6	K15	A.Y1	*Lactobacillus delbrueckii* 100% (Subsp. *bulgaricus* and subsp. *lactis* 99%)	*Lactobacillus delbrueckii* 98.6%
			B.Y2	*Lactobacillus delbrueckii* 99% (subsp. *bulgaricus* 99%)	*Lactobacillus delbrueckii* subsp. *bulgaricus* 95.2%
	7	K16	A.Y1	*Lactobacillus delbrueckii* 99% (subsp. *lactis* 99%)	*Lactobacillus delbrueckii* 94.2%
			B.Y2	*Lactobacillus delbrueckii* 99% (subsp. *bulgaricus* 99%)	*Lactobacillus delbrueckii* subsp. *bulgaricus* 95.2%
	8	K17	A.Y1	*Lactobacillus delbrueckii* 99% (subsp. *lactis* 99%)	*Lactobacillus delbrueckii* subsp. *bulgaricus* 58.4%
			B.Y2	*Lactobacillus delbrueckii* 99% (subsp. *bulgaricus* 99%)	*Lactobacillus delbrueckii* subsp. *bulgaricus* 94.6%
	9	K18	A.Y1	*Lactobacillus delbrueckii* 99% (subsp. *lactis* 99%)	*Lactobacillus delbrueckii* subsp. *bulgaricus* 86%
			B.Y2	*Lactobacillus delbrueckii* 99% (subsp. *bulgaricus* 99%)	*Lactobacillus delbrueckii* subsp. *bulgaricus* 95.8%
	10	KY1	A. NL1	*Kluyveromyces marxianus* 100%	*Kluyveromyces marxianus* 100%
			B. NL4	*Kluyveromyces marxianus* 100%	*Kluyveromyces marxianus* 100%
	11	KY2	A. NL1	*Kluyveromyces marxianus* 100%	*Kluyveromyces marxianus*

				Kluyveromyces lactis 100%	100%
			B. NL4	*Kluyveromyces marxianus* 100%	*Kluyveromyces marxianus* 100%
Homemade kefir 1	12	DDP	A. NL1	*Pichia membranifaciens* 83%	*Pichia membranifaciens* 49.7%
			B. NL4	*Pichia membranifaciens* 99%	*Pichia membranifaciens* 89.9%
	13	DRP	A. NL1	*Kluyveromyces marxianus* 100%	*Kluyveromyces marxianus* 100%
			B. NL4	*Kluyveromyces marxianus* 100%	*Kluyveromyces marxianus* 100% *Kluyveromyces lactis* 99%
	14	DDP	A. NL1	*Pichia membranifaciens* 100%	*Pichia membranifaciens* 98.9%
			B. NL4	*Pichia membranifaciens* 100%	*Pichia membranifaciens* 90%
	15	DW	A. NL1	*Clavispora lusitaniae* 100%	*Clavispora lusitaniae* 100%
			B. NL4	*Clavispora lusitaniae* 100%	*Clavispora lusitaniae* 100%
	16	MVS	A. Y1	*Lactobacillus helveticus* 100% *Lactobacillus kefiri* 99%	*Lactobacillus kefiri* 98.7%
			B. Y2	*Lactobacillus kefiri* 99%	*Lactobacillus kefiri* 97.3%
	17	RST	A. Y1	*Lactobacillus kefiranofaciens* 99%	*Lactobacillus kefiranofaciens* 97.1%
			B. Y2	*Lactobacillus kefiranofaciens* 100%	*Lactobacillus kefiranofaciens* 97.3%
The kefir Company coconut kefir	18	KA1	A. Y1	*Lactobacillus plantarum* 100%	*Lactobacillus plantarum* 98.3%
			B. Y2	*Lactobacillus plantarum* 100% *Lactobacillus pentosus* 100%	*Lactobacillus plantarum* 100% *Lactobacillus pentosus* 100%
	19	KA2	A. Y1	*Lactobacillus plantarum* 99% *Lactobacillus pentosus* 99%	*Lactobacillus plantarum* 59.3% *Lactobacillus pentosus* 59.5%
			B. Y2	*Lactobacillus plantarum* 99% *Lactobacillus pentosus* 99%	*Lactobacillus plantarum* 96.2% *Lactobacillus pentosus* 95.9%
	20	KN1	A. Y1	*Lactobacillus plantarum* 99% *Lactobacillus pentosus* 99%	*Lactobacillus plantarum* 66.3% *Lactobacillus pentosus* 66.3%
			B. Y2	*Lactobacillus plantarum* 99% *Lactobacillus pentosus* 99%	*Lactobacillus plantarum* 93.2% *Lactobacillus pentosus* 93.2%
	21	KN3	A. Y1	*Lactobacillus plantarum* 99%	*Lactobacillus plantarum* 62.5%
			B. Y2	*Lactobacillus plantarum* 99% *Lactobacillus pentosus* 99%	*Lactobacillus plantarum* 91.6%

	22	KN2	A. NL1	*Saccharomyces cerevisiae* 100%	*Saccharomyces cerevisiae* 99.8%
			B. NL4	*Saccharomyces cerevisiae* 100%	*Saccharomyces cerevisiae* 100%
	23	KL1	A. NL1	*Saccharomyces cerevisiae* 100%	*Saccharomyces cerevisiae* 100% Sake yeast Kyokai
			B. NL4	*Saccharomyces cerevisiae* 100%	*Saccharomyces cerevisiae* 100%
	24	KNL	A. NL1	*Saccharomyces cerevisiae* 100%	*Saccharomyces cerevisiae* 100% Sake yeast Kyokai
			B. NL4	*Saccharomyces cerevisiae* 100%	*Saccharomyces cerevisiae* 100%
Body Ecology	25	BA1	A. Y1	*Lactobacillus plantarum* 99%	*Lactobacillus plantarum* 78.1%
			B. Y2	*Lactobacillus plantarum* 99%	*Lactobacillus plantarum* 92.9% *Lactobacillus pentosus* 92.3%
	26	BA2	A. Y1	*Lactococcus lactis* 99%	*Lactococcus lactis* subsp. *Lactis* 99.3%
			B. Y2	No result	No result
	27	BL1	A. Y1	*Lactococcus lactis* subsp. *lactis* 99%	*Lactococcus lactis* subsp. *lactis* 99%
			B. Y2	*Lactococcus lactis* subsp. *lactis* 99%	*Lactococcus lactis* 96.8%
	28	BL2	A. Y1	*Lactococcus lactis* 99%	*Lactococcus lactis* subsp. *lactis* 99.6%
			B. Y2	*Lactococcus lactis* subsp. *lactis* 99%	*Lactococcus lactis* 96.5%
	29	BN1	A. Y1	*Lactobacillus plantarum* 99% *Lactobacillus pentosus* 99%	*Lactobacillus plantarum* 59.8% *Lactobacillus pentosus* 60%
			B. Y2	*Lactobacillus plantarum* 99% *Lactobacillus pentosus* 99%	*Lactobacillus plantarum* 96.1% *Lactobacillus pentosus* 95.7%
	30	BN2	A. Y1	*Lactococcus lactis* 99%	*Lactococcus lactis* subsp. *lactis* 98.3%
			B. Y2	*Lactococcus lactis* subsp. *lactis* 99%	*Lactococcus lactis* 96.5%
	31	BN3	A. Y1	*Lactococcus lactis* 99%	*Lactococcus lactis* subsp. *lactis* 99.3%
			B. Y2	*Lactococcus lactis* subsp. *lactis* 99%	*Lactococcus lactis* 96.7%
The Collective Kefir	32	CA1	A. Y1	*Leuconostoc pseudomesenteroides* 99% *Leuconostoc mesenteroides* 99%	*Leuconostoc pseudomesenteroides* 96.5% *Leuconostoc mesenteroides* subsp. *mesenteroides* 96.5%
			B. Y2	*Leuconostoc pseudomesenteroides* 100%	*Leuconostoc pseudomesenteroides* 98.6% *Leuconostoc mesenteroides*

					subsp. *mesenteroides* 98.6%
	33	CL1	A. NL1	*Torulaspora delbrueckii* 99%	*Torulaspora delbrueckii* 99%
			B. NL4	*Torulaspora delbrueckii* 100%	*Torulaspora delbrueckii* 100%
	34	CL2	A. Y1	*Streptococcus thermophiles* 100%	*Streptococcus salivarius* 100% *Enterococcus faecium* 100%
			B. Y2	*Streptococcus thermophiles* 100%	*Streptococcus salivarius* 99.6% *Enterococcus faecium* 99.6%
	35	CL3	A. Y1	*Leuconostoc mesenteroides* 99%	*Leuconostoc mesenteroides* 97.2%
			B. Y2	*Leuconostoc mesenteroides* 100%	*Leuconostoc mesenteroides* 98.9%
	36	CL3 ST	A. Y1	*Leuconostoc pseudomesenteroides* 99%	*Leuconostoc pseudomesenteroides* 96%
			B. Y2	*Leuconostoc pseudomesenteroides* 100%	*Leuconostoc pseudomesenteroides* 98.5%
	37	CL4	A. Y1	*Lactococcus lactis* 99%	*Lactococcus lactis* 94.2%
			B. Y2		
Homemade kefir 2	38	H1	A. NL1	*Kluyveromyces marxianus* 100%	*Kluyveromyces marxianus* 100% *Kluyveromyces lactis* 99%
			B. NL4	*Kluyveromyces marxianus* 100%	*Kluyveromyces marxianus* 100% *Kluyveromyces lactis* 99%
	39	H2	A. NL1	*Kazachstania unispora* 100%	*Kazachstania unispora* 99.1%
			B. NL4	*Kazachstania unispora* 100%	*Kazachstania unispora* 96.3%
	40	H3	A. NL1	*Kazachstania unispora* 100%	*Kazachstania unispora* 100%
			B. NL4	*Kazachstania unispora* 99%	*Kazachstania unispora* 97.4%
	41	H4	A. NL1	*Kazachstania unispora* 100%	*Kazachstania unispora* 100%
			B. NL4	*Kazachstania unispora* 99%	*Kazachstania unispora* 97.8%
	42	H5	A. Y1	*Lactobacillus kefiri* 99%	*Lactobacillus kefiri* 98.7%
			B. Y2	*Lactobacillus kefiri* 99%	*Lactobacillus kefiri* 97.2%
	43	HB3	A. Y1	*Lactobacillus kefiri* 99%	*Lactobacillus kefiri* 100%
			B. Y2	*Lactobacillus kefiri* 99%	*Lactobacillus kefiri* 97.3%
	44	HE2	A. Y1	*Lactobacillus kefiri* 99%	*Lactobacillus kefiri* 99%
			B. Y2	*Lactobacillus kefiri* 99%	*Lactobacillus kefiri* 97.4%
Mad Millie's kefir starter	45	ML1	A. NL1	*Torulaspora delbrueckii* 100%	*Torulaspora delbrueckii* 100%
			B. NL4	*Torulaspora delbrueckii* 99%	*Torulaspora delbrueckii* 94.8%
	46	MA1	A. Y1	*Leuconostoc mesenteroides* 99%	*Leuconostoc mesenteroides* 97%
			B. Y2	*Leuconostoc mesenteroides* 99%	*Leuconostoc mesenteroides* 96.8%
	47	MA2	A. Y1	*Lactococcus lactis* 99%	*Lactococcus lactis* subsp.

					cremoris 99%
			B. Y2	*Lactococcus lactis* subsp. *cremoris* 99%	*Lactococcus lactis* subsp. *cremoris* 96.4%
			A. Y1	*Lactococcus lactis* 99%	*Lactococcus lactis* subsp. *cremoris* 98.1%
			B. Y2	*Lactococcus lactis* subsp. *cremoris* 100%	*Lactococcus lactis* subsp. *cremoris* 100%
	48	MA3	A. Y1	*Leuconostoc pseudomesenteroides* 99%	*Leuconostoc pseudomesenteroides* 98.1% *Leuconostoc mesenteroides* subsp. *mesenteroides* 98.1%
			B. Y2	*Leuconostoc pseudomesenteroides* 99%	*Leuconostoc pseudomesenteroides* 96% *Leuconostoc mesenteroides* subsp. *mesenteroides* 96%
	49	ML2	A. Y1	*Lactococcus lactis* 99%	*Lactococcus lactis* subsp. *lactis* 99.3%
			B. Y2	*Lactococcus lactis* subsp. *lactis* 99%	*Lactococcus lactis* 97.5%
	50	ML3 W	A. Y1	*Lactococcus* sp. 99%	*Lactococcus* sp. 98.3% *Lactococcus raffinolactis* 98.3%
			B. Y2	*Lactococcus* sp. 99%	*Lactococcus* sp. 96.2% *Lactococcus raffinolactis* 81.1%
	51	ML4	A. Y1	*Lactococcus* sp. 100%	*Lactococcus* sp. 99.6% *Lactococcus raffinolactis* 99.6% *Lactococcus garvieae* 99.6%
			B. Y2	*Lactococcus* sp. 100%	*Lactococcus* sp. 96.5% *Lactococcus raffinolactis* 81.3%
	52	MN1	A. Y1	*Leuconostoc mesenteroides* 99%	*Leuconostoc mesenteroides* 97.5%
			B. Y2	*Leuconostoc mesenteroides* 99%	*Leuconostoc mesenteroides* 97.2% *Leuconostoc mesenteroides* subsp. *mesenteroides* 97.2%
	53	MN2	A. Y1	*Leuconostoc mesenteroides* 99%	*Leuconostoc mesenteroides* 96.3%
			B. Y2	*Leuconostoc mesenteroides* 99%	*Leuconostoc mesenteroides* 96.5% *Leuconostoc mesenteroides* subsp. *mesenteroides* 96.5%
Natures Goodness kefir	54	NM1	A. Y1	*Enterococcus faecium* 100%	*Enterococcus faecium* 100%
			B. Y2	*Enterococcus* sp. 99%	*Enterococcus faecium* 96.8%

Table 9 All of the microbes found and the codes given to them

Species	Number of isolates	Isolate code	Kefir origin
Lactobacillus delbrueckii	4	K15, K16, K17, K18	Homemade (1)
Lactococcus lactis subsp. *cremoris*	1	MA2	Commercial (1)
Leuconostoc mesenteroides	4	CL3, MA1, MN1, MN2	Commercial (2)
Lactobacillus kefiranofaciens	1	RST	Homemade (1)
Lactobacillus plantarum	6	KA1, KA2, KN1, KN3, BA1, BN1	Commercial (2)
Streptococcus thermophiles	6	K10, K13, K14, K1, K2, CL2	Commercial and homemade (2)
Lactobacillus kefiri	3	H5, HB3, HE2	Homemade (1)
Lactococcus lactis subsp. *lactis*	6	ML2, BN3, BA2, BN2, BL2, BL1	Commercial (2)
Leuconostoc pseudomesenteroides	3	CA1, CL3ST, MA3	Commercial (2)
Kluyveromyces marxianus	4	H1, DRP, KY1, KY2	Homemade (3)
Kazachstania unispora	3	H2, H3, H4	Homemade (1)
Saccharomyces cerevisiae	3	KL1, KN2, KNL	Commercial (1)
Torulaspora delbrueckii	2	ML1, CL1	Commercial (2)
Pichia membranifaciens	2	DDP	Homemade (1)
Clavispora lusitaniae	1	DW	Homemade (1)

All of the microbes identified have previously been found in milk kefir, except *Clavispora lusitaniae* which is normally associated with clinical infections, but has been isolated from cheese and other fermented milks in two studies; Mlimbila, Hosea, and Muruke (2013), and Prillinger, Molnar, Eliskases-Lechner, and Lopandic (1999). Therefore it was assumed it was a contaminant. *Torulaspora delbrueckii* has also not been found in any homemade kefirs, but was stated on the label (as its synonym *Candida colliculosa*) on the Mad Millie's kefir where it was identified from, and the Collective Kefir which does not state the microbes on the label. *Pichia membranifaciens* is also uncommon in kefir, being only found in one of the studies looked at (Miguel M. G., Cardoso, Magalhaes-Guedes, & Schwan, 2013), but was found in two different homemade kefirs tested. However a number of *Pichia* species have been found in kefir (Table 3), so it may not be a contaminant, but it is difficult to determine.

All of the other isolates are commonly found in kefir. Interestingly, a number of non-lactose fermenting yeasts were isolated in this study; *Torulaspora delbrueckii, Kazachstania unispora, Saccharomyces cerevisiae* and *Pichia membranifaciens.* It is likely that to grow in milk these yeast rely on a symbiotic relationship with other species present.

All of the species isolated were only found in one or two different sources, however *Kluyveromyces marxianus* was found in all the different homemade kefirs .It is unsurprising that it was the most common yeast, as it is one of the most isolated yeast in Table 4 and it is also a lactose fermenter.

The Kefir Company coconut water kefir advertise on the label: *Lactococcus lactis* subsp. *Lactis, Lactococcus lactis* subsp. c*remoris, Lactococcus lactis* subsp. *diacetylactis, Leuconostoc mesenteroides* subsp. *cremoris, Lactobacillus kefyr, Klyveromyces marxianus* var. *marxianus,* and *Saccharomyces unisporus* (The Kefir Company, 2011). The bacteria and yeast identified were *Lactobacillus plantarum,* and *Saccharomyces cerevisiae*. Neither of these species were stated on the label. However as the Kefir Company claim to be using the Body Ecology kefir starter (*Lactococcus lactis, Lactococcus cremoris, Lactococcus diacetylactis, Leuconostoc cremoris, Lactobacillus plantarum, Lactobacillus casei,* and *Saccharomyces boulardii* (Body Ecology, 2016)), both of these species are included on the label for Body Ecology, as *Saccharomyces boulardii* is a synonym of *Saccharomyces cerevisiae* (Kurtzman, Fell, & Boekhout, 2011). In the Body Ecology starter, the identified isolates were *Lactococcus lactis* and *Lactobacillus plantarum* both of which are on the label.

In the Mad Millie's freeze dried starter the label states that it contains the species; *Lactococcus lactis* subsp. *Lactis, Lactococcus lactis* subsp. c*remoris, Lactococcus lactis* subsp. *Lactis* biovar. *diacetylactis, Leuconostoc, Lactobacillus acidophilus, Bifidobacterium* species*, Streptococcus thermophiles,* and *Candidia colliculosa* (Mad Millie, 2016). From the isolates, *Torulaspora delbrueckii, Leuconostoc mesenteroides, Lactococcus lactis, Leuconostoc pseudomesenteroides* were identified. *Candidia colliculosa* is a synonym of *Torulaspora delbrueckii* (Kurtzman, Fell, & Boekhout, 2011), and the label does not state the species of *Leuconostoc,* so all of the species identified are stated on the label.

Natures Goodness failed to grow on a plate, however when it was grown overnight in UHT milk and then plated, the one isolate was identified as *Enterococcus faecium*. It was assumed that this was a contaminant from the UHT milk, and that the starter had been subjected to temperature abuse or something similar to kill the freeze dried microbes.

While *Lactobacillus kefiri* and *Lactobacillus kefiranofaciens* are common in homemade kefirs (Table 4), and were originally isolated from kefir, they are not advertised in any commercial kefirs. This may be due to these two bacteria introducing characteristics into the kefir that are not desired in commercial products. *Lactobacillus kefiranofaciens* has been found to aggregate (Wang S. , et al., 2012), and it was noticed in growing HB3 (*Lactobacillus kefiri*) had considerable

aggregation when being grown in medium. The kefir produced from the grain HB3 was isolated from also had noticeable 'flecks' or fine grains (distinct from the starter grains) which may not be desirable in a commercial product. Or these bacteria may produce a more 'traditional' tasting kefir which is not always desired by consumers.

4.2.1 Strain determination

For all of the *Lactococcus lactis* isolates (BA2, BL1, BL2, BN2, BN3, MA2, and ML2) it was decided to determine the subspecies based on growth and fermentation parameters. Method 3.3.1 Lactococcus strains was used to determine the subspecies of the isolates.

Table 10 Results from fermentation tests, + being a positive result, and – being negative

		Lactose	Maltose	Glucose	Arginine decarboxylase	Ribose	4%NaCl	Subspecies
ML2	*Lactococcus lactis*	+	+	+	+	+	+	*lactis*
MA2	*Lactococcus lactis*	+	-	+	-	-	-	*cremoris*
BN3	*Lactococcus lactis*	+	+	+	+	+	+	*lactis*
BN2	*Lactococcus lactis*	+	+	+	+	+	+	*lactis*
BA2	*Lactococcus lactis*	+	+	+	+	+	+	*lactis*
BL2	*Lactococcus lactis*	+	+	+	+	+	+	*lactis*
BL1	*Lactococcus lactis*	+	+	+	+	+	+	*lactis*

All the bacteria identified as *Streptococcus thermophiles* using BLAST were identified as *Streptococcus salivarius* using Seqmatch, however Seqmatch does not have any *Streptococcus thermophiles* in the database, but around 432 records of *Streptococcus salivarius* subsp. null. On a sucrose containing medium, such as Mitis-Salivarius agar, *Streptococcus salivarius* will produce 'levan' making large mucoid 'gum-drop' colonies, which can be used to differentiate *Streptococcus thermophiles* and *Streptococcus salivarius* (Vos, et al., 2009). As *Streptococcus salivarius* is associated with oral microflora, it was decided to streak saliva onto the agar to provide an example of the 'gum-drop' shape that differentiates *Streptococcus salivarius*, as described in method 3.3.2 Streptococcus determination.

Figure 15 Saliva on Mitis-Salivarius agar, mucoid gum-drop colonies can be seen

Figure 15 shows saliva streaked onto the agar, where there are distinctive large gummy looking colonies, whereas Figure 16 shows isolate K10 with small dark colonies. All of the isolates produced small dark colonies and were therefore identified as *Streptococcus thermophiles* rather than *Streptococcus salivarius.*

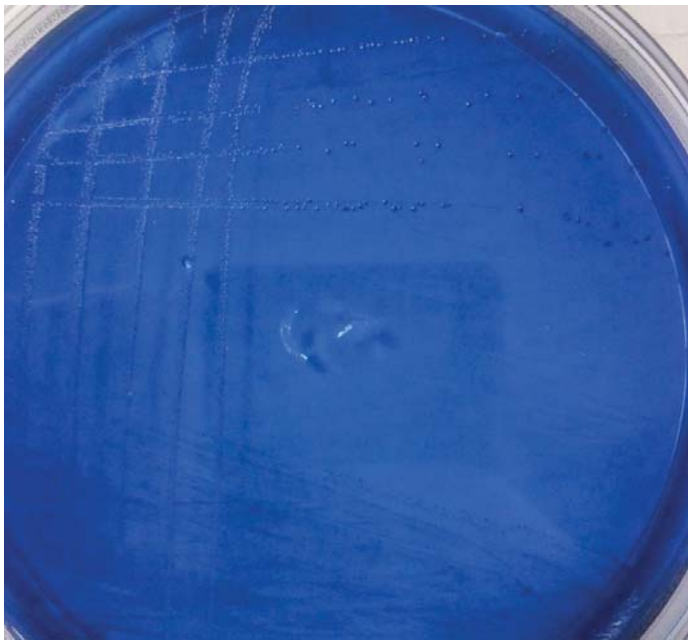

Figure 16 Isolate K10 on Mitis-Salivarius agar

4.3 Identified species

4.3.1 H2, *Kazachstania unispora*

The isolates H2, H3, and H4 were identified as *Saccharomyces unisporus,* which is synonymous for *Kazachstania unispora* (Kurtzman, Fell, & Boekhout, 2011), and H2 was selected for further testing, however as all of the isolates were identified from the same origin they are likely to be identical. *Kazachstania unispora* ferments glucose and galactose, but not lactose and will not grow in lactose liquid media. This indicates that in kefir it is reliant on the breakdown of lactose into galactose and glucose by other micro-organisms (Kurtzman, Fell, & Boekhout, 2011). It will grow at 25°C, 30°C, 37°C, but not at 45°C, and will grow in the presence of ethanol (Kurtzman, Fell, & Boekhout, 2011). It is auxotrophic as it cannot synthesize the essential vitamin thiamine (B1), so this needs to be present in the media for successful growth (Bhattacharya, Yan, Yadav, Tyagi, & Surampalli, 2013).

Kazachstania unispora in kefir and other fermented milk products is known to have a unique interaction with lactic acid bacteria, in which they both produce products which the other consumes. Notably *Kazachstania unispora* consumes glutamic and lactic acid, reducing the acidity of the kefir and enhances the growth of lactic acid inhibited bacteria (such as *Lactobacillus kefiranofaciens*) (Bhattacharya, Yan, Yadav, Tyagi, & Surampalli, 2013). As *Kazachstania unispora* is a non-lactose fermenting yeast, in kefir it would be expected to grow in the later stages of fermentation as it is reliant on other microbes to break down the lactose into galactose and glucose.

4.3.2 H1, *Kluyveromyces marxianus*

The isolates H1, DRP, KY1, and KY2 were identified as *Kluyveromyces marxianus,* isolated from all the homemade kefirs tested, and H1 was chosen as the representative isolate to use in further testing. It is a yeast which can ferment: glucose, galactose, lactose, raffinose, sucrose and inulin, but not maltose. It cannot grow in a vitamin free medium, and grows at 37°C (Kurtzman, Fell, & Boekhout, 2011). This ability to ferment a range of sugars makes it popular for industrial uses.

According to Lane and Morrissey (2010), *Kluyveromyces marxianus* can generate energy through either the Krebs cycle by oxidative phosphorylation or fermentation to ethanol, the balance between the two methods used is dependent on the environment (oxygen and sugar levels), and is strain specific in each case. However *Kluyveromyces marxianus* is considered to have less of a tendency to produce ethanol at a sugar excess in comparison to other yeasts such as *Saccharomyces cerevisiae.* Because of this, there is variation in the ethanol production between strains. *Kluyveromyces marxianus* is also known for having one of the fastest growth rates of any

eukaryotic microbe, with a doubling time of 70min. Because of this rapid growth, if *Kluyveromyces marxianus* is used in the production of kefir as a mono-culture, it may be beneficial to add at a later stage of the fermentation to reduce the quantity and consequent organoleptic effects, as well as reducing competition with other microbes. *Kluyveromyces marxianus* is thermotolerant, with the ability to grow up to 52°C. *Kluyveromyces marxianus* produces the enzymes: inulinase (breaks down inulin to form fructose), β-galactosidases, and pectinases. The production of pectinases mean that if it was decided to thicken the kefir with thickeners, pectin (such as in some Lifeway brand kefirs) should not be used if *Kluyveromyces marxianus* is present, it also may have an effect if any high pectin fruits are added as flavour.

Lane, et al., (2011) conducted a study focusing on the stress tolerance of different strains of *Kluyveromyces marxianus.* The tolerance of NaCl was found to have variation across the strains, but none of the strains grew on 1M NaCl. All the strains grew well at 42°C, however not all survived at 48°C. Of the 13 strains assessed, 4 reached stationary phase in 4-5 hours, and the other seven reached it in 8-10 hours when grown in lactose, producing ethanol in a range from 0.00-0.32 g/g (using HPCL analysis). It is thought that oxygen depletion leads to ethanol production in *Kluyveromyces marxianus* and therefore in the kefir production, stirring may reduce the amount of ethanol produced.

The lactose fermenter *Kluyveromyces marxianus* has the potential to produce the antimicrobial peptide nisin when mixed with *Lactococcus lactis*, this may be a useful tool in the reduction of pathogenic and spoilage bacteria especially due to its antilisterial properties (Shimizu, Mizuguchi, Tanaka, & Shioya, 1999; Delves-Broughton, Blackburn, Evans, & Hugenholtz, 1996).

4.3.3 KN2, *Saccharomyces cerevisiae*

The isolates KL1, KN2, and KNL were identified as *Saccharomyces cerevisiae*. All of these isolates were from the same sample and KN2 was chosen for further testing. It will ferment glucose, sucrose, galactose (variable) and raffinose, but not lactose. KN2 has variable growth at 37°C, and some strains are capable of producing 'killer' toxins which can reduce the growth of other microbes (Kurtzman, Fell, & Boekhout, 2011).

When mixed in a co-culture with *Lactobacillus kefiranofaciens* the lactic acid produced is reduced as it is consumed by *Saccharomyces cerevisiae*, and it stimulates growth and kefiran production by *Lactobacillus kefiranofaciens* (Beal & Helinck, 2014).

However in kefir, *Saccharomyces cerevisiae* is thought by Tamime (2006) to potentially be responsible for the sensory fault in kefir of having a very yeasty aroma, often accompanied by a

solvent-like and vinegar-like aroma. This is thought to be due to *Saccharomyces cerevisiae's* ability to multiply quickly and ferment strongly in the absence of oxygen.

Unfortunately there were a number of issues with the isolate KN2: while it grew well in 10mL of TSB, it failed to grow in the corresponding 1L bottle. After three attempts at growing the yeast in 1L bottles it was decided it was unfit for use in the commercial environment and so was abandoned as a potential starter culture. There were also some issues with suspected contamination of the frozen isolate, which again raised concerns with its suitability for use.

4.3.4 DDP, *Pichia membranifaciens*

The isolates DDP were identified as *Pichia membranifaciens,* and DDP was used for further testing. It is an environmental yeast most commonly found on grapes and in soil; it weakly fermented glucose, but not lactose, galactose, Raffinose, sucrose, maltose or trehalose (Kurtzman, Fell, & Boekhout, 2011). This was isolated from a homemade milk kefir, and is uncommon as it has only been found in one previous kefir study (Miguel M. G., Cardoso, Magalhaes-Guedes, & Schwan, 2013). However *Pichia fermentans* has been identified in at least three different studies (Table 3).

In the testing of DDP, there were a number of difficulties. In the bactrac, DDP gave very inconsistent results, as shown in Figure 37. It also only reached a cell count of around 5 x 10^6cfu/mL after the various incubation times (up to 80 hours). Because of the erratic bactrac results, it was decided to use the optical density method for determining the growth, shown in Figure 17. However DDP failed to grow much over 10^5cfu/mL after 120 hours. Because of this it was decided to not use DDP in the final isolate selection due to its impracticality of use on a commercial scale due to its long growth time and difficulty to grow, as well as its low cell count resulting in a starter with an impractically low cell count.

4.3.5 ML1 *Torulaspora delbrueckii*

The isolates ML1 and CL1 were identified as *Torulaspora delbrueckii,* with ML1 being chosen as the representative isolate for further testing. It can ferment glucose, but not lactose, and is variable for galactose, raffinose, sucrose, maltose and trehalose (Kurtzman, Fell, & Boekhout, 2011). It is a yeast commonly found on grapes and is used to ferment wine (van Breda, Jolly, & van Wyk, 2013). It is also used in the brewing industry, and produces low alcohol beers (2.66%) (Canoico, Agarbati, Comitini, & Ciani, 2016).

4.3.6 RST, *Lactobacillus kefiranofaciens*

RST was the only isolate identified as *Lactobacillus* kefiranofaciens, and was a slow grower in anaerobic conditions taking 5 days to form countable colonies on a pour plate, it did not grow in aerobic conditions and required MRS medium for growth. It is an obligatory homofermentative, it can ferment lactose, glucose, sucrose, and galactose (De Vos, et al., 2009).

Lactobacillus kefiranofaciens are one of the main producers of the polysaccharide kefiran, this polysaccharide is produced as both a capsular polysaccharide which coats the cell in a capsule, and an extracellular polysaccharide. Capsular kefiran helps protect the bacteria from the external environment, and is a useful prebiotic. Extracellular kefiran is useful for the textural effects it has on the fermented product, it can be used as a thickener, stabiliser, and emulsifier, and is thought to have anti-tumour activity (Chen, Hsiao, Hong, Dai, & Chen, 2012; Chiersilp, Shimizu, & Shioya, 2001).

Chiersilp, Shimizu, and Shioya, (2001) studied the production rates of kefiran and lactic acid in the growth and stationary phase, they found the growth phase at pH 5 lasted for 60 hours. During the growth phase, kefiran production was 8.8mg kefiran g^{-1} cell h^{-1}, and 0.7mg kefiran g^{-1} cell h^{-1} during the stationary phase. The rates of lactic acid production were 0.2278g kefiran g^{-1} cell h^{-1} during the growth phase and 0.0588g kefiran g^{-1} cell h^{-1} during the stationary phase. This means that in the production of kefir, once the stationary phase is reached, there will be little increase in the kefiran (and therefore thickness) and acid production during storage. Chiersilp, Shimizu, and Shioya, (2001) also found that lactose was initially fermented producing galactose and glucose, and once the lactose had decreased to a concentration of 20g/L the *Lactobacillus kefiranofaciens* began to ferment the glucose and galactose. At pH 7, no growth was observed, and at pH 4 growth stopped soon after inoculation. This means that in kefir production, *Lactobacillus kefiranofaciens* could potentially be reliant on another species to reduce the pH of the sheep's milk from its average pH of 6.51-6.85 (Park, Juarez, Ramos, & Haenlein, 2007).

However *Lactobacillus kefiranofaciens* can auto-aggregate while growing due to its exopolysaccharide production and biofilm forming ability. Wang S. , et al., (2012) found that *Lactobacillus kefiranofaciens* has a percentage auto-aggregation of 14.68±4.09 at pH 6.2, and 47.17±7.34 at pH 4.2. While this was not found to cause conventional kefir grains in a mixed culture, it still has the potential to produce unpleasant lumps.

4.3.7 HB3, *Lactobacillus kefiri*

The isolates HB3 and MVS were identified as *Lactobacillus kefiri,* with HB3 chosen for further testing. HB3 was a slow grower, taking 5 days for the pour plate colonies to become countable. It was also able to grow in both aerobic and anaerobic conditions, and on MRS medium. It will grow at 15°C but not 45°C, it will ferment lactose and glucose but not galactose or sucrose (Vos, et al., 2009).

It is considered to be a potential probiotic, and is thought to have anti-inflammatory effects as well as the potential ability to inhibit pathogenic bacteria (Carasi, et al., 2015; Carasi, et al., 2014; Zheng, et al., 2013; Likotrafiti, Valavani, Argiriou, & Rhoades, 2015). It also produces the exopolysaccharide kefiran (Micheli, Uccelletti, Palleschi, & Crescenzi, 1999).

4.3.8 KA1, *Lactobacillus plantarum*

The isolates KA1, KA2, KN1, KN3, BA1, and BN1 were identified as *Lactobacillus plantarum,* these five isolates are from two original commercial sources, and so KA1, KA2, and KN1 are likely to be the same, as well as BA1 and BN1. It is facultatively heterofermentative, and has potential as a probiotic with cholesterol-lowering activity (Huang, et al., 2013; Cebeci & Gurakan, 2003). It will stop growing at a pH of 4.6 (McDonald, Fleming, & Hassan, 1990). It will grow at 15°C but not 45°C (Vos, et al., 2009). It will ferment sucrose, glucose, galactose and lactose, and is found in many food products. It is considered a probiotic and is found in the human gastrointestinal tract as well as surviving passage through the stomach (Saulnier, Molenaar, Vos, Gibson, & Kolida, 2007).

Souza, Costa, Miglioranza, Furlaneto-Maia, and Oliveria, (2013) Studied the changes in microbial, physical, chemical, and sensory aspects of *Lactobacillus plantarum* fermented in sweetened milk. After fermenting 4% starter in autoclaved reconstituted milk with added sugar, they found the CFU count started at 1.32 x 10^{10}cfu/mL at day 0, 3.31 x 10^{10}cfu/mL at day 14, and 8.32 x 10^{8}cfu/mL after 70 days of storage at 4°C. The pH was found to be 4.2 at time 0, and decreased slightly over the storage period. The percent of lactic acid was also measured, and found to be 0.70% decreasing to 0.66% over time. The sensory acceptability of the product was 84.3% at day 0, and decreased to only 75.7% after 70 days. This gives an example of the potential CFU and pH reached in milk, however it was supplemented with 10% refined sugar which would be expected to increase the CFU and therefore decrease the pH.

4.3.9 K15, *Lactobacillus delbrueckii*

The isolates K15, K16, K17, and K18 were identified as *Lactobacillus delbrueckii*, all of these isolates were received in freeze dried vials, and were assumed to be all from the same homemade kefir source and are therefore likely to be the same. According to Vos, et al. (2009) it is obligatory homofermentative and will grow at 45°C but not 15°C, and has four subspecies*; delbrueckii, bulgaricus, indicus* and *lactis*, with a major difference being the ability to ferment lactose, with *lactis* and *bulgaricus* being lactose fermenters. They are all found in fermented dairy products, with *bulgaricus* a common starter in yoghurt production.

Bulgaricus is commonly used in conjunction with *Streptococcus thermophiles* as the starter for yoghurt manufacture in a mutualistic relationship. Hassan, Frank, Schmidt, and Shalabi, (1996) studied the growth of *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptococcus thermophiles* in milk for yoghurt development, and found the single-strain fermentation reached a pH of 4.6 after fermentation.

4.3.10 MA1, *Leuconostoc mesenteroides*

The isolates CL3, MA1, MN1, and MN2 were all identified as *Leuconostoc mesenteroides.* MA1, MN1, and MN2 were all isolated from the same source so are likely to be the same. MA1 was chosen as the representative isolate for further testing. It is a facultative anaerobe, which grows between 5°C and 30°C, with its optimum growth between 21°C and 25°C. *Leuconostoc mesenteroides* will metabolise: glucose, fructose, galactose, sucrose and generally lactose. It produces slime in sucrose solutions (Vos, et al., 2009). This slime is dextran, and as sucrose levels increase the dextran production increases resulting in a highly viscous product. It will stop growing at a pH of 5.4-5.7 (McDonald, Fleming, & Hassan, 1990).

Leuconostoc mesenteroides rarely acidifies and curdles milk (De Vos, et al., 2009). There are three subspecies, *Leuconostoc mesenteroides* subsp. *mesenteroides, Leuconostoc mesenteroides* subsp. *dextranicum,* and *Leuconostoc mesenteroides* subsp. *cremoris* (De Vos, et al., 2009).

Leuconostoc are heterofermentative cocci (oven oval) and usually form pairs and chains and are gram positive. They are approved as GRAS organisms – generally recognised as safe, and are considered to be a bacteriocin producer. Bacteriocin producers can be considered as 'protective' cultures as they can reduce the growth of potential pathogens such as *Listeria monocytogenes* (Tamime, 2006). It will grow on MRS agar. In an experiment done by Fatma and Benmechernene (2013) on 83 *Leuconostoc* isolates from raw camel milk, 87% of the isolates could grow in a 3% NaCl solution, and they preferred a pH of 6.5 to 4.8, and none would grow at 4°C or 45°C, however they could withstand heating to 55°C for 15 minutes.

4.3.11 CA1, *Leuconostoc pseudomesenteroides*

The isolates CA1, CL3st, and MA3 were identified as *Leuconostoc pseudomesenteroides* with CA1 being used for further testing. It is a gram positive spherical cell; it grows at 10°C and 37°C. Most strains produce acid from lactose, sucrose, glucose and galactose, and it is often isolated from dairy sources and food (De Vos, et al., 2009). *Leuconostoc pseudomesenteroides* is also shown to produce mannitol in the presence of fructose or sucrose, and mannitol can aid in the drying process of starter cultures due to its osmo-protecting properties (Grobben, et al., 2001).

4.3.12 CL2, *Streptococcus thermophiles*

K10, K13, K14, K1, K2, and CL2 were identified as *Streptococcus thermophile,* K10, K13, K14, K1, and K2 were all freeze dried isolates from 1983 assumed to be from the same homemade kefir and therefore likely to be the same. CL2 was used as the representative sample for further testing. It would not grow on MRS media, and tended to produce both small and large colonies on an agar streak. It is a factitively anaerobic bacterium, it breaks lactose down into glucose and galactose, the glucose is fermented into lactic acid, and the galactose cannot be metabolised (Vos, et al., 2009). It can grow at 42°C up to 50°C, but will not grow at 10°C. It is commonly found alongside *Lactobacillus delbrueckii* subsp *bulgaricus* in yoghurt as they have a positive interaction with each other. In milk, *Streptococcus thermophiles* produce formic acid, folic acid, and CO_2. Formic acid and folic acid are growth requirements for a number of bacteria, or increase the growth rate, such as *Lactobacillus delbrueckii* subsp *bulgaricus*. While some strains can produce cell envelope proteinases, it is not common among *Streptococcus thermophiles;* however *Lactobacillus delbrueckii* subsp *bulgaricus* does produce cell envelope proteinases, which will break down the casein for *Streptococcus thermophiles*, further increasing their symbiotic relationship. In Hassan, Frank, Schmidt, and Shalabi, (1996) study, the single strain fermentation of *Streptococcus thermophiles* in milk reached a final pH of 4.6, when mixed with *Lactobacillus delbrueckii* it reached a final pH of 4.2.

4.3.13 MA2, BL1, *Lactococcus lactis*

The isolates MA2, ML2, BN3, BA2, BN2, BL2, and BL1 were all identified as *Lactococcus lactis*. While MA2 and ML2, as well as BN3, BA2, BN2, BL2, and BL1 were from the same sources, the advertised species in the sources included various subspecies so it was assumed that there was some sub species variation between these isolates. However all but MA2 were identified as *Lactococcus lactis* subsp *lactis,* with MA2 being identified as *Lactococcus lactis* subsp *cremoris*.

MA2 and BL1 were used as the representative samples for *Lactococcus lactis* subsp *cremoris* and *Lactococcus lactis* subsp *lactis* respectively.

It is a gram positive coccus, it will grow at 10°C but not 45°C, and will cease to grow at a pH of about 4.5 (Vos, et al., 2009) (Kosikowski & Mistry, 1997). It consists of two subspecies; *Lactococcus lactis* subsp *cremoris* and *Lactococcus lactis* subsp *lactis.* Both are common cheese starters, with *Lactococcus lactis* subsp *cremoris* commonly used as a single strain starter for cheddar in the New Zealand and Australian cheese industry (Kosikowski & Mistry, 1997).

Ruas-Madiedo, Tuinier, Kanning, and Zoon, (2002) studied the growth of *Lactobacillus lactis* subsp. *lactis* in milk. They tested the growth of 4 strains under different temperatures and inoculation rates. The temperatures used were 20°C, 25°C, and 30°C, with inoculation rates ranging from 0.1% to 5%; they found all the strains could reach a pH of 4.4 after 16-18 hours of incubation (excluding one at pH 4.8 at 20°C). The cell counts all ranges between 3.2 x 10^8cfu/mL and 1.4 x 10^9cfu/mL, without any significant differences between the cell counts at the different incubation temperatures.

4.4 Starter culture preparation

It was required to have a starter culture with a known cell count for further use of the isolates in the development of a kefir.

It was decided to use a single isolate from each species identified and to use these for the rest of the experimental work and development. The isolates chosen are shown in Table 11 below.

Table 11 Isolates selected for further testing

Isolate code	Species	Media
K15	*Lactobacillus delbrueckii*	MRS
MA2	*Lactococcus lactis* subsp. *cremoris*	M17
H1	*Kluyveromyces marxianus*	M17
H2	*Kazachstania unispora*	M17
MA1	*Leuconostoc mesenteroides*	MRS
ML1	*Torulaspora delbrueckii*	M17
RST	*Lactobacillus kefiranofaciens*	MRS (anaerobic)
KA1	*Lactobacillus plantarum*	M17
KN2	*Saccharomyces cerevisiae*	MRS
CL2	*Streptococcus thermophiles*	M17
HB3	*Lactobacillus kefiri*	MRS
BL1	*Lactococcus lactis* subsp. *lactis*	M17
CA1	*Leuconostoc pseudomesenteroides*	MRS
DDP	*Pichia membranifaciens*	MRS

4.4.1 Impedance measurements

It is required to know the growth rate of the species for developing a starter culture with the highest possible cell count. Impedance can be used to assess the growth of bacteria and yeasts based on the conductivity and capacitance of a media over time. As the microbes grow, the impedance increases resulting in a growth curve measured by a BacTrac™ 4100. The BacTrac™ measures the change in conductivity of the growth media, called the M-value, and the change in capacitance on the electrode surface, called the E-value (Walker, Ripandelli, & Flint, 2005).

Method 3.4 Impedance was used to assess the isolates growth in MRS and M17 L+G. It was noticed that a number of isolates had not grown well in either media based on visual observations, so 1mL was added instead of 0.1mL, these isolates were; RST, CL2, HB3, K15, and

MA2. The bactrac was run for 65 hours. The results can be seen in Appendix 6.3 Impedance graphs, with blue lines representing M17 and yellow MRS, and the initial plate count given next to the corresponding media (these have not been adjusted for the 0.1 in 10mL dilution). Most of the isolates did not produce a growth curve with either media, even though growth was easily observable. This is likely due to the fact that impedance relies on the presence of salts in the media, so not all media is suitable for use in the BacTrac™.

It was decided to use 10mL Columbia broth with 1mL added of autoclaved reconstituted 10% skim sheep's milk and 0.1mL 25% filter sterilized sucrose solution, as described by Walker, Ripandelli, and Flint (2005), as well as Triptone Soy Broth (TSB). This was done to serve as a comparison to the low results from M17 and MRS as Columbia (COL) and TSB broths are well established media for use in the bactrac. Method 3.4 Impedancewas repeated, however with COL and TSB replacing M17 and MRS in the bactrac broth, and the media prescribed in Table 11 was used for the initial incubation and the bactrac was run for 24 hours. This method was then repeated; however the bactrac was run for 75 hours. This method was then repeated for a final time, but with the initial incubation time being 48 hours, and all 4 media used in the bactrac vials, and left in the bactrac for 65 hours. The results from these tests can be seen in Appendix 6.3 Impedance graphs.

The E value results were plotted against time for each test, as shown in Appendix 6.3 Impedance graphs, and tended to give a growth curve of a steep incline followed by a slow decrease, the highest point was considered to be the point where the highest number of viable cells, with the important information being the time taken to reach this point. This point is unique to each media, as the microbes grow at different rates in each media. It is also affected by the initial inoculation, as with a higher inoculation ratio the shorter the initial lag phase. This caused some difficulty in the evaluation of the time to maximum growth as there was considerable variation in some of the inoculation cell counts. Because of this variation, it was decided to take an approximate value for the media with the least variation in values and easiest to determine peaks The media selected and the time taken for the impedance to level out indicating peak cell count are shown in Table 12. A 1.5 hour warm up time was used on the bactrac where the machine warms up without measuring data, so 1.5 hours needs to be added onto the incubation time to reach peak cell count.

The media from Table 12 for each isolate was then used for method 3.4 Impedanceagain; however 1mL of the initial incubated media was added to a 1L bottle of the media. This was done as the real-time bactrac curves in the 10mL vials were assumed to represent the growth in the 1L bottles. The bactrac curve was watched for levelling off, with the expectation to coincide

with the times given in Table 12. When the bactrac curves levelled off, the 1L bottles were refrigerated at 4°C before being used in Method 3.6 Starter culture preparation.

Table 12 Time taken or each isolate to reach peak impedance

Isolate code	Time from graph (hours)	Total time (hours)	Highest cell count	Media
K15	17	18.5	$9x10^8$ MRS 48hrs	COL
MA2	8	9.5	$1x10^8$ M17 24hrs	TSB
H1	15, 33	16.5, 34.5	$3x10^7$ M17 24hrs	TSB
H2	20	21.5	$2x10^7$ M17 48hrs	TSB
MA1	7 (TSB), 23 (M17)	8.5 (TSB), 24.5 (M17)	$4x10^8$ M17 24hrs	TSB, M17
ML1	31	32.5	$8x10^7$ M17 48hrs	TSB
RST				
KA1	16 (MRS), 30 (TSB)	17.5 (MRS), 31.5 (TSB)	$6x10^8$ M17 48hrs	MRS, TSB
KN2				
CL2	8	9.5	$6x10^7$ M17 48hrs	COL
HB3				
BL1	6	7.5	$2x10^9$ M17 24hrs	COL
CA1	14	15.5	$9x10^8$ MRS 24hrs	MRS
DDP				

The isolates HB3, DDP, and RST did not produce consistent growth curves in the bactrac, as shown in appendix 6.3 Impedance graphs.

As the impedance method failed to work for the three isolates, an alternative method was required. It was decided to grow the isolates in 1L UHT milk for each isolate, MRS broth for HB3, and RST, and M17 broth for DDP. Initially they were grown in 10mL of MRS and M17 and added to the 1L mediums. The growth of the isolates in the non-milk medium was monitored using optical density.

4.4.2 Optical density measurements

Optical density is the measurement of light passing through a medium, the more opaque the medium the higher the optical density, with water being zero. This can be used to assess the growth rate of an isolate growing in a liquid medium based on how cloudy it becomes over time which represents the increase of cells in the media (it should be noted that this measures all cell count, not just viable cells).

The isolates HB3 (*Lactobacillus kefiranofaciens*), RST (*Lactobacillus kefiri*), and DDP (*Pichia membranifaciens*) were not able to be measured using the impedance method. RST failed to grow in the initial incubation stage prior to being added to the bactrac tube therefore no impedance was measured, this was likely due to RST requiring a longer incubation time. HB3 grew in the bactrac tubes; however HB3 aggregates in the medium, which may have affected the ability to measure the impedance. Due to these issues using the bactrac, another method was required to monitor the growth of the isolates in a 1L broth. Optical density was used to monitor the growth, as described in Method 3.5 Optical density. To prevent a potential issue with HB3 aggregating, all the samples were stirred vigorously with a stirrer bar autoclaved with the initial medium before being measured. After Method 3.5 was completed, the samples were then used in Method 3.6 to prepare the starter cultures.

While DDP successfully grew in 10mL M17 broth, when grown in 1L broths there was no visual growth using both the bactrac or OD methods. It was also attempted to incubate it in UHT cow's milk, however again no growth was observed visually and with plate counts over 5 days. It can be seen in Figure 17 below that the plate counts only increased from 10^4 to 10^5 after 5 days. This cell count is too low for preparing a starter, and the time taken to achieve this low cell count is too long for practical industry use. *Pichia membranifaciens*, is not commonly found in kefir (Table 3), so it was decided to remove this isolate from further testing because of its inability to grow and lack of consumer awareness as it is not a commonly advertised species.

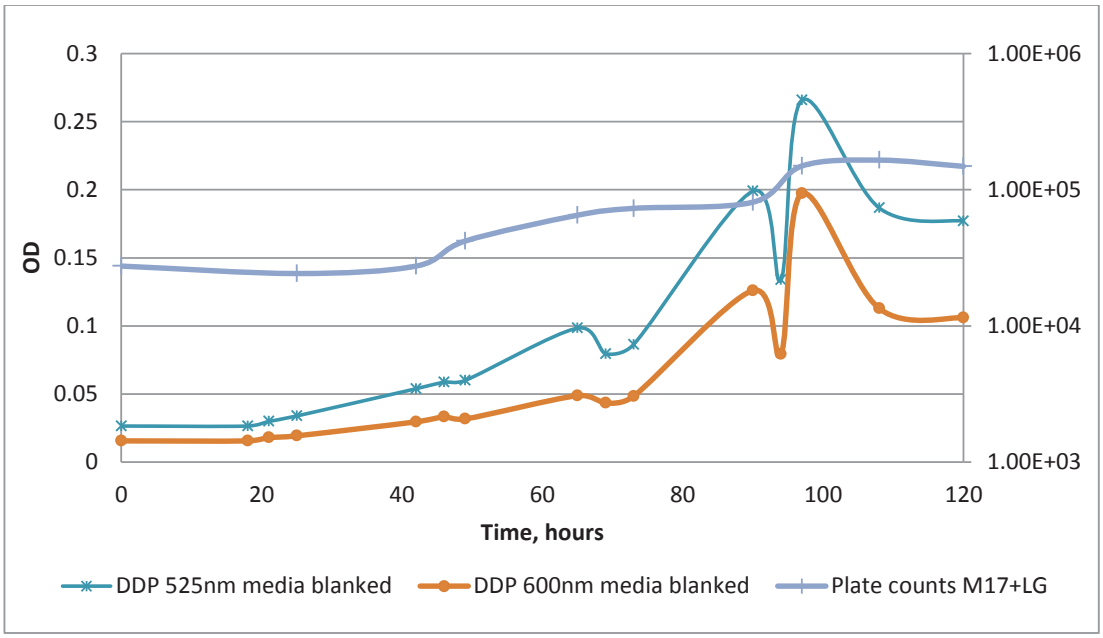

Figure 17 Optical density and cell counts over time at 30'C for DDP

For the first trial using RST and HB3, the peptone water used for washing the bacteria in the final stage was unfortunately contaminated. This meant the test had to be repeated. However, instead of testing the OD three times a day and cell count twice, OD and cell counts were done once a day, and compared to the previous test to ensure it was growing at a similar rate. The results are shown in Figure 18 and Figure 19 below.

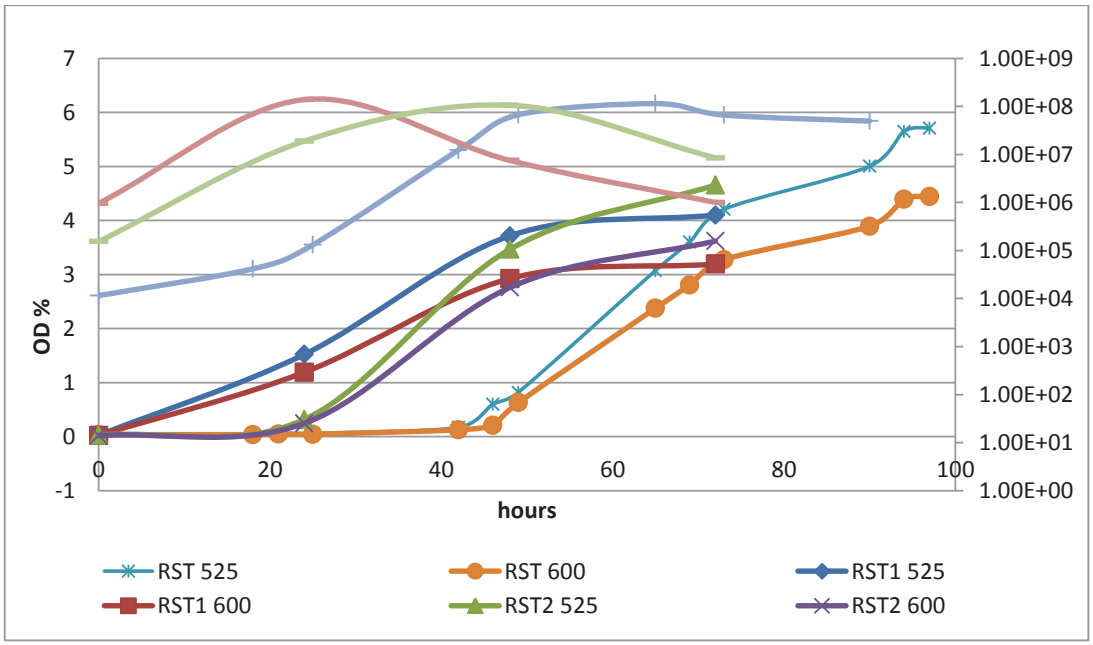

Figure 18 Optical density and cell counts over time at 30'C for RST

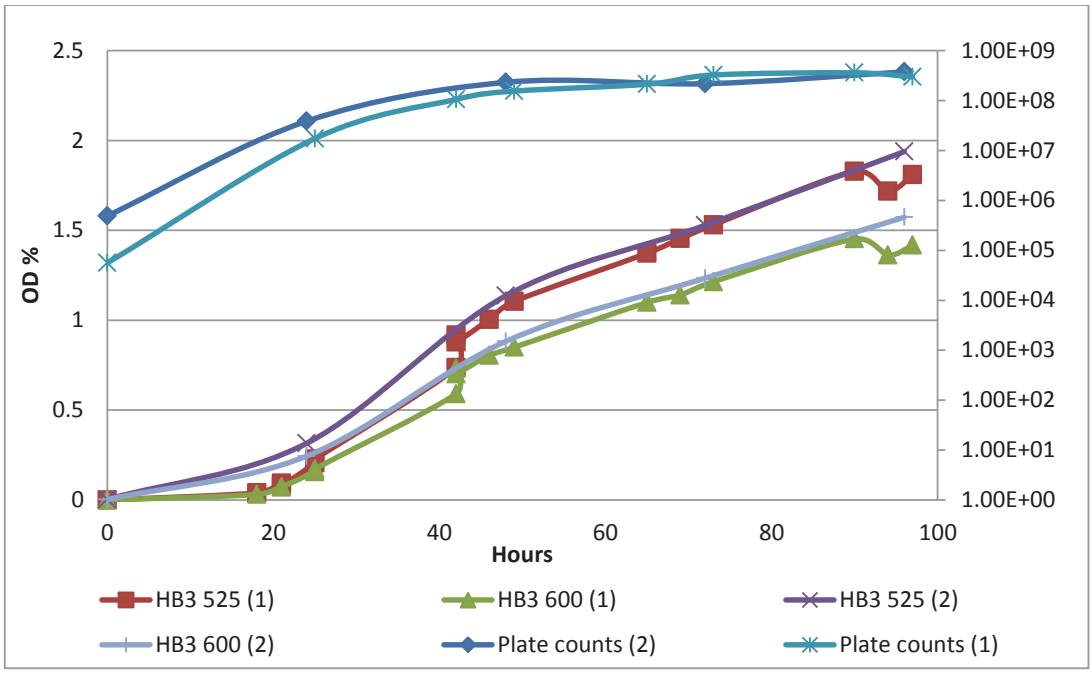

Figure 19 Optical density and cell counts over time at 30'C for HB3

It can be seen in Figure 19 above that HB3 produced very consistent results between the two different samples. However Figure 18 shows variation between the samples RST1, RST2 and RST for the optical density and plate counts. Three different samples were tested for RST, sample RST1 and RST2 have fairly similar results, however RST has a much longer lag phase. It is also odd that the plate counts for all the RST tests reach peak cell count before peak optical density. This variation in trials may make RST less suitable for use as a starter.

4.4.3 Inoculation ratio

The inoculation ratio is an important factor in the processing of fermented products. To determine the inoculation rate to use, the cell count of traditional kefir directly after inoculation was measured as described in Method 3.7 Inoculation ratio of kefir grains. The freshly inoculated milk had a cell count of 8.85×10^6cfu/mL in M17 and 6.6×10^6cfu/mL in MRS. The fermented kefir had a cell count of 4.35×10^8cfu/mL in M17 and 1.3×10^9cfu/mL in MRS.

This was compared to information on the inoculation ratio used in manufacture; 450mL of 1×10^9cfu/mL culture added to 6000L, using $C_1V_1=C_2V_2$, $^{450 \times 10^9}/_{600000} = V_2$, $V_2=7.5 \times 10^5$cfu/mL, this means that after inoculation, the CFU in the media prior to fermentation should be around 7.5×10^5cfu/mL. (Andrew Patrick, Personal Communication, May 25, 2016). Australia New Zealand Food Standards Code (2015) also requires the minimum starter culture

concentration to be 10^6cfu/g. Based on this range of values for initial inoculation cell count, it was decided to use $1x10^6$cfu/mL for the initial inoculation of the sheep milk for each isolate.

Immediately after Methods 3.4 Impedance and 3.5 Optical density were done, Method 3.6 Starter culture preparation was used to prepare frozen starter cultures for each isolate. The final cell count for each sample can be seen below in Table 13. MA1 was grown in both M17 and TSB, however M17 had a higher cell count in the frozen culture, so this was used instead of TSB.

Table 13 Cell count for frozen started culture

Isolate		Cell count in frozen sample	mL in 100mL for $1x10^6$
H1	*Kluyveromyces marxianus*	$1.68x10^8$	0.595
MA1	*Leuconostoc mesenteroides*	$1.93x10^{10}$	0.005
ML1	*Torulaspora delbrueckii*	$3.05x10^8$	0.328
K15	*Lactobacillus delbrueckii*	$8.8x10^9$	0.036
CA1	*Leuconostoc pseudomesenteroides*	$1.66x10^{10}$	0.006
H2	*Kazachstania unispora*	$3.75x10^8$	0.267
CL2	*Streptococcus thermophiles*	$1.5\ x10^8$	0.669
BL1	*Lactococcus lactis* subsp. *lactis*	$1.8x10^{10}$	0.006
MA2	*Lactococcus lactis* subsp. *cremoris*	$8.4x10^7$	1.19
KA1	*Lactobacillus plantarum*	$1.04x10^{10}$	0.01
HB3	*Lactobacillus kefiri*	$5.65x10^9$	0.018
RST	*Lactobacillus kefiranofaciens*	$9.7x10^7$	1.031

Due to some issues with the growths of *Pichia membranifaciens* (DDP) and *Saccharomyces cerevisiae* (KN2) they were not included in the later stages of the project. DDP failed to grow in 1L media after multiple attempts. This was deemed to be unacceptable for commercial production and so DDP was discarded. KN2 had a number of contamination issues, and as this is a product to be consumed it was decided not to risk the potential of using a contaminated starter.

4.5 Assessing isolate growth in sheep's milk

4.5.1 Isolates growth in gamma sterilized sheep's milk

For the development of a kefir, the isolates were assessed for their growth characteristics in sheep's milk to select the species expected to produce a promising kefir product.

The criteria for the final kefir are:

- To have a low ethanol content (less than 0.5%)
- To have a low pH, between 4.5-5.5
- To have a high cell count, over 10^6 at the time of consumption to be considered a probiotic
- Have a thicker texture than milk
- Have some effervescence
- Have some probiotic potential
- Produce lactic acid for a sour yoghurt flavour
- Produce diacetyl for a buttery flavour
- To not separate or form lumps
- Must contain a yeast.

So to determine the best mix of species, the isolates were assessed based on these criteria for their growth in sheep's milk to select the species expected to produce a promising kefir product. This was done by:

- HPLC to measure the ethanol, lactic acid, diacetyl, and sugar content (Method 3.9 HPLC)
- pH measurement
- Plate counts
- Visual assessment of texture as well as syneresis and rheology testing (Methods 3.11 Syneresis and 3.12 Rheology)
- Gas production testing (Method 3.10 Gas production).

A sterile sheep's milk was required to ensure the growth was representative of the isolate; however some of the isolates may struggle to grow in milk without the symbiotic environment kefir provides. At a total solids concentration representative of sheep's milk (18%), the sheep's milk created a gel structure when autoclaved, and separated after UHT processing. Because of this the sheep's milk powder was gamma sterilized at the MSD animal health facility in Upper Hutt. Unfortunately it is illegal in New Zealand to consume gamma sterilized produce, so taste

testing was unable to be performed on the fermented milk. The sterilization process also slightly altered the smell of the powdered milk, likely due to the high fat concentration of the powder being oxidized, however this was assumed to not have an effect on the growth of the micro-organisms.

The isolates were grown in gamma sterilized sheep's milk as described in Method 3.8 Growth in sheep's milk to test the gas production using Method 3.10 Gas production to use for further tests. Plate counts were done upon initial inoculation to determine the exact cell count, and after 24 hours fermentation. After 24 hours each duplicate sample was assessed: using HPLC as described in Method 3.9 HPLC, syneresis as described in Method 3.11 Syneresis, rheology as described in Method 3.12 Rheology, and the pH was measured.

The remaining incubated milk was then separated into 5mL Eppendorf tubes, and after 7, 14, and 52 days storage at 4°C two tubes of each isolate were removed and used for method 3.9 HPLC, plate counts and pH measurement. These results are shown in the sections below, and were used to select the species to use in the kefir.

The rheology and syneresis were not tested in the storage tests as there was very little variation in the isolates, and so visual assessment of separation was done. MA2, BL1, and KA1 were the only isolates that appeared to have a slightly thicker texture than sheep's milk; however the rheology test was not sensitive enough to determine this. There was also no separation or lump formation observed during the storage time.

30°C was chosen for the temperature of fermentation instead of the commonly used 25°C. This was done for a number of reasons: convenience due to the availability of a large 30°C incubator, and increased growth of isolates at this temperature. UHT cow's milk was also inoculated with 10% Lifeway Kefir and incubated at 25°C, 30°C, and 37°C for 24 hours. The kefir at 25°C had the same observable texture as milk, the 37°C kefir had a too thick yoghurt texture, and the 30°C kefir had a texture very similar to the original Lifeway product, this can be seen in Appendix 6.5. This indicated that Lifeway fermented their product at 30°C. It was also thought that the recommended 25°C is mostly considered for household kefir as average room temperature, and not based on optimum growth or commercial production.

4.5.1.1 HPLC results

The lactose, glucose, galactose, lactic and acetic acids, diacetyl and ethanol quantity was measured using HPLC in duplicate samples of each isolate. This was done after 24 hours, 7 days, 14 days, and 52 days to assess the changes over storage. The standards used were pure and of a

known concentration, thus giving a single peak in the chromatogram. The time the peak appears is the retention time, and this is used to identify the compound in the fermented milks. The quantity of the compound was determined by comparing the area under the peak of the known concentration to the unknown concentration. The average retention time, concentration, and area for the standards are shown in Table 14 and the results for each compound are shown in Figure 21 to Figure 23 below, and Figure 30 to Figure 33 in Appendix 6.2.

Table 14 Standards used with average retention time and area, based on approximately 14 runs for each standard

Sample	Average retention time (Detector A 210nm) min	Average area (Detector A 210nm) % (outliers removed)
Lactose 5%	7.99	9157000
Glucose 10%	9.33	17118000
Galactose 10%	9.88	18694000
Lactic acid 1%	12.73	1162000
Acetic acid 1%	14.73	734000
Diacetyl 10%	16.05	10085000
Ethanol 10%	21.32	4602000

Figure 21 shows the lactose fermentation over the storage period. The sheep's milk had an initial lactose concentration of 5.8% lactose, so all of the isolates fermented lactose. H1 had the largest initial decrease in lactose and the largest decrease over time. This is to be expected as H1 (*Kluyveromyces marxianus*) is known for its ability to ferment lactose and is used for this purpose in the ethanol production industry. It was also the highest ethanol producer, as shown in Figure 30, producing over 1.8% ethanol after 52 days storage which is higher than the 0.5% limit. However the results for H1 may not be entirely accurate, although care was taken to ensure the same amount was centrifuged for each sample; H1 was extremely bubbly and so may have resulted in some loss of product due to gas displacing the liquid, which would result in slightly lower results.

The other two yeasts assessed, which are known non-lactose fermenters, H2 and ML1 (*Kazachstania unispora* and *Torulaspora delbrueckii*) were the isolates responsible for the least amount of lactose fermentation. It is also interesting to see that there was continued breakdown of lactose for the first 7 days of storage, and then the breakdown slowed down considerably. This continued breakdown is not consistent with Leite A. M., et al., (2013) (Figure 20) whom found a continued breakdown of lactose in kefir of 0.2 percentage points as opposed to the 0.4 – 1 percentage point loss in the pure isolates. Of the four different isolate

combinations for kefir (see section 4.7), two (K1 and K2) also showed this decrease in lactose after 7 days, however K3 and K4 did not. This is likely due to the low pH reached of 4.85 after 24 hours: Leite A. M., et al., (2013) showed that such low pHs halt the growth of the microbes and thus their ability to ferment lactose.

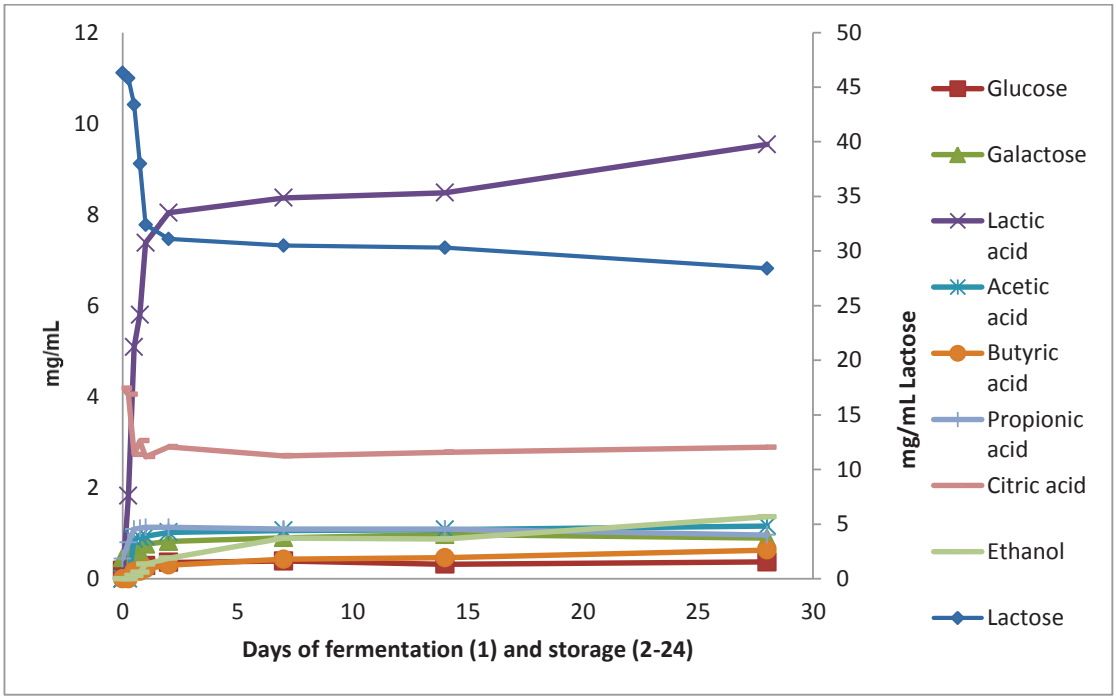

Figure 20 Organic acids and sugars produced in kefir during fermentation and storage for 14 days, measured by HPLC (Leite A. M., et al., 2013)

The rate of lactose breakdown is also indicative of growth, and interestingly some species that are reported to not grow at 4°C have a lower rate than those that do grow at low temperatures. An example is *Lactococcus lactis* subsp. *cremoris* (MA2), which does not grow at 4°C, and has been reported to have a lower rate of lactose breakdown over 7 days than *Lactococcus lactis* subsp. *lactis* (MA1) which does grow at 4°C (Vos, et al., 2009).

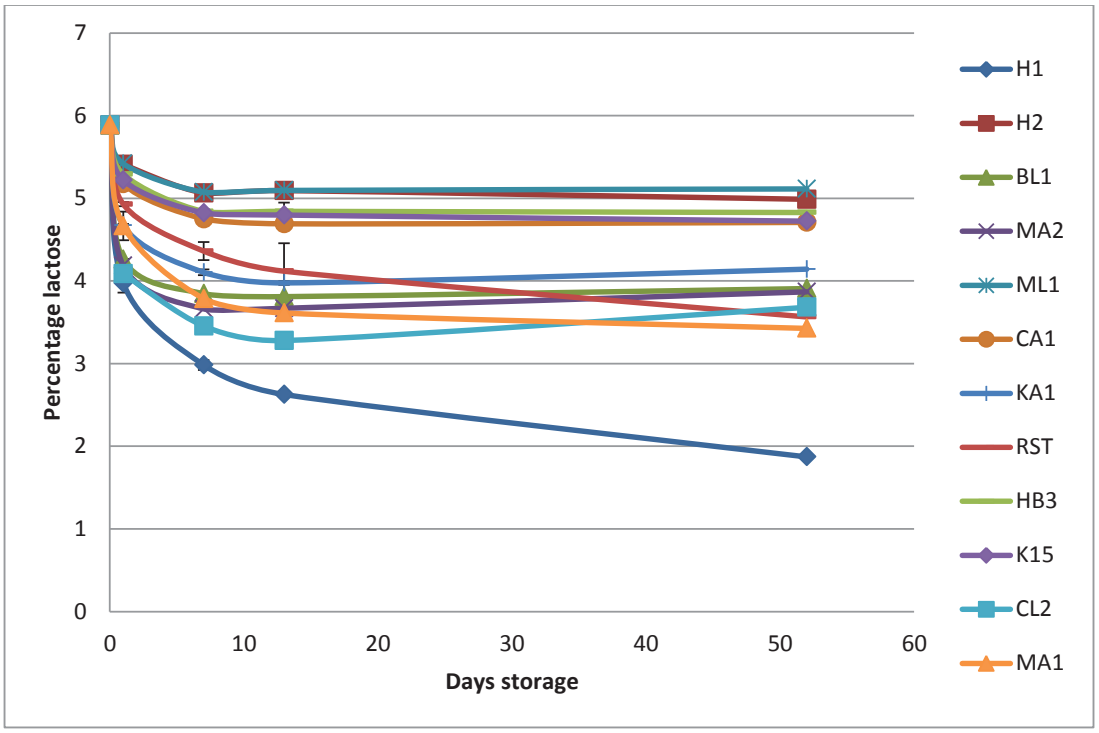

Figure 21 Averaged HPLC results for lactose from each isolate grown in gamma sterilized sheep's milk over 52 days storage at 4°C.

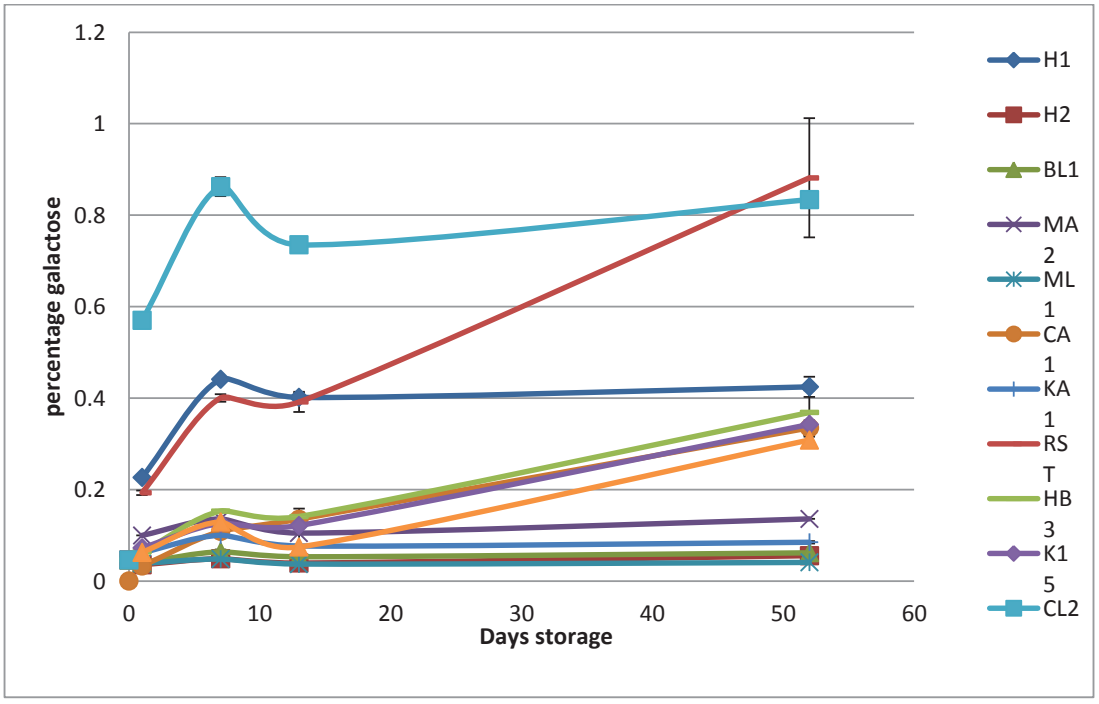

Figure 22 Averaged HPLC results for galactose from each isolate grown in gamma sterilized sheep's milk over 52 days storage at 4°C.

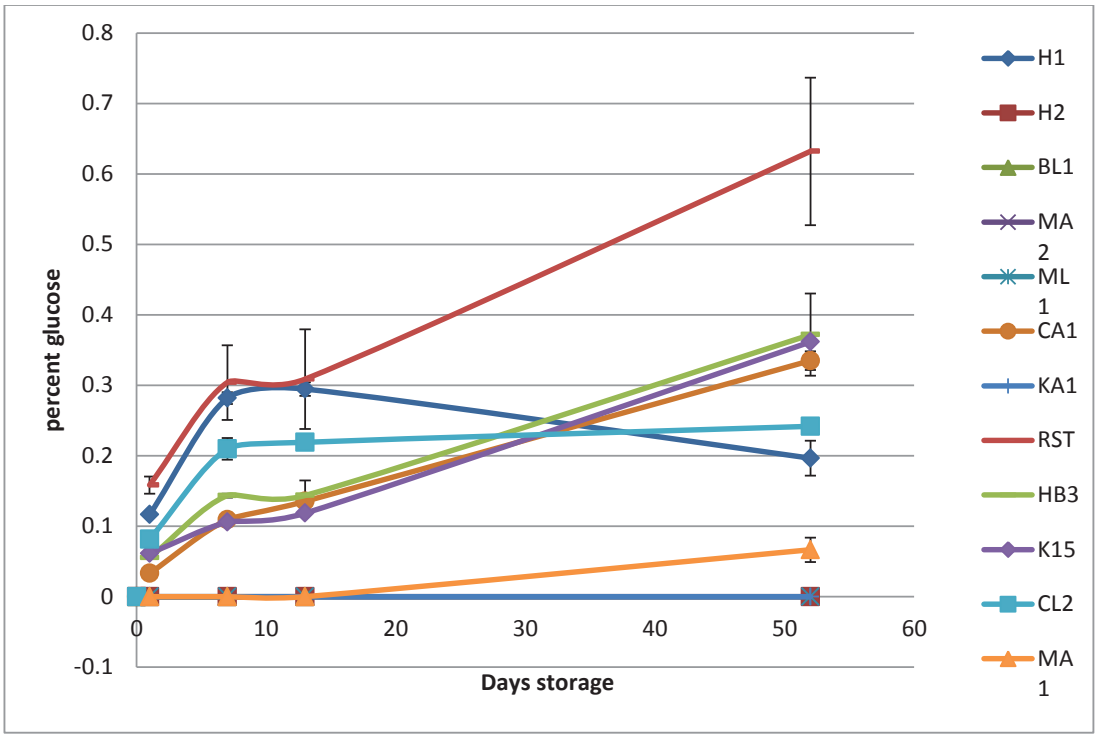

Figure 23 Averaged HPLC results for glucose from each isolate grown in gamma sterilized sheep's milk over 52 days storage at 4°C.

Figure 23 and Figure 22 show the changes in glucose and galactose over time. It is interesting to note the number of isolates that increased in both of these sugars between days 14 and 52 (RST, HB3, CA1, MA1, and K15). This is unusual as glucose and galactose is produced by the breakdown of lactose, however the change in lactose did not correspond to the change in glucose and galactose.

In yoghurt, the concentration of lactic acid is around 0.9% (Cheng, 2010). Both BL1 and MA2 had very similar lactic acid production to each other and yoghurt, starting at around 0.75%, increasing to around 1.1% after 7 days, and then decreasing to 0.8% after 13 days. CL2, MA1 and KA1 also produced between 0.2% and 0.8% over the 13 days, with the other isolates producing between 0 and 0.1%.

Diacetyl is found in yoghurts at a range of 0.2mg/kg to 3mg/kg (Cheng, 2010) which is 0.00002% to 0.0003% and this is significantly lower than the results obtained.

MA2 (0.004%), MA1 (0.0025%) and KA1 (0.01%) were the only isolates to produce over 0.002% diacetyl, however over the storage period RST and HB3 produced around 0.004% after 7 days. After 14 days, the diacetyl had decreased in KA1 to 0.008%, and increased in MA2 and RST to around 0.006%. The initial production of diacetyl was used in the selection of isolates for the final product.

The acetic acid was also measured, as this can be produced when the production of lactic acid is exhausted and the lactic acid is then metabolized further into acetic acid (Sedewitz, Schleifer, & Gotz, 1984). This can be seen in KA1 and MA1 in Figure 32 in Appendix 6.2, where the concentration of acetic acid increased to 0.2% after the 52 days. This is slightly unusual as the carbohydrate lactose had not been completely depleted with around 3.5-4% remaining (Figure 21), however it is thought that the production of acetic acid increases in an aerobic environment for *Lactobacillus plantarum* (KA1) so this or the presence of extracellular enzymes may be responsible for the increase of acetic acid (Sedewitz, Schleifer, & Gotz, 1984).

4.5.1.2 pH and cell count of isolates in sheep's milk

The pH of the isolates in sheep's milk was measured after 24 hours fermentation, and after 6, 14, and 52 days of storage at 4°C, with the results shown in Table 15 Most of the results were consistent between duplicates within ±0.1pH. However RST varied between pH5.5 and pH6.2 after 52 days, RST also had the most variation between duplicates in all the tests, indicating that it is either an unreliable culture or that one of the duplicates became contaminated.

The isolate with the lowest pH after 24 hours was MA2 at pH4.6, and this stayed stable over the storage time. MA2 also had a significant decrease in cell counts over time (Figure 24) from over 10^8cfu/mL to fewer than 10^3Cfu/mL, which is below the required 10^6cfu/mL. This decrease is likely due to the low pH.

KA1 and MA1 decreased over time, by pH0.3 and pH0.8 respectively. The remaining isolates remained fairly constant over the storage time. MA2, BL1, KA1, CL2, and MA1 all lowered the pH to pH5.5 or below after 24 hours.

Table 15 pH of isolates over time

isolate	no.	Initial pH	pH after 24 hours fermentation	pH after storage at 4°C		
				6 days	14 days	52 days
RST	**1**	6.4	6.2	6.1	5.9	5.5
	2	6.4	6.2	6.3	6.3	6.2
MA2	**1**	6.4	4.6	4.7	4.6	4.7
	2	6.4	4.6	4.6	4.6	4.7
BL1	**1**	6.4	5.0	5	5	5
	2	6.4	5.0	4.9	5	5
KA1	**1**	6.4	5.4	5.4	5.3	5.1
	2	6.4	5.4	5.4	5.3	5.1
HB3	**1**	6.4	6.4	6.4	6.4	6.2
	2	6.4	6.4	6.4	6.4	6.3
CL2	**1**	6.4	5.4	5.5	5.5	5.4
	2	6.4	5.5	5.5	5.5	5.4
H2	**1**	6.4	6.4	6.3	6.4	6.3
	2	6.4	6.4	6.4	6.4	5.8
CA1	**1**	6.4	6.1	6.1	6.2	6.1
	2	6.4	6.1	6.1	6.1	6.1
K15	**1**	6.4	6.2	6.1	6.2	5.9
	2	6.4	6.2	6.1	6.2	5.9
ML1	**1**	6.4	6.4	6.4	6.5	6.4
	2	6.4	6.4	6.4	6.5	6.4
MA1	**1**	6.4	5.6	5.3	5.1	4.8
	2	6.4	5.5	5.2	5.1	4.7
H1	**1**	6.4	5.9	5.8	6.1	6
	2	6.4	6.1	5.8	6	6.1

The initial cell count varied between 10^5cfu/mL and 10^7cfu/mL, there was some variation from the 1x10^6cfu/mL aim. However due to the very small amounts of the starter culture added some variation was expected from not all the starter leaving the pipette due to the thick consistency, even though back flushing of the pipette was done to reduce this. All of the yeasts (H1, H2, and ML1) reached the lowest cell count after 24 hours, this was expected for ML1 (*Torulaspora delbrueckii*) and H2 (*Kazachstania unispora*) as they are non-lactose fermenting species. H1

(*Kluyveromyces marxianus*) can ferment lactose, and had the highest cell count of the three yeast at 10^7cfu/mL as expected. Three of the isolates MA1, KA1, and BL1 all produced a cell count after 24 hours over 10^9cfu/mL. BL1 decreased after 52 days to 10^7cfu/mL, but this was still above the required 10^6cfu/mL. The only isolates that failed to remain above the required 10^6cfu/mL were MA2 and CA1.

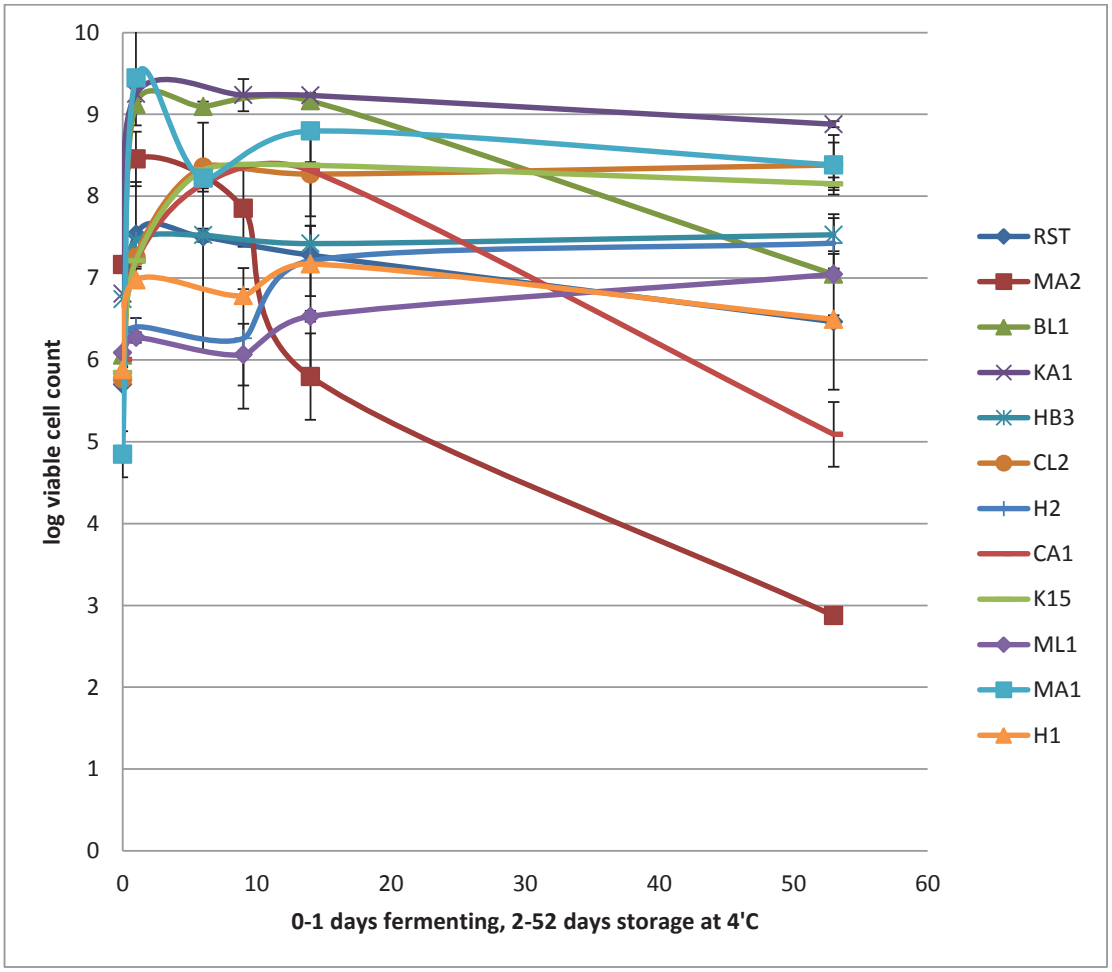

Figure 24 Log average cell count for each isolate over fermentation and storage at 4'C

4.6 Selection of isolates for combination

The criteria for the final kefir are:

- To have a low ethanol content (less than 0.5%)
- To have a low pH, between 4.5-5.5
- To have a high cell count, over 10^6cfu/mL at the time of consumption to be labelled
- Have a thicker texture than milk
- Have some effervescence

- Have some probiotic potential
- Produce lactic acid for a sour yoghurt flavour
- Produce diacetyl for a buttery flavour
- To not separate or form lumps
- Must contain a yeast.

The results for the tests based on these criteria are shown in Table 16, being ascribed a '+' if the isolate met the criteria or a '–' if the isolate failed the criteria. Groups of isolates were chosen for the fermentation of a final kefir product. The selection was based on the results obtained from the pure isolate milk fermentation, and how they could be combined to produce a kefir based on the criteria.

Table 16 Isolates selection criteria results

Isolate	Species	Cell count over $1x10^7$ after 24 hours	Cell count over $1x10^7$ after 2 weeks	pH below 5.5	Diacetyl production	Lactic acid production	Gas production	Texture	Ethanol less than 0.5%
K15	*Lactobacillus delbrueckii*	+	+	-	-	-	-	-	+
MA2	*Lactococcus lactis* subsp. *cremoris*	+	-	+	+	+	-	+	+
H1	*Kluyveromyces marxianus*	-	+	-	-	-	+	-	-
H2	*Kazachstania unispora*	-	-	-	-	-	+	-	+
MA1	*Leuconostoc mesenteroides*	+	+	+	+	+	-	-	+
ML1	*Torulaspora delbrueckii*	-	-	-	-	-	-	-	+
RST	*Lactobacillus kefiranofaciens*	+	-/+	-	-	-	-	-	+
KA1	*Lactobacillus plantarum*	+	+	+	+	+	-	+	+
CL2	*Streptococcus thermophiles*	+	+	+	-	+	-	-	+
HB3	*Lactobacillus kefiri*	+	+	-	-	-	-	-	+
BL1	*Lactococcus lactis* subsp. *lactis*	+	+	+	-	+	-	+	+
CA1	*Leuconostoc pseudomesenteroides*	+	+	-	-	-	-	-	+

The chosen groupings were;

Group 1; MA1, H2, HB3, KA1, BL1.

Group 2; MA1, H2, RST, KA1, BL1.

Group 3; MA1, H2, HB3, KA1, BL1, MA2.

Group 4; MA1, H2, HB3, KA1, BL1, CL2.

KA1, MA1, and BL1 were chosen as they met the largest number of criteria, and KA1, *Lactobacillus plantarum,* is a well-established probiotic with the others being considered to have probiotic potential. H2 was included as the required yeast, and as a gas producer without a high ethanol content. MA2 is included in one group to determine the effect of a lower pH, as it reached a pH of 4.6. It was a concern that this would produce a too sour product; however the low pH would produce a more food safe product which may be an advantage. HB3 or RST was desired to be included due to the marketing factor of including a bacteria with the term *kefir* in the name. CL2 meets a large number of criteria; however it had a very ropy texture which has the potential to produce an unappealing mouthfeel. To assess what effect the ropy texture had, CL2 was included in the final group 4. RST, CL2, and MA2 also have the lowest number of cells/mL in the starter, so are not ideal for commercial uses as they require a larger addition of starter to the milk. The 4 groups only contain one differing species to assess the effect of that particular isolate. The other isolates were not included because they did not meet enough criteria or did not have a unique property. While H1 was the greatest gas producer, it was not included due to its high ethanol content (0.5-1%).

In the single isolate sheep milk fermentation, MA2's cell count dropped from an average of 2.85 x 10^8cfu/mL after 24 hours, to 6.25 x 10^5cfu/mL after 14 days, and after 52 days the cell count was under 10^3cfu/mL. This is a significant die-off, especially in comparison to other isolates that remained at 10^8cfu/mL after 52 days. MA2 also lowered the pH of the sheep's milk to pH 4.6 after 24 hours, which was the lowest of all the isolates (the next lowest was BL1 at pH 5.0), this may have had an effect on the viability of the cells. This may be an issue for the kefir as it is required to have a cell count over 10^6cfu/mL.

4.7 Kefir development

For the development of a final kefir product, the 4 groups described in section 4.6 Selection of isolates for combination were fermented in sheep's milk, as described in Method 3.13 Kefir fermentation. The 4 groups were initially tested for textural and flavour acceptability, and then assessed using HPLC (Method 3.9 HPLC), pH, and cell counts.

Table 17 Starter culture concentration, and amount required in 10mL of milk

Isolate	cells/mL in starter	mL starter in 10mL milk
MA1	$1.93x10^{10}$	0.0005
H2	$3.75x10^{8}$	0.0267
BL1	$1.8x10^{10}$	0.0006
KA1	$1.04x10^{10}$	0.001
HB3	$5.65x10^{9}$	0.0018
MA1	$1.93x10^{10}$	0.0005
RST	$9.7x10^{7}$	0.1031
CL2	$1.5\ x10^{8}$	0.0669
MA2	$8.4x10^{7}$	0.119

A casual tasting of the four kefirs was undertaken to determine if there were any major differences in the groups. Each kefir was handed in a random order to 11 individuals for their assessment of acceptability and to rank them based on preference. Each individual was asked in isolation from one another to prevent their opinions being altered through discussion.

Table 18 Rankings of each kefir group wherein a ranking of 1 indicates most preferred and 4 indicates least preferred

Kefir 1	Kefir 2	Kefir 3	Kefir 4	Other comments
1, yeasty	3	2	4, Did not drink	
2	1	4, Unappealing flavour	3 , Unappealing texture	
1, nice flavour, cucumber	2, Best texture	3, Unappealing flavour	4, Unappealing texture	
3, Too cheesy tasting	1, Good texture	2, OK	4, Unappealing texture	
1, Most refreshing, cucumber	2, Too strong flavour, peanuty	3, Too strong flavour	4, Unappealing texture	
2	3	1	4, Did not drink	Difficult to determine between 1, 2, and 3
1	3	4	2, unappealing texture	2 and 3 too strongly flavoured
1	4	3	2, unappealing texture	2 and 3 too strongly flavoured
3	1	4	2	4 texture was unappealing but preferable to flavour of 1 and 3
3, Too thin	1	4, Unappealing flavour and aftertaste	2, More filling, like a dessert	
2	3	1	4, Liked 4, but not as a drink – more for cereal or smoothie	
20	**24**	**31**	**35**	**Total**

Table 18 above shows the results of the tastings, with the last row showing the sum of the rankings to give an indication of the preferred kefir. The total scores clearly show two groups – kefir 1 and 2 being preferred with the scores of 20 and 24 respectively, and kefir 3 and 4 being less preferred with scores of 31 and 35 respectively.

Kefir 2 was considered to have the best texture, and kefir 1 the best taste, with 2 and 3 often considered too strong tasting.

Kefir 4 was firmly rejected by a number of tasters, some refusing to drink it, based on its ropy slimy texture as shown in Figure 25. This outcome was considered to be likely due to it being unlike any commonly consumed yoghurt or milk products. However 5 tasters ranked the flavour higher and considered the unappealing nature of the texture to be less detrimental than the flavours of some of the other kefirs. If the final kefir product was designed as a fruit smoothie, the addition of CL2 could benefit the texture of a smoothie.

Figure 25 K4 being poured after fermentation and chilling

Table 19 pH, cell count and syneresis of kefirs

Kefir sample	% through filter	% through filter after 14 days	Cell count 24 hours	pH
K1	69.63	13.92	3.05×10^9	4.76
K1s	71.26	88.78	3.05×10^9	4.71
K2	23.35	6.45	2.75×10^9	4.57
K3	4.51	5.88	1.04×10^8	4.58
K4	77.04	11.99	1.18×10^9	4.55

While the syneresis testing was done using a method similar to those used by a number of studies (Paucean, Socaciu, Vodnar, & Mudura, 2010; Sahan, Yasar, & Hayaloglu, 2008; Wu, Hulbert, & Mount, 2000), it was deemed to be fairly inaccurate and not representative of

syneresis. This is shown in Figure 26 and Table 19 where after 14 days a low value of syneresis is given (being the percent which passed through a suction filter); however the image clearly shows an unappealing yellow whey on the surface, a sign of syneresis. This is an unacceptable timeframe for the kefir to split.

Figure 26 K4 after 14 days exhibiting separation

The HPLC results for the four kefirs are shown in Figure 27, in comparison to homemade grain kefir and commercial kefir from 'the collective' (both of which were made with bovine milk). The sheep's kefirs varied the most in lactose, and had a similar overall profile to the homemade kefir. However the commercial kefir had twice as much lactic acid, and 2.5% galactose in comparison to the almost 0% galactose found in the other kefirs. This increase in galactose will likely increase the sweetness of the drink, and compensate for the increased acidity of the higher lactic acid content and pH 4.2. CL2 and CA1 were both isolated from the collective kefir (*Streptococcus thermophiles* and *Leuconostoc pseudomesenteroides* respectively, along with the other isolated species *Torulaspora delbrueckii, Lactococcus lactis,* and *Leuconostoc mesenteroides*) and both of them produce galactose, however at around 0.8% and 0.3% respectively, which is far off the 2.5% present in the collective kefir. Also *Leuconostoc mesenteroides* and *Lactococcus lactis* are reported to ferment galactose so this high value of galactose is unusual (Vos, et al., 2009).

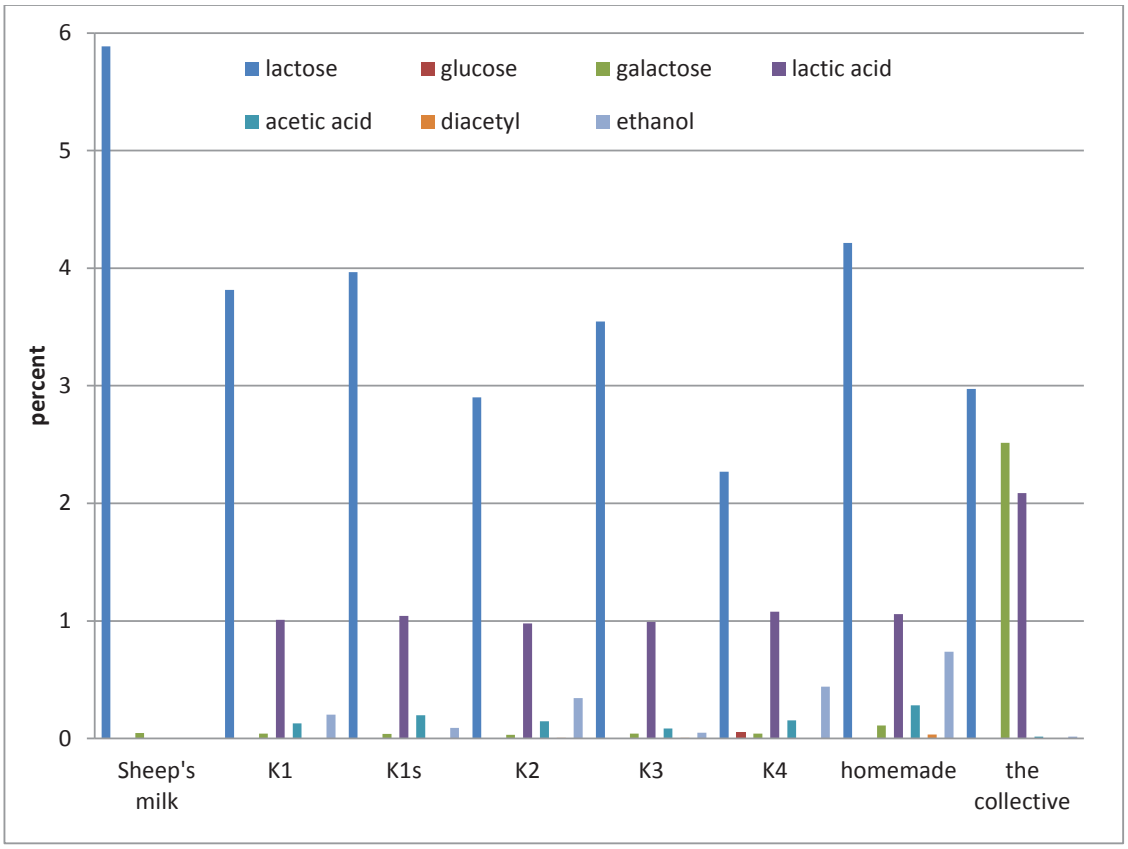

Figure 27 HPLC results for the 4 culture options in sheep's milk in comparison to homemade and commercial cow's milk kefirs

Kefir 1 was most preferred for taste; however it lacked the preferred thicker texture of kefir 2. Because it was noted that the Lifeway kefir produced a thicker curd when re-inoculated in milk (as shown in Appendix 6.5), it was thought this might be the same for kefir 1. Thus kefir 1 was repeated but with the incubation temperature of 37°C.

Kefir 1 and kefir 2 were repeated at 37°C to determine the effect on the thickness of the kefir. Unfortunately there was no change in the thickness of either kefir. They also developed sharper more cheesy flavours, and a soapy aftertaste, which was unpleasant and lost the more refreshing 'cucumber' taste. The plate counts were also lower at this temperature, being around 7×10^8cfu/mL for the samples.

From the plate counts, there appeared to be very little variation in the colony formation. This was thought to indicate that one of the isolates was taking over the fermentation and preventing other species from growing. Based on the growth speeds from the impedance measurements, it was thought that this faster growing isolate was either BL1 (7.5 hours) or MA1 (8.5 hours). This theory could explain why kefir 1 did not thicken up, as the isolate KA1's growth was slowed down, whereas in sheep's milk alone KA1 produced thick milk. There was also an issue with the

lack of effervescence, so increasing the growth of H2 (the only yeast present) could produce more bubbles, however it would need to not produce a more yeasty 'bready' flavour. Another option to increase the effervescence was to ferment the kefir in sealed bottles to prevent the CO_2 produced from escaping.

One solution to the thickness problem was to add the isolates at different times in the fermentation. KA1 had a growth time of 17.5 hours in MRS media, and H2 had 21.5 hours in TSB media. Assuming similar growth speeds in sheep's milk, if BL1 and MA1 were added at around 9 hours prior to the 24 hour fermentation end time, KA1 and H2 would have reached peak growth and so the problem of disproportionate growth of one isolate would still be an issue. So the isolates BL1 and MA1 could be added after 6 hours, by which time KA1 and H2 would be beginning their exponential growth phase, as shown on Figure 41 and Figure 45 in Appendix 6.3 Impedance graphs. However this method would be impractical to do commercially based on the timing required and so is unfeasible.

Another solution would be to alter the amount of isolate added for the initial inoculation. Adding more KA1 and less MA1 would ideally alter the lag phase of the isolates encouraging them to grow either slower or faster. It can be seen in the impedance graph for MA1 (Figure 41 in Appendix 6.3 Impedance graphs) that COL and TSB reached the exponential phase much faster than COL 3 and TSB 3. COL and TSB had 2 x 10^8cfu/mL in the initial medium (0.1mL of this was added to 10mL medium in the bactrac – resulting in the bactrac starting at a concentration of 2 x 10^6cfu/mL), whereas COL 3 and TSB 3 had an initial starter of 4 x 10^6cfu/mL (4 x 10^4cfu/mL in the bactrac). The lower initial concentration took around 19 hours to reach peak growth in comparison to the approximately 8 hours of the higher initial concentration. KA1 and BL1 had fairly consistent starter concentrations so this comparison cannot be made for KA1. Based on this, it was decided to trial batches of kefir 1 again.

This was done using five different samples:

K1 A; 1 x 10^6 of all the isolates
K1 B; a lower concentration of MA1, 1 x 10^5 cfu/mL
K1 C; a lower concentration of BL1, 1 x 10^5 cfu/mL
K1 D; a lower concentration of MA1 and BL1, 1 x 10^5 cfu/mL
K1 E; a higher concentration of KA1, 1 x 10^7 cfu/mL.

This was done using Method 3.13 Kefir fermentation, and fermented in both open containers (covered in clingfilm) and in sealed 250mL carbonation appropriate bottles. Unfortunately there was no discernible difference in taste or texture between the 5 samples. However the bottled

samples did produce an effervescent kefir, which was much more appealing and made the thinner texture of K1 less of a concern as the bubbles with the slight cucumber taste and thinner texture produced a more refreshing beverage, such as kefir is supposed to be. It should be noted that fermenting in bottle produces a more anaerobic environment, and stirring a more aerobic, however there was very little change in final cell count between the bottle fermented (K1 A-E Table 20), loosely covered (K1 Table 19), and stirred (K1s Table 19). Unfortunately it could not be determined whether particular species were growing at different rates, but as there was no discernible difference between these samples this is inconsequential.

Table 20 Cell count after 24 hours fermenting and cooled, K1 A-E

sample	Cfu/mL after 24 hours
K1 A	4.75×10^9
K1 B	3.85×10^9
K1 C	3.6×10^9
K1 D	3.05×10^9
K1 E	3.4×10^9

To ensure that 24 hours is an appropriate fermentation time, the pH over time was measured during the fermentation. If 24 hours is a good length of time, the resulting graph should be a flattened backwards 's' shape, with a slow initial change of the lag phase, followed by a rapid decrease, then slowing down again as the microbes growth slows and produces less lactic acid. This shape can be seen in Figure 28, showing that 24 hours is an acceptable fermentation time. K1 37 was fermented at 37°C, and K1 D and A at 30°C. A higher fermentation temperature should result in faster growth, and this can be seen in Figure 28, as the K1 37 line drops lower initially.

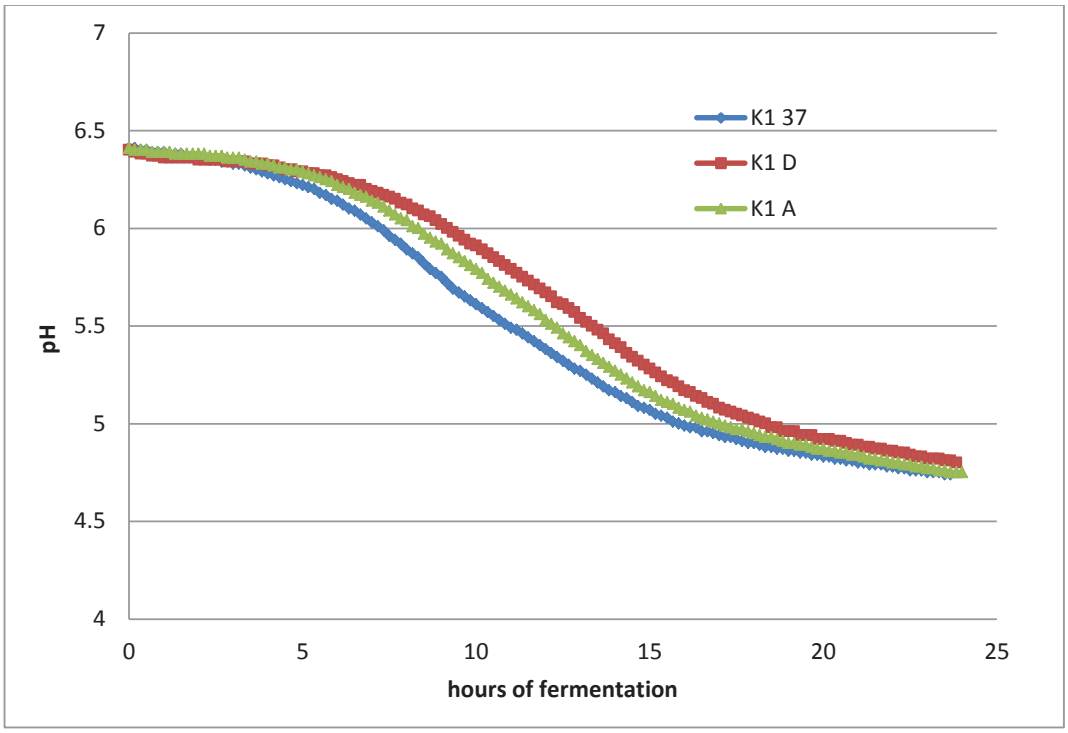

Figure 28 Change in pH over fermentation time for various K1 kefirs

A final plate count was done on K1s, K1 A, and K1 E to check the cell count over storage time at 4°C. After 3 weeks, K1 A and K1 E both had a cell count of 1.6 x 10^9cfu/mL, and after 5 weeks K1s had a cell count of 9.7 x 10^8cfu/mL. This is to be expected based on the results for the individual species, and is high enough to still be included on the labelling.

Some ropy strains can sometimes be induced by fermenting at a lower than optimum temperature (Kosikowski & Mistry, 1997), so a final test of fermenting K1 A at 25°C was done. This unfortunately did not produce a thicker product, and the same soapy taste from the 37°C test was detected.

K1, consisting of 1x10^6 of MA1, H2, HB3, KA1, and BL1 (*Leuconostoc mesenteroides, Kazachstania unispora, Lactobacillus kefiri, Lactobacillus plantarum,* and *Lactococcus lactis* subsp. *lactis*) fermented at 30°C for 24 hours and was deemed to be the most successful kefir. It had a cell count over 10^6 after 5 weeks, and was pleasantly effervescent and had a refreshing taste. Being bottle fermented also allows for easier manufacture as no stirring is required and heating and cooling can be done in bottle.

5. Conclusions

The microbial composition of commercial and homemade kefirs differs. *Saccharomyces cerevisiae, Torulaspora delbrueckii, Lactococcus lactis* subsp*. lactis, Leuconostoc mesenteroides, Lactobacillus plantarum, Lactococcus lactis* subsp*. cremoris,* and *Leuconostoc pseudomesenteroides* were found in exclusively commercial kefirs. *Kluyveromyces marxianus, Kazachstania unispora, Pichia membranifaciens, Clavispora lusitaniae*, *Lactobacillus delbrueckii, Lactobacillus kefiranofaciens,* and *Lactobacillus kefiri,* were found exclusively in homemade kefir. *Streptococcus thermophiles* was the only species identified in both homemade and commercial kefirs. This discrepancy between commercial and homemade kefir is reflected in the literature assessed.

The absence of *Lactobacillus kefiri* from the commercial kefir assessed is not in agreement with the CODEX definition of kefir, and a number of the homemade kefirs in the studies assessed also did not adhere to the CODEX definition. It is also required for a kefir to contain yeast to be considered kefir, and only five of the nine commercial kefirs considered contained yeast. From this it can be concluded that the CODEX definition is not representative of kefir.

Of the methods used in the literature assessed, it was clear that to achieve an accurate description of the species present in kefir and kefir grains, both culture dependent and culture independent (RNA sequencing) techniques need to be used. For the assessment of yeast, ITS sequencing is more successful than RNA sequencing at identifying low frequency species in kefir. Only culture dependent methods were used in this research as isolates were required to be grown for use as starter cultures.

An acceptable sheep milk kefir can be produced using a mix of *Kazachstania unispora, Lactococcus lactis* subsp*. lactis, Leuconostoc mesenteroides, Lactobacillus plantarum,* and *Lactobacillus kefiri.* The final product has a refreshing sour taste and effervescence, with a pH of 4.6, and a cell count above $3x10^9$cfu/mL which decreases to above $9x10^8$cfu/mL after five weeks, which is over the 10^6 cfu/mL required for labelling purposes. *Kluyveromyces marxianus* is not appropriate for the use in kefir as it produces over 1.8% ethanol after 52 days, higher than the 0.5% limit. *Lactococcus lactis* subsp. *lactis* was also not appropriate for use in kefir due to cell numbers decreasing below the 10^6 cfu/mL required for labelling purposes after 14 days storage.

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6. Appendix

6.1 Taranaki survey

On the 22 February I attended and spoke at the Food Futures Taranaki 2016 conference. I took 2 different kefir samples to give to attendees and asked them to fill out a questionnaire (Figure 29) to indicate their preference and give any comments. It was decided that the simple preference test was easy to do in the causal setting and was less time consuming. It was important to see what the individuals thought without prompting, so no sensory terminology was included (such as acidity). The samples were set up on a table at lunch break, and people were free to come and try the two samples whenever. Unfortunately this meant people were discussing their responses with each other, and as the table was outside the samples warmed up during the testing. There was also no water or crackers provided for between samples, so the order in which the individual tried the samples will have had an effect on their responses. These issues will have introduced bias into the survey. The major bias will be in influencing peoples initial response, as their expectations will have changed based on what other people had told them, which was unfortunate in this survey as it was designed to see what peoples initial thoughts were without prompt.

The preference test was designed around the comparison of a traditional kefir (grains) to a more modern kefir (freeze dried starter), with the expectation that people will prefer the more mild and familiar modern kefir due to its similarity to drinking yoghurt. Sample 1 was made using kefir grains, and sample 2 was made using Mad Millie's freeze dried starter (a commercial starter). They were incubated at room temperature (25°C) for 22 hours in 15% whole milk powder solution, stirring at 100RPM. 22 hours was chosen as the kefir grains develop a distinct cheesy aroma after extended fermentation, and 22 hours was chosen to help mitigate this. The final pH levels for the samples were, sample 1; pH 4.51, and sample 2; pH 4.39. They were both strained through a sieve to remove lumps, and stored in a refrigerator overnight before being sampled.

Sheep's milk kefir questionnaire

Which sample do you prefer? **1** **2** (please circle)

What do you like/dislike about them, and any other comments?

Thank you for your feedback

Figure 29 Taranaki visit questionnaire

The preferences and comments gathered from this trip are shown in Table 21. There were a total of 41 participants, with 2 not indicating a preference. 24/39 people preferred sample 2 (Mad Millie's starter), and 15/39 people preferred sample 1 (grains). Sour/acidic/sharp was the most common response, with 16/41 comments mentioning it. However there was a range of responses to the sourness, with some preferring the sourness, and variation in which sample was deemed sourer. 9/41 expressed dislike for either sample or considered them an acquired taste.

Table 21 Responses from the Taranaki questionnaire, including common responses, (?) indicates a word that could not be deciphered.

Preferred sample				Common responses					
	1	**2**	**comments**	cheese/rotten	yoghurt/Greek	sour/ sharp/acidic	sweet	bitter	dislike
1	1		I like number 1 because it tastes tall, number two is wider tasting but it's still oki doki						
2		1	1 doesn't taste like a dairy product to me						
3	1		2 just a bit more sure(?)						
4		1	2 sweeter 1 cheesier	1			1		

5	1		1 is creamy and more like a yoghurt which felt more familiar		1				
6		1	not keen on either a bit sharp			1			1
7		1	2- liked the sour, yoghurt like flavours, 1- a bit more nutty (soy-like)		1	1			
8	1		Less = sour!			1			
9	1		2 seemed more bitter					1	
10			Not sure, an unusual taste to my palate and I couldn't decide which I preferred - but I would be (?) To train myself to like either if I knew it was doing great good for my gut						1
11	1		cling to your mouth						
12	1		the acid in 1 made it more interesting, 2 tasted a bit 'soapy' and bland			1			
13	1		2 tasted stronger (reminded me of eating crayons at school)						1
14	1		don't really like either						1
15		1	flavour + texture						
16		1	2 more sour - preferred			1			
17		1	Both are slightly sour. Similar to Greek products		1	1			
18	1		2 is sweeter, 1 has an earthy taste				1		
19		1	not as sour			1			
20	1		1 smoother 2 slight retunasal(?) aftertaste but smooth						
21		1	I like the lightness of texture						
22		1	At first I thought I preferred 1 because 2 tasted sharper/tarter but in continuing to taste samples alternately I preferred 2. 1 is a lot like a basic Greek yoghurt and quite palatable		1	1			
23		1	1 tastes more fermented or 'off' to me or sour perhaps, 2 preferred 2 for its mild taste, yummy! My children would	1		1			

			eat this one (they don't eat mine)						
24		1	2 didn't taste like kefir :) it was more yoghurtish and didn't have the classic 'rotten' kefir taste that I am familiar with, 1 had the 'rotten' taste, which I don't really enjoy and my kids and husband won't touch	1	1				
25		1	1 has a subtle, smoky flavour. Probably not as palatable (Asian taste)						
26		1	little less sour that 1			1			
27		1	More like yoghurt nice front of mouth and good sustained taste, 2 too much cheese!	1	1				
28			for Russians/ not for Chinese						1
29	1		like the sweeter flavour similar to fermented mares milk				1		
30		1	less acidic			1			
31	1		slight bitterness not bad					1	
32		1	2 less sour			1			
33	1		dislike taste						1
34		1	1 astringent, both have acquired taste			1		1	1
35		1							
36		1	2 is stronger						
37		1	not my cup of tea						1
38		1	I like the tanginess of 2, wasn't too keen on 1 at first, more of an acquired taste			1			
39	1		probably a slightly more familiar taste - hint of natural yoghurt		1				
40		1	the second has a nice 'sour' taste that gave me a pleasant experience			1			
41		1	1 was a but 'musty' 2 a bit sharper neither were my taste but I could learn to like			1			1
	15	**24**	**Total**	**4**	**7**	**16**	**3**	**3**	**9**

6.2 HPLC

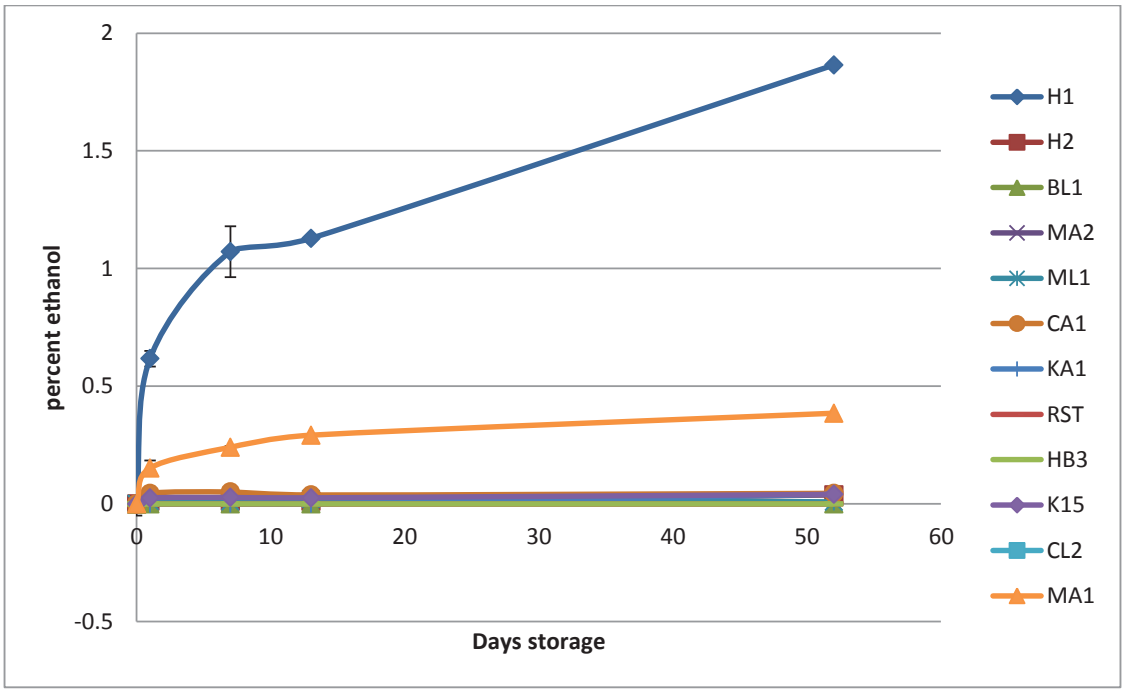


Figure 30 Averaged HPLC results for ethanol from each isolate grown in gamma sterilized sheep's milk over 52 days storage at 4°C.

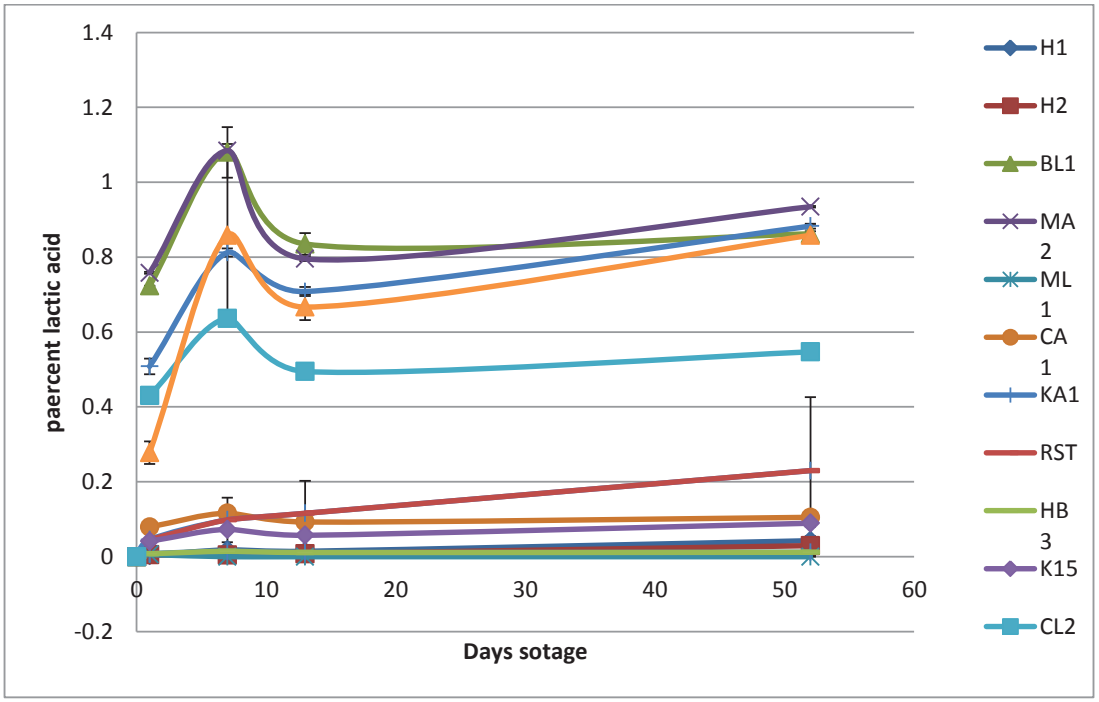


Figure 31 Averaged HPLC results for lactis acid from each isolate grown in gamma sterilized sheep's milk over 52 days storage at 4°C.

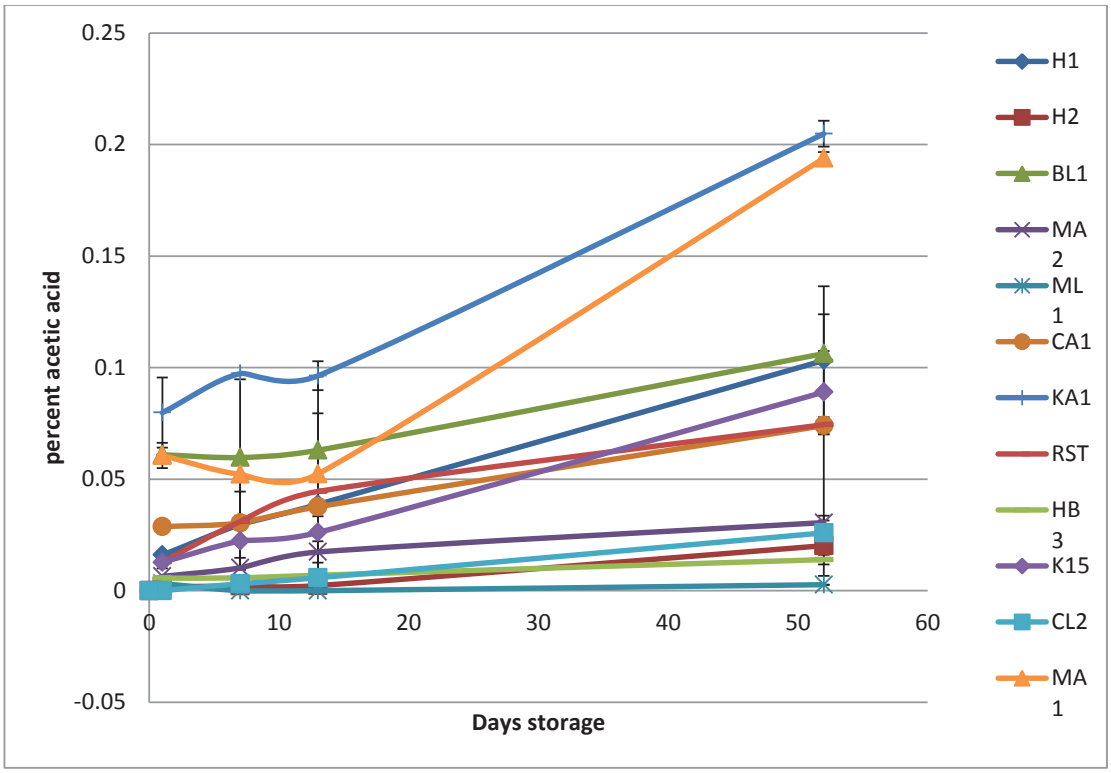


Figure 32 Averaged HPLC results for acetic acid from each isolate grown in gamma sterilized sheep's milk over 52 days storage at 4°C.

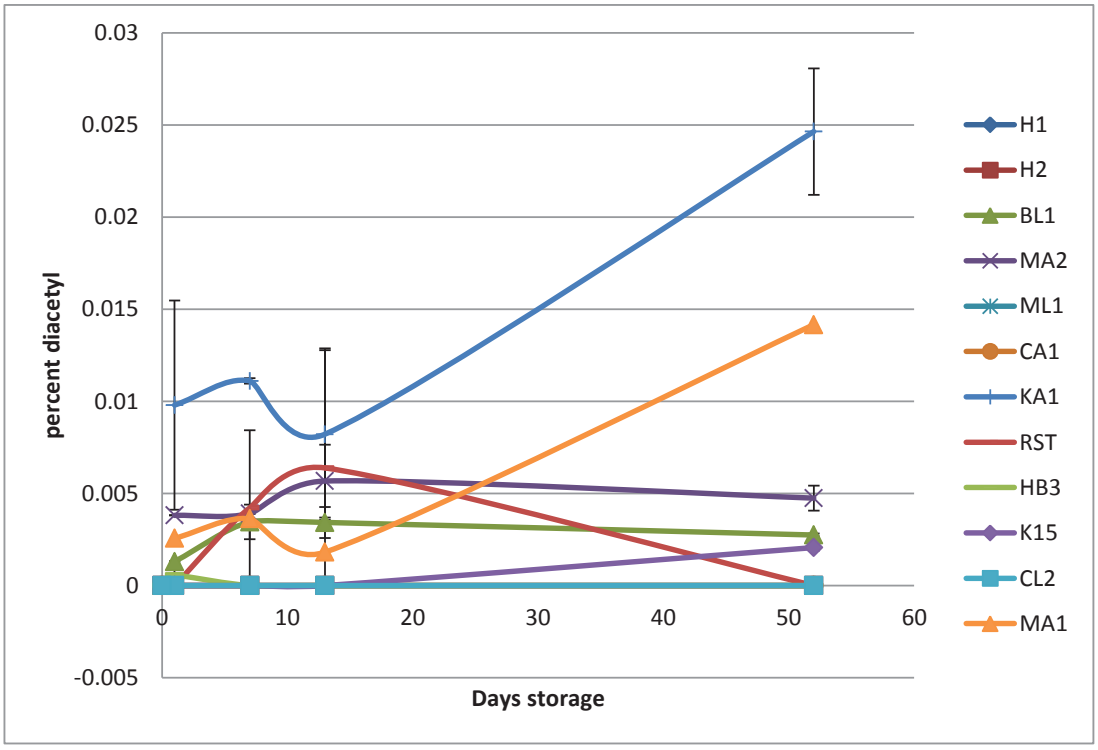


Figure 33 Averaged HPLC results for diacetyl from each isolate grown in gamma sterilized sheep's milk over 52 days storage at 4°C.

6.3 Impedance graphs

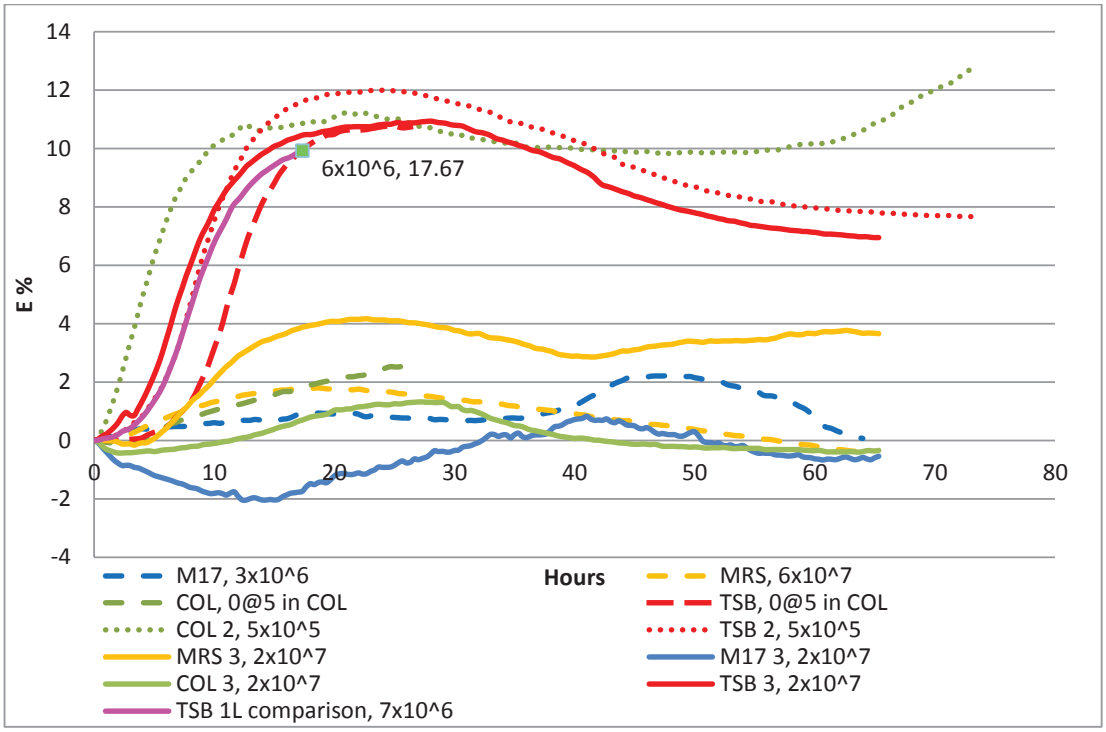


Figure 34 Change in capacitance on the electrode surface (E%) over time for H2 *Kazachstania unispora,* values in legend indicate cfu/mL in initial inoculum.

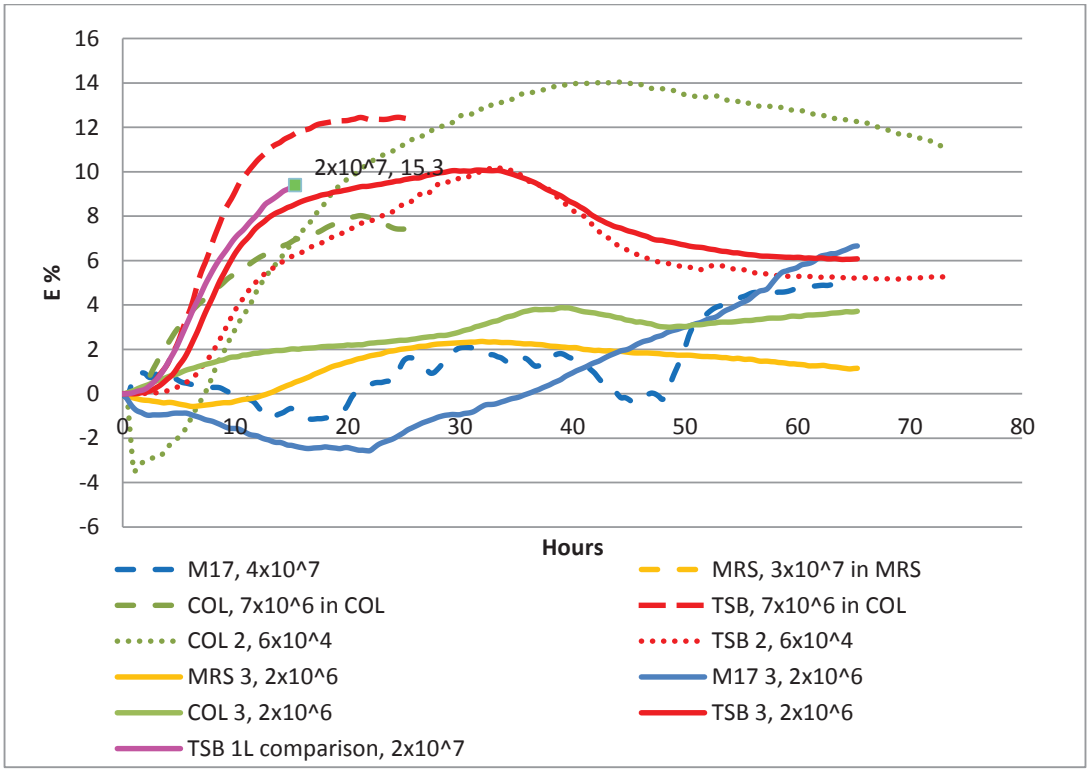


Figure 35 Change in capacitance on the electrode surface (E%) over time for H1 *Kluyveromyces marxianus,* values in legend indicate cfu/mL in initial inoculum.

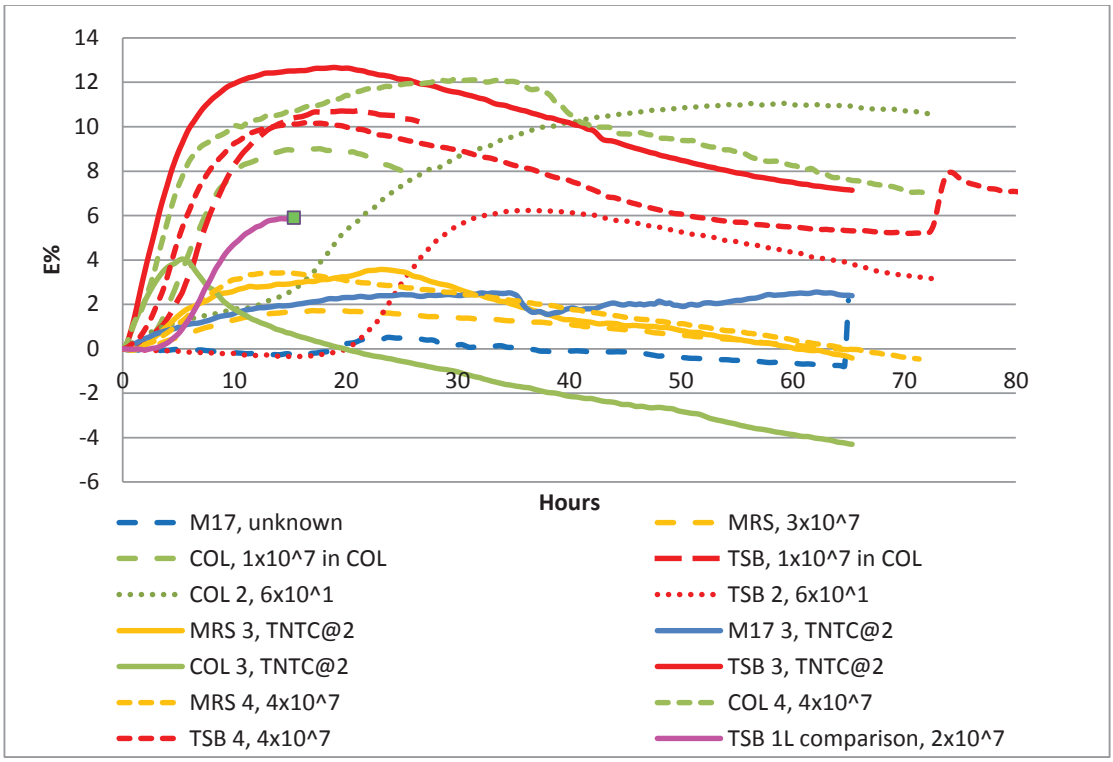


Figure 36 Change in capacitance on the electrode surface (E%) over time for KN2 *Saccharomyces cerevisiae,* values in legend indicate cfu/mL in initial inoculum.

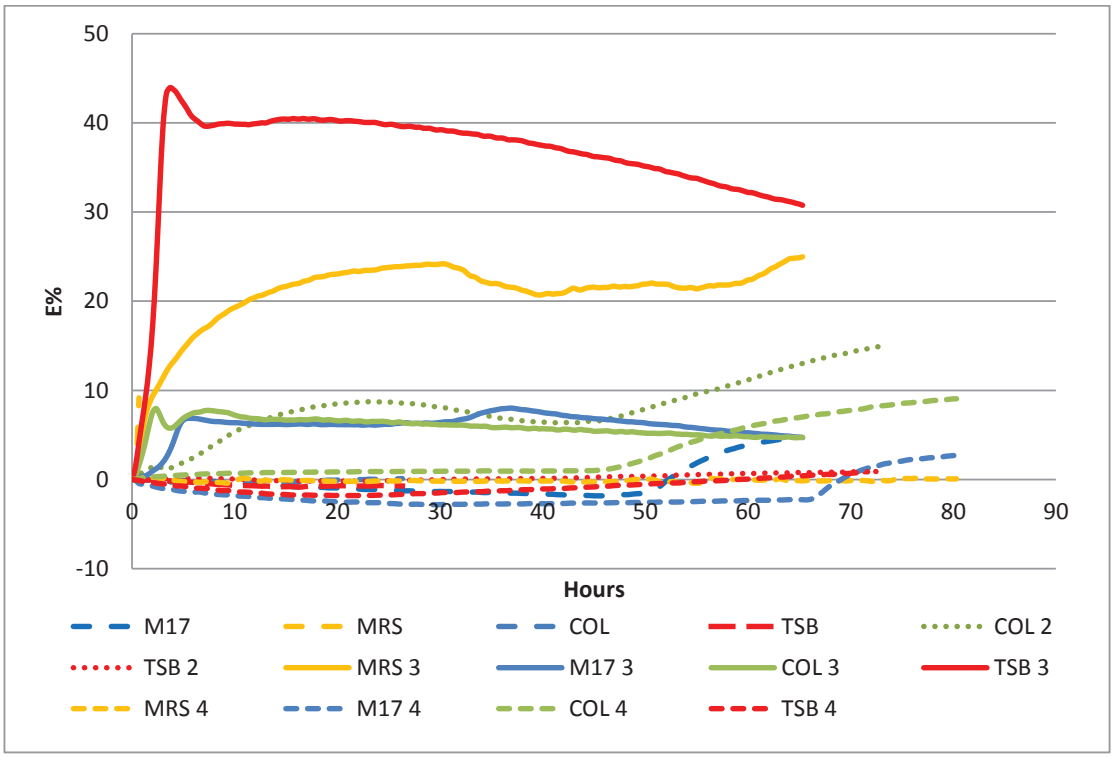


Figure 37 Change in capacitance on the electrode surface (E%) over time for DDP *Pichia membranifaciens,* values in legend indicate cfu/mL in initial inoculum.

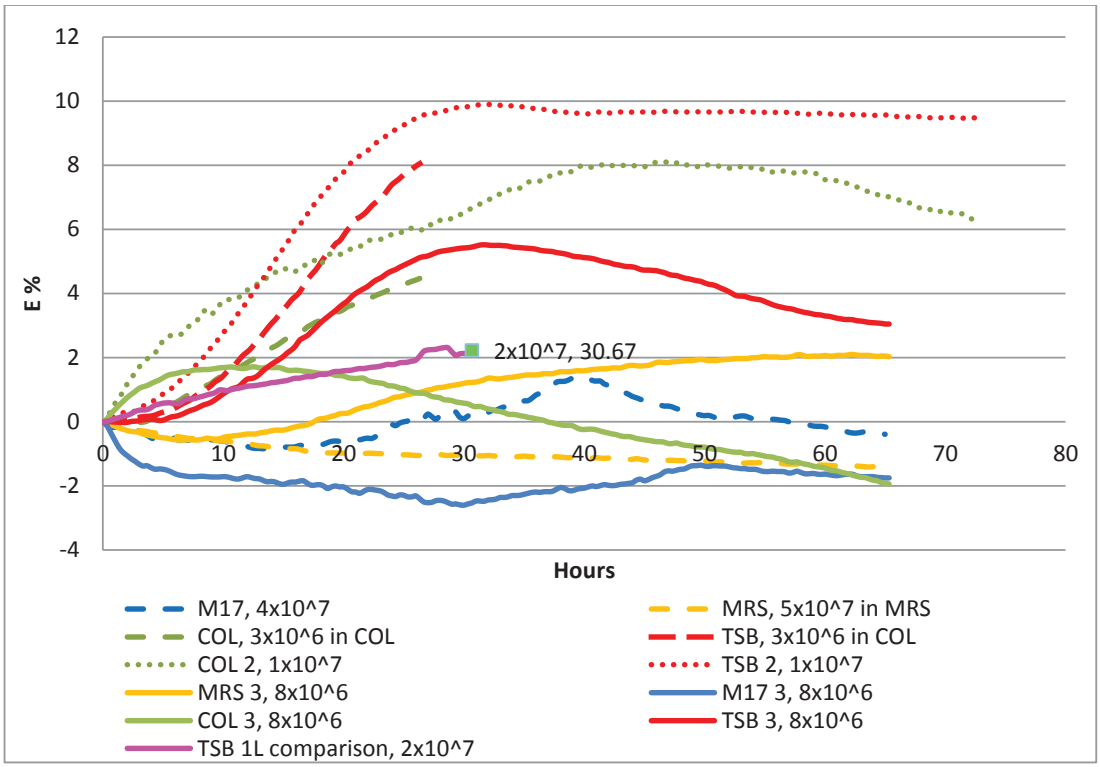


Figure 38 Change in capacitance on the electrode surface (E%) over time for ML1 *Torulaspora delbrueckii,* values in legend indicate cfu/mL in initial inoculum.

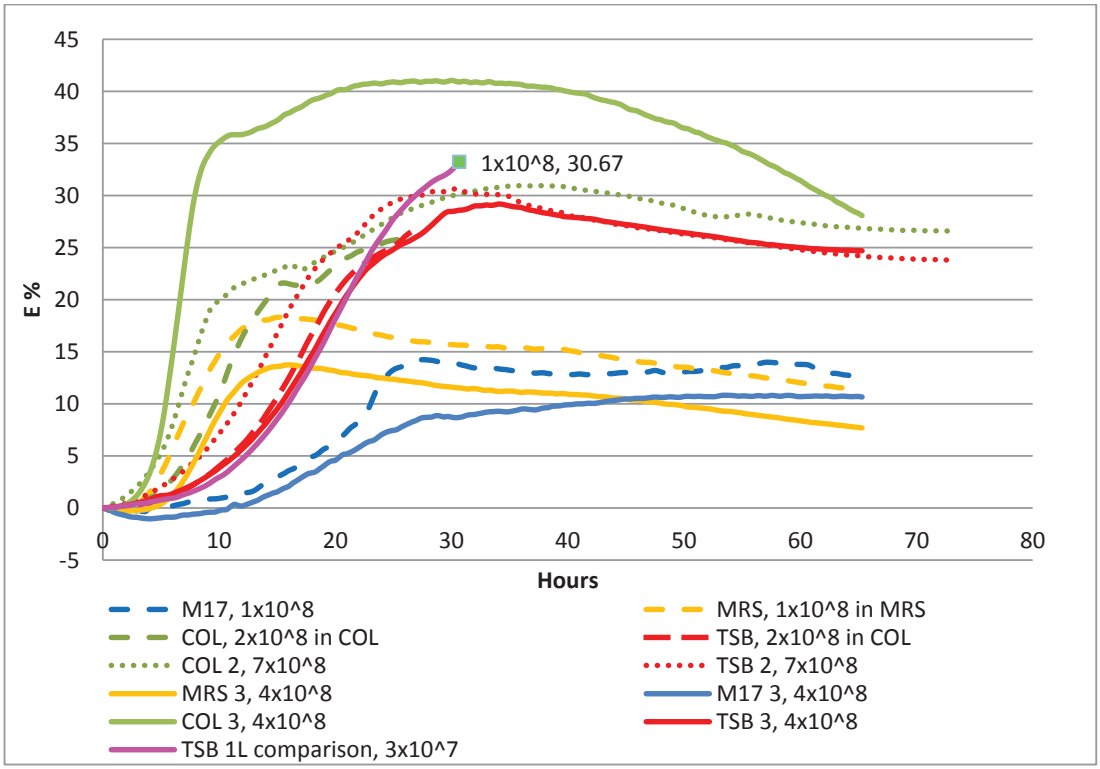


Figure 39 Change in capacitance on the electrode surface (E%) over time for KA1 *Lactobacillus plantarum,* values in legend indicate cfu/mL in initial inoculum.

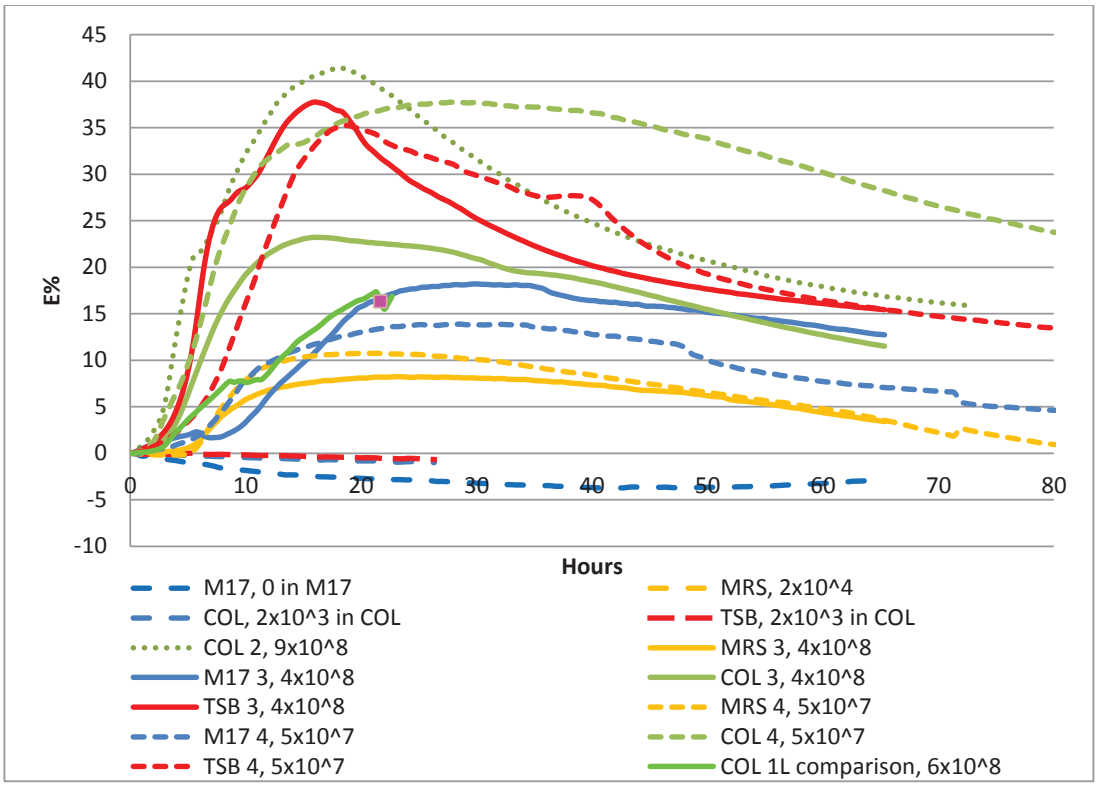


Figure 40 Change in capacitance on the electrode surface (E%) over time for K15 *Lactobacillus delbrueckii,* values in legend indicate cfu/mL in initial inoculum.

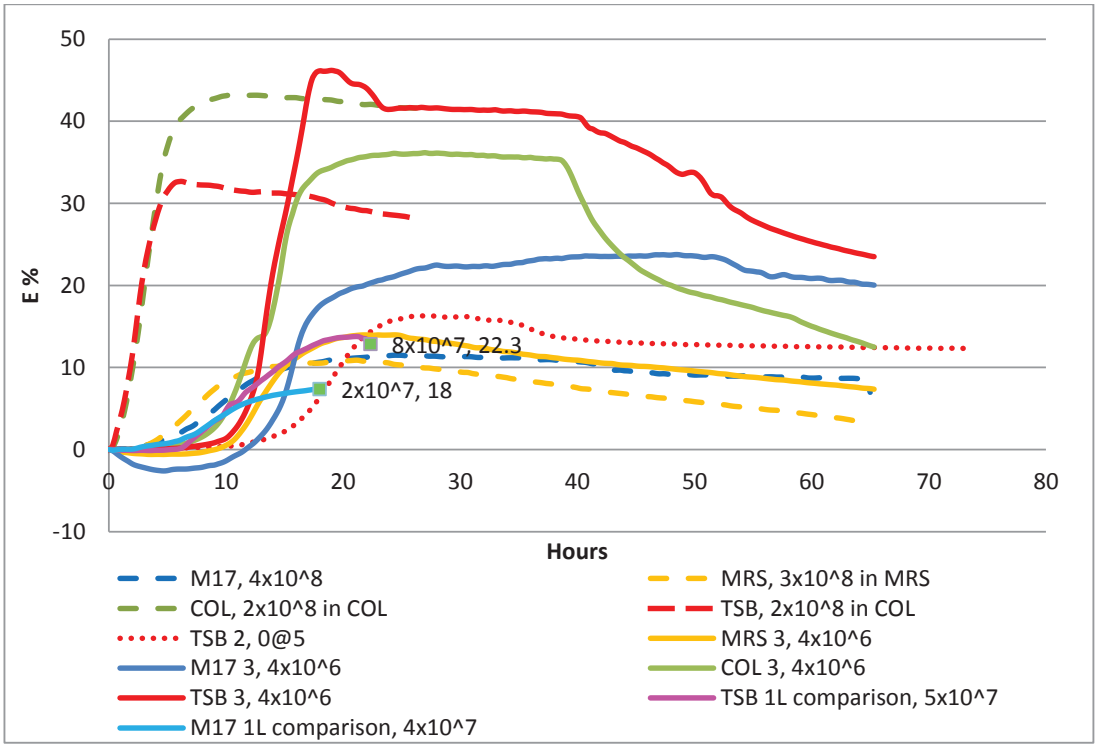


Figure 41 Change in capacitance on the electrode surface (E%) over time for MA1 *Leuconostoc mesenteroides,* values in legend indicate cfu/mL in initial inoculum.

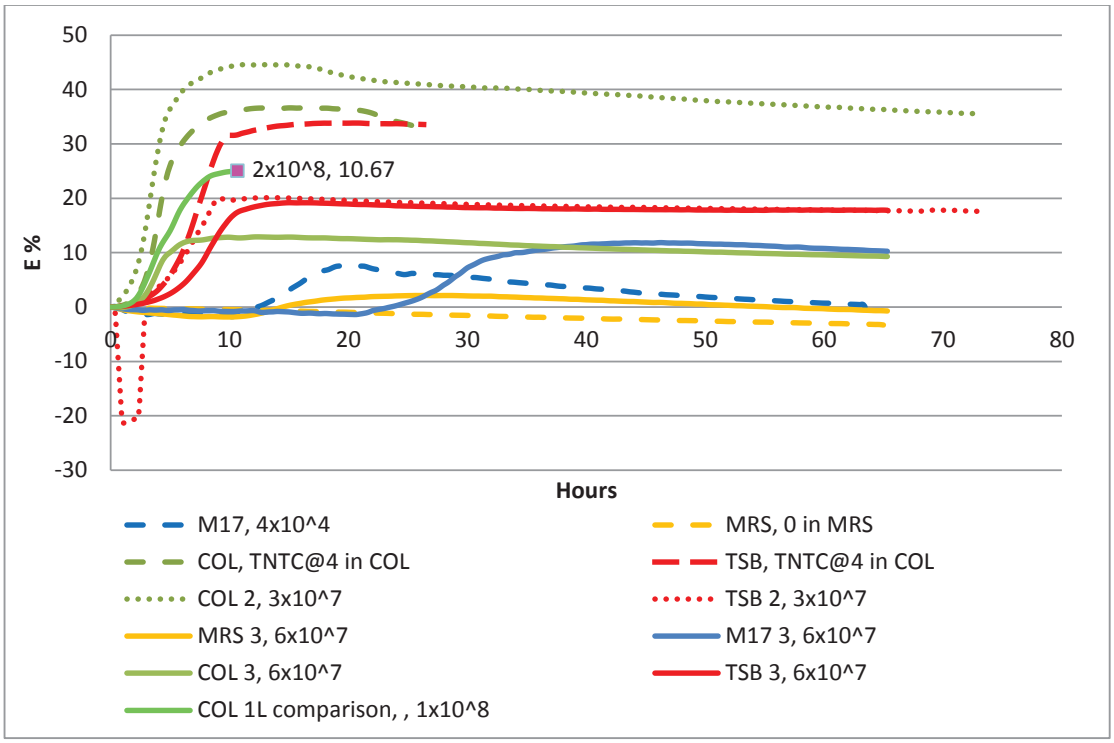


Figure 42 Change in capacitance on the electrode surface (E%) over time for CL2 *Streptococcus thermophiles* , values in legend indicate cfu/mL in initial inoculum.

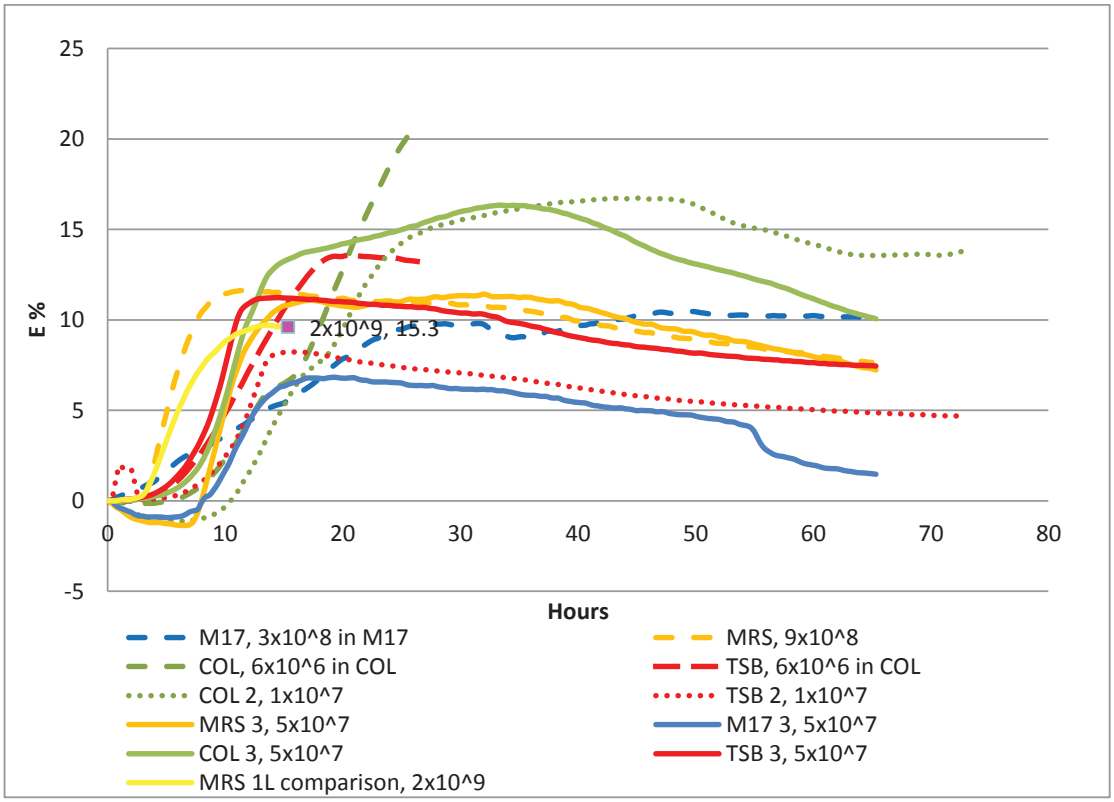


Figure 43 Change in capacitance on the electrode surface (E%) over time for CA1 *Leuconostoc pseudomesenteroides,* values in legend indicate cfu/mL in initial inoculum.

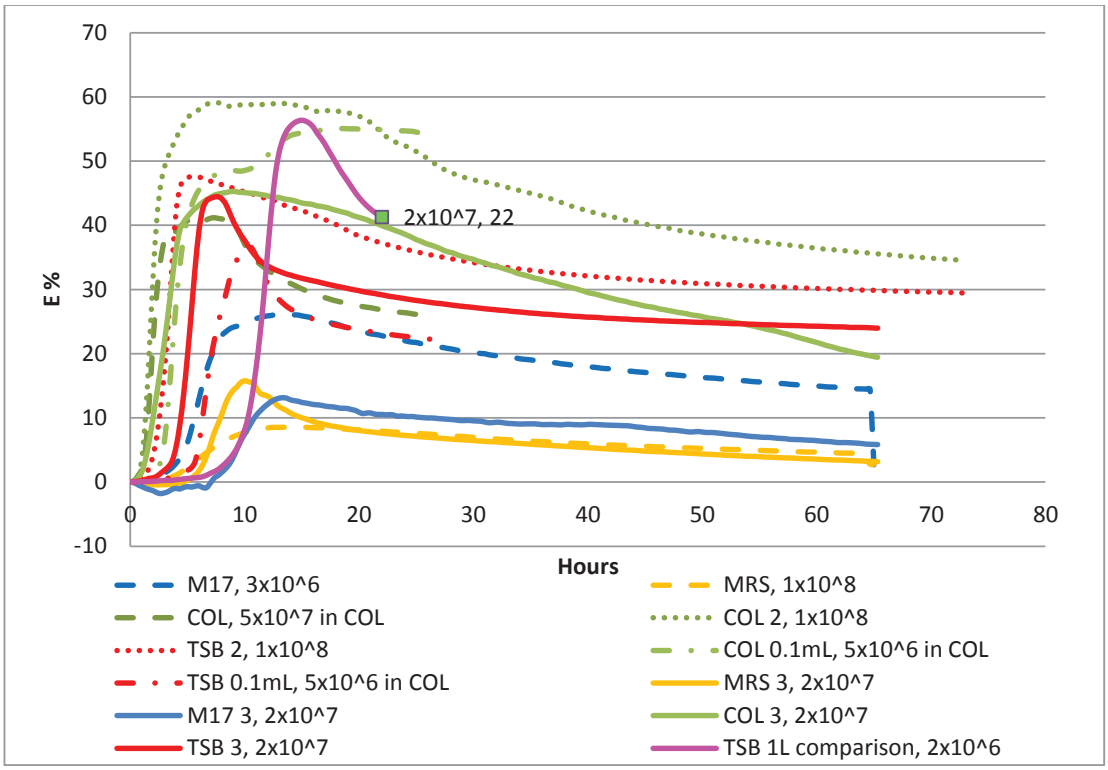


Figure 44 Change in capacitance on the electrode surface (E%) over time for MA2 *Lactococcus lactis* subsp. *cremoris,* values in legend indicate cfu/mL in initial inoculum.

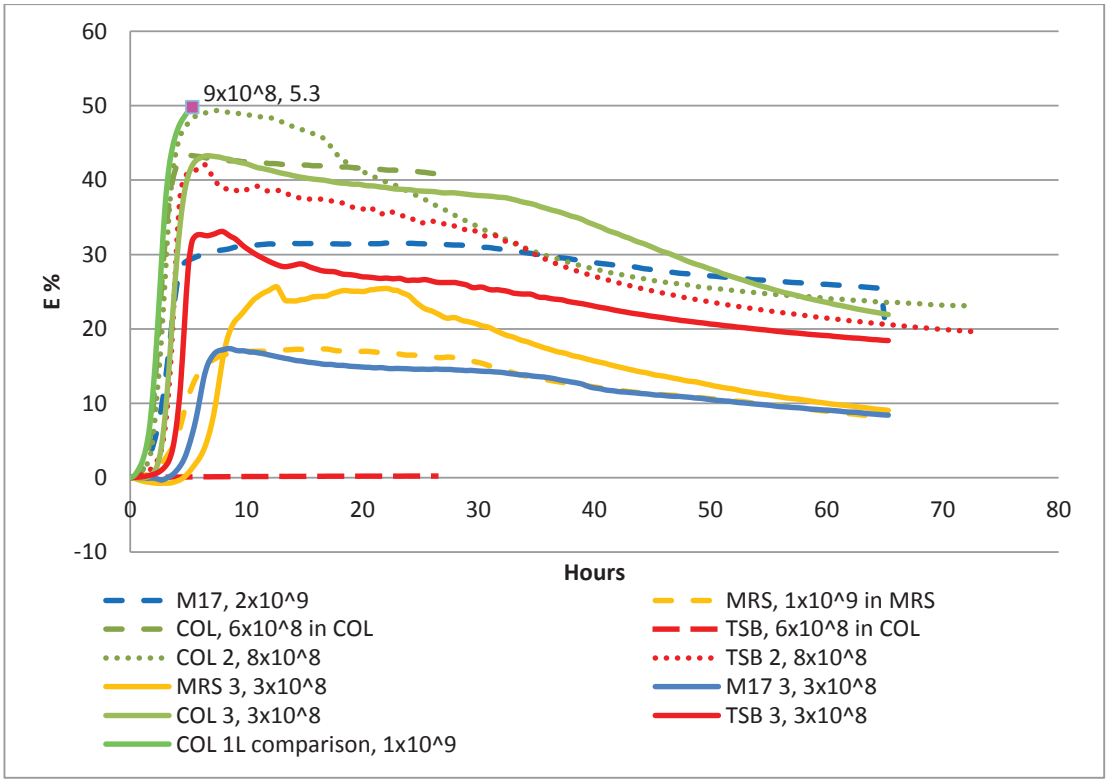


Figure 45 Change in capacitance on the electrode surface (E%) over time for BL1 *Lactococcus lactis* subsp. *lactis,* values in legend indicate cfu/mL in initial inoculum.

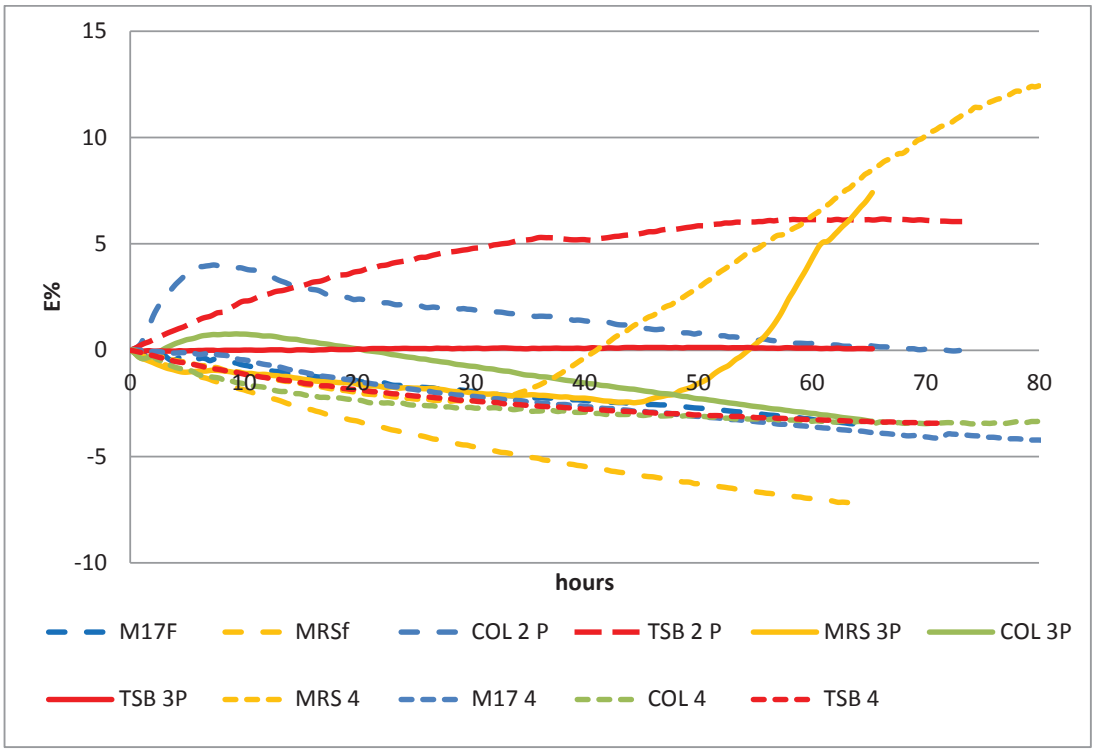


Figure 46 Change in capacitance on the electrode surface (E%) over time for RST *Lactobacillus kefiranofaciens*

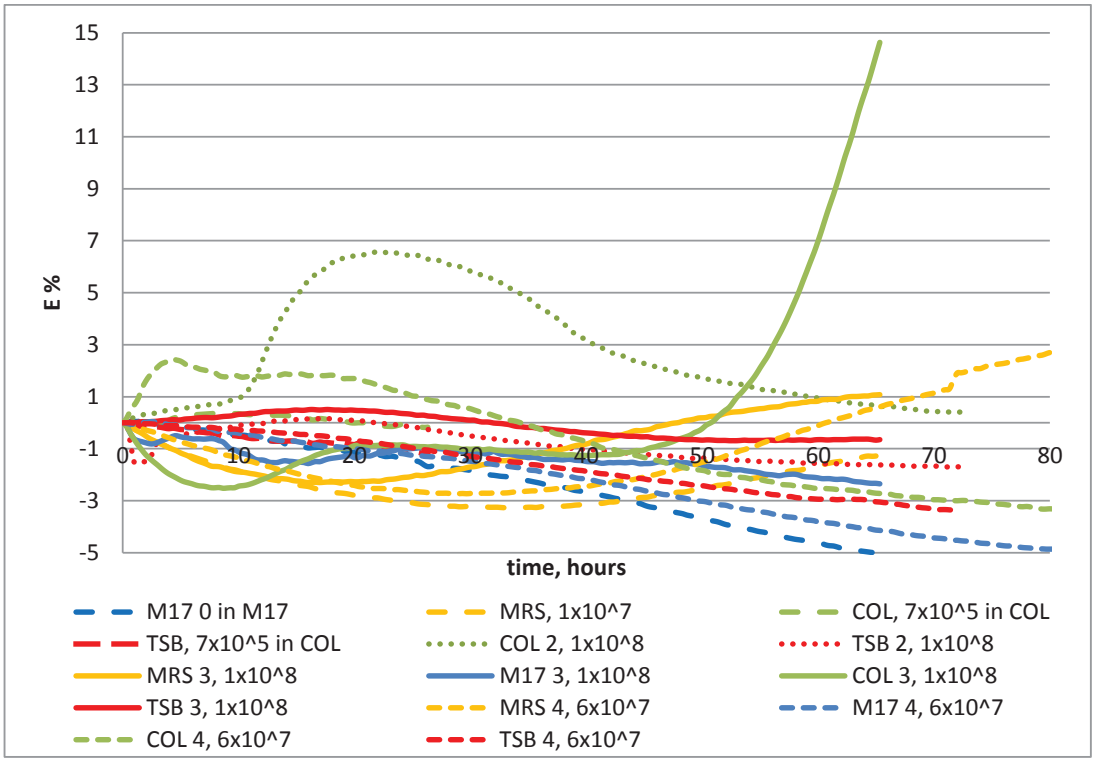


Figure 47 Change in capacitance on the electrode surface (E%) over time for HB3 *Lactobacillus kefiri,* values in legend indicate cfu/mL in initial inoculum.

6.4 The micro-organisms advertised in commercial kefir

Lifeway kefir cow milk kefir contains: *Lactococcus lactis, Lactobacillus rhamnosus, Streptococcus diacetylactis, Lactobacillus plantarum, Lactobacillus casei, Saccharomyces florentinus, Leuconostoc cremoris, Bifidobacterium longum, Bifidobacterium breve, Lactobacillus acidophilus, Bifidobacterium lactis, Lactobacillus reuteri* (Lifeway kefir, 2016).

The kefir company coconut water kefir contains: *Lactococcus lactis* subsp. *Lactis, Lactococcus lactis* subsp. c*remoris, Lactococcus lactis* subsp. *diacetylactis, Leuconostoc mesenteroides* subsp. *cremoris, Lactobacillus kefyr* (thermophilic), *Klyveromyces marxianus* var. *marxianus, Saccharomyces unisporus,* in a dextrose carrier (The Kefir Company, 2011).

Clover Stornetta Farm's cow milk kefir contains: *Streptococcus thermophiles, Lactobacillus delbrueckii* subsp. *bulgaricus, Lactobacillus casei, Lactobacillus acidophil* (Clover Stornetta Farms Inc, 2015).

Evolve cow milk kefir 'smoothie' contains: *Lactococcus lactis* subsp. *Lactis, Lactococcus lactis* subsp. c*remoris, Lactococcus lactis* subsp. *Lactis biovar*. *diacetylactis, Leuconostoc mesenteroides* subsp. *cremoris, Lactobacillus acidophilus, Lactobacillus delbrueckii* subsp. *bulgaricus, Lactobacillus delbrueckii* subsp. *lactis, Lactobacillus rhamnosus, Lactobacillus casei, Bifidobacterium lactis, Streptococcus thermophiles.* (Evolve Kefir, N.D)

Wallaby Yogurt Company cow milk kefir contains: *Streptococcus thermophilus*, *Propionibacterium freudenreichii* subsp. *shermanii*, *Lactobacillus delbrueckii* subsp. *bulgaricus, Lactobacillus casei, Lactobacillus acidophilus, Bifidobacterium lactis, Lactobacillus rhamnosus, Lactococcus lactis* subsp. *lactis, Lactococcus lactis* subsp. *cremoris, Lactobacillus paracasei* subsp. *paracasei, Lactococcus lactis* subsp. *lactis* biovar. *diacetylactis, Leuconostoc mesenteroides* subsp. *cremoris, Lactobacillus delbrueckii* subsp. *lactis.* (Wallaby Yogurt Company, 2016)

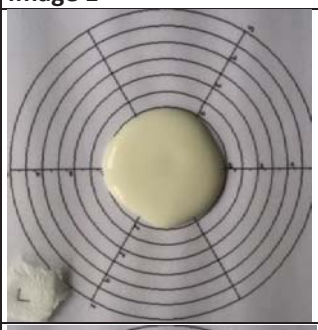
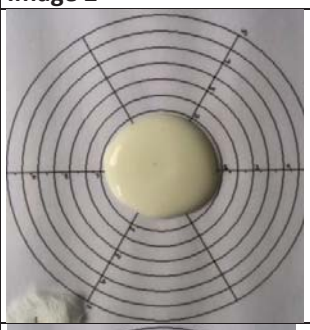
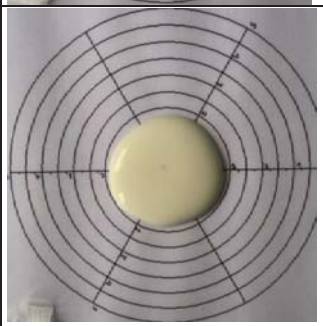
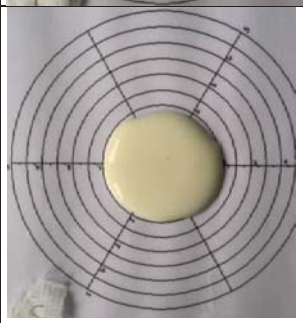
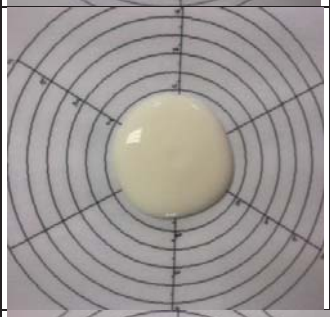
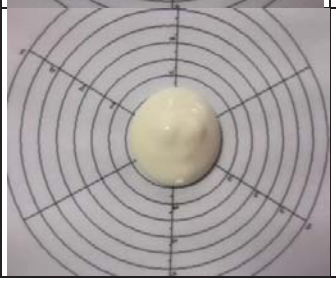
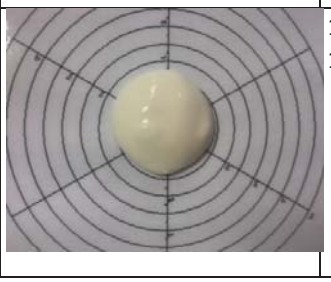
Mad Millie's freeze dried kefir starter contains: *Lactococcus lactis* subsp. *Lactis, Lactococcus lactis* subsp. c*remoris, Lactococcus lactis* subsp. *Lactis biovar*. *diacetylactis, Leuconostoc, Lactobacillus acidophilus, Bifidobacterium* species*, Streptococcus thermophiles,* and *Candidia colliculosa* (Mad Millie, 2016)

Body ecology freeze dried kefir starter cultures contains: *Lactococcus lactis, Lactococcus cremoris, Lactococcus diacetylactis, Leuconostoc cremoris, Lactobacillus plantarum, Lactobacillus casei,* and *Saccharomyces boulardii* (Body Ecology, 2016).

Trader Joe's cow milk kefir: *lactobacillus casei, Lactococcus lactis, Lactobacillus acidophilus, Lactobacillus plantarum, Lactobacillus rhamnosus, Bifidobacterium longum, Leuconostoc cremoris, Bifidobacterium breve, Streptococcus diacetylactis, Saccharomyces florentinus, Lactobacillus reuteri, Bifidobacterium lactis* (Trader Joe's, 2015)

Green Valley Organics cow milk kefir: *Lactobacillus bulgaricus, Lactobacillus acidophilus, Lactobacillus casei, Lactobacillus rhamnosus, Lactococcus lactis, Lactococcus diacetyl lactis, Lactococcus cremoris, Streptococcus thermophiles, Leuconostoc cremoris, Bifidobacterium bifidum* (Green Valley Organics, 2016)

6.5 Rheology tests

Sample	Image 1	Image 2	Score	pH	Observations
Lifeway			2, 1.8	3.78	Quite acidic tasting and not effervescent. Smooth cream-like texture.
The Collective			1.8, 2	4.20	Thick, smooth and pleasant texture. Thicker mouthfeel than lifeway. Effervescent and mildly acidic.
Lifeway incubated at room temperature				5.14	Very thin, similar to unfermented milk.
Lifeway incubated at 30°C			2.75, 2.9	3.97	
Lifeway incubated at 37°C			1.5, 1.5	3.77	Very thick, like pourable yogurt.