Antimicrobial properties of lactic acid bacteria isolated from fermented foods and drink

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MSc by Research

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DECLARATION

I Kathryn Tyson declare that the work contained in this submission is all my own work and to the best of my knowledge has not been carried out previously. All other results other than my own are clearly cited and referenced. No sections of the work referred to in this dissertation have been submitted in support of an application for another degree or qualification to this university or any other institute of learning.

Signature ----------

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ABSTRACT

Lactic acid bacteria isolated from fermented food have shown to be antimicrobial in activity towards known pathogens. As resistance to standard antibiotics becomes more prevalent, there is always the need to discover new sources of effective antimicrobials. There have been numerous studies on identifying lactic acid bacteria and in most cases, the bacterocin has been extracted and used to test against the known pathogens. In this study, bacteria were isolated from fermented foods such as sour beer, kefir and kombucha using selective agars. These were then identified using the Gram stain method and API kits. The isolation procedure had limited results and only a few bacteria were isolated and identified. These were: *Lactobacillus Plantarum* (73.4% likelihood) *and Pediococcus acidilactici* (99.9% likelihood) from sour beer and *Lactococcus lactis* (83.15% likelihood) from kefir. The identified organisms were tested for antimicrobial activity using the disc method on agar and the pathogen *Escherichia coli*. *Lactococcus lactis* isolated from kefir was found to possibly exhibit antimicrobial activity. This was then tested against other pathogens such as *Salmonella Spp* and *Staphylococcus aureus*. The isolate of *Lactococcus lactis* was shown, to have some possible antimicrobial activity against *Salmonella Spp* and *Staphylococcus aureus*.

Table 1. Measurement of growth zones around the discs.

Bacteria on plate	**20µl of Isolate of *Lactococcus lactis***
E.coli	12mm (Very strong growth)
Salmonella	12mm
Staph aureus	10mm

When growth of the pathogens was measured in the presence of the isolate, *Lactococcus lactis,* using turbidity readings. There was a slight reduction in turbidity when the isolate, *Lactococcus lactis* was present, compared to the control of Ringer's solution for *E.coli*, E.Coli C822037and *Staph aureus*., as shown in table 2. However, there is actually more turbidity suggesting growth of *Salmonella* when the isolate is present.

Table 2. Differences between readings when Ringers solution present or isolate present

	***Lactococcus lactis* Isolate + Tryptone soya broth**	**Ringers + Tryptone soya broth**	**Difference**
C - *E.Coli*	1.312	1.510	0.198
D – *E.Coli* C822037	1.283	1.419	0.136
E - *Salmonella*	1.354	1.162	-0.192
F – *Staph aureus*	0.594	0.651	0.057

Scanning electron microscope pictures show that when the *Lactococcus lactis* was present with the pathogen *E.coli* or *E.coli* C822037 the biofilm on the pathogen was changed or possibly reduced. The biofilm on an organism helps with pathogenicity and its own protection, if damaged it could make the pathogen weak and possibly inactive. No conclusive evidence was gained that showed that the *Lactococcus lactis* affected the *Salmonella* Spp or *Staphylococcus aureus*.

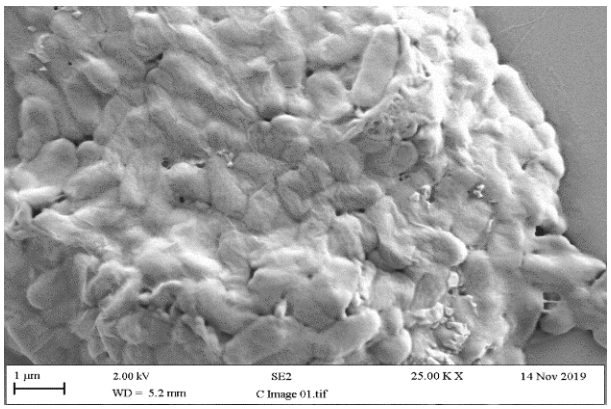

Figure 9. SEM image of isolate and *E.coli*

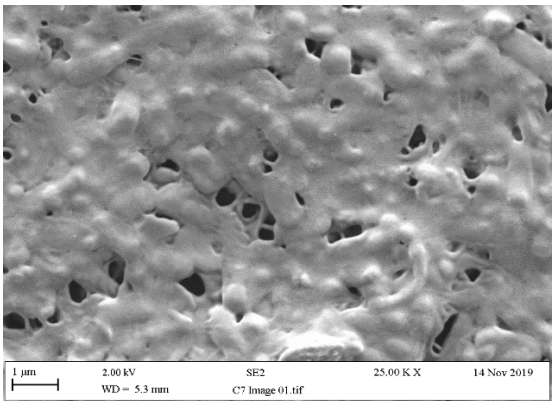

Figure 10. SEM image of Ringer's solution and *E.coli*

In Figure 10, the E.coli in the Ringer's solution has a sticky/string like biofilm present, which has been reduced when the isolate was added (Figure 9.). Rod shaped cells can be seen in both images but look flatter and more merged in Figure 9.

Further studies need to be carried out to show what affect the isolate has had on the biofilm and if the actual bacteria cells of the pathogen have been damaged. In addition, the nature of the biofilm needs to be established. Where the *E.coli* and *Lactococcus lactis* are present together there could be a biofilm present that has a different appearance to the sticky one produced by the *E.coli* only. Where multiple bacteria are present, the formation of a biofilm can be enhanced.

1. INTRODUCTION

1.1.1 Background

Lactic acid bacteria have been found to exhibit antimicrobial properties and these have been isolated from various foods. There is a continual demand to identify new antimicrobial agents to be used against known pathogens as organisms are continually becoming resistant to traditional antibiotics.

The project initially started by isolating microorganisms from a starter culture of sour beer (Sour power). More and more breweries are producing sour beers. Their creations not only mimic the more traditional sours of Europe but also break new ground on 'wild' versions of modern styles and have also sparked a renaissance for near-extinct tart beers. (Weikert, 2016). This was a starter culture that a brewery had kept over a period of time and was interested in its contents and activity. As results from this were limited, to try and obtain more isolates, other fermented foods including kefir and kombucha were used. These were chosen as they were obtained from known reliable companies that have worked with the University in the past. The kefir was obtained from a small new company from Manchester. The kombucha was produced by a very successful expanding company in Cheshire. The names of these companies are not included in this thesis due to confidentiality reasons.

Sour Power is a starter culture used in a local Manchester brewery to produce sour beers. Through information from the brewery, it is thought to contain strains of *Pediococcus* (a genus of Gram-positive lactic acid bacteria, placed within the family of *Lactobacillus*), at least three other strains of *Lactobacillus* (a genus of Gram-positive lactic acid bacteria) and *Saccharomyces* (a genus of fungi that includes many species of yeasts). There are also wild yeasts present that are not of a particular concern as they are killed in the cooking process. The three main species usually found in the production of sour beers are *Brettanomyces, Lactobacillus* and *Pediococcus*. (Kate Bernot 2015)

Lactobacillus is a bacterium that uses the sugars in the wort and rather than converting them to alcohol, converts them to lactic acid. This lowers the beers pH, making it sour. It has a relatively clean taste for drinkers since the bacteria does not produce much except lactic acid. It is responsible for the 'tang' in German style beers such as Berliner Weisse (Bernot, 2015).

Pediococcus is a bacterium, which like *Lactobacillus* produces lactic acid and lowers the pH. However, the introduction of *Pediococcus* into beer tends to make it harsher, in taste, than

the introduction of L*actobacillus*. Whilst *Lactobacillus* produces a clean sourness, *Pediococcus* can contribute other aromas and flavours. (Kate Bernot 2015)

The *Lactobacillus* strains are of the most interest in how they subjectively affect a good sour beer. Whilst there has been studies on the effect of the bacteria on the beer little research has been carried out on the antimicrobial properties they possess.

As this starter culture is very old, it is of interest to identify the organisms present and research if they exhibit antimicrobial properties against known pathogens. These organisms could possibly be probiotic through foods, beverages and dietary supplements. Probiotics are live microorganisms that may be able to help, prevent and treat some illnesses (Betty Kovacs Harbolic, MS, RD).

Kefir is a fermented drink usually made from milk. It is considered to have considerable health benefits. Numerous probiotic bacteria have been found in kefir, including lactic acid bacteria such as *Lactobacillus acidophillus, Lactobacillus delbrueckii subsp bulgaricus, Lactobacillus heleticus, Lactobacillus kefiranofaciens, Lactococcus lactis and Leuconostoc* species. (Olivera et al., 2013). Similarly, Kombucha is also believed to have health benefits and is produced by fermenting sweetened tea using a culture of bacteria and yeasts. It is mainly a mixture of acetic acid bacteria and yeasts in a zoogleal, which is a complex group of organisms in a symbiotic relationship that produce a slime growth mat. (Ernst, 2003). In this study, the presence of any lactic acid bacteria present will be investigated.

Isolated lactic acid bacteria from the fermented foods will be tested for any anti-microbial activity against common pathogens such as *E.coli*, *Salmonella* and *Staphylococcus aureus*. The whole organism will be used rather than the extracted bacteriocin. The effect of isolated microorganisms on the biofilm of pathogens will be observed under the electron microscope.

The formation of biofilm is believed to be a survival strategy and fossilised biofilm has been found that is 3.5 billion years old (Reffuveille et al., 2017).Most bacteria do not live as single cells but as a group in a matrix composed of polysaccharides, extracellular DNA, proteins and lipids. It is believed that bacteria in a biofilm are 210 to 1000 times more resistant to conventional antibiotics than free-floating bacteria. This figure depends on the strain, type of antibiotic and nature of the study (Reffuveille, F.et al., 2017). The intracellular aggregate that is created produces a slime layer called a matrix where the bacteria stick to each other and the surface. It is the result of an organised community. It is three- dimensional and appears mushroom shaped, containing water or fluid channels. These channels are vital in delivering nutrients into the biofilm's deep layers. The structures are species specific (Reffuveille, F.et al., 2017).

There are five recognised stages of biofilm formation: (i) Initial attachment; (ii) irreversible attachment; (iii) early development of biofilm architecture (micro colony formation); (iv) maturation; and (v) dispersion (Salas-Jara et al., 2016) In the first stage adhesion depends on the bacterial cell and surface and is reversible. Cell morphology changes to form the biofilm, becomes attached securely to the surface and the process becomes irreversible the third stage sees the cells form into micro colonies and EPS formation. At the fourth stage, the biofilm becomes organised and becomes mushroom shaped. Finally, in the fifth stage the biofilm becomes detached from the colony (Salas-Jara, M,J et al., 2016).

Biofilm formation in mixed biofilm communities is an intra and inter species product that requires dynamic interactions. Many infections involve biofilms which are difficult to eliminate when using conventional antibiotics.(Fang et al., 2018). This makes the understanding of how these biofilms interact and work important. Presently, this not well understood compared to microorganisms in single species biofilm (Fang et al 2018).

Work carried out my Fang et al. (2018) showed that different species of *E.coli* had different effects on other strains of *E.coli*, *Salmonella* and *Staphylococcus aureus* biofilms. One strain, Probiotic *Escherichia coli* Nissle 1917, caused an effect on the production of the other biofilms by producing an external bi functional (protease and Chaperone) periplasmic protein produced outside the cells. It inhibited *E.coli* biofilm population 14 fold compared to *E.coli* single species biofilm and 1,100 fold for *S.aureus* and 8,300 for *Salmonella* epidermis. Commensal *E.coli* did not exhibit any inhibitory effect towards other bacterial biofilm. In addition, some bacteria co- exist to encourage biofilm formation e.g. *Enterococcus faecalis* forms an ideal environment for the growth of *E.coli*. Probiotic organisms may use different mechanisms to control the biofilm formation of other bacteria due to the complexity of biofilm regulations. This should be explored further. The opposite side of the interaction is how pathogens affect the physiology and biofilm growth of probiotics and is equally important in understanding the molecular interactions in mixed bacterial communities. Understanding the mechanisms between probiotics and pathogens will provide knowledge for combating persistent biofilm associated bacterial infections.

Numerous studies have shown that biofilm formation by lactic acid helps promote colonisation and a longer presence on the host. It also impedes colonisation of pathogenic bacteria through various ways including immunomodulation. It is strain specific (Salas-Jara et al., 2016).

1.2 Purpose of Study

The knowledge and understanding generated from this research will be available to the brewing industry science community, the brewing industry and the public who are becoming more interested in the origins and history of beer and brewing worldwide. It will also be available to producers of fermented foods such as kefir and Kombucha, who are increasing interest in their health and possible antimicrobial benefits.

Microorganisms identified as having antimicrobial properties will be available to have further research carried out on them with regard to their probiotic properties and introduction into food and drink.

1.3 Aims and Objectives of the study

Aims

The aim of the project was to isolate and identify the microorganisms present in a selection of fermented food and drink and to establish if any of these have antimicrobial properties.

Objectives

- To isolate and identify microorganisms in fermented foods such as beer, kefir and kombucha, using Gram stain, selective agar and API kits.
- Investigation of antimicrobial activity of the isolated microorganisms against selected pathogens.
    - Determine the minimum inhibitory concentration (MIC) and the effects on morphology of the pathogens using scanning electron microscopy (SEM).

1.4 Research Strategy

The methods carried out to identify potential sources of microorganisms exhibiting antimicrobial activity from fermented foods were to:

- Isolate the microorganisms in the fermented foods using selective agar.
- Identify the microorganisms (*Lactobacillus* species being of particular interest) using Gram stain and API kits.
- Test isolated microorganisms for antimicrobial properties against selected known pathogens.

- Observe the isolated organisms and known pathogens under the scanning electron microscope and identify any changes in morphology of the pathogens.

2.0 Literature Review

2.1 Introduction

In reviewing the literature on *Lactobacillus species* and lactic acid bacteria in general, it has become evident that there has been quite a substantial amount of work on the probiotic and antimicrobial properties of this species. The organisms have been isolated from a variety of sources including various fermented foods, dairy products and animal and human gastrointestinal tracts. It is recognised that some of these microorganisms may be useful as antimicrobials. Since they occur naturally, they may be a more popular choice than using manmade substances and chemicals. The sharp increase in antibiotic resistance imposes a global threat to human health and the need to identify new effective antimicrobial alternatives, which can be used to extend the shelf life of food and inhibit the growth of foodborne pathogens. There is a rising interest in the use of probiotics against bacterial pathogens. For example, work carried out on probiotics from the species *Bifidobacterium* and *Lactobacillus* exhibited antimicrobial action on pathogenic *E.coli* resistant to at least five antibiotics, Ceftazidime, Ampicillin, Clarithromycin, Amoxicillin + Clavulanic Acid and Ceftriaxone (Leite et al., 2015).

Lactic acid bacteria are classified by identifying two fermentation pathways of hexose (a monosaccharide with six carbon atoms). The main genera of this group are *Lactobacillus, Leuconostoc, Pediococcus, Lactococcus and Streptococcus*. Other less common genera are *Aerococcus, Carnobacterium, Enterococcus, Oenococcus, Sporolactobacillus, Tetragenococcus, Vagococcus and Weissella*. These all belong to the order Lactobacillales (Ganzle; 2015). Lactic acid bacteria are cocci or rod shaped and anaerobic but can withstand and grow in the presence of oxygen (facultative anaerobe). Lactic acid bacteria are catalase negative. Catalase is a common enzyme found in most living organisms that are exposed to oxygen. It turns hydrogen peroxide into water and oxygen. (Ganzle; 2015) Strains of lactic acid bacteria are the most common microorganisms used as probiotics. Probiotics are identified as providing live, potentially beneficial bacterial cells to the gut environment of both humans and animals (Sonomoto and Yokota; 2011). Research has dealt with diarrhoea, inflammatory bowel disease and irritable bowel syndrome but probiotics in the future may be used to treat gastrointestinal diseases and possibly as a delivery system for vaccines, immunoglobulins and other treatments (Ljungh and Wadstrom; 2009). Exopolysaccharides, from lactic acid bacteria, that offer health and sensory benefits beyond just nutritional components have become of interest. However, there is a wide variation in molecular structures of exopolysaccharides. The mechanisms by which physical changes in foods and their bioactive effects are carried out varies. (Welman; 2009).

Lactic acid bacteria are an order of Gram-positive organisms. They are acid tolerant and generally do not produce spores. They are a group of bacteria that share common metabolic and physiological characteristics. As lactic acid bacteria can withstand acidic conditions they can survive and compete with other bacteria in fermentation conditions, as organic acids such as lactic acid are produced. Most lactic acid bacteria cannot respire and therefore the media used in the laboratory to encourage growth needs to contain carbohydrates. They are used extensively in the food industry for fermentation to make a variety of fermented foods such

as bread, yoghurt, beer, kombucha and kimchi. Lactic acid bacteria are considered one of the best ways to manufacture natural, safe and healthy foods. (Li et al; 2016).

2.2 Properties of lactic acid bacteria

Some lactic acid bacteria have been identified to show antimicrobial properties as they produce bactericidal bioactive peptides called bacteriocins and enzymes that are capable of controlling the formation of biofilm and inhibiting the growth of pathogenic organisms (Karska-Wysocki et al., 2010). The formation of biofilm helps microorganisms survive. It contains a diverse community of bacteria and fungi and provides an equilibrium and stability for microbial communities to form clusters, mature and grow.

Bacteriocins are not antibiotics. The main difference being that bacteriocins are only active against strains of related species or particularly the same species, whereas antibiotics have a wider activity spectrum and even if their activity is restricted this does not show any preferential effect on closely related strains. In addition, bacteriocins are produced during the primary stages of growth and are ribosomally synthesized whereas antibiotics are usually secondary metabolites (Zacharof and Lovitt; 2012).

Bacteriocins used in the food industry as natural preservatives are generally considered safe (Zacharof and Lovitt, 2012). The use of antimicrobial compounds (e.g. bacteriocins) to fight against pathogens and food spoilage has proved to be effective. Nisin, the first and most well know bacteria bacteriocin has for over 50 years been approved to be used in over 40 countries (Miao et al., 2015). Klaenhammer et al. (2012) noted that lactic acid bacteria are believed to have health promoting benefits for humans and animals. Cotter et al. (2013) stated that that they are antibiotics that have the potential to be used against multi drug resistant pathogens. Cavera et al. (2015), Lu et al. (2014) acknowledge that various bacteriocins have been isolated to inhibit both Gram positive and Gram-negative pathogens.

Klaenhammer (1993) stated that bacteriocins are present in species of the genus *Lactobacillus*, including *Lactobacillus acidophilus* which produce lactacin B or F and *Lactobacillus Casein* B80 that produce casein 80. Using a mixture of these lactic acid bacteria against Methicillin Resistant *Staphylococcus aureus* (MRSA), they inhibited growth, exhibiting antibacterial activity. Thus, concluding that in lactic acid bacteria there are some components that can inhibit the growth of MRSA. (Karska-Wysocki et al., 2010). In addition, studies found that *Clostridium difficile disteria* could be controlled by using mixed cultures of *Lactobacillus.* (Beausoleil et al., 2007).

Other research has shown the antimicrobial effect of bacteriocins from lactic acid bacteria. These include *Lactobacillu*s strains from commercially available food in Gulbarga market

produced potential probiotics for the prevention of bacterial gastro-intestinal infection and other related enteric infections. (Prabhurajeshwar and Chandrakant, 2018).

A substance named CO26H11N3 produced by lactic acid bacteria was found to have a broad antimicrobial spectrum even to multidrug resistant pathogens. (Zhang et al., 2017). This has the potential to be a new preservative or 'antibiotic' as does a newly discovered bacteriocin produced by *Lactobacillus plantarum* A-1, plantaricin ASM1 (PASM1), which showed stability in neutral and weak alkaline conditions. It is heat-stable but digested by trypsin and inhibits the growth of other lactic acid bacteria, such as *Lactobacillus*, *Leuconostoc* and *Enterococcus*. PASM1 showed stability in a wide pH range compared to nisin and therefore has a possible application in the food industry (Hu et al., 2017).

A bacteriocin produced from *Lactobacillus plantarum* ZJ008, which was isolated from fresh milk, showed broad-spectrum antimicrobial behaviour against Gram- positive and Gram-negative bacteria, particularly *Staphylococcus spp*. The activity of the bacteriocin was bactericidal but it did not cause cells lysis but pore formation on the surface of the bacterial membrane. These results suggest that the bacteriocin could be very useful in controlling and inhibiting *Staphylococcus* species in the food industry (Biswas et al., 2017).

Using scanning electron microscopy and transmission electron microscopy methods, results showed that bacteriocin BM1157, obtained from probiotic *Lactobacillus crustorum* MN047 killed *Listeria monocytogenes* by biofilm destruction and pore formation. This was also verified by crystal violet dye and lactic dehydrogenase release tests. In addition, the BM1157 inhibited the growth of *Listeria monocytogenes* in milk and exhibited broad-spectrum antimicrobial activity in *Escherichia coli* and *Staphylococcus aureus* (Yi et al., 2018).

A bacteriocin BMP11, isolated from *Lactobacillus Crustorum* MNO47, was found to be effective against two significant food poisoning bacteria *Listeria monocytogenes* and *Cronobacter sakazakii.* The results of scanning electron microscope (SEM) and transmission electron microscope (TEM) indicated that BMP11 destroyed the integrity of cell envelopes of pathogens with cell wall perforation and cell membrane permeability. BMP11 also exhibited anti-biofilm formation activity and the inhibition of the growth of *Listeria monocytogenes* in milk. Therefore, BMP11 had promising potential as an antimicrobial to control foodborne pathogens in dairy products (Yi et al., 2018).

Leite et al., 2015 demonstrated that probiotics isolated from six strains belonging to the genera *Lactobacillus* and *Bifidobacterium* strains reduced the biofilm formation of two multi resistant *E.coli*.

The biofilm of the organism *Bacillus subtilis,* supports the resistance of antimicrobial agents. Thus, new substances that eliminate biofilms are important. Sarikhani et al., 2018 looked at

the effect a bacteriocin from *Lactobacillus acidophilus* ATCC 4356 had on *Bacillus subtilis* BM19 in the presence and absence of Hbsu. Hbsu is a nucleoprotein that is involved in several processes in the cells of the organism, such as replication, transcription, cell division, recombination and repair. It is involved in the formation of the biofilm. Results showed that purified bacteriocin from *L*. *acidophilus* ATCC 4356, in the absence of Hbsu, was more effective in inhibiting the growth of *B*. *subtilis* than when Hbsu was present.

Adverse growth conditions such as low temperatures, lack of or limited nutrients and lack of moisture encourage bacteriocin production. This is probably due to slow growth, enzyme reactions and more availability of amino acids. (Barbosa et al., 2016).

A gastric carcinogenic pathogen, *Helicobacter pylori*, resistant to antibiotics and therefore a concern to the World Health organisation was shown to be susceptible to a probiotic from the *Lactobacillus fermentum* UCO-979c strain (encapsulated in carrageenan) (Gutierrez-Zamorano, 2019.)This was under simulated gastric conditions, either fasting or a standard diet, pH 3.0 or under and microaerophilic conditions with agitation. Results showed that the probiotic was more effective under fasting (harsher acid environment) than administering it associated with the diet. Thus, demonstrating that the probiotic should be consumed whilst fasting and that pH does affect its effectiveness (Abdelhamid et al; 2018). A lower pH would be expected to help effectiveness as lactic acid bacteria prefer acidic conditions for growth.

Ideally, probiotic bacteria should be able to tolerate harsh conditions, as they have to be able to withstand the gut environment. They must be able to arrive where they are going to exhibit probiotic activity and should be active against or protected from pathogens by producing an antimicrobial substance such as a bacteriocin or a metabolite, like organic acids. Ideal characteristics are tolerance to low pH, bile salts, antibiotics sensitivity and hydrophobicity. Studies revealed that *Lactobacillus sp.* G3_4_1TO2 is a potential probiotic bacteria and produced the enzyme amylase. Further research needs to be carried out on Lactobacillus sp. G3_4_1TO2 for it to be used in the food industry. However, suggested uses of amylolactic acid bacteria, such as this, can be used in the development of certain foods such as cereal based foods, fermented foods like European sour rye bread, Asian salt bread, sour porridges, dumplings and non-alcoholic beverages. Starch and amylolactic acid bacteria together produces a cost effective fermentation process (Tallapragada et al., 2018).

Other enzymes produced by lactic acid bacteria include proteases, peptidases, polysaccharides, ureases, lipases, amylases, esterases and phenoloxidases. These also come from a variety of other sources including fungi, yeasts, plants and animals. Usually amylases are obtained from microorganisms because they tend to be more stable than extractions from plants and animals, exhibit properties that are more desirable and are easier to use in bulk production and thus more economical. (Tallapragada et al., 2018).

Investigations have shown that bacteriocins alone in food probably will not ensure complete safety, especially in controlling Gram-negative bacteria (Gutierrez et al., 2010). Antibacterial peptides produced by bacteria that are safe to use in food are much more effective when combined with nanoparticles. Gutierrez et al., 2010 found that some nanoparticles are antimicrobial due to activity of their large surface area in contact with the microorganisms. This interaction causes a broad range of probable antimicrobial activities, useful in food processing and packaging. Investigations are still in their infancy and extensive safety and acceptability tests are needed before they can be used for large-scale production and consumption (Sidhu and Nehra; 2017).

The Bacteriocin-M1-UVs300 produced by *Lactobacillus plantarum* M1-UVs300, isolated from fermented sausage was found to exhibit antimicrobial activity against Gram positive bacteria and Gram negative bacteria. It was found to be heat resistant and active between pH 2-8, sensitive to proteolytic enzymes but not sensitive to α-amylase. These findings indicated that bacteriocin-M1-UVs300, is a novel bacteriocin with a broad inhibitory spectrum, and that it had the potential to act as a natural preservative in the food industry. (An et al. 2017). Studies with *Lactobacillus paracasei* by (Bengoa et al; 2018), showed that the probiotic activity was also not affected by heat. Here, temperature could be used to improve the technological properties of the product.

The number of organisms present in the food affects effective antimicrobial activity. It is also influenced by the method of application, the food components, pH and temperature (Lin and Pan 2017).

Table 3. Summary table of Lactic Acid bacteria found to show antimicrobial properties

Lactic Acid Bacteria	**Bacteriocin**	**Active against microorganism**	**Possible use in the food industry**
Lactobacillus acidophilus	Lactacin B or F	Methicillin resistant *Staphylococcus aureus*	Yes
Lactobacillus casein B80	Casein 80	Methicillin resistant staphylococcus aureus	Yes
Lactobacillus plantarum A-1	Plantaricin ASM1 (PASM1)	*Lactobacillus, leuconostoc* and *Enterococcus*	Stability in wide range of pH compared to nisin.
Lactobacillus plantarum ZJ008	*Lactobacillus plantarum* ZJ008	Variety of Gram positive and Gram negative organisms. especially *Staphylococcus spp*	Yes
Lactobacillus plantarum M1-UVs300	Lactobacillus plantarum M1-UVs300	Variety of Gram positive and Gram negative organisms	Yes

Lactobacillus crustorum MN047	Bm1157	*Listeria Monocytogenes, Escherichia coli Staphylococcus aureus*	Yes
Lactobacillus crustorum MN047	BMP11	*Listeria Monocytogenes, Cronobacter sakazakii*	Yes possible in dairy products
Lactobacillus acidophilus ATCC 4356	*Lactobacillus acidophilus* ATCC 4356	*Bacillus subtilis*	Yes
Lactobacillus fermentum UCO-979c	*Lactobacillus fermentum* UCO-979c	Helicobacter pylori	Possible – further research needed

2.3. The use of Lactic acid bacteria in the food industry

Bacteriocins can be applied in a purified or in a crude form, or through the use of a product previously fermented with a bacteriocin producing strain as an ingredient in food processing or incorporated through a bacteriocin producing strain (starter culture). (Zacharof and Lovitt, 2012) Lactobacillus has been used for a long time in the dairy industry and other bio-industries (Karska-Wysocki et al., 2010; Lin and Pan, 2017). It is believed to be the oldest method of food preservation and can prolong the shelf life of the food (Lin and Pan, 2017). When using lactic acid bacteria there are three different approaches:

A. Inoculating bacteriocin producing lactic acid bacteria into the food as it is processed.

B. Applying the pure bacteriocin directly onto the food product.

C. Using a previously fermented product from a strain of bacteria that produces a bacteriocin. (Lin and Pan, 2017).

In recent years, there has been an increased interest in discovering and using new lactic acid strains that when put into food will be a health benefit. The flavour of fermented milk products is dependent on the lactic acid bacteria present and their proteolytic system. Most isolated from milk use multiple amino acids for growth and break down of casein. (Beganovic et al., 2013). Therefore, most of the lactic acid bacteria isolated from fermented dairy products have multiple amino acids auxotrophy (inability to synthesize) and their growth in protein rich medium depends on a complex proteolytic system for the degradation of casein (Beganovic et al.,2013). There is demand for new antimicrobial products as nisin, for example,

is commonly used but is only effective against Gram positive organisms usually *Listeria Monocytogenes*. When added it is an additional cost whereas plantaricins produced by *Lactobacillus plantarum* can be utilized in situ fortification and have an antimicrobial effects on the pathogens (Li et al., 2016).

2.3.1 Examples of studies using lactic acid bacteria from fermented foods

Isolated *Lactobacillus plantarum,* NTU102 from homemade Korean- style pickled cabbage showed that the cell- free supernatant of this bacterium had antimicrobial properties. This substance Rc 13988kii BC was effective against *Vibrio parahaemolyticus* BCRC 12864 and *Cronobacter sakuza* concluding that this could have the potential to be used as natural preservative/food additive. (Lin and Pan, 2017).

Lactobacillus coryniformis which was originally isolated from Jiangshui Cai, a fermented vegetable made with Chinese cabbage, from China produces a bacteriocin called Lactocin MXJ 32A. (Lu et al., 2018), when purified it had broad antimicrobial properties against many Gram positive and Gram negative foodborne pathogens including some antibiotic resistant foodborne pathogenic strains. It works by pore formation of the cytoplasmic membrane of targeted cells. (Lu et al., 2018). *Lactobacillus casei* was also isolated from Jianshui Cai. The purified bacteriocin (LiN333) showed antimicrobial activity in a range of temperatures 60˚C to 121 ˚C and PH levels of PH 3 to 9. The minimum inhibitory concentration against *E.coli* and *S.aureus* was 15 µg/ml. Therefore, this bacteriocin shows that potentially it could be used to preserve food especially as it is stable across a range of temperatures and pH (Ullah et al., 2017).

Champagne et al. (2010) isolated L*. helveticus* KLDS1.8701 strain from traditional sour milk in Sinkiang in China. It has been found that *Lactobacillus helveticus* is present in several fermented foods and also is used as a probiotic with many health-promoting properties. (Li et al., 2015). Chen et al., (2015), identified the complete genome sequence of this organism and showed its genetic basis on which adhesion, exopolysaccharides (EPS) production, acid and bile tolerance and bacteriocin production behaved.

A natural antimicrobial substance from *Lactobacillus paracasei subsp tolerans* FX-6, isolated from Tibetan kefir (traditional fermented milk from Tibet, China) was found to contain a bacteriocin with broad-spectrum activity effective on fungi and bacteria (both Gram positive and Gram- negative). The shelf life of fresh pork was found to be extended to 12 days when the bacteriocin was applied (Miao et al., 2015).

Antimicrobial peptide F1, a novel antimicrobial peptide from Tibetan kefir, have shown strong antimicrobial activity against several bacteria and fungi. Studies showed that

antimicrobial peptide F1 contained 18 amino acids which increased the outer and inner membrane permeability of E. coli, and the leakage of the cytoplasmic β-galactosidase and potassium ions was detected in the process. (Miao et al., 2014). These findings showed that the antimicrobial peptide F1 targeted and killed the E.coli in several ways.

Lactobacillus paracasei Subsp. *Tolerans* FX-6 isolated from Tibetan kefir was shown to increase its antimicrobial activity under optimized conditions. This investigation used response surface methodology (RSM), a method to optimize process conditions, which can determine the influence of various factors and their interactions on the organisms. These substances also exhibited a broad spectrum antimicrobial activity against a variety of both Gram positive and Gram negative bacteria and fungi. The antimicrobial substances were highly tolerant to enzyme and heat treatment and showed stronger antimicrobial activity than nisin. Thus, the conditions, which the *Lactobacillus* are present and are grown, can significantly affect the microbial effectiveness; in this case by more than 80%. These results demonstrated a novel approach to producing an effective antimicrobial substance, with possible uses as a food preservative. (Miao et al., 2014).

Crude *Lactobacillus* cultures, isolated from four commercial Zimbabwean dairy products (Probrand sour milk, Kefalos Vuka cheese and Chibuku opaque beer) and three strains of *L. plantarum* from Balkan cheeses (*CLP1, CLP2 or CLP3*) exhibited high antibacterial activities against pathogenic strains of *E.coli* which cause paedriatic diarrhoea (Chingwaru and Vidmar, 2017).

Another novel bacteriocin (Caseicin TN-2) was identified from *Lactobacillus casei* TN-2 isolated from fermented camel milk (Shubat) of Xinjiang Uygur Autonomous region of China. (Lu et al., 2014) was shown to have a broad antimicrobial spectrum against both Gram positive and Gram-negative organisms, which included some antibiotic resistant foodborne pathogens. It was sensitive to proteases, such as trypsin and papain. Scanning electron microscopy (SEM) and transmission electron microscopy (TEM) analyses was carried out to investigate the effect of Caseicin TN-2 on the target cells. It was demonstrated that the bactericidal mode of action was pore formation in the cytoplasmic membrane (Lu et al., 2014).

Bacteriocin, plantaricin SLG1, produced from *Lb. plantarum* SLG1 and isolated from yak cheese showed a wide range of antimicrobial activity against many food-borne spoilage and pathogenic bacteria, as well as some fungi. Results using a scanning electron microscopy showed that the mode of action was bactericidal and that plantaricin SLG1 was able to damage the cell membrane integrity of the pathogens (Pei et al., 2018)

Chhang, a traditional fermented beverage produced in Lahaul and Spiti district of Himachal Pradesh, is made from the spontaneous fermentation of rice by adding a traditional inoculum called ‘Phab’. Traditional fermented beverages are not very well explored and it is thought are

rich sources of rare/novel probiotic strains with potential of various health benefits. Studies of the probiotics activities of the isolated lactic acid bacteria, from this traditional fermented beverage, were carried out. The results obtained showed that the probiotic strain *Lactobacillus plantarum* F22 isolated from chhang was capable of tolerating high bile salt, able to survive in simulated low gastric pH and showed a broad antimicrobial spectrum against a wide range of food borne/spoilage causing bacteria. The isolate was also found sensitive to most common antibiotics, had strong auto aggregation and hydrophobicity. Therefore, *Lactobacillus plantarum* F22 has been proved to be highly effective, and therefore a potential agent to be used in the development of new pharmaceuticals and functional food preparations. (Handa and Sharma, 2016).

Experiments to look at the activity of microorganisms during Kahudi fermentation (a traditional rapeseed fermented food product of Assam, India) showed lactic acid bacteria counts of up to up to 10^9 CFU/g on the final day of fermentation (Day 11). During this time, the pH of the sample dropped from 6.8 (Day 1) to 3.95 (Day 11). Twelve isolates of lactic acid bacteria selected based on colony morphology were identified as *E. durans* (6 isolates), *L. plantarum* (2 isolates), *L. fermentum* (2 isolates), and *L. casei* (2 isolates). This study showed that *Kahudi* has potential as a non-dairy fermented probiotics product. (Goswami et al., 2017).

Lactobacillus strains that produced bacteriocin were isolated from some Nigerian indigenous fermented foods and beverages. These were ogi, fufu, garri and nono. The *Lactobacillus* identified were *L.acidophilus, L.casei, L.fermentum, L.lactis and L.plantarum.* Screened against a variety of known pathogens, it was found that each fermented food had its own microbial interaction, showing narrow to moderate antimicrobial activity. (Ogunshe et al., 2007).

Eighty-four isolates of Lactic acid bacteria were obtained from two traditionally fermented Indian products Tungtap (a fish product) and Tungrymbai (a soybean product). From these, eleven isolates produced potential antimicrobial bacteriocins. Their antimicrobial activity was tested alone and in combination with commercially available antimicrobial agents such as cefotaxime, polymyxin B, imipenem and tigecycline, which are effective against pathogens. They were tested for antibacterial and synergistic activity against β-Lactamase-producing nosocomial bacteria*, Streptococcus pyogenes, Enterococcus faecalis, Escherichia coli, Klebsiella pneumoniae and bacillus cereus.* Purified bacteriocins from *Lactobacillus, Pediococcus* and *Enterococcus* inhibited the growth of all the pathogens and β-lactamase harbouring clinical pathogens with significant inhibitions when compared with antibiotics alone. (Biswas et al., 2017)

Lactobacillus alimentarius FM-MM4, isolated from Nanx Wudl (a traditional Chinese fermented meat) was found to produce a bacteriocin, Lactocin MM_4 which exhibited broad-spectrum antimicrobial activity against both Gram positive and Gram negative food-borne

pathogens, as well as several yeasts. Extraction of Lactocin MM_4 was by ethyl acetate and purified through cation exchange chromatography and semi-preparative high-performance liquid chromatography (RP-HPLC). (Hu et al., 2017).

Lactobacillus sakei ST22Ch, ST153Ch and ST154Ch were isolated from traditional pork product from Northwest of Portugal. They inhibited the growth of *Enterococcus* spp., *Listeria* spp., *Escherichia coli*, *Klebsiella* spp., *Pseudomonas* spp., *Staphylococcus* spp., and *Streptococcus* spp. The mode of action of the bacteriocins was bactericidal, as shown against *Enterococcus faecium*. Antimicrobial activity was reduced after treatment of the bacteriocins with proteolytic enzymes, but not when they were exposed in presence of α-amylase, suggesting that they are not glycosylated. Most activity of the bacteriocins was recorded during the early stationary phase of growth and remained stable only for a short period, followed by a decrease. Bacteriocins ST22Ch, ST153Ch and ST154CH have a narrow spectrum of activity, are heat resistant and stable between pH 2.0 and 10.0, not adsorbing to the surface of the producer cell and are produced at higher levels during the stationary phase of fermentation in the presence of 2% (w/v) D-glucose. Different levels of bacteriocins ST22Ch, ST153Ch and ST154Ch were produced in presence of a combination of tryptone, meat extract and yeast extract. This suggests that the three bacteriocins may be produced at high levels during all phases of (fermented) meat processing. The antibacterial spectrum of activity of these strains (ST22Ch, ST153Ch and ST154Ch) indicates their potential for use in a mixed starter culture for the fermentation of meat products. Further research is needed on the safety, food technology and specific flavours (Todorov et al., 2013).

2.3.2. Other Sources of Lactic Acid Bacteria

Lactic acid bacteria have been isolated from a variety of other sources as well as fermented foods. Such as *Lactobacillus paracasei* SD1, a strain from the human oral cavity, found to exhibit a broad spectrum of antimicrobial activity against oral pathogens, thus suggesting that it could be used in prevention and treatment of oral disease. The active compound of the bacteriocin was obtained using ammonium sulphate precipitation and then chloroform and gel filtration (Wannun et al., 2014.)

A bacteriocin purified and obtained from *Lactobacillus murinus* AU06, a bacteria isolated from marine sediments, exhibited a broad spectrum antimicrobial activity against fish pathogens . It was found that the bacteriocin production was highest at 35 °C and PH 6.0. The bacteriocin exhibited a broad inhibitory spectrum against both Gram positive and negative bacteria The antimicrobial activity of the purified bacteriocin was completely inhibited when treated with proteinase K, pronase, chymotrypsin, trypsin, pepsin and papain. This study concluded that the bacteriocin is of potential interest for food safety and may have future use in food

preservative due to its ability to inhibit a wide-range of pathogenic bacteria (Elayaraja et al., 2014).

Two hundred and thirty-four lactic acid bacteria were isolated from Brazilian food products and fifty-one were found to be able to survive at pH 2.0. The bacteria used for further investigation were the ones found to be either highly tolerant to bile, capability of auto – aggregation or hydrophobic abilities. Of these bacteria, some showed adhesion their ability to work in a similar way to commercial probiotics. This showed that the probiotic characteristics were strain-specific and that the selected isolates of species *Lactobacillus plantarum* and Lactobacillus *brevis* had potential probiotics properties. (Ramos et al., 2013)

In experiments by Liu et al., (2016). One hundred and eighteen lactic acid bacteria were isolated from food products and only one (*Lactobacillus plantarum*) produced an inhibitory effect when tested against *Pseudomonas fluorescens* AS1.1802. Further work showed the inhibitory effect to be caused by a novel plantaricin Q7, which became inactive in the presence of proteolytic enzymes such as proteinase K, pepsin, trypsin, papain, and pronase E. This work also again showed that some of these compounds can withstand high temperatures and a range of pH. Plantaricin was still active after incubation at 121°C for 20 min with a pH from 3 to 12. It exhibited antimicrobial activity against species of *Pseudomonas, Listeria, Salmonella, Shigella, Staphylococcus aureus and E.coli.* Again, this work suggests that Plantaricin Q7 has the possibility be used in food products to inhibit the growth of both food spoilage organisms and pathogens.

Lactobacillus sakei R1333 isolated from smoked salmon was found to produce a bacteriocin, which is antimicrobial towards strains of *Streptococcus, Lactococcus* and *Listeria.* This was shown to be caused by cell lysis, enzyme, and DNA leakage. Results of biochemical tests showed that L*. sakei* R1333 is a potential producer of sakacin G. This is the first time that sakacin G produced by *L. sakei* has been reported as being isolated from smoked salmon (Todorov et al., 2011).

One hundred and six lactic acid bacteria were isolated from pastırm (a Turkish dry-cured meat product), obtained from fourteen different manufacturers. DNA sequencing was performed to identify these lactic acid bacteria isolates. Four bacteria genus, *Weissella*, *Lactobacillus*, *Pediococcus* and *Leuconostoc*, were detected. *Lactobacillus sakei* was the highest isolate identified in twelve of fourteen samples. (Oz et al., 2017).

Barbosa et al., (2016), explored the biochemical and genetic features of a bacteriocin produced by Lactobacillus plantarun MBSa4, which was isolated from an Italian type salami that is made in Brazil. This is a two-peptide bacteriocin and was found to be active against

several *Listeria monocytogenes* from different serotypes. *Lactobacillus plantarum* MBSa4 was also found to produce an antifungal agent. (Barbosa et al., 2016).

2.4. Fermented Foods

2.4.1 Sour Beer

Sour beers are made by allowing wild yeasts and strains of bacteria into the brew, unlike traditional beer production, which is carried out in a sterile environment (Koch and Allyn, 2011). The most common microorganisms involved in the making of sour beer are *Lactobacillus, Brettanomyces* and *Pedicoccus* (Bernot, 2015). These make the beer taste tart. As wild yeasts are used the process can be very unpredictable and difficult to standardize. *Brettanomyces i*s a strain of yeast that is probably the most commonly used agent in sour beers (Josh Weikert, 2016). It serves the same purpose as *Saccharomyces* does, fermenting beer but working more slowly. Thus, a beer that could ferment within days or weeks with other *Saccharomyces* can take months or even years to display its full character. There are different strains of *Brettanomyces,* each that produce its own flavour ranging from tropical pineapple, and fruity peach to intense flavours described as sweaty horse blanket, dirt, earth and barnyard. (Kate Bernot 2015)

The beer can take a long time to ferment, several months or years to mature (Koch and Allyn, 2011).

Lactobacillus is a bacterium that uses the sugars in the wort and rather than converting them to alcohol, converts them to lactic acid. This lowers the beers pH, making it sour. It produces a clean taste since the bacteria does not produce much except lactic acid. It is responsible for the 'tang' in German style beers (Bernot, 2015).

Pediococcus is a bacterium, which like *Lactobacillus* produces lactic acid and lowers the pH. However, the introduction of *Pedicoccus* into beer tends to make it harsher, in taste. Whilst *Lactobacillus* produces a clean sourness, *Pediococcus* can contribute other aromas and flavours. (Kate Bernot 2015)

There are many types of sour beer and the one used in this study is a sour beer based on the traditional Berliner Weisse, a popular alcoholic drink in Berlin. It is a weak beer made sour by *Lactobacillus* bacteria. (Papazian, 2003).

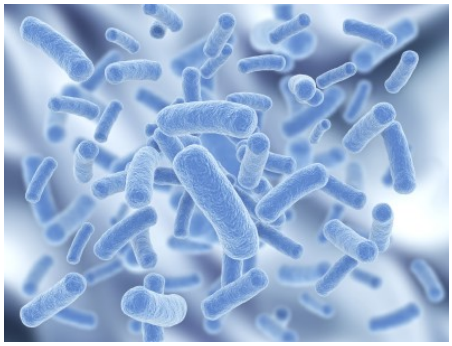

Figure 1. Lactic acid bacteria isolated from sour beer

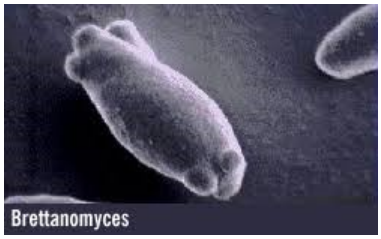

Figure 2. Brettanomyces bacteria isolated from sour beer

Figure 3. Yeasts isolated from sour beer

Figure 4. Microorganisms in sour beer production

2.4.2. Kombucha

Kombucha is a fermented, slightly alcoholic, lightly effervescent, sweetened black or green tea drink. (Mayo Clinic, 2018. 'A mug of kombucha for your health'. Online; Dutta and KrPaul, 2019). The live bacteria in the tea are thought to be probiotic (Bauer and Brent, 2017). It is believed to have health benefits and is produced by fermenting sweetened tea using a culture of bacteria and yeast called a scoby or known as a 'mother' or 'mushroom'. The soup being the remaining liquid. The microorganisms in a scoby vary. It is a mixture of acetic acid bacteria and yeasts in a zoogleal, which is a complex group of organisms in a symbiotic relationship that produce a slime growth mat. Lactic acid bacteria are not common. The yeasts *Brettanomyces, Zygosaccharomyces, Saccharomyces* and *Pichia* are usually present, and bacteria nearly always include *Gluconacetobacter* species. The bacteria oxidizes alcohols produced by yeasts to acetic acid (Jarrell et al., 2000 and Jonas et al., 1998). Other bacteria include *Acetobacter xylinum* (Sinir, 2019). Over-fermentation generates high amounts of acids similar to vinegar (Mayo Clinic, 2018. 'A mug of kombucha for your health'. Online). The pH of the drink is typically about 2.5. Ideally, the fermentation period is between 7–12 days to obtain a good tasting kombucha. Eventually, sugar is converted to organic acids and ethanol (Sinir, 2019). The bacterial diversity has been found to be higher in the soup than in the biofilm with a peak on the seventh day of fermentation and the biochemical properties changed with the progression of the fermentation. (Chakravorty et al., 2016). Also, experiments fermenting the tea at different temperatures found significant differences in the microorganisms present (DeFilippis et al., 2018).

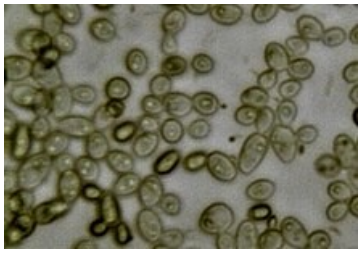

Figure 5. Yeast and bacteria in kombucha magnified 400 times

Figure 6. A Scoby used for brewing kombucha

Figure 7. A scoby used in the production of Kombucha

2.4.3. Kefir

Kefir is considered a miraculous food towards human health (Erdogan et al., 2019). It is a fermented drink usually made from milk with numerous attributed health claims. This is due to the presence of a complex mix of bacteria and yeast cultures in a exopolysaccharide matrix (Gut et al., 2019). Investigations have shown that two yeasts isolated from Kefir *Saccharomyces unisporus* and *Kluyveromyces lactis* have potential probiotic properties. (Gut et al., 2019). In work carried out on the bacterial and fungal microflora of four Turkish kefir grains it was found that, *Lactobacillus kefiranofaciens* was the dominant bacterial strain and *Dipodascaceae* family the dominant yeast strain. However, other strains were present (Dertili and Con, 2017). Kefir can be drunk, used in recipes or kept and undergo a secondary fermentation. It has a shelf life of up to thirty days at ambient temperature. (Moteqi et al., 1997). It is made by adding kefir grain to milk to cause fermentation. It is stored in the dark, in jars. Fermentation is 12 to 24 hours at 20 °C to 25 °C. The solution is strained, and the grains reused for another fermentation. It is lactose that is fermented, and it makes a sour, carbonated, slightly alcoholic drink. Much like runny yoghurt (Corona et al., 2016). Lactose is broken down into ethanol and carbon dioxide at the late stage of fermentation and this is

what makes it different from yoghurt and most sour milk products. Therefore, kefir contains very little lactose. (Farnworth, 2005). The probiotic bacteria found in kefir include, *Lactobacillus acidophillus, Bifidobacterium bifidum, Streptococcus thermophilus, Lactobacillus delbrueckii subsp bulgaricus, Lactobacillus heleticus, Lactobacillus kefiranofaciens, Lactococcus lactis and Leuconostoc* species. The *Lactobacilli* numbers can range from one million to one billion colonies per millimeter. Yeasts include *Kluyveromyces marxianus, Kluyveromyces lactis, Saccharomyces fragilis, Saccharomyces cerevisiae, Torulaspora delbrueckii and Kazachstania unispora* (Leite et al., 2013).

Figure 8. Kefir grains, a symbiotic matrix of bacteria and yeast

2.5. Inference from literature review

To conclude, lactic acid bacteria have shown to be antimicrobial to a variety of pathogens, because they disrupt the integrity of cells by affecting the biofilm and cell lysis of the bacteria. This is demonstrated when just using the bacteriocin and other times when using the whole organism. They appear to be more effective under stressful conditions and in the presence of nanoparticles.

Fermented foods provide a rich and varied source of lactic acid bacteria, many of which exhibit antimicrobial activity against known pathogens. Studies, again, have shown effectiveness when using both the isolated bacteriocin and the crude organism. There is a wide variety of sources to isolate lactic acid bacteria and there are probably many that have not been discovered yet. By isolating organisms from fermented foods in this project, it may be possible to identify a strain of lactic acid bacteria that exhibits antimicrobial properties and can be used to reduce pathogens in food.

3.0 METHODOLOGY

3.1. Introduction

Microorganisms were isolated and identified from several fermented food products. Initially Sour Power starter culture from sour beer was tested but as limited results were obtained; other products such as kefir and kombucha were included.

From information gained from the brewery, the starter culture was expected to contain strains of *Pediococcus*, at least three strains of *Lactobacillus, Saccharomyces* and wild yeasts. Kefir was also expected to contain lactic acid bacteria. However, the kombucha was unlikely to have high numbers of lactic acid bacteria due to the process usually producing acetic acid.

The method of isolation used was to grow the bacteria on general and specific agars and then identify them by using biochemical tests. Isolated colonies were taken, streaked out on to agar to gain purity and taken for identification.

Once identified any lactic acid bacteria were tested to see if they exhibited antimicrobial activity against selected pathogens. *E.coli* (*Escherichia coli* ATCC™ 25922), *Staphylococcus aureus* (*Staphylococcus aureus* subsp. aureus ATCC™ 6538P) and *Salmonella* Spp (*Salmonella enterica subsp. enterica serovar Typhimurium* ATCC™ 14028$^{T)}$). The pathogens used were from Cultiloop freeze-dried cultures that have a typical bacterial loading of about 10^6 colony-forming units per millilitre (cfu/ml) and a 24-hour incubation yields a broth culture with a turbidity value of about six on the McFarland standard. *E.coli* C822037, laboratory stock, isolated from animal/ human source was also used.

O.1µl of culture was inoculated in to 9 ml of nutrient broth (Oxoid UK,) and incubated for 24 to 48 hours at 37°C, depending on the pathogen. The viability of the cultures was confirmed by plating out onto plate count agar (Oxoid UK, CM 0325).

The effect of the amount of time the lactic acid bacteria were incubated with the pathogens was tested over time as was the minimum inhibitory concentration of the lactic acid bacteria. Zones of growth were measured on pathogen-seeded plates.

From the literature review, it is evident that numerous lactic acid bacteria have proved to be antimicrobial to varying degrees and that many are more effective when additives are present. (Gutierrez et al., 2010). Also, that most work has been carried out when the bacteriocin has been isolated (Zacharof and Lovitt, 2012). Here the intact organism alone has been tested against the pathogens to measure effectivity. An electron microscope was used to view the morphology of the isolated microorganisms and pathogens.

3.2. Isolation of the Microorganisms

The fermented food products tested were sour beer, kefir and kombucha.
Samples of the Sour power starter culture were transported from the brewery to the laboratory in sterile 250 ml glass containers and stored in the fridge. Within 24 hours, the starter culture was diluted down using standard serial dilutions of 9ml of sterile Ringers solution (Oxoid, BR0052G) to 1 ml of starter culture using sterile pipettes. These were plated out onto selective agar. 0.1ml of each dilution of microbial solution was pipetted onto duplicate plates, spread, with a sterile spreader and incubated (Harrigan et al., 1966). Plates were counted and typical colonies selected from the plates of the highest dilution showing growth. One colony from each plate was re-streaked twice on to the same agar to ensure purity
Initially, Tryptone soya agar (Oxoid UK, CM0131) was used to encourage the growth of the general microbial flora. The plates were incubated aerobically at 30°C for 48 hours. To encourage better growth. 10% lactic acid was added to the Tryptone soya agar (Oxoid UK, CM0131) to reduce the pH down to pH 3 (TSA adjusted). This was to try to establish conditions for growth similar to the pH in the sour beer. Wallerstein Laboratory Nutrient Agar (Oxoid UK, CM0501) was used for the examination of materials encountered in brewing and the industrial fermentations containing mixed flora of yeasts and bacteria. It is suitable to differentiate wild yeasts from brewing yeasts. Plates were incubated aerobically and anaerobically at 30°C. Raka Ray agar (Oxoid UK, CM0777) was used to isolate lactic acid bacteria in beer and brewing processes by the presence of phenylethanol and cycloheximide. Two methods were used here: the standard spread plate method and the overlay method, where the petri dish is covered in a thin layer of agar and left to dry. The selected dilution was spread over the agar and then the remaining agar poured over the top. Plates were incubated aerobically and anaerobically at 30°C.

To obtain further isolation of colonies using the old starter culture and new starter culture of Sour Power (Sour power 16/3, A and B). Colonies were taken off the Raka Ray overlay plates 10^{-2}, 10^{-3} and Raka Ray surface plates from the initial starter culture of Sour Power16/3 and put into Tryptone soya broth and incubated anaerobically at 30°C for 48 hours to try and encourage growth. Both the Sour Power A and B starter cultures were put onto De Man, Rogosa Sharpe agar and incubated at 30°C anaerobically for 48 hours. From these overnight broths of the Sour Power starter culture 16/3 and Sour Power B starter culture they were streaked onto De Man, Rogosa Sharpe agar and Tryptone soya agar plates. The De Man, Rogosa Sharpe agar was incubated anaerobically at 30°C for 48 hours and the Tryptone soya agar aerobically for 48 hours. Results from this showed only yeasts were present on the plates. There was no evidence of lactic acid species. Gram stains were carried out on several colonies to confirm this, all showing as Gram negative. A stock culture of *Lactobacillus* was grown

overnight in malt extract broth, pH 4.9 and streaked onto the selective media plates to check selectivity and effectiveness.

To maintain cultures further incubation and plating out of colonies from Sour Power B and Sour Power starter 16/3 on De Man, Rogosa Sharpe agar and from Sour power starter culture A on Raka Ray agar were carried out. Any colonies looking like potential lactic acid bacteria were tested initially by Gram stain.

To isolate yeasts and moulds, Malt Extract agar (Oxoid UK, CM0059) was used. This agar inhibits bacterial overgrowth whilst permitting selective isolation of fungi and yeasts. Plates were incubated aerobically at 25°C.

As a standard / control *Lactobacillus, casei* was streaked out, from the laboratory stock, onto Tryptone soya agar to check that that the colonies looked similar to the isolated ones.
The results from the Gram stain shown in table indicate that they are all yeasts (the two types as before) for the colonies from Sour Power B and Sour power 16/3 Counts were very low at 10^{-3} whereas there would be expected to be growth at 10^{-6} for yeasts as these originate from starter cultures. Colonies that had an appearance of *Lactobacillus Spp* were present on the control plates of TSA. The colonies from Sour Power A on the RR plates were tested with an API kit and were identified as *Lactococcus ordinate* (*Lactis Ssp* 99.7%). However this organism did not survive further subbing.

The brewery could not confirm how many times the starter cultures had been subbed. It was confirmed that the starter cultures Sour power 16/3, Sour Power A and B starter culture were very old.
Another Sour power starter culture was obtained named C and plated out on to De Man, Rogosa Sharpe agar and Raka Ray agar.
The possible Lactobacillus colonies (small translucent colonies) were re-streaked three times back onto De Man, Rogosa Sharpe agar and Raka Ray agar. These were then Gram stained and identified using the API kits.

The Kefir sample was delivered, to the laboratory in a sterile glass bottle and refrigerated immediately. Serial dilutions were carried out within 24 hours of receiving it, using Ringers solution and the method as for the Sour Power starter cultures above.

Batches of Kombucha obtained from production, were delivered in sterile glass bottles as would be sold in retail. Again, serial dilutions were carried out from 10^{-1} to 10^{-9} as above.

A general selective agar for *Lactobaccilli w*as used for these fermented foods as a medium specific for the beer brewing industry was not needed; De Man, Rogosa Sharpe agar (Oxoid UK, CM0361), designed to favour the luxuriant growth of *Lactobacilli*. It contains sodium acetate which supresses the growth of many competing bacteria. Tryptone soya agar (Oxoid

UK, CM 0131), adjusted was also used and plates for both agars were incubated at 30°C anaerobically for 24 to 48 hours. Plates were inspected for different colonies such as morphology, size and colour. Pure strains of bacteria selected from the streaked plates, particularly ones exhibiting the appearance of lactic acid bacteria (small, translucent colonies) went on to be Gram stained.

Summary of samples used:

Four samples, taken at different times from different vessels, of the Sour Power starter culture were tested due to initial limited results. To distinguish between the various samples of the Sour Power starter culture, they were named as:

- Sour Power 16/03
- Sour Power A
- Sour Power B
- Sour Power C

One sample of kefir used and three of Kombucha were used:

- Kefir
- Kombucha
- Kombucha A
- Kombucha B

Summary of isolation of microorganisms from the food and drink

- Delivery of food/ drink sample
- Store for maximum of 24 hrs
- Serial dilutions
- Plate onto selective agar
- Incubate aerobically/ anaerobically
- Plate counts
- 1 colony from plate re-streaked twice

Cultures of microorganisms, isolated from all the fermented foods were kept fresh and alive by using Tryptone Soya Broth (Oxoid UK, CM0129) a medium used for cultivating aerobes and facultative anaerobes including some fungi. Malt Extract Broth (Oxoid UK, CM0059) was used for yeasts and moulds. For longer periods, cultures were maintained on agar slants or frozen.

3.3. Identification of the isolated microorganisms

Isolated colonies were Gram stained by fixing the bacteria on a washed and dried microscope slide and then staining using:

- Crystal violet for 1 minute
- Removing and rinsing with water
- Iodine for 1 minute
- Removing and rinsing with water
- Rinsing with acetone until running clear (holding the slide at an angle) for a maximum of 5 seconds
- Rinsing with water for a minimum of 20 seconds
- Safranin for 1 minute
- Removing and rinsing with water
- Examining under the microscope. Gram positive bacteria stain purple and Gram-negative bacteria pink.

Gram positive, both rods and cocci bacteria were identified using a 50CH (Biomerieux, France) API kit for *Lactobacillus* and related genera. During incubation, the carbohydrates are fermented to acids, which produce a decrease in the pH detected by a change in colour. The results make up the biochemical profile, which is used in the identification software to identify the strain. The first tube is used as a negative control. Two factors are demonstrated, one being oxidation of colour change and the other assimilation shown by growth in the cupule. Selected colonies, from streaked plates, to ensure purity, were put into the 50CHL growth medium and measured to the correct concentration, using the McFarland test for turbidity of 2.0. This solution was added to the cupules, in the API strips, up to the black marker point and then overlaid with oil to produce anaerobic conditions. These were incubated at 37°C for 48 hours and then checked and recorded for colour change. The results were entered into APIWEB (the API software), which identified the organisms with a percentage certainty.

Yeasts were identified using the API kit 20C AUX (Biomerieux, France) but no further investigation was carried out with these as the project concentrated on antimicrobial bacteria. Isolates identified by Gram stain and API kits as *Lactobacillus plantarum 1, Pediococcus acidilactici* and *Lactococcus lactis*, were selected for further study.

Several attempts at isolation of bacteria from an original sample of Kombucha (A) were carried out and then a new sample was used (B). Samples of Kombucha A and B were plated out onto Tryptone soya agar PH adjusted and De Man, Rogosa Sharpe agar and incubated anaerobically at 30°C for 48 hours.

Three colonies from plates 10^{-5} and 10^{-7} from the De Man, Rogosa Sharpe agar and colonies from plates 10^{-5} Raka Ray were re streaked on to De Man, Rogosa Sharpe agar and Raka Ray

agar respectively and then Gram stained. Gram positive rods and cocci were present. These were tested with API kits. To confirm results and consistency, 1m of each of the starter cultures from16/3, A, B and kefir were put into 9ml of TSB, incubated for 48 hours anaerobically at 30°C. These broths were then streaked on to TSA adjusted plates.

3.4. Testing of Isolated Microorganisms for Antimicrobial Properties

3.4.1. Disc method

Tests were carried out to identify which isolated lactic acid bacteria showed signs of antimicrobial behaviour against *E.coli* by using inoculated discs and pathogen -seeded plates. Modified method used of (Hudzicki, 2009). Initially the screening was to identify if the isolates had any effect against *E.coli,* further standard tests and measurements were carried out to confirm this. In addition, other pathogens such as *Staphylococcus aureus* and *Salmonella Spp* were then also tested.

Tryptone soya agar (Oxoid UK, CM0131) adjusted plates were inoculated with 100µl of dilution of *E coli* $^{-6}$, spread with a sterile spreader and left to dry in the 37°C incubator for 5 minutes. There were 6 discs on each plate. Tested were:

12 discs x TSB + pellet of test isolate, re-suspended

12 discs X supernatant of test isolate

A drop of each isolate identified from the fermented foods was put on a disc (Samples labelled as A1, A2, B1, B2, C1, D1, D2, E1, E2, F1 and F2)

1. A1 from Sour Power B
2. A2 from Sour Power B
3. B1 from Sour Power B
4. B2 from Sour Power B
5. B2 from Sour Power B
6. C1 from Sour Power C 1
7. D1 from Sour Power C 1
8. D2 from Sour Power C 1
9. E1 from Kefir 1
10. E2 from Kefir 1
11. F1 from Kefir 1
12. F2 from Kefir 1

A and B = *Lactobacillus Plantarum*

C and D = *Pediococcus acidilactici*

E and F = *Lactococcus lactis*

The plates were incubated at 37 °C for 24 to 48 hours and zones of inhibition or growth observed. Also plated out was the *E.coli* to establish how many organisms were present.

In further experiments other pathogens, *Salmonella, Staphyloccus aureus* were also used. Sterile discs placed on the surface of the plates were inoculated with a known amount of the overnight suspension of the isolated lactobacillus species (10µl and 20µ). They were incubated at 37°C. Zones around the discs were identified and measured. This stage and subsequent stages was only carried out using the isolate that had shown signs of antimicrobial activity i.e. *Lactobacillus lactis isolated from the kefir.*

The method used to prepare the isolate for antimicrobial testing was to take the isolates that has been re streaked twice onto Tryptone soya agar (Oxoid UK, M0131) adjusted and put into Tryptone soya broth (Oxoid UK, CM0129). These were incubated anaerobically for 24 hours. The broth was spun down, after incubation, using a centrifuge. 1ml of broth was put in each vial and spun at speed 8 for 10 minutes twice. The supernatant was inoculated onto the discs using a sterile pipette. The solid was inoculated into 9ml of Tryptone soya broth (Oxoid UK, CM0129), using a sterile loop and incubated for a further 24 hours. This was pipetted into the pathogen and incubated for 24 hours and then plated onto nutrient agar (Oxoid UKI, CM003). This gave the number of pathogens in the broth.

Plated out were:

- Broth + isolate
- Broth + pathogen
- Broth with isolate and pathogen

Both the supernatant and pellet re suspension were used to establish if there was any difference in results. There is a possibility that cells could break off into supernatant and would therefore act in a different way. These results were compared to identify if there was any difference.

The disc method was repeated to obtain quantitative results using the Kirby Bauer method for discs (Hudzicki, J. 2009). A swab of pathogenic bacteria with a 0.5 turbidity on the McFarland standard was spread onto a plate. The discs were inoculated with 20µl of isolate. The plates was split into 6 and discs were used with and without isolate. Several isolates were used but no zones were identified.

3.4.2. Effect of the isolate on the growth of the pathogens

Organisms were taken from streaked plates and grown overnight in Tryptone soya broth (Oxoid UK, CM0129) as were the pathogens, *E.Coli*, *Salmonella* and *Staphylococcus aureus*. To establish the number of bacteria present, serial dilutions were carried out and plated out on to plate count agar (Oxoid UK, CM 0325). 0.1ml of the pathogen broth culture and 1.0ml of the isolate broth culture were then inoculated into 9 ml Tryptone soya broth (Oxoid UK, CM 0129), plated out on to plate count agar, and incubated aerobically at 37°C to establish initial numbers of the pathogenic bacteria. This mixed broth was then incubated aerobically at 2, 4, 6 and 24 hours at 37°C. After incubation at these times, the solutions were serially diluted down, plated out onto plate count agar and incubated aerobically at 37°C, to establish growth over a period. This showed the effect of the lactic acid bacteria on the growth of the pathogen. Plates were counted and number of colonies recorded. All colonies were counted. This assumed that the lactic acid bacteria would not grow aerobically on the plates over the short period. To grow the lactic acid bacteria, a selective agar and anaerobic incubation of at least 48 hours was needed as established at the start when isolating the lactic acid organisms. However, there is an assumption that the lactic acid may be growing to some extent if it were to be effective against the selected pathogens. Once identification was completed, selected organisms were screened for their antimicrobial activity using MIC (Minimum inhibitory concentration). This establishes the lowest concentration of an antimicrobial that will inhibit the visible growth of a microorganism after overnight incubation.

Using a 96 well Microplate, the vertical axis had 20µl amounts from A to H:

A – Lactic acid isolate
B – Overnight culture of *E.Coli*
C – *E,coli* + Lactic acid isolate
D – Overnight culture of *Salmonella*
E – *Salmonella* + Lactic acid isolate
F – Overnight culture of *Staphylococcus aureus*
G – *Staphylococcus aureus* + Lactic acid isolate
and the horizontal axis 1 to 12 (10µl of the Lactic acid isolate, dilutions 10^{-1} to 10^{-11}). The tray was incubated for 48 hours at 37°C.

Turbidity was observed, by looking for cloudy colouration in the wells, to establish the minimum inhibitory concentration needed to stop the growth of the pathogen.

3.4.3. Method to establish antimicrobial action

Pathogens *E.coli*, *E.coli* C822037, *Salmonella Spp* and *Staphylococcus aureus* were grown in Tryptone soya broth and incubated aerobically overnight at 37 °C. The sample of lactic acid bacteria isolate was inoculated into Tryptone soya broth and incubated anaerobically at 30°C. After overnight incubation, the bacteria were streaked on to selective agar to obtain fresh, pure colonies. *E.coli* and E.coli C822037 were put on to Eosin methylene blue agar (EMB) and incubated aerobically at 37°C. *Staphylococcus aureus* was put onto Baird- Parker agar BPA and incubated aerobically at 37°C. *Salmonella Spp* was put on to Bismuth sulphite agar (BSA) and incubated aerobically at 37°C. The lactic acid isolate (E) was put on to TSA- adjusted and incubated anaerobically at 30°C Individual colonies were taken off the plates and put into Ringer's solution to achieve a turbidity of 0.5 McFarland standard. These were serial diluted and plated out on to selective agars to confirm the number of bacteria present.

Using a 96 well Microplate, wells were inoculated with 25µl of each pathogen. This was carried out in triplicate. To each well was added 25µl of the Lactic acid bacteria and 50µl of tryptone soya broth. This was repeated using a broad-spectrum test antibiotic - Penicillin Streptomycin (Sigma – Aldrich, 10,000units of penicillin and 10mg of streptomycin/mL) in place of the isolate (E2 originating from Kefir sample 1) . Also, tested was the lactic acid bacteria in double volume with no pathogen present and the pathogen with just Ringer's solution and broth.

Table 4. **Layout of 96 well Microplate**

	1	2	3	4	5	6	7	8	9	10	11	12
A	100µl TSB	100µl TSB	100µl TSB									
B	100µl Isolate E2	100µl Isolate E2	100µl Isolate E2									
C	25µl *E.coli* + 50µl TSB +25µl E2	25µl *E.coli* + 50µl TSB +25µl E2	25µl *E.coli* + 50µl TSB +25µl E2	25µl *E.coli* + 50µl TSB +25µl Antibiotic	25µl *E.coli* + 50µl TSB +25µl Antibiotic	25µl *E.coli* + 50µl TSB +25µl Antibiotic	25µl *E.coli* + 50µl TSB +Ringer's solution	25µl *E.coli* + 50µl TSB +Ringer's solution	25µl *E.coli* + 50µl TSB +Ringer's solution			
D	25µl *E.coli* C822037 + 50µl TSB +25µl E2	25µl *E.coli* C822037 + 50µl TSB +25µl E2	25µl *E.coli* C822037 + 50µl TSB +25µl E2	25µl *E.coli* C822037 + 50µl TSB +25µl Antibiotic	25µl *E.coli* C822037 + 50µl TSB +25µl Antibiotic	25µl *E.coli* C822037 + 50µl TSB +25µl Antibiotic	25µl *E.coli* C822037 + 50µl TSB +25µl Ringer's solution	25µl *E.coli* C822037 + 50µl TSB +25µl Ringer's solution	25µl *E.coli* C822037 + 50µl TSB +25µl Ringer's solution			
E	25µl *Salmonella* + 50µl TSB +25µl E2	25µl *Salmonella* + 50µl TSB +25µl E2	25µl *Salmonella* + 50µl TSB +25µl E2	25µl *Salmonella* + 50µl TSB +25µl Antibiotic	25µl *Salmonella* + 50µl TSB +25µl Antibiotic	25µl *Salmonella* + 50µl TSB +25µl Antibiotic	25µl *Salmonella* + 50µl TSB +25µl Ringer's Solution	25µl *Salmonella* + 50µl TSB +25µl Ringer's Solution	25µl *Salmonella* + 50µl TSB +25µl Ringer's Solution			
F	25µl *Staphylococcus aureus* + 50µl TSB +25µl E2	25µl *Staphylococcus aureus* + 50µl TSB +25µl E2	25µl *Staphylococcus aureus* + 50µl TSB +25µl E2	25µl *Staphylococcus aureus* + 50µl TSB +25µl Antibiotic	25µl *Staphylococcus aureus* + 50µl TSB +25µl Antibiotic	25µl *Staphylococcus aureus* + 50µl TSB +25µl Antibiotic	25µl *Staphylococcus aureus* + 50µl TSB +25µl Ringer's solution	25µl *Staphylococcus aureus* + 50µl TSB +25µl Ringer's solution	25µl *Staphylococcus aureus* + 50µl TSB +25µl Ringer's solution			
G	50µl TSB +50µl E2	50µl TSB +50µl E2	50µl TSB +50µl E2	50µl TSB +50µl Ringer's solution	50µl TSB +50µl Ringer's solution	50µl TSB +50µl Ringer's solution						
H	50µl TSB +50µl Antibiotic	50µl TSB +50µl Antibiotic	50µl TSB +50µl Antibiotic									

Key

Test isolate + Pathogen + Broth

Antibiotic control + Pathogen + Broth

Ringer's solution + Pathogen + Broth

Controls with no pathogen

The plate was incubated aerobically at 37°C for 24 hours and the wells were checked for turbidity using a turbidity meter. A mixture of each of the three wells was plated out of each pathogen plus the lactic acid bacteria E and each pathogen plus Ringer's solution on to the above selective agar. Plate counts could not be carried out at this stage as the mixture in the wells was not consistent. Some had sediment at the bottom of the wells. In addition, a sample of these was prepared for slides for the electron microscope.

3.4.4. Observation of the effect of the isolate on pathogens under the electron microscope.

The effect of the isolate on *E.coli*, *E.coli* C822037, *Salmonella* and *Staphylococcus aureus* was observed under the scanning electron microscope. This works by producing images of the sample by scanning the surface with a focused beam of electrons. The electrons and atoms in three sample interact and produce information about the surface and composition of the sample. The microscope can produce images with a resolution better than 1 nanometre. Due to the very narrow electron beam, SEM images have a large depth of field and producing a three-dimensional appearance, which is useful for understanding the surface structure of a sample. (Stokes, 2008).) It is a very useful tool for this project, as we want to see how the isolate has affected the cells and bio film. By observing the images and comparing the pathogens on their own and then with the isolate we can and determine if the morphology of the cells have changed, if they are damaged and how extensively. The biofilm can be compared to what changes, if any, have taken place and if the type and structure of the biofilm is the same or different. The shape of the pathogens should be rod shaped and the isolate *(Lactococcus lactis*) cocci shaped. The biofilm would be expected to look stringy or sticky and join the bacterial cells together in a mass.

The following fixation method, obtained from the electron microscope laboratory, was used to prepare the slides for the electron microscope: 450µl of the sample was prepared in 450µl of 2.5% glutaraldehyde in 0.1 M phosphate buffer solution (PBS) and incubated at 4°C overnight. The sample was then washed in 0.1 M PBS twice by spinning the sample down in a centrifuge for 5 minutes at full speed and then adding 500µl of PBS. The sample was washed in 20% methanol for 30 minutes, then in 40% methanol for 30 minutes and again with 60%, 80% and finally 100% methanol for 30 minutes. The isolate in the 100% methanol was the put onto microscope slides and left to dry in a vacuum assisted desiccator overnight. The slides were then observed under the electron microscope.

4.0 RESULTS, DISCUSSION & CONCLUSION

4.1 Introduction

Lactococcus lactis, isolated from kefir, proved to show some signs of antimicrobial activity against pathogens, *E.coli*, *E.coli* C822037 and *Salmonella* with limited activity against *Staphylococcus aureus.*

There were several problems with the sour power starter culture. Not many bacteria were present, and several samples had to be taken before any results were obtained. There was success eventually, but not vast amounts of microorganisms were isolated. The samples where organisms were isolated and tested were from Sour Power 16/3, Sour power A, B, and C. Isolates were obtained from the kefir and kombucha to a varying degree of success. As the *Lactococcus lactis,* from the kefir, was the only organism showing antimicrobial activity this isolate was used for further investigation and no further work was carried out on the isolates from the other foods.

4.2. Results/Conclusions

4.2.1 Isolation and identification of LAB from fermented food and drinks

4.2.1.1 Isolation and identification of LAB from Sour power

Table 5. Sour Power starter culture 16/3 plate counts

Agar Plate	**10^{-2}**	**10^{-3}**	**Number of organisms /ml From plates with 30 to 300 colonies**
TSA Aerobic 24 hr	No growth	No growth	
TSA Aerobic 48hr	Growth unable to count	2 types of white/ cream colonies	
WL Aerobic 24hr + 48hr	No growth	No growth	
WL Anaerobic 24hr	26	2	
WL Anaerobic 48hr	26	2	
RR	No growth	No growth	

Anaerobic 24hr + 48hr			
RR Anaerobic Layer 24hr +48hr	Growth unable to count	Growth unable to count	
Malt Extract Agar (MEA) Aerobic 24hr	33	3	3.3×10^3
Malt Extract Agar (MEA) Anaerobic 48hr	Growth unable to count	Growth unable to count	

Most plates were impossible to count however, using Gram staining methods and the API kits, the colonies taken from the plates below were gram positive and identified as:

From the 10^{-7} RR overlay plate the bacteria identified was *Aerococcus vividans*

From the 10^{-2} RR surface plate the bacteria identified was *Lactobacillus curivatus*

The yeasts identified were:

From the 10^{-4} WL plate the yeast identified was *Candida kefyr*

From the 10^{-4} WL plate the yeast identified - *Candida sphenca*

From the 10^{-3} WL plate the yeast identified – *Saccharomyces cervis*

Table 6. Check of selective agar using a stock culture of *Lactobacillus* Spp.

Agar	**Colonies**
MRS (De Man, Rogosa Sharpe Agar)	Growth of small colonies
MEA (Malt Extract Agar)	No growth
RR (Raka Ray Agar)	Small colonies
WL (Wallerstein Laboratory Nutrient Agar	Good growth of yeasts
TSA (Tryptone Soya Agar)	Limited growth of colonies

These results showed that the MRS agar, RR agar and TSA were effective because colonies were present. The MEA would not expect to support the growth of *Lactobacillus* Spp only yeasts and moulds. The WL agar did have growth of yeasts on it, this was probably due to contamination from the air as no yeast colonies should have been present in the stock culture of *Lactobacillus* Spp.

Table 7. Gram stain results for isolates from Sour Power samples

Media and sample colony	
MRS agar dilution 10^{-3} Sour Power B	Gram -ve cocci small cream/ white round colonies = yeasts Gram +ve large rods = yeasts

MRS agar dilution 10^{-2} Sour Power16/3	Gram -ve cocci small cream/ white round colonies = yeasts (as above)
RR dilution 10^{-2} Sour Power A	Small translucent colonies

Table 8. Starter culture Sour Power C on MRS

Dilution	**MRS agar (Number of colonies and description)**	**RR agar (Number of colonies and description)**
10^{-1} to 10^{-5}	Possible Lactobacillus - too many to count	Possible Lactobacillus - too many to count
10^{-6} to 10^{-9}	Yeasts	Yeasts

4.2.1.2 Isolation and identification of LAB from kombucha

Table 9. Plate dilutions of Kombucha and Kombucha A and B on De Man, Rogosa Sharpe Agar (MRS agar

	Dilution	**dilution**	**dilution**	**Number of bacteria/ml**	
	10^{-2}	10^{-3}	10^{-4}		
Kombucha A			52	5.2×10^5	
Kombucha B		200		2.0×10^5	
Kombucha	57			5.7×10^3	

The white colonies isolated were Gram positive and then tested with the API kits. Further plating out in triplicate of Kombucha was carried out.

Table 10. Plate counts from a kombucha sample on to Tryptone Soya Agar Adusted

	Dilution	**Number of bacteria/ml**
	10^{-2}	
Kombucha	54	5.4×10^3

Table 11. Streaked out plates from Kombucha starter culture grown for 48 hrs anaerobically

Kombucha sample	**Agar**	**Results/ Appearance**
K1 x2	TSA	Yeasts/ moulds – No Lactic acid bacteria
K1 x2	MRS	Yeasts/ moulds – No Lactic acid bacteria
K2 x2	TSA	Small pale cream colonies –possible lactic acid bacteria
K2 x2	MRS	Small pale cream/ translucent colonies –possible lactic acid bacteria

K2A x 2	TSA	No growth
K2A x 2	MRS	No growth
K2B x 2	TSA	Small pale cream colonies –possible lactic acid bacteria
K2B x 2	MRS	Small pale cream/ translucent colonies –possible lactic acid bacteria
K4 x 2	TSA	No growth
K4 x 2	MRS	No growth
K4A x 2	TSA	Yeasts/ moulds – No Lactic acid bacteria
K4A x 2	MRS	Yeasts/ moulds – No Lactic acid bacteria
K5 x 2	TSA	No growth
K5 x 2	MRS	No growth
K5A x 2	TSA	No growth
K5A x 2	MRS	No growth
K6 x 2	TSA	Yeasts/ moulds – No Lactic acid bacteria
K6 x 2	MRS	Yeasts/ moulds – No Lactic acid bacteria
K6A x2	TSA	Yeasts/ moulds – No Lactic acid bacteria
K6A x 2	MRS	Yeasts/ moulds – No Lactic acid bacteria
K7 x 2	TSA	Small translucent colonies – possible Lactic acid bacteria
K7 x 2	MRS	No growth
K7A x 2	TSA	No growth
K7A x 2	MRS	No growth

Key

K - Original Kombucha Sample

KA - Kombucha Sample A

KB - Kombucha Sample B

Numbers 1 to 7 - Plate Number

TSA – Tryptone Soya agar

MRS - De Man, Rogosa Sharpe Agar

Table 12. Gram stain Results

Kombucha sample	**Agar**	**Gram Stain Result**
K2	Tryptone Soya agar TSA	Positive
K2	De Man, Rogosa Sharpe Agar MRS	Positive
K2B	Tryptone Soya agar TSA	Positive
K2B	De Man, Rogosa Sharpe Agar MRS	Positive

Identification of colonies grown from the Kombucha starter culture were inconclusive when tested on API kits. It was quite probable that lactic acid bacteria would not be isolated from kombucha because the main bacteria in Kombucha are acetic acid. Due to time, colonies isolated from Sour power 16/3, Sour power C and kefir were concentrated on for identification of antimicrobial properties. The isolation of lactic acid bacteria proved quite difficult. Numbers and variety were low. The results were affected by the use of old cultures and at times the overgrowth of yeasts. The largest problem was to keep consistency as much of the work entailed subbing the bacteria on. It was very difficult to have enough continuous days to do the work and bacteria had to be subbed on or stored for longer than desired periods in the fridge. From this, there was always the risk that the cultures became contaminated.

4.2.1.3 Isolation and identification of LAB from kefir

Table 13. Results for Kefir after 48-hour incubation anaerobically at 30°C

Dilution	**De Man, Rogosa Sharpe Agar MRS agar (Number of colonies and description)**
10^{-1} to 10^{-7}	Too many to count
10^{-8}	7 small white/ cream translucent colonies
10^{-9}	0

No conclusive colony counts because there was not between 30 and 300 colonies present

Table 14. Results of streaked plates from Sour Power and Kefir

	Fermented Food	**Description of Colonies**
A	Kefir	Small colonies possibly Lactic acid bacteria
B	Sour Power 16/3	Small colonies possibly Lactic acid bacteria
C	Sour Power C	Yeast and fungi, some possible Lactic acid bacteria
D	Sour Power A	Yeasts discarded plates no small colonies
E	Sour Power B	Yeasts discarded plates no small colonies

The colonies from the Sour power 16/3, C and Kefir were then streaked again on to PH adjusted TSA and MRS agar. Duplicate plates of each and two colonies taken off each plate.

Table 15. Colonies streaked on to PH adjusted Tryptone Soya Agar(TSA) and De Man, Rogosa Sharpe Agar (MRS Agar)

Source	PH adjusted TSA	MRS
Sour Power (16/3) 1	White colonies (yeast looking)	White colonies (yeast looking)
Sour power (16/3) 2	Small translucent cream colonies	Small translucent cream colonies
Sour power C 1	Small translucent white colonies	Small translucent white colonies
Sour power C 2	Small translucent white colonies and mould	Small translucent white colonies and mould
Kefir 1	Small translucent white colonies and mould	Small translucent white colonies and mould
Kefir 2	Small translucent white colonies and mould	Small translucent white colonies and mould

These colonies were then Gram stained and the Sour Power (16/3) 2, C 1 and 2 and Kefir 1 and 2 were all found to be all Gram positive. These then went on to be identified with API strips.

Table 16. API kits results

Source	PH adjusted TSA	MRS agar	% Likelihood
A. Sour Power 2	*Lactobacillus Plantarum*	*Lactobacillus Plantarum*	73.4
B. Sour Power 2	*Lactobacillus Plantarum*	*Lactobacillus Plantarum*	73.4
C. Sour Power 1	*Pediococcus acidilactici*	*Pediococcus acidilactici*	99.9
D. Sour Power 1	*Pediococcus acidilactici*	*Pediococcus acidilactici*	99.9
E. Kefir 1	*Lactococcus lactis*	*Lactococcus lactis*	83.4
F. Kefir 1	*Lactococcus lactis*	*Lactococcus lactis*	82.9

These bacteria were then used to see if they displayed any anti- microbial properties. Each bacteria was tested twice in the experiments'. A1 and A2 etc.

4.2.2. Disc method.

The plates below show how the isolate has an effect on the surrounding pathogen. The first set of plates, below, show the discs that were inoculated with the isolated bacteria that was spun down and then re -suspended in broth. The second set of plates show the discs inoculated with the isolated bacteria and the broth it was grown in, inoculated directly on to the discs.

Key for photographs see Appendix 4

1. A1 from Sour Power B
2. A2 from Sour Power B
3. B1 from Sour Power B
4. B2 from Sour Power B
5. B2 from Sour Power B
6. C1 from Sour Power C 1
7. D1 from Sour Power C 1
8. D2 from Sour Power C 1
9. E1 from Kefir 1
10. E2 from Kefir 1
11. F1 from Kefir 1
12. F2 from Kefir 1

A and B = *Lactobacillus Plantarum*

C and D = *Pediococcus acidilactici*

E and F = *Lactococcus lactis*

The results showed that the isolate from Kefir 1 had growth around the disc, thus either growing over the top of the pathogen or growing in place of the pathogen. This happened the same if the isolate was left in the broth or spun down. Thus, concluding that the isolate is, as effective left in the broth and spinning down has no real advantage. This isolate was identified as *Lactococcus lactis,* from these results *Lactobacillus Plantarum* and Pediococcus *acidilactici* did not exhibit growth around the discs from either the broth or when spun down, concluding that they were not showing any antimicrobial activity against *E.coli.* This experiment was repeated using the isolate *Lactococcus lactis* on both TSA plates and nutrient agar plates seeded with *E.coli*, turbidity of 0.5 on the Mc Farland standard. 20 µl of the isolate was added to the disc as above. The growth round the discs was measured after incubation at 37°C for 48 hours as shown in Table 15.

Table 17. Measurements of the growth zones around the discs 1.

Disc with isolate *Lactococcus lactis*	**Nutrient Agar1**	**Nutrient Agar2**	**TSA 1**	**TSA 2**
1	10mm	10mm	10mm	12mm
2	6mm	11mm	12mm	9mm
3	6mm	10mm	9mm	11mm
Average	7mm	10mm	10mm	11mm

4.2.2.1. Effect of *Lactococcus lactis* (isolated from Kefir) on different pathogens

Pathogens *E.coli*, *Salmonella* and Staphylococcus *aureus* were then used in the seeded plates to see the effectiveness of the isolates 9, 10, 11 and 12 *Lactococcus lactis* (E2 from kefir 1) *on* them.

Table 18. Measurement of growth zones around the discs 2.

Bacteria on plate	**10µl of Isolate**	**20µl of Isolate**
E.coli	No visible zone	12mm (Very strong growth)
Salmonella	8mm	12mm
Staph aureus	9mm	10mm

The results show that there is growth around the discs on all the plates of the three pathogens with the effect being the strongest on the *E.coli* when 20 µl of isolate was used, the smaller amount proving ineffective. Growth was visible at inoculation of 10 µl for *Salmonella* and *Staphylococcus aureus.* However, results could vary if the amount of pathogens present or strengths or amounts of isolate were altered. Much more work could be carried out here to look at the effect of concentration of isolate on the pathogens. The effect of the number of pathogens present, other pathogens and a combination of pathogens present. The bacteriocin could be extracted to see how it works on its own and additives could be added as found in the literature review the lactic acid bacteria tend to exhibit stronger antimicrobial action when used in the presence of additives.

The isolate does appear to show some strength against the pathogens as it looks like it is growing over the top. However, further investigation needs to be carried out to see if there is actual reduction in pathogen growth or if in fact the isolate and pathogens are growing together. There are certainly no clear zones showing complete inhibition of the pathogen growth in any of the three-pathogen plates. These experiments were repeated and this time there were no growth or zones around the discs. This could have been due to the test

Lactobacillus lactis not been as strong or stronger/ new pathogens as these were from new batches of strips. The method used was the Kirby Bauer method where the pathogen and isolate were made up to the strength of the Mc Farland standard of 0.5 and spread on to agar plates using a swab. This was to try to gain a consistent method. The McFarland standard of 0.5 is the density of bacterial suspension of 1.5×10^8 colony forming units/ml. Isolates used were E1, E2, F1, F2, all samples of *Lactococcus lactis*. E1 and the pathogens were also plated out to check numbers. Showing from the plates that there were approximately 8×10^4 colony forming units/ml present for the isolate. The pathogens present were also in lesser numbers than the McFarlane scale would indicate. See tables 17 and 18.

Table 19. Numbers of E1 on seeded plates

Dilution	E1	Number of bacteria/ml
10^{-3}	86	8.6×10^4
10^{-4}	8	

Table 20. Numbers of pathogens on seeded plates

Dilution	E.coli	E.coli C822037	Salmonella	Staph. Aureus
10^{-5}	Too many to count	Too many to count	Too many to count	22
10^{-6}	Too many to count	50 5.0×10^7 bact/ml	62 6.2×10^7 bact/ml	1

These results showed that there was no effect on the pathogens from the Lactic acid isolate. Repeated experiments showed poor results with regard to zones. An alternative method of MIC and plating out was carried out observing growth over time.

4.2.2.2. Effect of *Lactococcus lactis* (isolated from Kefir) over time on the growth of the pathogens

The first time this experiment was carried out, it did not give conclusive results as the stock pathogens were contaminated. This method was repeated, and the results are shown in Appendix 5.Tables 27-33

From the results for *E.Coli* and *Salmonella Spp* overtime there was no effect on the growth of the pathogen when the isolate was present. Time 0 hours was the control i.e. the initial number of bacteria present. The pathogen continued to grow over the tested period and there was no reduction in colonies present. From the results for *Staphylococcus aureus*,

overtime there was some effect on the growth of the pathogen when the isolate was present. Time 0 hours was the control i.e. the initial number of bacteria present. The pathogen continued to grow over the tested period, but growth did slow down between 4 and 6 hours.

Discussion

From these results the effect on the amount of time the *Lactococcus lactis* isolate is in the presence of the pathogens *E.Coli* and *Salmonella Spp* does appear to have no effect.

4.2.3. Minimum Inhibitory Concentration (MIC).

Table 21.The results for the MIC.

Organisms	1	2	3	4	5	6	7	8	9	10	11	12
A	+	-	-	--	-	-	-	-	-	-	-	-
B	+	-	-	-	-	-	-	-	-	-	-	-
C	+	+	+	-	-	-	-	-	-	-	-	-
D	+	-	-	-	-	-	-	-	-	-	--	-
E	+	+	+	-	-	-	-	-	-	--	-	--
F	+	-	-	-	-	-	-	-	-	-	-	-
G	+	+	+	-	-	-	-	-	-	-	-	-
H	Row not used											

Key to results

+ = Cloudy/ Visible growth

- = reduction in visible growth

Key to organisms

A Just E1 (*Lactobacillus lactis* isolate)

B *E.Coli*

C *E.coli* + E1 (*Lactobacillus lactis* isolate)

D *Salmonella Spp*

E *Salmonella* = E1 (*Lactobacillus lactis* isolate)

F *Staphyloccus aureus*

G *Staph* + E1 (*Lactobacillus lactis* isolate)

Table 22. E1 and E.coli

Dilution	E1	Number of bacteria/ml	E.coli	Number of bacteria/ml
10^{-5}	76	7.6×10^{6}		
10^{-7}			64	6.4×10^{8}

The Minimum inhibitory concentration for the E1 (*Lactobacillus lactis* isolate) to be effective against the pathogen is at the dilution 10^{-3}

4.2.4. Measurement of turbidity of the isolate and pathogen compared to the turbidity using a wide spectrum antibiotic and a control of Ringers solution.

Table 23. Initial bacteria counts on selective agar.

Dilution	MRS agar (Isolate – Small translucent colonies)	EMB (E.Coli - dark purple colonies)	EMB (E.Coli C822037 – purple colonies)	BSA (*Salmonella Spp* – purple colonies)	BPA (*Staphylococcus aureus* – small black colonies)
10^{-5}	Too many colonies to count	60 (6.0 x $x10^{6}$/ml)	Too many colonies to count	31 (3.1 x $x10^{6}$/ml)	Too many colonies to count
10^{-6}	42 (4.2 $x10^{7}$/ml)	5	43 (4.3 $x10^{7}$/ml)	4	27 (2.7 $x10^{7}$/ml)

Table 24. Averages of turbidity readings (OD) after 24 hour aerobic incubation at 37°C

Well	1-3	4-6	7-9	10-12
A	0.736	0.038	0.036	0.037
B	0.054	0.037	0.038	0.044
C	1.312	0.081	1.510	0.038
D	1.283	0.075	1.419	0.037
E	1.354	0.080	1.162	0.037
F	0.594	0.085	0.651	0.040
G	0.400	0.295	0.039	0.037

H	0.068	0.038	0.037	0.039

Table 25. Averages of turbidity readings (OD) after 48 hour aerobic incubation at 37°C

Well	1-3	4-6	7-9	10-12
A	0.151	0.036	0.036	0.034
B	0.051	0.035	0.035	0.035
C	1.521	0.071	1.331	0.034
D	1.382	0.076	0.990	0.036
E	1.671	0.082	1.482	0.036
F	0.916	0.076	0.353	0.036
G	0.230	0.197	0.037	0.035
H	0.058	0.037	0.037	0.037

Key

Test isolate + Pathogen + Broth

Antibiotic control + Pathogen + Broth

Ringers Solution + Pathogen + Broth

Empty wells

A-H well number

Controls with no pathogen

Table 26. Differences between readings (OD) when Ringers solution present or isolate present

	E2 + TSB	Ringers + TSB	Difference
C - *E.Coli*	1.312	1.510	0.198
D – *E.Coli* C822037	1.283	1.419	0.136
E - *Salmonella*	1.354	1.162	-0.192
F – *Staph aureus*	0.594	0.651	0.057

Table 26, shows a slight reduction in turbidity when the isolate is present compared to the control of Ringer's solution for *E.coli*, E.Coli C822037and *Staph aureus*. However, there is actually more turbidity suggesting growth of *Salmonella* when the isolate is present. A1 well looks to be contaminated as it is a much high reading than A2 and A3 which have exactly the same broth at this stage, the pathogens do not seem to be significantly reduced by the test Lactic acid bacteria. They are slightly lower readings than the wells containing Ringers rather than the isolate. The test antibiotic has shown to work significantly well, and all the readings

are low G4 is also high than expected. All the controls give low readings. Because the findings with the isolate in are not significantly different, it is impossible to conclude that the isolate is working as an effective antimicrobial. The micro well plate was incubated for a further 24 hours aerobically at 37°C, as bacteria such as *Salmonella* Spp usually take longer than 24 hours to give good growth. When looking at the table the turbidity for *Staphylococcus aureus* + isolate + broth does seem lower, however when analysing the controls *Staphylococcus aureus*+ ringers + broth is also lower than the other pathogens . This could be due to poor mixing of the bacteria in suspension, as across the three wells there is variation. Again, with this experiment different media, temperatures, concentrations and times could have been tested. In addition, different pathogens could have been used. The experiment needed to be duplicated and perhaps a better way to count the organisms used as plate counts were not effective due to low amounts of culture and the inability to mix the solutions in the cells to give an accurate sample and reading. The turbidity machine was working at its limit and would have struggled with any higher turbidity readings.

4.2.5 Discussion of the Isolation and identification, antimicrobial activity and effect of time of Lactic acid bacteria from fermented food and drink against pathogens.

A limited number of lactic acid bacteria were isolated and identified. There were several problems with the sour power starter culture. Not many bacteria were present, and several samples had to be taken before any results were obtained. There was success eventually, but not vast amounts of microorganisms were isolated. The samples where organisms were isolated and tested were from Sour Power 16/3, Sour power A, B, and C. Isolates were obtained from the kefir and kombucha to a varying degree of success. When the organisms were isolated from two different media, using Gram stain and API kits there were varying percentage likelihoods that this was the exact organism. Here further tests such as PCR could have been carried out for increased certainty.

Lactobacillus Plantarum was isolated from Sour power 2 with a likelihood of 73.4%. The most common bacteria found in sour beer are *Lactobacillus* and *Pediococcus* (Bernot, 2015). *Lactobacillus Plantarum* has been isolated from several fermented foods and shown to have antimicrobial properties, such as in homemade Korean- style pickled cabbage (Lin and Pan, 2017), Nigerian indigenous foods (ogi, fufu, garri & nono) (Ogunshe et al., 2007). It was also found in Kahudi fermentation (Goswami et al., 2017), Balkan cheeses (Chingwaru and Vidmar, 2017) and from yak cheese (Pei et al., 2018). It has also been isolated from Chhang, a traditional fermented beverage from Himachal Pradesh and this strain of *Lactobacillus Plantarum* is thought to be a potential agent to be used in the development of new pharmaceuticals and functional food preparations. (Handa and Sharma, 2016). The Bacteriocin-M1-UVs300 produced by *Lactobacillus plantarum* M1-UVs300, isolated from fermented sausage was found to exhibit antimicrobial activity against Gram positive bacteria

and Gram negative bacteria and that it had the potential to act as a natural preservative in the food industry. (An et al. 2017).

Pediococcus acidilactici was isolated and identified from Sour Power 1 with a 99.9% likelihood. *Pediococcus species* have been used occasionally in dairy products and have been shown to with stand gut conditions, however probiotic research is in its infancy compared to strains from the genera lactobacillus. Some strains produce pediocins (Holland et al., 2011).

In these investigations neither *Lactobacillus Plantarum* nor *Pediococcus acidilactici* exhibited antimicrobial properties. However, much more work could be carried out by testing against other pathogens. Also, it must be remembered that the number of organisms present in the food affects effective antimicrobial activity. It is also influenced by the method of application, the food components, pH and temperature (Lin and Pan 2017). Therefore there are many variables that could be applied to check if there are any conditions where these organisms would exhibit some antimicrobial activity or if indeed these strains are not active. Further work could be carried out on other pathogens. Although, *Lactobacillus Plantarum* isolated here from the sour power16/3 did not show antimicrobial properties against *E.coli* other research has shown that a bacteriocin produced from *Lactobacillus plantarum* ZJ008, which was isolated from fresh milk, showed broad-spectrum antimicrobial behaviour against Gram-positive and Gram-negative bacteria, particularly *Staphylococcus spp*. The activity of the bacteriocin was bactericidal but it did not cause cells lysis but pore formation on the surface of the bacterial membrane. These results suggest that the bacteriocin could be very useful in controlling and inhibiting *Staphylococcus* species in the food industry (Biswas et al., 2017). A substance named CO26H11N3 produced by lactic acid bacteria was found to have a broad antimicrobial spectrum even to multidrug resistant pathogens. (Zhang et al., 2017). This has the potential to be a new preservative or 'antibiotic' as does a newly discovered bacteriocin produced by *Lactobacillus plantarum* A-1, plantaricin ASM1 (PASM1), which showed stability in neutral and weak alkaline conditions. It is heat-stable but digested by trypsin and inhibits the growth of other lactic acid bacteria, such as *Lactobacillus*, *Leuconostoc* and *Enterococcus*. PASM1 showed stability in a wide pH range compared to nisin and therefore has a possible application in the food industry (Hu et al., 2017).

Bacteriocins used in the food industry as natural preservatives are generally considered safe (Zacharof and Lovitt, 2012). The use of antimicrobial compounds (e.g. bacteriocins) to fight against pathogens and food spoilage has proved to be effective. Cotter et al. (2013) stated that that they are antibiotics that have the potential to be used against multi drug resistant pathogens. Cavera et al. (2015), Lu et al. (2014) acknowledge that various bacteriocins have been isolated to inhibit both Gram positive and Gram-negative pathogens.

Other research has shown the antimicrobial effect of bacteriocins from lactic acid bacteria. These include *Lactobacillu*s strains from commercially available food in Gulbarga market

produced potential probiotics for the prevention of bacterial gastro-intestinal infection and other related enteric infections. (Prabhurajeshwar and Chandrakant, 2018).

Lactococcus lactis was isolated from kefir 1 and is commonly found in kefir (Leite et al., 2013). It has desirable features such as it is considered safe, it has probiotic properties, the absence of inclusion bodies and endotoxins, surface display and extracellular secretion technology and a diverse selection of cloning and inducible vectors (Song et al., 2017). It is commonly associated with the dairy industry but was originally isolated from plants and only became active once gaining entry into the gut via animals (Bolotin et al. (2001).) It has been used for centuries in fermentation to make cheese, yoghurt and other dairy products. It has been isolated from traditional Sardinian dairy products (Cosentino et al. 2012) and other foods such as mushroom substrate (Bolcan et al. 2017).

No lactic acid bacteria were isolated from the kombucha however this is probably not surprising as they tend not to be common in Kombucha (Jarrell et al., 2000 and Jonas et al., 1998).

The production of the acid from *Lactobacillus lactis* helps flavour in food and can help to preserve it. Some enhance this by producing bacteriocins. It has become the model Lactic acid bacteria for genetic engineering over the past two decades and has become a successful microbial cell factory. As well as a major role in fermentation recent studies have shown the potential of *Lactobacillus lactis* as a bio-preservative against *Listeria Monocytogenes* (Unlu et al. 2016) and (Bolocan et al. 2017). Biocins have the potential to be useful for a variety of clinical uses (Cirkovic et al. 2016). They can be used to reduce biofilm. Examples of bacteriocins include Lacticin 3147 56, Lacticin Q/Z and Lsb B 58.However the best well known is Lantibiotic (nisin). A *Lactobacillus lactis* is also so used to produce ethanol for biofuels. Its ability to survive passage through the gut unlike lactobacillus species as it does not colonize the gut it can be used to potentially treat inflammatory bowel disease. It has also the potential to deliver cancer vaccines (Son et al. 2017). The results show that *Lactococcus lactis* exhibited antimicrobial activity against the pathogens *E.coli, E.coli* C822037, *Salmonella spp* and to some extent *Staphylococcus aureus.* However, the length of time the pathogens were in the presence of the isolate did not show any real significance.

Many other factors could be investigated to establish the effectiveness of the isolate as an antimicrobial agent. Further tests need to be carried out to establish if the isolates would ever be effective in the gut when competing with other bacteria, stomach acids etc. and also if different foods would alter its effectiveness. As established in the literature review other additives tend to make the isolated bacteriocins work more effectively as antimicrobials. It would need to be established if this would apply to the whole bacteria. The amounts and which additives / micro particles would be effective would need to be investigated. Work

could be carried out on just isolating the bacteriocin from the *Lactobacillus lactis* and measuring its effectivity and comparing this to when the intact bacterium is used. The isolate needs be tested at a wider range of concentrations over a longer test period to establish if either of these factors have any effect and the isolate could be grown in various nutrient broths and over various times to establish if there are varying results.

4.2.6 Observations from the electron microscope

The images from the electron microscope show the pathogen and isolate and then the control of the pathogen and Ringer's solution. The images of the isolate with no pathogen present did not produce any clear images of bacteria. These images would have been useful to see if any biofilm was present and if so, how that changed when the pathogens were present. Some lactic acid bacteria are capable of controlling the formation of biofilm and inhibiting the growth of pathogenic organisms (Karska-Wysocki et al., 2010).

When comparing the two pictures of the E.coli with isolate and E.coli with Ringer's solution it can be seen that there has been some change.

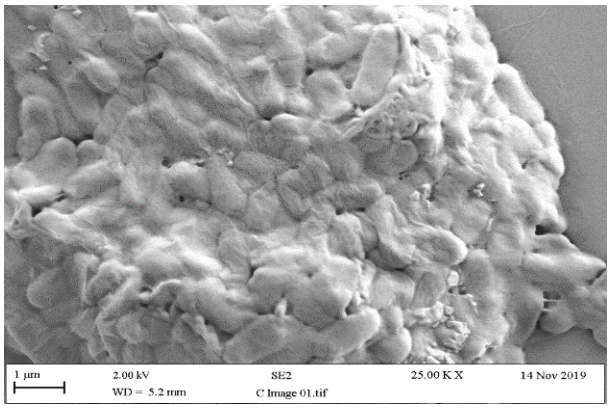

Figure 9. SEM image of isolate and *E.coli*

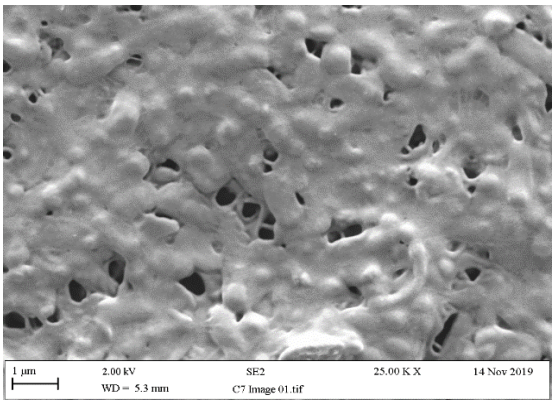

Figure 10. SEM image of Ringer's solution and *E.coli*

In Figure 10, the E.coli in the Ringer's solution has a sticky/string like biofilm present, which has been reduced when the isolate was added (Figure9).Rod shaped cells can be seen in both images but look flatter and more merged in Figure 9.

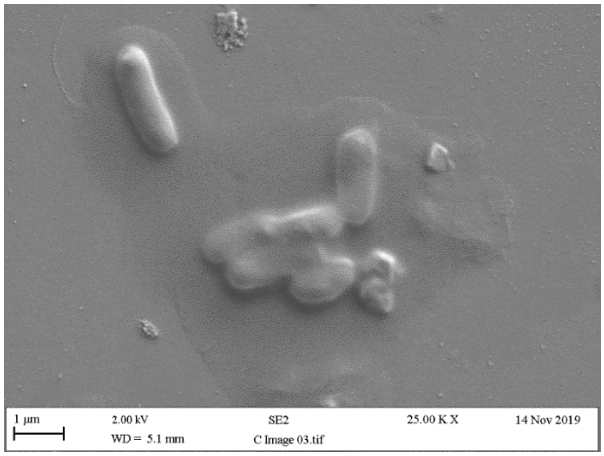

Figure 11. SEM image of isolate and *E.coli*

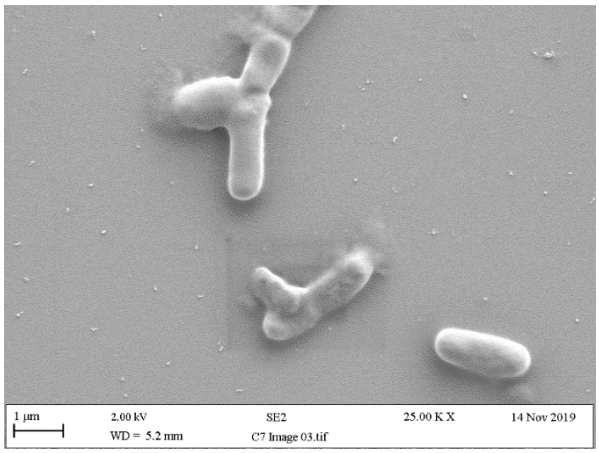

Figure 12. SEM image of Ringer's Solution and *E.coli*

In Figure 11 and 13, the rod shaped E.coli cells look more merged than when they are in Ringer's solution in Figure 12 and 14. In Figure 13 it is difficult to see any *Lactobacillus lactis* cocci shaped cells.

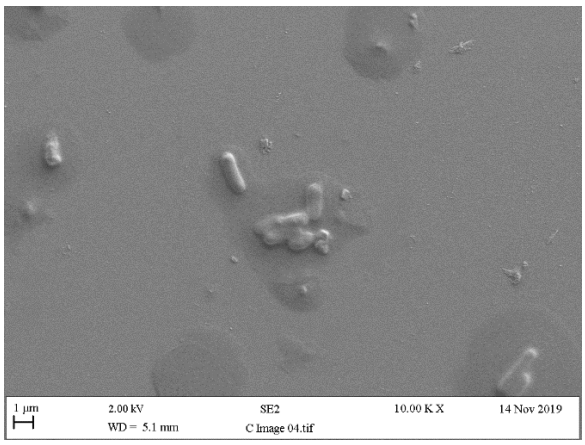

Figure 13 SEM image of isolate and *E.coli*

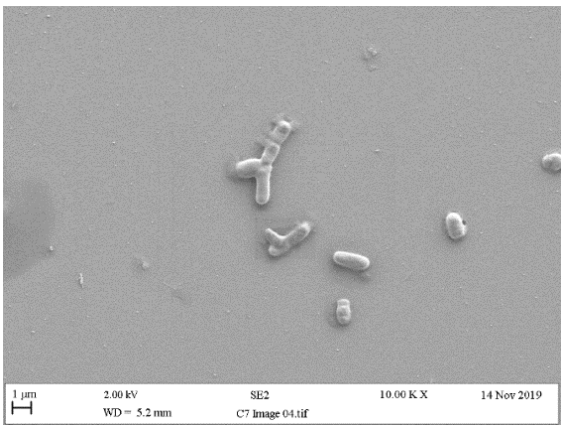

Figure 14. SEM image of Ringer's Solution and *E.coli*

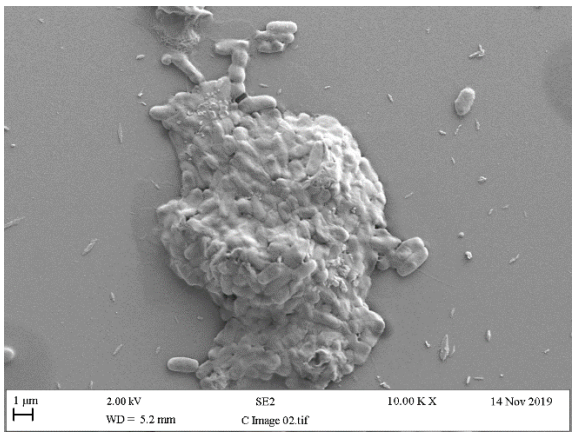

Figure 15. SEM image of isolate and *E.coli*

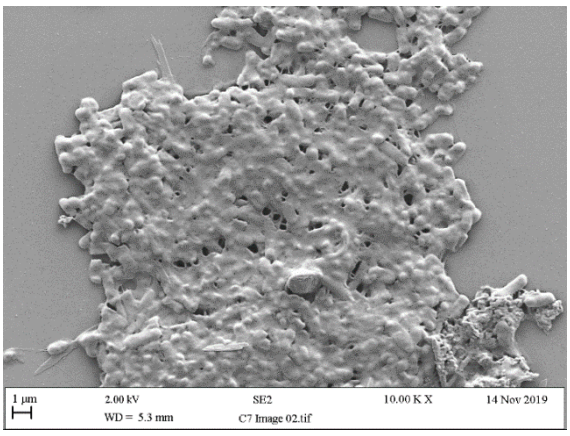

Figure 16. SEM image of Ringer's Solution and *E.coli*

Figure 16 shows gaps and strands where there is just *E.coli* present, possibly biofilm. Where the isolate and E.coli are present in figure 15 the cells are more merged together and there are no obvious stringy areas and less holes on the surface. The results for *E.coli* C822037 are very similar to the above strain of *E.coli*. The possible sticky biofilm is not as prevalent in the sample containing the isolate. Thus, concluding that the isolate is having some effect on the E.coli.

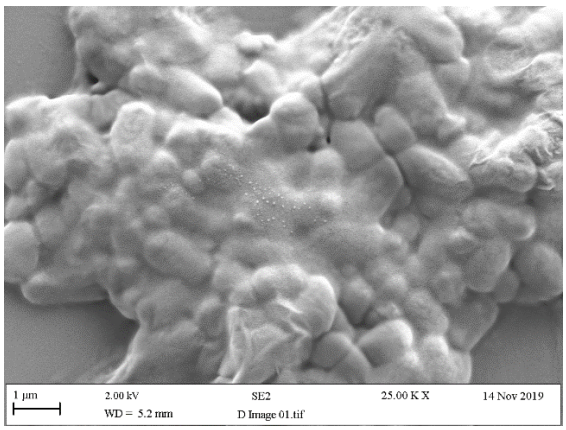

Figure 17. SEM image of isolate and *E.coli C822037*

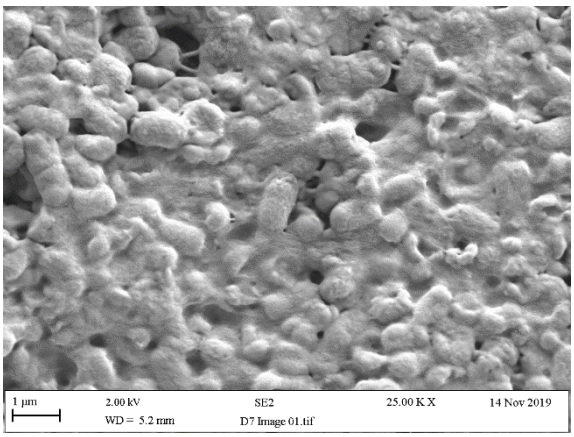

Figure 18. SEM image of Ringer's solution and *E.coli C82203*

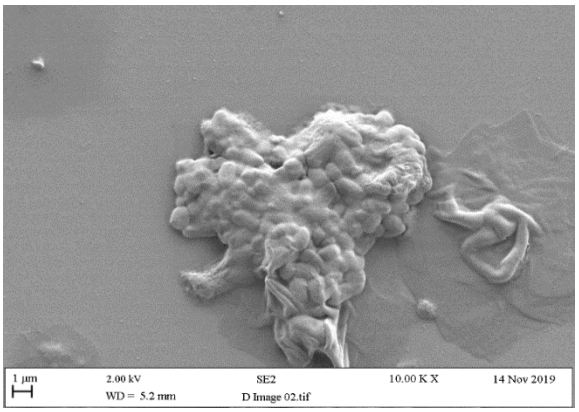

Figure 19. SEM image of isolate and *E.coli C822037*

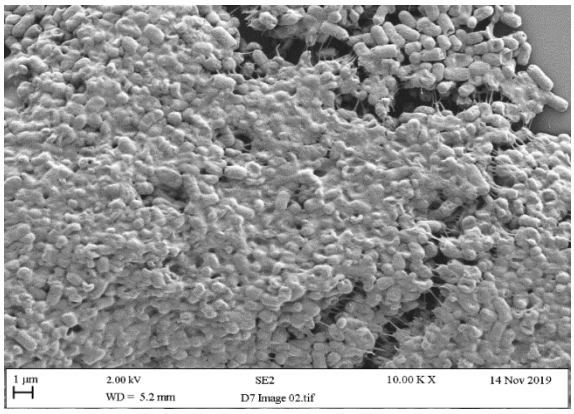

Figure 20. SEM image of Ringer's solution and *E.coli C822037*

Figures 18 and 20 show a sticky looking film when only *E.coli C822037* was present. Whereas in Figures 17 and 19 they show merged cells, rod shaped with no strands between the cells when both the isolate and *E.coli* were present. A few cocci shaped cells can also be seen.

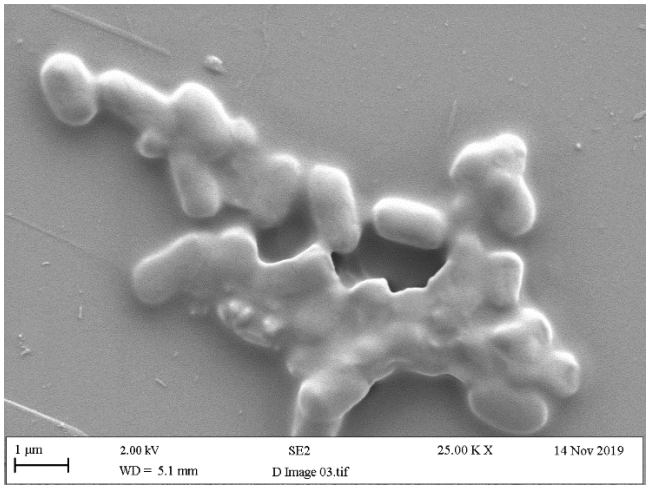

Figure 21. SEM image of isolate and *E.coli C822037*

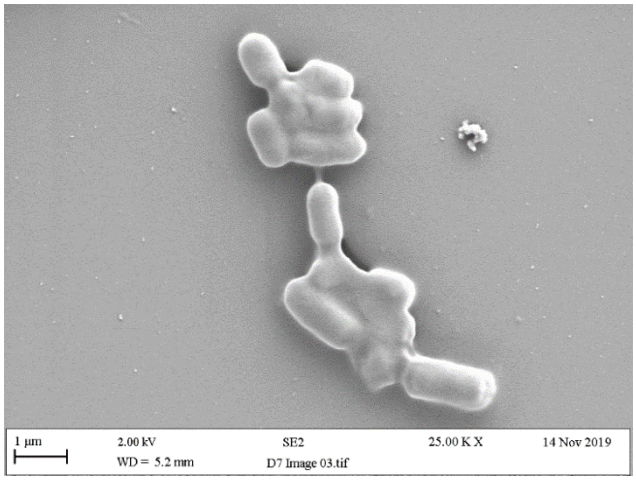

Figure 22. SEM image of Ringer's solution and *E.coli C822037*

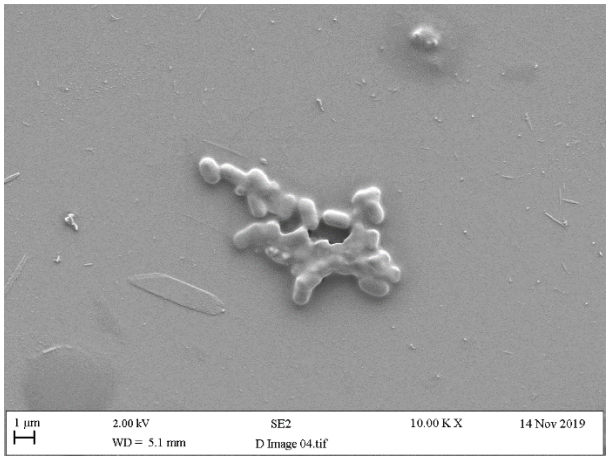

Figure 23. SEM image of isolate and *E.coli C822037*

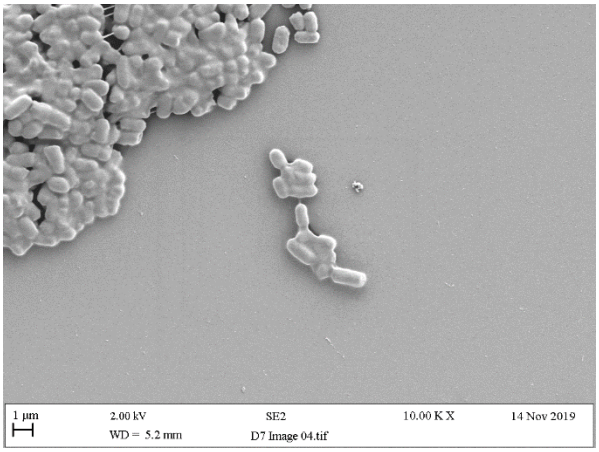

Figure 24. SEM image of Ringer's solution and *E.coli C822037*

Figures 21, 22, 23 and 24 do not show much difference in cell shape or biofilm. However, the cells in figure 23 do look slightly more merged than the ones in Figure 24.

The isolate and *Salmonella* showed cocci shaped bacteria present and some rods, whereas with the *Salmonella* and Ringer's solution rods can be seen. It is difficult to conclude from these images if the isolate has removed or damage the rods of *Salmonella* . There does look to be a reduction in rods with the isolate present but further images need to be taken to obtain the correct conclusion.

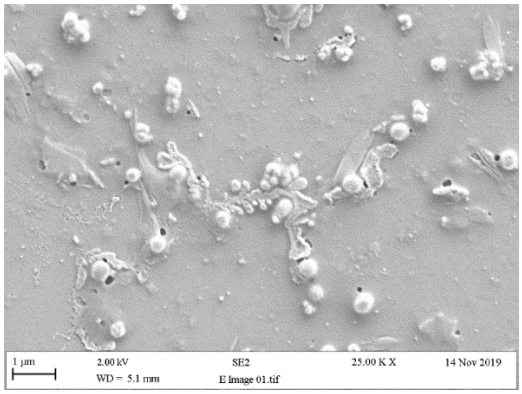

Figure 25.

SEM image of Isolate and *Salmonella Spp*

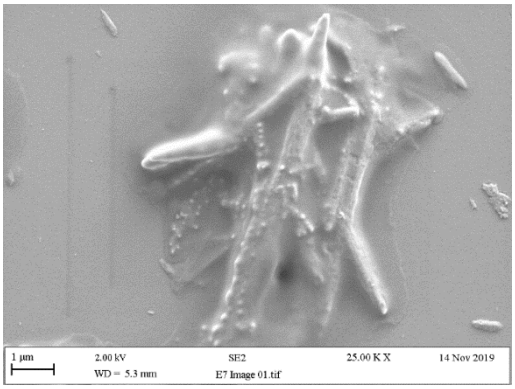

Figure 26.

SEM image of Ringer's solution and *Salmonella Spp*

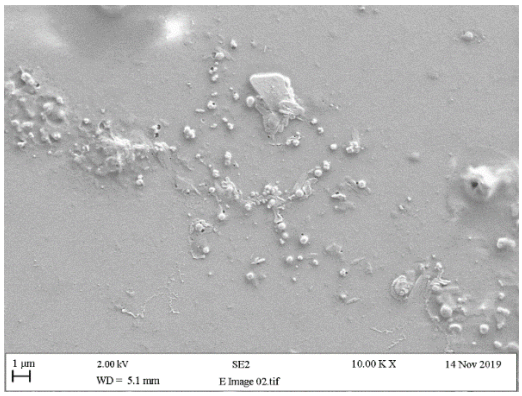

Figure 27.

SEM image of Isolate and *Salmonella Spp*

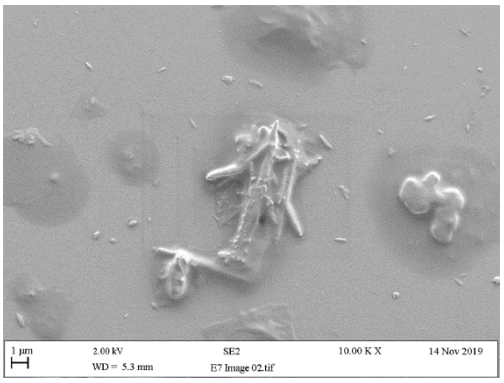

Figure 28.

SEM image of Ringer's solution and *Salmonella Spp*

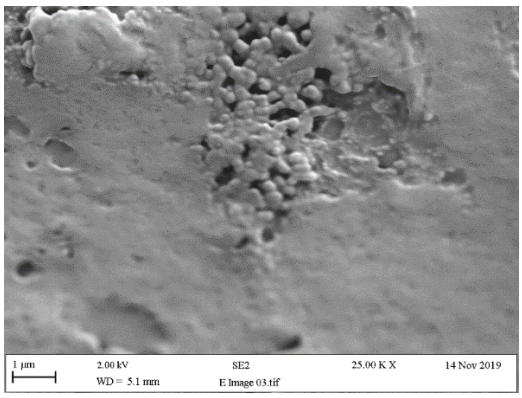

Figure 29.

SEM image of Isolate and *Salmonella Spp*

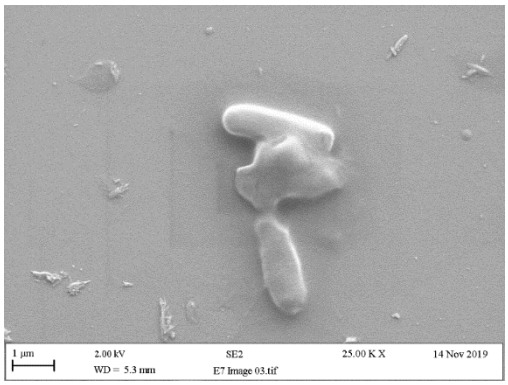

Figure 30.

SEM image of Ringer's solution and *Salmonella Spp*

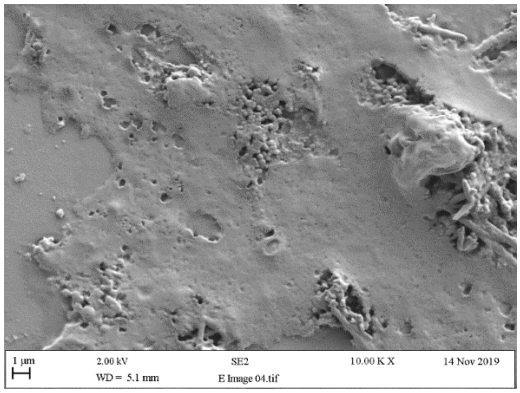

Figure 31.

SEM image of Isolate and *Salmonella Spp*

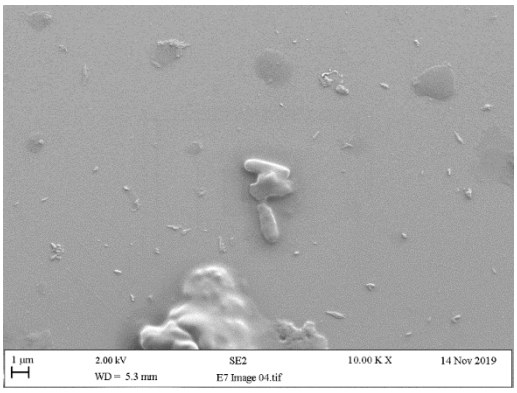

Figure 32.

SEM image of Ringer's solution and *Salmonella Spp*

The isolate and *Staphylococcus aureus* images show surprising results that show possible contamination. Here rods can be seen which should not be present as both the isolate *Lactococcus lactis* and *Staphylococcus aureus* are cocci shaped. No real conclusions can be obtained from these images.

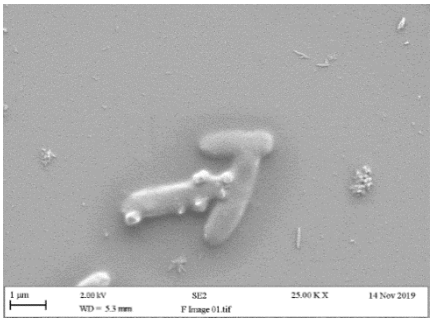

Figure 33.

SEM image of Isolate and *Staphylococcus aureus*

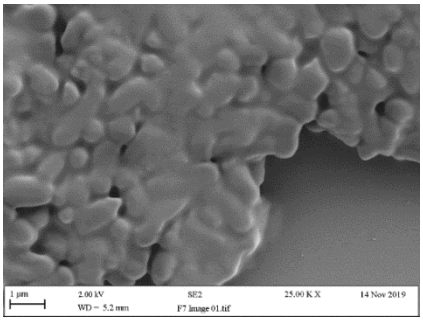

Figure 34.

SEM image of Ringer's solution and *Staphylococcus aureus*

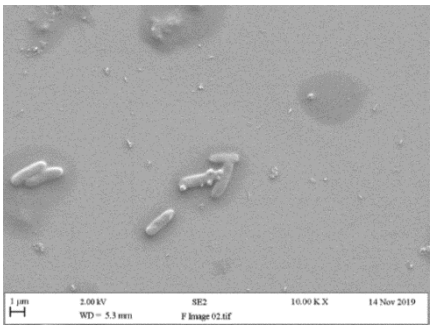

Figure 35.

SEM image of Isolate and *Staphylococcus aureus*

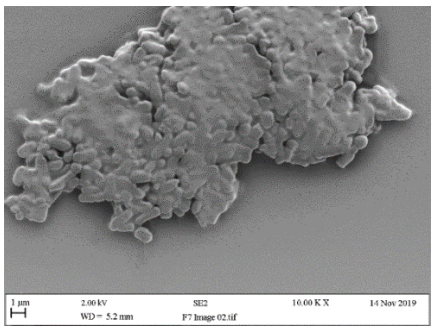

Figure 36.

SEM image of Ringer's solution and *Staphylococcus aureus*

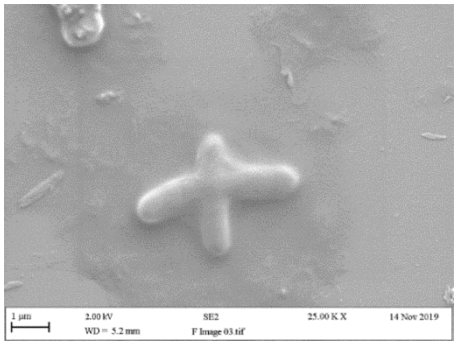

Figure 37.

SEM image of Isolate and *Staphylococcus aureus*

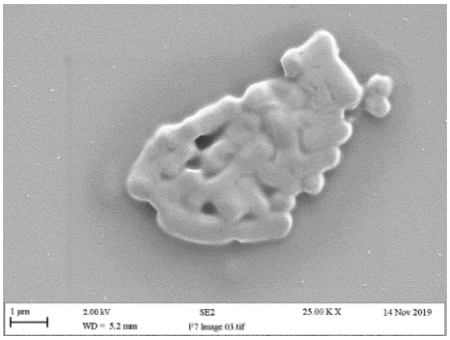

Figure 38.

SEM image of Ringers solution and *Staphylococcus aureus*

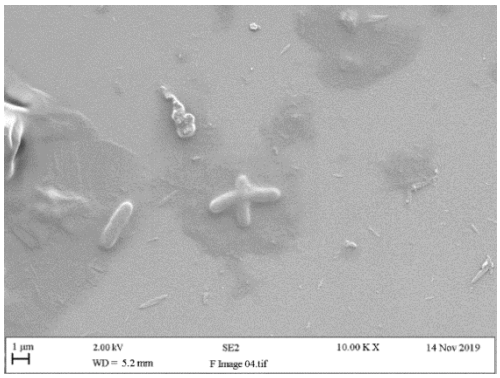

Figure 39.

SEM image of Isolate and *Staphylococcus aureus*

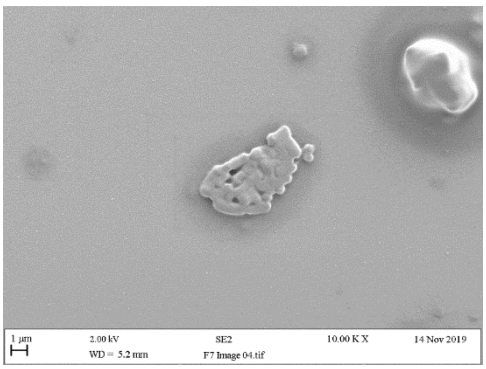

Figure 40.

SEM image of Ringers solution and *Staphylococcus aureus*

4.2.1 Discussion from observation of electron microscope

The images for *E.coli* and *E.coli* C822037 show a change in cell morphology and appearance of biofilm when the isolate *Lactobacillus lactis* is present. The rod shaped cells appear to be flatter and more merged together when the isolate is present. From these results, it is not possible to establish if the biofilm has been reduced or just looks different in appearance. In addition, as the *Lactococcus lactis* could possibly produce a biofilm, the images may show a mixed biofilm or indeed just the biofilm from *Lactococcus lactis.*

Further images need to be taken of the slides containing *Salmonella* to obtain conclusive results and no conclusions could be gained from the slides containing *Staphylococcus aureus*. However, both these organisms do produce biofilms. *Salmonella i*s associated with intestinal tract infections. Nosocomial infections are a major health problem and 80% are due to biofilm-associated infection, *Staphylococcus aureus* is the leading bacteria that causes this. The antimicrobial resistance of this bacterial community is accentuated because it can be formed by superbugs such as methicillin resistant *S.aureus* (MRSA). (Reffuveille, F et al., 2017). Biofilms can be unbreakable structures and there are theories that antibiotics cannot penetrate the biofilm. This is unlikely as there are many inward water channels. Another

theory is that in the biofilm there are antibiotic degrading enzymes, which could destroy the antibiotics. With the isolate in this project that could possibly be the case, but further studies would be helpful to establish which enzymes are secreted. Bacteria in the matrix have a slow metabolism antibiotic used to specifically target these have not been successful. (Reffuveille, F et al., 2017).

4.3 Conclusion

The results show that a variety of microorganisms, including Lactic acid bacteria were isolated from the selected fermented food and drink, sour beer, kefir and kombucha. Low numbers and variety of microorganisms proved to be problematic with the Sour beer and kombucha. The microorganisms identified were: *Lactobacillus Plantarum and Pediococcus acidilactici* from sour beer and *Lactococcus lactis* from kefir.

The overall conclusion is that *Lactobacillus lactis* isolated from a sample of kefir does exhibit some antimicrobial properties against *Escherichia coli.* The biofilm on the pathogen appears to be changed and possibly reduced in the presence of *Lactococcus lactis.* There was no conclusive evidence that *Lactococcus lactis* affected *Salmonella Spp* or *Staphylococcus aureus,* although initial disc method tests did show some antimicrobial activity. *Lactobacillus Plantarum and Pediococcus acidilactici* did not exhibit antimicrobial properties in these tests but further investigations could be carried out on a wider range of pathogens. The effect of the *Lactococcus lactis isolate* over time on the pathogens seemed to have little effect. Work carried out on probiotics from lactic acid bacteria exhibited antimicrobial action on pathogenic *E.coli* resistant to at least five antibiotics, Ceftazidime, Ampicillin, Clarithromycin, Amoxicillin + Clavulanic Acid and Ceftriaxone (Leite et al., 2015). Probiotics are identified as providing live, potentially beneficial bacterial cells to the gut environment of both humans and animals (Sonomoto and Yokota; 2011). Probiotics in the future may be used to treat gastrointestinal diseases and possibly as a delivery system for vaccines, immunoglobulins and other treatments (Ljungh and Wadstrom; 2009). Thus this isolate of *Lactococcuss lactis* isolated from a sample of kefir could have beneficial effects as a probiotic and antimicrobial agent. Kefir has been claimed to be a drink beneficial to the human gut and a probiotic drink. It is considered a miraculous food towards human health (Erdogan et al., 2019) and is a fermented drink usually made from milk with numerous attributed health claims. This is due to the presence of a complex mix of bacteria and yeast cultures in an exopolysaccharide matrix (Gut et al., 2019). This initial research seems to support this. However, much more work needs to be carried out including testing the isolate against other pathogens and eventually testing how it reacts in a gut environment. Also, if it works more effectively on its own or with other micro nutrients and if the effect would be increased if the bacteriocin was extracted from the bacteria.

5.0 RECOMMENDATIONS

The results from this project indicate that *Lactococcus lactis* isolated from Kefir does exhibit some antimicrobial properties. The pathogens it is most effective against is *E.coli* and possibly to a lesser extent *Salmonella Spp.* The results against *Staphylococcus aureus* are inconclusive although the initial investigations did show signs of activity.

There is much more work that can be carried out to investigate how well this isolate of *Lactococcus lactis* can work and be used. These actions include establishing the minimum and maximum concentration of bacteria needed to be effective against the pathogens, the maximum number of pathogens present that can be reduced by the isolate, the range of pathogens that are affected by the isolate and the ideal temperatures for effective antimicrobial action. It would also be interesting to establish which additives could be used alongside the isolate to improve performance. A whole range of tests could be carried out on how the isolate works in the animal and human body and specifically the gut.

Further investigations would be useful to determine how the isolate actually reduces or changes the biofilm on the pathogens. It would be interesting to establish how damaged the pathogen cell has become or if just the biofilm has changed. Also, with this change in biofilm if the pathogen is any less effective and if so by how much.

To confirm that these results show a true representation of the *Lactococcus lactis* isolate the above experiments need to be repeated under varying conditions and then more images on the scanning electron microscope carried out.

A scanning electron microscope and transmission electron microscope method could be used to show how any pathogenic cells were destroyed e.g. damage to the integrity of the cell wall, pore formation, anti- biofilm formation and the verification could be carried out by using crystal violet dye and lactic dehydrogenase release tests (Yi et al., 2018). The active compound of any bacteriocin present could possibly be detected using ammonium sulphate precipitation and then chloroform and gel filtration (Wannun et al., 2014).

This *Lactococcus lactis* has affected the pathogen's biofilm. Some strains of *Lactococcus lactis* can produce a biofilm themselves but tend to be poor at forming them by themselves. The production of biofilm can be increased when other organisms are present (Kives, et al., 2005). Therefore when observing the slides containing *Lactococcus lactis* (the isolate) and pathogen together further research is needed to establish what biofilm is present. The slides containing both types of *E.coli* and the *Lactococcus lactis* look as if less biofilm is present. The cells look more merged and there looks to be no or reduced area of stringy, sticky looking strands.

However, it is possible that biofilms from two organisms are present on the slides which has changed the resulting appearance.

In this study, the concern is with how the *Lactococcus lactis* isolate affects the pathogens by either damaging the biofilm or cell lysis. However, some lactic acid bacteria produce a biofilm that can be beneficial to themselves when resisting attack from pathogens. Thus, it is vital in further work to identify the biofilms present and establish if they are produced by both bacteria and how they interact.

Good viscoelasticity (viscous and elastic properties) in biofilms facilitates survival by promoting larger and stronger biofilms. When exposed to shear forces viscoelasticity properties promote fluid like behaviour of the biofilm and subsequent expansion by viscous flow. This enables resistance to both mechanical and chemical methods of removal (Rupp, C, J et al., 2005) .The actual physical properties could be analysed, and the biofilms compared. There are several ways that the mechanical properties of biofilms can be analysed. The most common are mechanical indentation or the application of shear stresses. Using these two methods, a number of different mechanical analyses can be performed. For analyses using mechanical indentation, a *normal force* is applied to the biofilm surface. For analyses using shear stresses, a *shear force* is applied to the biofilm surface using either spinning disk rheology or a flow cell system. Both can give micro and macro analysis of the biofilm properties (Vincent, J, and F 2012). (Rupp, C, J et al., 2005). Measurement indicate that single cells are mechanically stiff whereas bacterial biofilms show viscoelasticity. Biofilm viscoelasticity contributes to the virulence of chronic biofilm infections (Gloag, E. S. 2019).

Biofilm is a very varied material and production on one type of surface may not indicate biofilm formation on a different surface (Lajhar, A. et al.2018). Laboratory strains of *E.coli* tend not to show significant ability to attach to solid surfaces and to form biofilms (Castonguay, M-H et al. 2006). Again research could be carried out using the isolate *Lactococcus lactis* on different surfaces.

From the literature review, it is evident that some of the probiotic bacteria are more effective when other additives are present. Therefore, this could be investigated to see if this is the case with *Lactococcus lactis*. Unlike antibiotics, which target a single metabolic pathway, novel agents usually attack multiple sites on bacteria.(Cao,Y et al. 2019) Novel non-antibiotic antimicrobial agents, including silver nanoparticles or novel antimicrobial proteins may bind to and oxidize thiol groups, block DNA replication, alter bacterial gene expression, denature enzymes, induce reactive oxygen species (ROS), or damage bacterial membranes. (Cooper et al., 2018 and Naseri - Nosar and Ziara, 2018). Examples of antimicrobial nanoparticles include silver, zinc oxide and titanium dioxide. These agents can exert strong antimicrobial effects on a range of bacteria (Mishra et al., 2017).The second group is antimicrobial proteins, including antimicrobial peptides and enzymes derived from naturally occurring organisms such as

insects and bacteria. These have shown antibacterial properties, which make them of interest for the food industry and for biomedical applications (Yoon et al., 2012).

Another investigation into how effective the isolate is, is to look at the effect of Quorum sensing on the pathogen. This is a mechanism for communication between bacteria. It alters gene expression (Hoiby et al. 2010) and regulates the production of extracellular polymeric substances such as polysaccharides, proteins, lipids and extracellular DNA which make up 50% to 90% of the biofilm. (Billings et al., 2015). Gram-positive bacteria use a peptide-based quorum sensing system, known as the *agr* system.

The effectiveness of *Lactococcus lactis* could depend on the presence of antimicrobial peptides and proteins, not only do these enzymes attack bacteria directly, but they also inhibit the formation of biofilms (Cao, 2019). Their actions are not fully understood but major targets are thought to be the bacterial cell membrane and intracellular molecules. Antimicrobial enzymes are found in many organisms as a part of their innate defence mechanisms against bacterial invasion. The major types of antimicrobial enzymes are proteolytic enzymes and polysaccharide-degrading enzymes e.g. Lysozyme (Cao, Y. 2019)

The more effective an antimicrobial agent is, the more space it leaves for surviving bacteria to repopulate (Journals ASM.org). Although, the results from this study did not show significant effect of time on the pathogen population it would be interesting to carry out further studies to see at what point the number of pathogenic bacteria actually increase again. As the time required for surviving bacteria to repopulate the biofilm is significant and could be taken as a measure for effectiveness of the antimicrobial treatment. It depends on several factors such as the concentration, duration, presence of other substances, times etc.

This study has shown initial indications that the isolate *Lactococcus lactis* isolated from Kefir could be used as an antimicrobial agent, particularly against *E.coli.* However, there is much more research that could be carried out to gain a better understanding of how this works, its relationship with the pathogen and particularly with reference to biofilms.

DNA sequencing could be used to establish the exact nature of the *Lactococcus lactis,* as presently it has only been identified using morphology, Gram stain and API kits. To isolate a larger selection of lactic acid bacteria from fermented foods a wider selection of foods could be used. In addition, alternative selective media and different times and temperatures could be explored.

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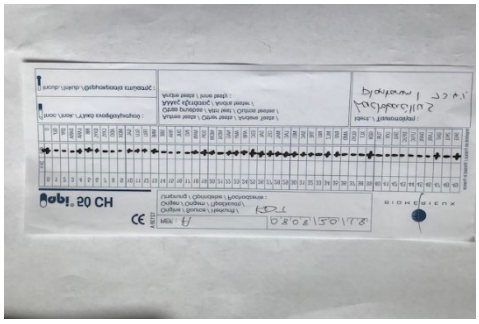
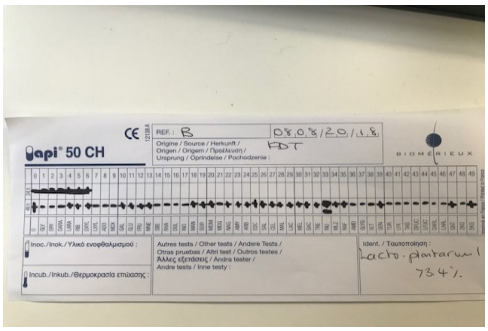
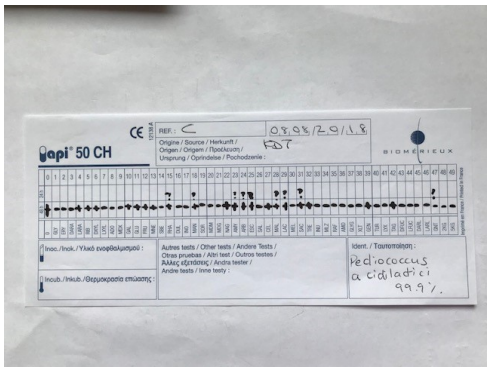
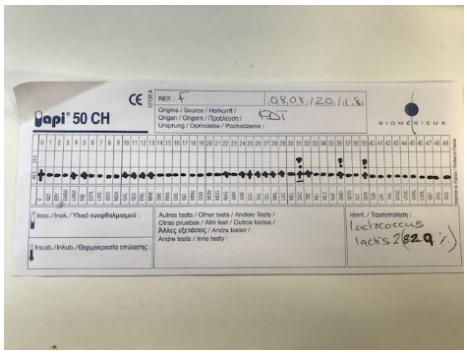
APPENDIX

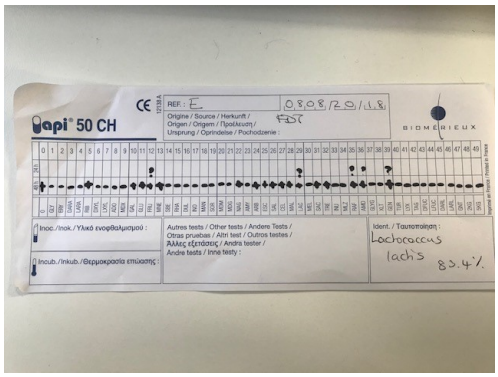
Appendix 1. API Example Sheets Figures 41 to 51

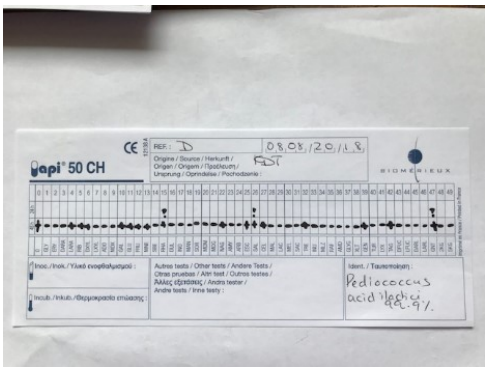


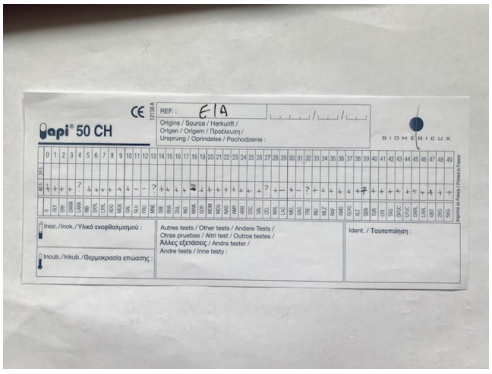








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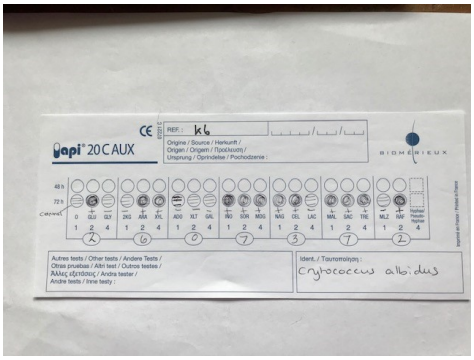
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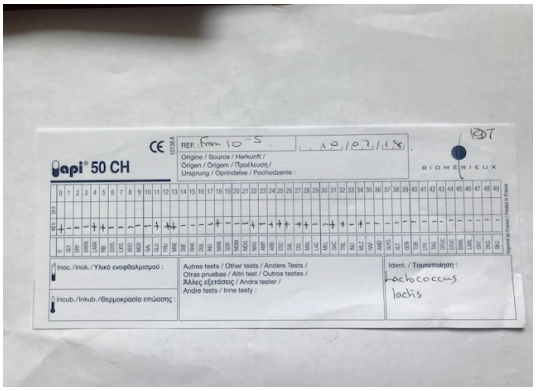
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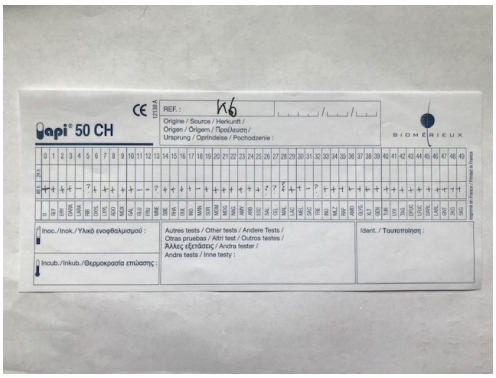
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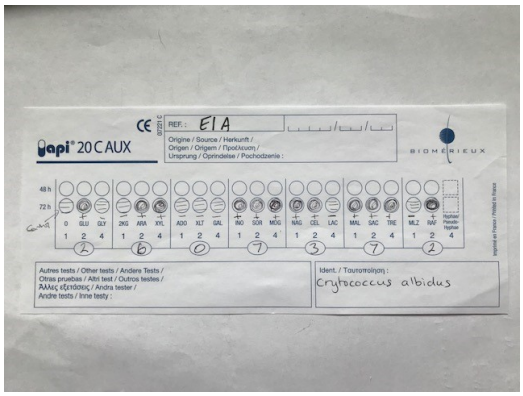
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Appendix 2. Images of Microorganisms plates Figures. 52 to 57

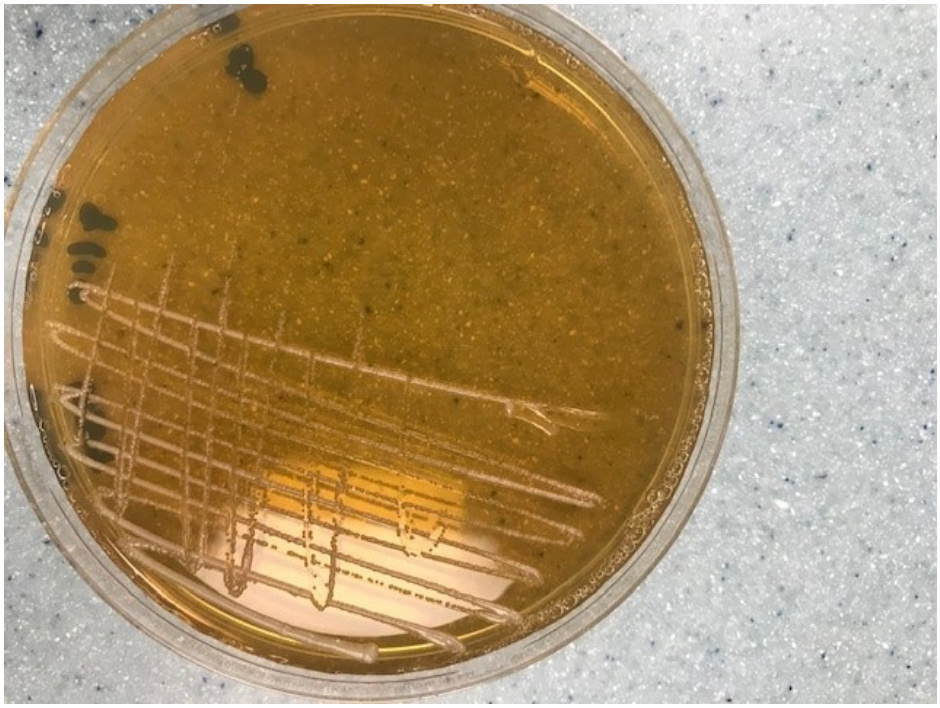

***Figure 52.Lactococcus lactis* on MRS agar**

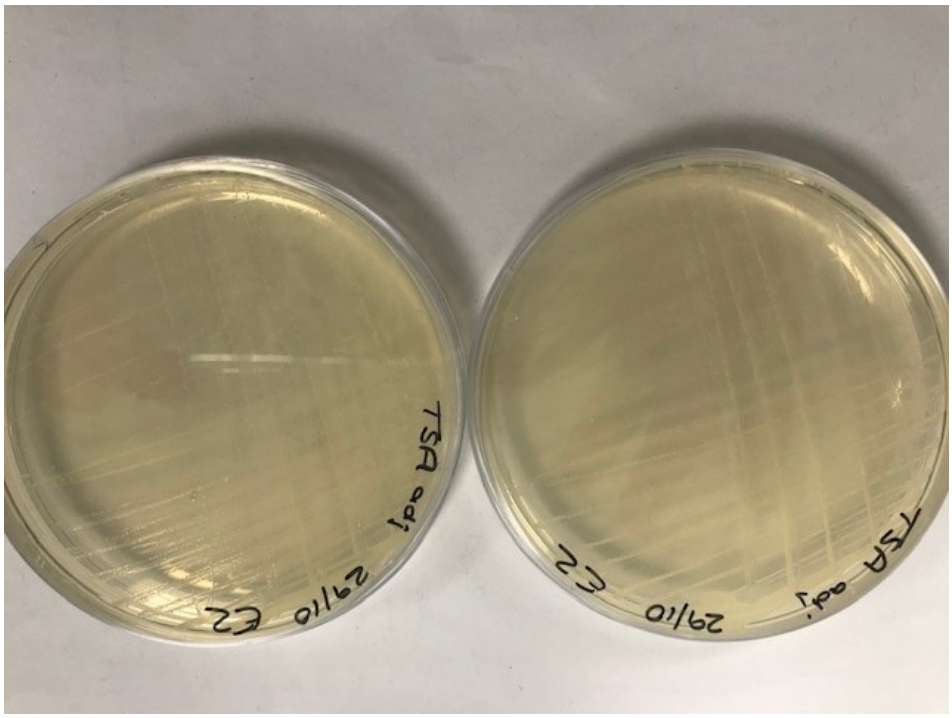

Figure 53 *Lactococcus lactis* on TSA

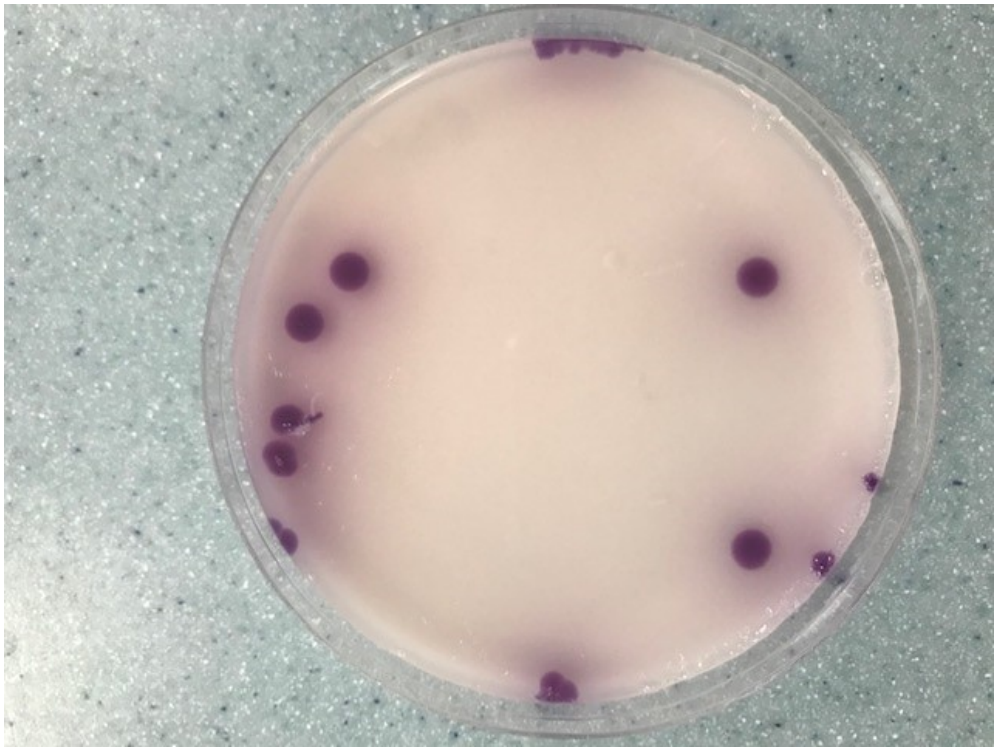

Figure 54. ***Salmonella Spp on BSA***

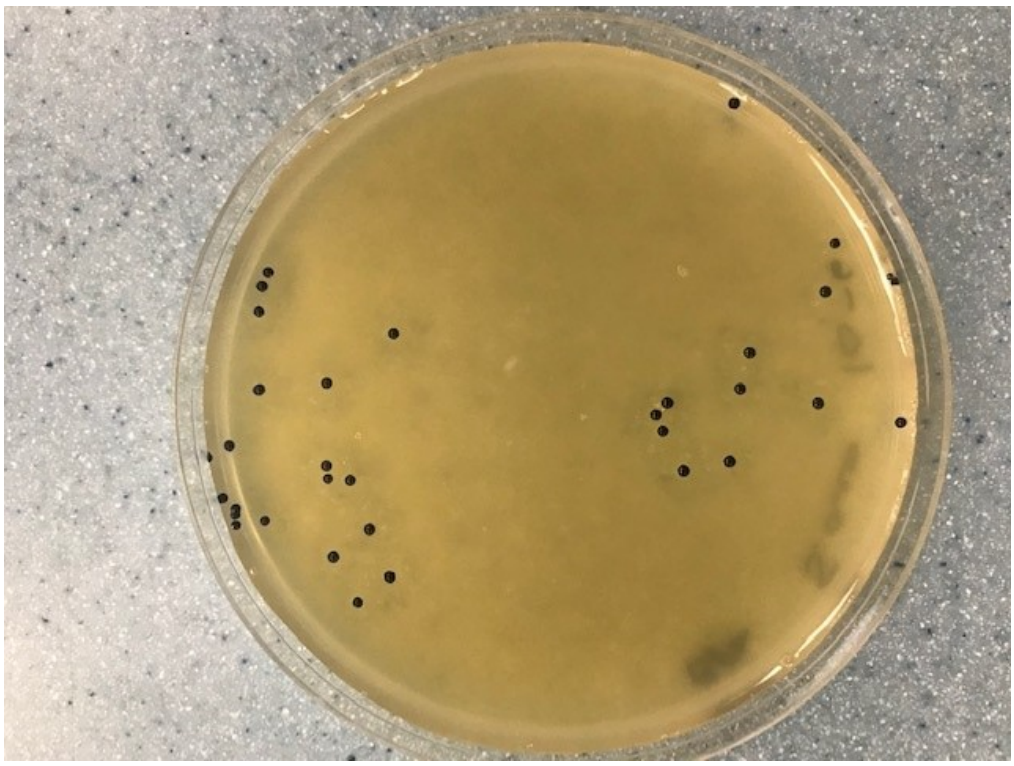

Figure 55. ***Staphylococcus aureus on BPA***

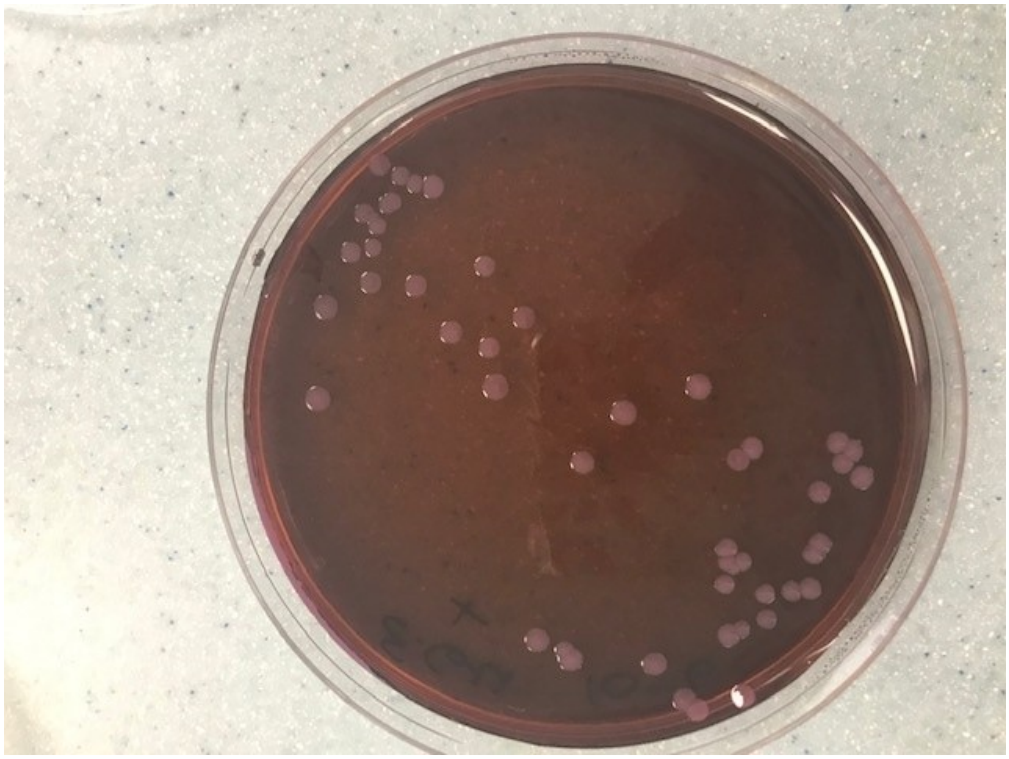

Figure 56 *E.coli* on EMB Agar on BPA

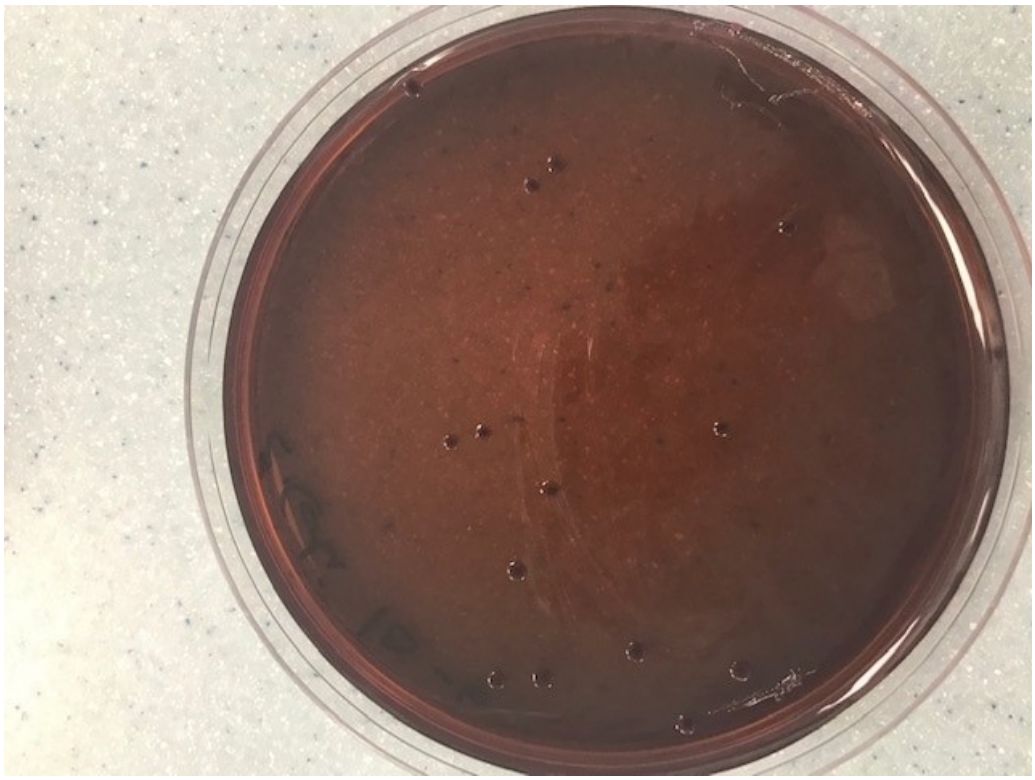

Figure 57. *E.coli* C822037 *on EMB Agar*

Appendix 3 Gram Stains.

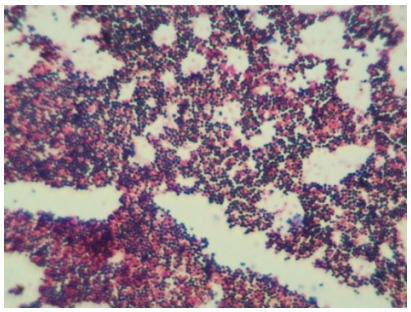

Figure 58. Gram positive bacteria *Lactococcus lactis*, Gram staining showing typical cell shape and characteristics. Oil immersion. Microscopy magnification x 1000.

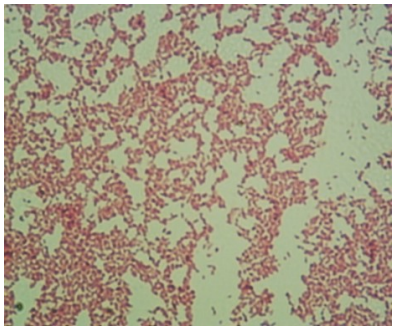

Figure 59. Gram negative bacteria. Species not identified. Gram staining showing typical cell shape and characteristics. Oil immersion. Microscopy magnification x 1000.

Appendix 4. Disc Plates. Figures 60 to 87

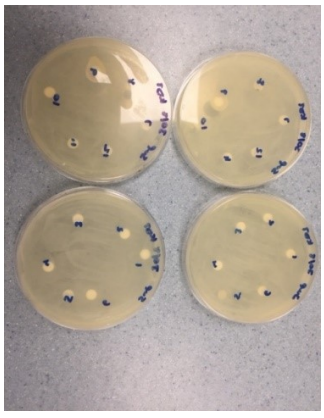

Figure 60. *E.coli* seeded agar plates, with re-suspended pellet on the discs.

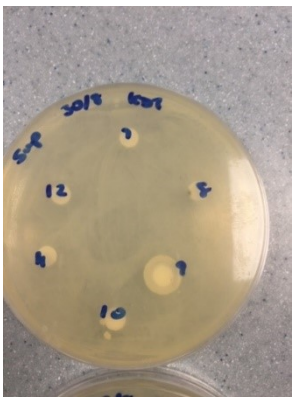

Figure 61. Close up photograph of *E.coli* seeded agar plate, with re-suspended pellet on the discs 7 to 12 showing zones of growth around 9, 10, 11 and 12.

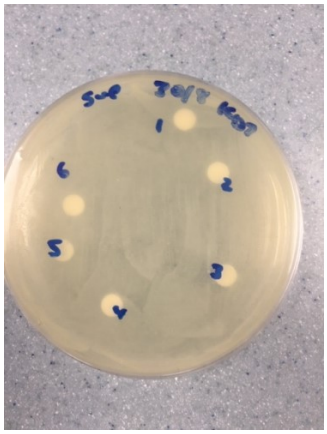

Figure 62. Close up photograph of E.coli seeded agar plates with re-suspended pellet on the discs 1 to 6

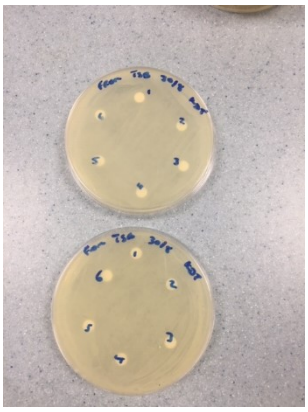

Figure 63. *E.coli* seeded agar plates with the bacteria in the original broth on the discs 1 to 6.

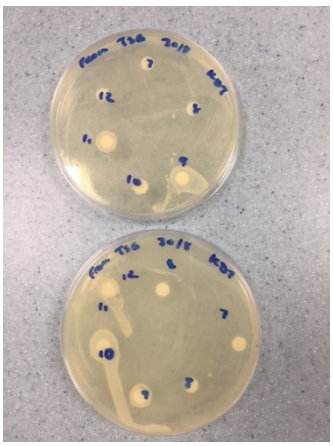

Figure 64. *E.coli* seeded agar plates with bacteria in the original broth on the discs, showing growth around 9, 10, 11, and 12.

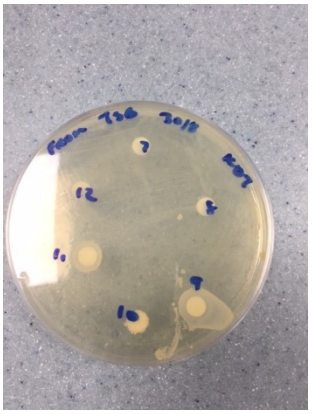

Figure 65 Close up photograph of *E.coli* seeded agar plates with bacteria in the original broth on the discs, showing growth around 9, 10, 11, and 12.

The results showed that the isolates numbered 9, 10, 11 and 12 had growth around the disc, with number 12 having the least growth

4

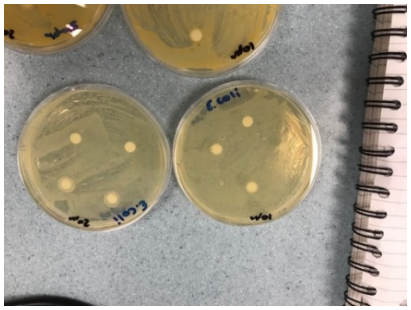

Figure 66. Seeded agar plates of *E.coli* with 10µl and20µl of isolate on the discs.

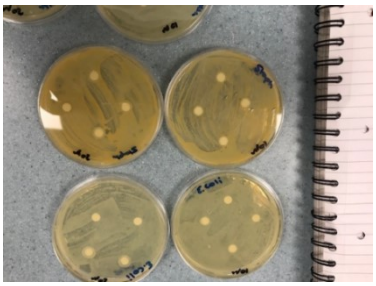

Figure 67 Seeded agar plates of *E.coli* and *Staphylococcus aureus* with 10µl and20µl of isolate on the discs.

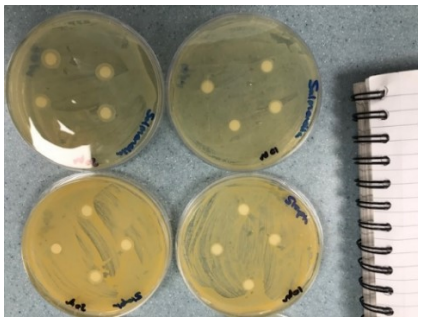

Figure 68. Seeded agar plates of *Salmonella Spp* and *Staphylococcus aureus* with 10µl and 20µl of isolate on the discs

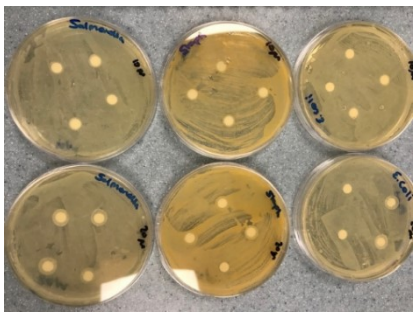

Figure 69. Seeded agar plates of *Salmonella Spp*, *Staphyloccus aureus* and E.coli with 10µl and 20µl of isolate on the discs.

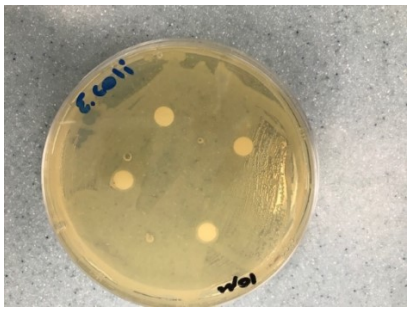

Figure 70 Seeded agar plates of *E.coli* with 10µl of isolate on the discs

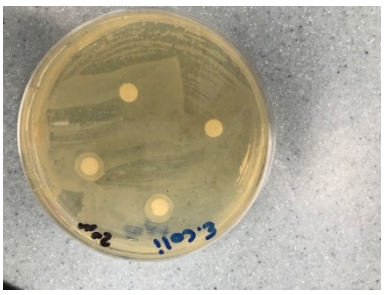

Figure 71 Seeded agar plates of *E.coli* with 20µl of isolate on the discs

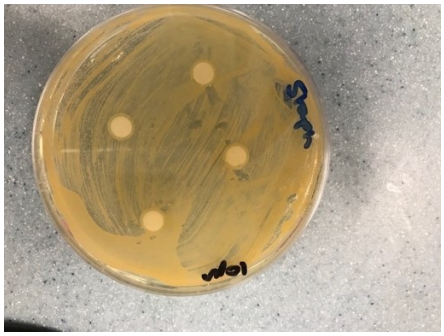

Figure 72. Seeded agar plates of *Staphylococcus aureus* with 10µl of isolate on the discs.

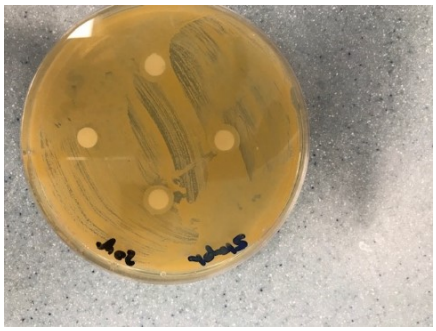

Figure 73. Seeded agar plates of *Staphylococcus aureus* with 20µl of isolate on the discs

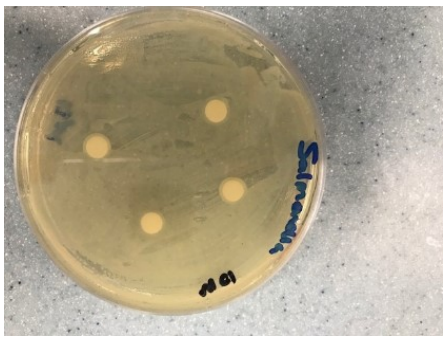

Figure 74. Seeded agar plates of *Salmonella Spp* with 10µl of isolate on the discs.

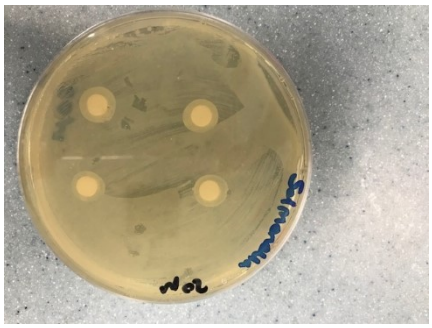

Figure 75. Seeded agar plates of *Salmonella Spp* with 20µl of isolate on the discs, showing distinct growth around the discs.

Figure 76. *E.Coli* seeded agar plates, with isolate (E2) on the disc

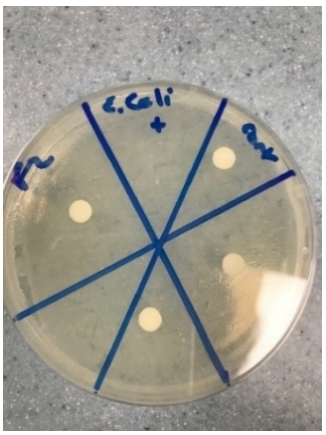

Figure 77. *E.Coli C822037* seeded agar plates, with isolate (F2) on the discs

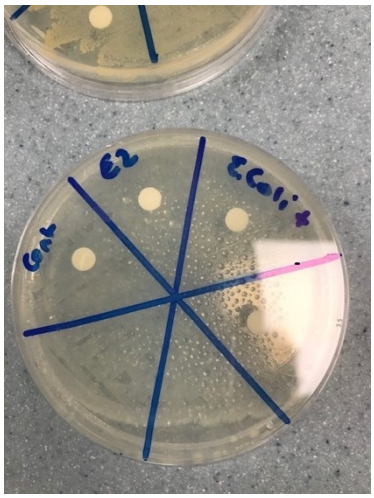

Figure 78. *E.Coli* C822037seeded agar plates, with isolate (E2) on the discs

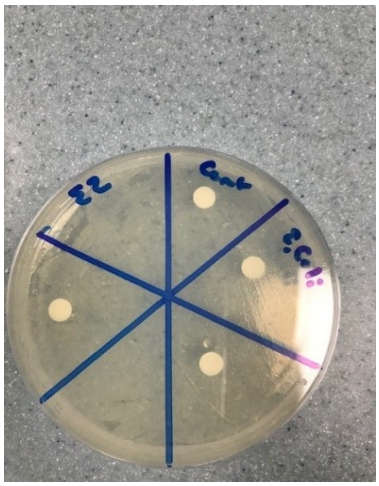

Figure 79. *E. coli* seeded agar plates, with isolate (E2) on the discs

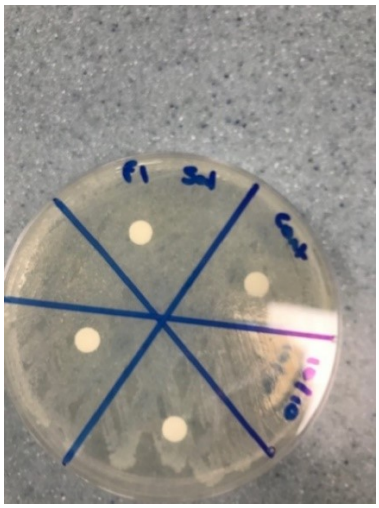

Figure 80 *Salmonella Spp* seeded agar plates, with isolate (F1) on the disc

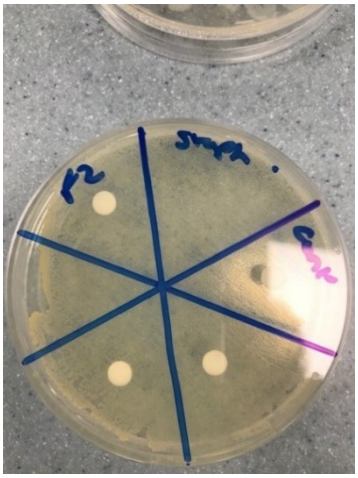

Figure 81. *Staphylococcus aureus* seeded agar plates, with isolate (F2) on the discs. Plate 1.

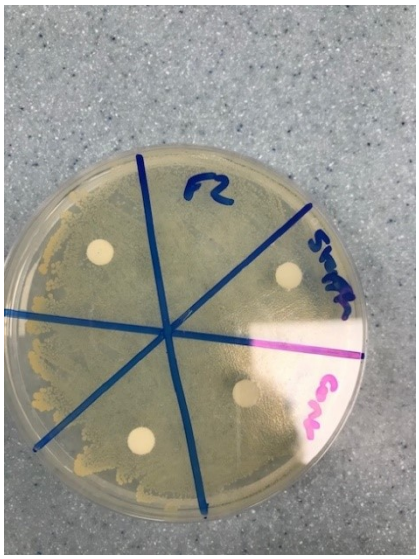

Figure 82. *Staphylococcus aureus* seeded agar plates, with isolate (F2) on the discs. Plate 2

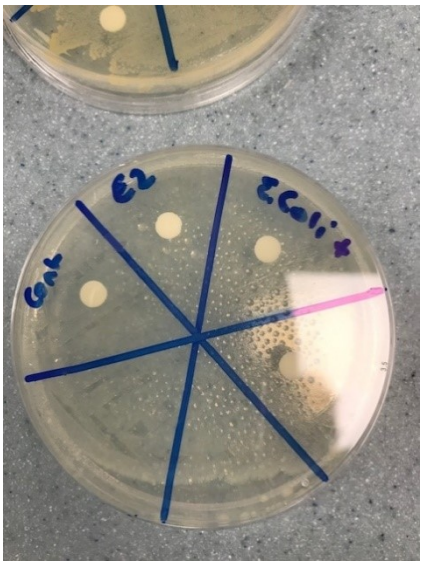

Figure 83. *E.Coli C822037* seeded agar plates, with isolate (E2) on the discs

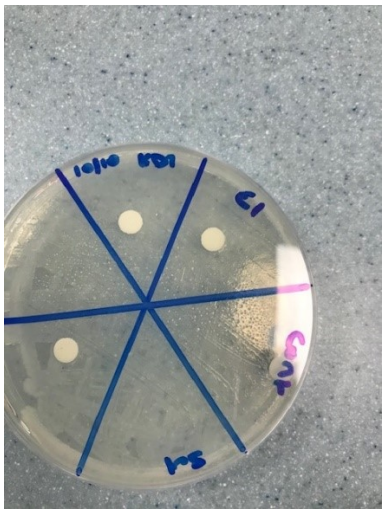

Figure 84. *Salmonella Spp* seeded agar plates, with isolate (E1) on the discs.

Figure 85. *E.Coli C822037* seeded agar plates, with isolate (E1) on the discs.

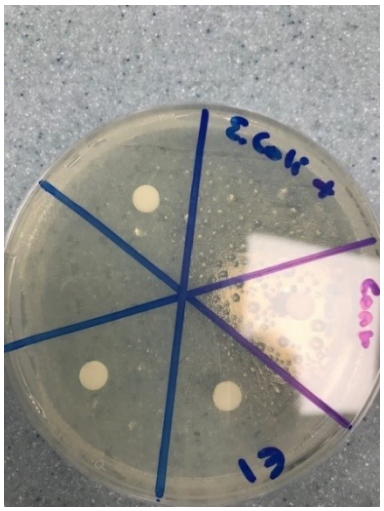

Figure 86. *E.Coli C822037* seeded agar plates, with isolate (E1) on the discs. Plate 2

Figure 87. *E.Coli C822037* seeded agar plates, with isolate (F2) on the discs. Plate 2.

Appendix 5. Tables 27 to 33 for The effect of *Lactococcus lactis* (isolated from Kefir) over time on the growth of the pathogens

Table 27. *E.Coli* + Isolate

	0hrs	**2hrs**	**4hrs**	**6hrs**	**24hrs**
10^{-4}	264	Too many to count	Too many to count	Too many to count	Too many to count
10^{-5}	27	Contaminated	Too many to count	Too many to count	Too many to count
10^{-6}	3	16	Too many to count	Uncountable	Too many to count
10^{-7}	0	2	Uncountable	6	8
10^{-8}	0	0	0	0	0

Table 28. *Salmonella* + Isolate

	0hrs	**2hrs**	**4hrs**	**6hrs**	**24hrs**
10^{-4} to 10^{-5}	Too many to count	Too many to count	Too many to count	Too many to count	Too many to count
10^{-6}	20	Too merged	Too merged	Too merged	Too merged
10^{-7}	2	6	0	0	0
10^{-8}	0	0	0	0	0

Table 29. *Staphylococcus aureus* + Isolate

	0hrs	**2hrs**	**4hrs**	**6hrs**	**24hrs**
10^{-4}	Too many to count	Too many to count	Too many to count	Too many to count	Too many to count
10^{-5}	69	Too many to count	Too many to count	Too many to count	Too many to count
10^{-6}	7	Uncountable	Uncountable	Uncountable	Uncountable
10^{-7}	0	6	50	68	70
10^{-8}	0	0	4	9	8

From the results for *Staphylococcus aureus*, overtime there was some effect on the growth of the pathogen when the isolate was present. Time 0 hours was the control i.e. the initial number of bacteria present. The pathogen continued to grow over the tested period, but growth did slow down between 4 and 6 hours.

Tables 31 to 34 show the number of bacteria present in the samples used.

Table 30. E1

	0hrs
10^{-4} to 10^{-5}	Too many to count
10^{-6}	160
10^{-7}	11
10^{-8}	1

Table 31. *E.coli*

	0hrs
10^{-4} to 10^{-6}	Too many to count
10^{-7}	218
10^{-8}	26

Table 32. *Salmonella*

	0hrs
10^{-4} to 10^{-6}	Too many to count

10^{-7}	106
10^{-8}	10

Table 33. *Staphylococcus aureus*

	0hrs
10^{-4} to 10^{-6}	Too many to count
10^{-7}	469
10^{-8}	46

Appendix 6. Turbidity Readings

User: USER
Path: C:\Program Files (x86)\BMG\Omega\User\Data\
Test ID: 20
Test Name: QUICK ABS
Date: 01/11/2019
Time:
11:09:18
ID1: Kathryn 1
Absorbance Absorbance values are displayed as OD

Raw Data (450)

	1	2	3	4	5	6	7	8	9	10	11	12
A	1.994	0.113	0.102	0.04	0.036	0.038	0.033	0.039	0.036	0.04	0.034	0.037
B	0.054	0.056	0.053	0.036	0.038	0.037	0.038	0.037	0.038	0.035	0.036	0.062
C	1.485	1.26	1.192	0.08	0.075	0.088	1.539	1.401	1.591	0.041	0.037	0.037
D	1.448	1.303	1.099	0.082	0.076	0.068	1.571	1.542	1.143	0.039	0.036	0.035
E	1.498	1.268	1.297	0.077	0.077	0.086	1.15	1.117	1.22	0.039	0.036	0.036
F	0.583	0.56	0.638	0.095	0.077	0.082	0.429	0.127	1.398	0.038	0.041	0.041
G	0.082	0.502	0.617	0.738	0.074	0.073	0.038	0.04	0.038	0.04	0.037	0.035
H	0.067	0.07	0.069	0.038	0.038	0.038	0.039	0.037	0.036	0.039	0.039	0.039

User: USER
Path: C:\Program Files (x86)\BMG\Omega\User\Data\
Test ID: 21
Test Name: QUICK ABS
Date: 04/11/2019
Time:
09:22:39
ID1: Kathryn 2
Absorbance Absorbance values are displayed as OD

Raw Data (450)

	1	2	3	4	5	6	7	8	9	10	11	12
A	0.286	0.086	0.081	0.038	0.036	0.035	0.036	0.037	0.035	0.037	0.034	0.032
B	0.06	0.048	0.045	0.037	0.034	0.035	0.036	0.036	0.032	0.035	0.033	0.038
C	1.904	1.341	1.318	0.071	0.069	0.073	1.537	1.915	0.541	0.034	0.033	0.035
D	1.704	1.222	1.221	0.081	0.074	0.072	1.396	1.373	0.202	0.038	0.035	0.036
E	1.965	1.401	1.648	0.084	0.084	0.077	1.274	1.618	1.555	0.035	0.037	0.037
F	1.161	0.98	0.607	0.089	0.068	0.07	0.456	0.364	0.238	0.034	0.033	0.041
G	0.075	0.318	0.298	0.468	0.058	0.066	0.037	0.04	0.035	0.035	0.036	0.034
H	0.048	0.067	0.06	0.036	0.036	0.038	0.036	0.04	0.036	0.036	0.033	0.041