PUTATIVE ANTIBACTERIAL ACTIVITY OF LACTIC ACID BACTERIA ISOLATED FROM LOCAL GOAT MILK

NUR SHAHDINA HANI BINTI MOHD ZAKI

FACULTY OF SCIENCE
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NUR SHAHDINA HANI BINTI MOHD ZAKI

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ABSTRACT

Antimicrobial compounds excreted by microorganisms have attracted lots of attention among researchers in the field of food microbiology as an alternative for natural preservatives and antibiotics. To compete for nutrients and space in a particular habitat, bacteria often produce natural antimicrobial compounds such as organic acids, antifungal, siderophores and bacteriocins. Hence, the aim of this study is to identify the bioactive antimicrobial compounds deriving from lactic acid bacteria (LAB) isolated from local goat milk. The screening of antimicrobial activity from 24 bacteria isolated from goat milk led to the isolation of *Enterococcus faecium* FGa, *Enterococcus faecium* RGB, *Bacillus cereus* FG1 and *Serratia marcescens* FG3. These strains show good inhibition zones against varying types of foodborne pathogens such as *Staphylococcus aureus* SA7001, *Shigella boydii* SB1003, *Shigella dysenteriae* SD1007 and *Salmonella typhimurium* SM4001. The antimicrobial substances in cell-free culture supernatant of all four isolates were stable against *S. aureus* at different temperatures, pH and also when exposed to proteolytic enzymes except pepsin for *E. faecium* FGa and *E. faecium* RGB. The mode of action for isolates was determined as bactericidal where they lysed the cells of *S. aureus* which induced the unbalanced growth of its cell cycle. Since LAB are our main aim, both *E. faecium* FGa and RGB have undergone further safety evaluation. Both strains show good probiotic properties since they can survive well under conditions simulating the human GI tract and do not show any potential virulence characteristics. Enterocin A has been detected through PCR amplification in one of our *E. faecium* strains which is *E. faecium* FGa. This indicates it as one of the possible compounds that is responsible for antimicrobial activity. *E. faecium* FGa has also undergone Q-TOF LC/MS

analysis and several metabolites that may also contribute to antimicrobial activity have been detected which are netilmicin, maleic acid and 2-furonic acid. Hence, *E. faecium* FGa can be used as a potential bio-preservative or protective culture in our food industry.

Keywords: Lactic acid bacteria, antibacterial activity, probiotic, safety properties

AKTIVITI ANTIBAKTERIA YANG PUTATIF BAGI ASID LAKTIK BAKTERIA YANG DIASINGKAN DARI SUSU KAMBING TEMPATAN

ABSTRAK

Sebatian antimikroba yang dikeluarkan oleh mikroorganisma telah menarik banyak perhatian sebagai alternatif untuk pengawet semula jadi dan antibiotik. Bagi bersaing untuk mendapatkan nutrien dan ruang di habitat tertentu, bakteria sering menghasilkan sebatian antimikroba semula jadi seperti asid organik, antikulat, siderophores dan bakteriosin. Oleh itu, tujuan kajian ini adalah untuk mengenalpasti potensi sebatian antimikroba bioaktif yang berasal dari strain bakteria asid laktik (LAB) yang diperolehi daripada susu kambing tempatan. Pemeriksaan aktiviti antimikroba dari 24 bakteria yang diasingkan dari susu kambing menyebabkan pengasingan *Enterococcus faecium* FGa, *Enterococcus faecium* RGB, *Bacillus cereus* FG1 dan *Serratia marcescens* FG3 yang menunjukkan zon perencatan yang baik terhadap pelbagai jenis patogen bawaan makanan seperti *Staphylococccus aureus, Shigella* boydii SB1003*, Shigella dysenteriae* SD1007 dan *Salmonella typhimurium* SM4001. Bahan antimikroba dalam supernatan kultur bebas sel dari keempat-empat isolat ini menunjukkan bahawa mereka stabil terhadap *S. aureus* pada suhu dan pH yang berbeza dan juga apabila terdedah kepada enzim proteolitik kecuali pepsin bagi *E. faecium* FGa dan *E. faecium* RGB. Kaedah tindakan oleh isolat-isolat ini terhadap *S. aureus* ditentukan sebagai bakterisida di mana mereka memecahkan sel-sel membran *S. aureus* yang seterusnya menyebabkan pertumbuhan kitaran selnya tidak seimbang. Oleh kerana LAB adalah tujuan utama kami, kedua-*dua E. faecium* FGa dan RGB telah menjalani penilaian keselamatan lebih lanjut di mana kedua-dua strain menunjukkan sifat probiotik yang baik kerana ia dapat bertahan dengan baik dalam keadaan yang mensimulasikan saluran GI manusia dan ia tidak menunjukkan ciri-ciri virulensi yang berpotensi. Enterocin A telah dikesan di salah satu strain *E. faecium* kami iaitu *E. faecium* FGa melalui penguatan PCR yang menunjukkannya sebagai salah satu

kemungkinan sebatian yang bertanggungjawab untuk aktiviti antimikroba. *E. faecium* FGa juga telah menjalani analisis Q-TOF LC / MS dan beberapa metabolit yang juga dapat menyumbang kepada aktiviti antimikroba telah dikesan iaitu netilmicin, asid maleik dan asid 2-furonik. Oleh itu, *E. faecium* FGa boleh digunakan sebagai budaya pengawet atau pelindung bio yang berpotensi dalam industri makanan kita.

Kata kunci: Bakteria asid laktik, aktiviti antibakteria, probiotik, sifat keselamatan

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LIST OF SYMBOLS AND ABBREVIATIONS

α	:	alpha
β	:	beta
^{o}C	:	degree celcius
γ	:	gamma
μ	:	micro
AMP	:	ampicillin
BLAST	:	basic local alignment search tool
CFCS	:	cell-free culture supernatant
CDC	:	centers for disease control and prevention
C	:	chloramphenicol
CIP	:	ciprofloxacin
MRS	:	de man, rogosa and sharpe
DGGE	:	denaturing gradient gel electrophoresis
GI	:	gastrointestinal
GRAS	:	generally recognized as safe substances
g	:	gram
h	:	hour
HCl	:	hydrochloric acid
H_2O_2	:	hydrogen peroxide
K	:	kanamycin
kDa	:	kilodalton
LAB	:	lactic acid bacteria
LB	:	luria-bertani
MRSA	:	methicillin-resistant *S. aureus*
mg/ml	:	miligram per milimeter

mm	:	milimeter
M	:	molar
MDRP	:	multidrug resistant *P. aeruginosa*
nm	:	nanometer
N	:	normality
OD	:	optical density
PBP	:	penicillin-binding protein
PBS	:	phosphate-buffer saline
PCR	:	polymerase chain reaction
pH	:	potential of hydrogen
Q-TOF LC/MS	:	quadrupole time-of-flight liquid chromatography/mass spectrometry
qPCR	:	quantitative polymerase chain reaction
rpm	:	revolutions per minute
NaCl	:	sodium chloride
NaOH	:	sodium hydroxide
sp.	:	species
S	:	streptomycin
TGGE	:	temperature gradient gel electrophoresis
TE	:	tetracyclin
V	:	vancomycin
VRE	:	vancomycin-resistant enterococci
VRSA	:	vancomycin-resistant *S. aureus*
v/v	:	volume per volume

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CHAPTER 1: INTRODUCTION

1.1 Introduction

Antimicrobial agents have become a powerful tool to treat and reduce the occurrence of infectious diseases around the world. Due to inappropriate dosage, insufficient use of antibiotic and rapid mutation of bacteria, the function of antibiotic is no longer effective, thus, leading to the evolution of antimicrobial resistance among bacteria. According to the Centers for Disease Control and Prevention (CDC), antimicrobial resistance is the ability of microorganism to resist the effects of drugs without stopping their growth (CDC, 2015). In the United States, antibiotic resistance is the most worrying issue of all. The estimated minimum number of illnesses and deaths due to antibiotic resistance is approximately 2,000,000 illnesses and 23,000 deaths (CDC, 2015). Recently, new antibiotics are being developed to curb this problem. Still, none of them seem to work effectively towards antibiotic-resistant bacteria, for instance, *Pseudomonas aeruginosa, Staphylococcus aureus* and *Shigella* sp. The emergence and rapid evolution of antimicrobial resistance in bacteria have made common medical practices such as surgery and chemotherapy harder for practitioners and patients. Not only do the patients have to suffer the persistent infection inside an already immunosuppressed body, but they also have to carry a load of medical costs from having to undergo additional treatment and a prolonged stay in the hospital. This epidemic highlights the importance of discovering novel antimicrobial agents.

Lactic acid bacteria are categorized as Gram-positive cocci or rods, aerotolerant, and able to produce small organic substances that can give specific organoleptic attributes to the products (Caplice & Fitzgerald, 1999). These microorganisms can be found in milk, meat and fermented products such as in fermented vegetables, fruits and beverages. LAB used or/and produced in fermented food are usually for improving the nutrients, changing

the organoleptic properties of the products and inhibiting the growth of pathogenic, thus increasing the shelf life of food products. The ability of LAB to inhibit the growth of pathogenic bacteria making them generally recognized as safe (GRAS) and/or probiotics (Mayo et al., 2010). The property of probiotic strain that makes it desirable is the ability to produce antimicrobial substances. Due to the ability of LAB in inhibiting pathogenic bacteria growth, they can preserve foods and fermented products for a very long time. They can be used as a natural competitive microbiota. LAB in general have a very good preservative effect because of their production of several active metabolites such as organic acids (lactic, acetic, propionic, butyric and formic acids) which can enhance their action by reducing the pH of the media, and other substances such as hydrogen peroxide, ethanol, acetoin, diacetyl, antifungal compounds, bacteriocins (nisin, lacticin, pediocin, enterocin and others) and bacteriocin-like inhibitory substances (BLIS) can also be found in LAB (Perin et al., 2013; Reis et al., 2012).

Enterococci is one of the lactic acid bacteria that may be frequently associated with fermented foods that can be used to improve safety and extend the shelf life of food, including fermented milk, cheeses, and sausages (Álvarez et al., 2020; Del Rio et al., 2019). Moreover, enterococci can produce bacteriocins known as enterocin. Enterocins which represent one of the antimicrobial compounds have the ability to inhibit food spoilage or pathogenic bacteria like *Listeria* sp., *Staphylococcus aureus* or *Bacillus* sp (Furlaneto-Maia et al., 2020; Qiao et al., 2020). Hence, enterocins stand a chance to be used as food preservatives. These led to the value of enterococci like *E. faecium* as a commercial probiotic due to their desirable beneficial activities (Beirão et al., 2018; Holzapfel et al., 2018). As to be used as a commercial probiotic, several aspects, including the safety of the strains and their functional and technological properties, have to be considered. The criteria for the selection of probiotics include competitive exclusion of a wide range of pathogens, tolerance to gastrointestinal conditions, and lack of

pathogenicity (Hanchi et al., 2018). Some of *Enterococcus* strains have been questioned for their safety to be used as probiotics which are highly concerning. This was due to some of the strains are resistant to many antibiotics that could cause nosocomial infections like endocarditis, bacteremias, urinary tract infections and a reservoir for resistance gene (Gao et al., 2018; Puchter et al., 2018). Therefore, each particular strain needs to be carefully analyzed by proper selection criteria of probiotic bacteria for food applications.

This study investigates the potential antibacterial compounds from selected lactic acid bacteria that have significant antibacterial effects against food-borne pathogen and to develop an alternative to current antibiotics in view of the increasing global antibiotic resistance. The project focused on the identification of these antibacterial compounds and the characterization of these antibacterial compound producing strains which include the investigation of their suitability as probiotic bacteria.

1.2 Objectives:

1) To screen and isolate bacteria from raw local goat milk with antagonistic activity against pathogenic strains.
2) To identify the selected bacteria and characterize their antimicrobial metabolites produced.
3) To determine the suitability of the chosen isolates as probiotic bacteria by investigating their viability under conditions simulating the human GI tract and their potential virulence factors.
4) To determine the potential compounds that contribute to antimicrobial activity from the chosen isolate using PCR gene detection and LCMS-QTOF.

CHAPTER 2: LITERATURE REVIEW

2.1 Antimicrobial Resistance among Bacteria

Antibiotic or antimicrobial drugs have been known to become the cause for abrupt changes in treating any infectious disease and as a fate of mankind. Yet, any infection with drug-resistant microorganisms still is the main issue in a clinical setting. The first known antimicrobial agent, salvarsan, was found by Ehrlich in 1990 to treat syphilis. A few years later, Domagk and other scientists had developed a synthetic compound called sulphonamides. In 1928, penicillin was discovered by Fleming. He discovered that the growth of *Staphylococcus aureus* was suppressed by the presence of blue mould zone surrounding the culture plate. Since then, the antibiotic was developed and become widely used during World War II. In addition, streptomycin was found in soil bacterium, *Streptomyces griseus*. From that, tetracycline, chloramphenicol, macrolide and vancomycin were developed. Initially, penicillin was known to effectively inhibit the growth of *S. aureus,* but after a certain period, *S. aureus* became penicillin-resistant, as it produced penicillinase, thus leading to the development of methicillin. In 1960, cephems were developed and had been classified into several generations based on their antimicrobial spectra. The first-generation cephams were very effective towards Gram-positive bacteria and *Escherichia coli.* Next, second-generation cephams had increased the antimicrobial spectrum, including Gram-positive bacteria, Gram-negative bacteria and *Enterobacteriaceae*. The third-generation cephams were able to cover Gram-negative bacteria and *Pseudomonas aeruginosa*. Carbapenem is an antibiotic class that is very effective towards Gram-positive bacteria, Gram-negative bacteria and anaerobes.

Instead of the vast development of antibiotics, the antimicrobial resistance issue seems very common in clinical settings. *Staphylococcus aureus* is the most resistant bacteria in the clinical field. It acquired resistance towards sulphonamides when in use. So, they used

penicillin to treat diseases, yet the strain had become increasingly resistant since the 1950s. Thus, penicillin-stable methicillin was developed. Methicillin-resistant *S. aureus* (MRSA) was isolated in the UK in 1961 and started to become a problem during the 1990s. MRSA became resistant to β-lactam antibiotics via the acquisition of penicillin-binding protein (PBP) 2' gene. This gene was known to be involved in the synthesis of cell wall that had a low binding affinity towards β-lactam antibiotics. In addition, vancomycin-resistant *S. aureus* (VRSA) was reported in the US. It obtained the resistance genes horizontally from vancomycin-resistant enterococci. *Streptococcus pneumoniae* was known to be susceptible to penicillin, but penicillin-intermediate *S. pneumoniae* and penicillin-resistant *S. pneumoniae* were found during the 1960s and 1970s, respectively. Apart from that, ampicillin was used to inhibit the growth of *Haemophilus influenzae*. During the 1980s, some of the species were found to produce β-lactamase, which led to resistance towards ampicillin. *Pseudomonas aeruginosa* was known to be resistant to all antimicrobial classes, including carbapenems, quinolones and aminoglycosides, and becoming multidrug-resistant *P. aeruginosa* (MDRP). There were various drug resistance mechanisms of MDRP, including reduced membrane permeability due to reduction of the outer membrane protein (D2 porin), mutation of the quinolone target especially DNA gyrase, over expression of efflux pump, production of metallo-β-lactamase of carbapenem-hydrolyzing enzyme and production of aminoglycoside modification enzyme. The resistance genes were acquired through conjugative plasmids (Saga & Yamaguchi, 2009).

2.2 Bacteria in Goat Milk

In healthy udder cells, the milk is considered sterile until it becomes colonized by microbes from various sources, such as milking tools, teat apex, water, feed, air, soil, and other factors. Bacteria that are commonly detected on the teat surface are *Solobacterium, Arcanobacterium* and *Clavibacter* sp. Goat milk represents almost 2.1% of global milk

production. It is known to be better than cow milk because it is less likely to induce allergies. In terms of nutrition, goat milk has a higher value of iron bioavailability, a higher content of fatty acids, smaller fat globules and forms a softer curd during the fermentation process, which is easier to digest.

Goat milk has a very rich and complex microbiota. This complexity of goat milk is responsible for the particular characteristics in fermented dairy products. This led to several studies about their characterization in order to select potential starter cultures based on their technological properties (Badis et al., 2004; Schirru et al., 2012). The microbiota of raw goat milk is exceptionally compelling due to its diversity and the existence of several bacteriocin-producing bacteria (Psoni et al., 2007; Schirru et al., 2012). In goat milk, lactic acid bacteria were dominated, including *Lactococcus, Leuconostoc, Enterococcus, Enterobacteriaceae*, yeasts and moulds. In addition, goat milk collected during winter is more likely to be dominated by *Lactococus* and *Pseudomonas*, in summer by *Klebsiella* and *Pantoea agglomerans*, and in autumn by *Staphylococcus, Corynebacteria, Acinetobacter baumannii, Chryseobacterium indologenes* and yeasts. Such variations are caused by several factors, including the difference in feed, animals' health state and weather conditions. The presence of *Lactococcus* sp. in raw milk does contribute to the function as a starter-culture in the cheese-making industry. In cheese production, the primary process is acidification through the production of L-lactate, proteolysis, conversion of amino acids into flavoring compounds, fat metabolism and citrate utilization. Other than that, *Lactobacillus* serves an important role in industrial dairy applications. *Lactobacillus helvaticus* can grow at high temperatures. Rapid autolysis of such strain will induce the release of intracellular enzymes, thus contributing to the flavor and reducing the cheese's bitterness. The usual way of isolating the bacteria from the milk sample is done by using culture-dependent method. Culture-dependent methods are using agar-based methods for bacteria isolation,

then followed by the method of phenotypic or genotypic for further identification. Apart from detecting the microbial species using culture-dependent method, the use of culture-independent method, for instance, DGGE, TGGE and qPCR might increase the bacterial discovery in raw milk (Quigley et al., 2013). Callon et al. (2007) used both culture-dependent and culture-independent methods in isolating bacteria from 118 goats' milk samples to detect the presence of microbial diversity of bacterial population in the milk (Table 2.1).

Culture-dependent*	Culture-independent†
Acinetobacter baumannii	*Acinetobacter baumannii*
Arthrobacter species	*Arthrobacter species*
Bacillus thuringiensis/cereus	*Bacillus thuringiensis/cereus*
Brachybacterium paraconglomeratum	*Brachybacterium paraconglomeratum*
Brevibacterium stationis	*Brevibacterium stationis*
Chryseobacterium indologenes	*Chryseobacterium indologenes*
Citrobacter freundii	*Citrobacter freundii*
Corynebacterium variable	*Corynebacterium variable*
Delftia acidovorans	*Delftia acidovorans*
Enterococcus faecalis/ saccharominimus	*Enterococcus faecalis/ saccharominimus*
Exiguobacterium	*Exiguobacterium*
Jeotgalicoccus psychrophiles	*Jeotgalicoccus psychrophiles*
Kocuria rhizophila/Kristinae carniphila	*Kocuria rhizophila/Kristinae carniphila*
Lactobacillus casei	*Lactobacillus casei*
Lactococcus lactis/garvieae	*Lactococcus lactis/garvieae*
Leuconostoc mesenteroides	*Leuconostoc mesenteroides*
Microbacterium oxydans	*Microbacterium oxydans*
Micrococcus species/caseolyticus	*Micrococcus species/caseolyticu*
Pantoea agglomerans	*Pantoea agglomerans*
Pseudomonas species/putida/ aeruginosa/fulgida	*Pseudomonas putida/aeruginos*
Salinicoccus species	*Salinicoccus species*
Staphylococcus epidermidis/ simulans/caprae/equorum	*Staphylococcus epidermidis/ caprae/simulans/equorum*
Stenotrophomonas maltophilia	*Stenotrophomonas maltophilia*
Streptococcus mitis	*Streptococcus mitis*
	Dietzia maris
	Enterobacter species/absuria
	Hahella chejuensis
	Klebsiella milletis/oxytoca
	Ornithinicoccus species
	Rothia species

Table 2.1: Bacterial population in raw goat milk using culture-dependent and culture-independent method

The most common bacterial populations detected were highlighted in red.
The less common, but frequently isolated bacterial populations detected were highlighted in blue.
The occasional bacteria that are detected were highlighted in black.
The bacteria that were detected by only one of the methods were underlined, while other bacteria can be detected by both methods.
References used for data in this table are from Callon et al. (2007), Goetsch et al. (2011) and Quigley et al. (2013).

2.3 Lactic Acid Bacteria (LAB)

Lactic acid bacteria (LAB) are classified as Gram-positive bacteria that are non-spore-forming, non-motile and rod / coccus-shaped organisms. LAB can produce a variety of antibacterial agents and are divided into several classes, including organic acids, diacetyl, hydrogen peroxide, and antimicrobial peptides such as bacteriocins. The utility of these natural antimicrobial substances is observed with a broad spectrum of inhibitory activities, however, only in limited types of food-based upon the environmental pH, microbial adaptation and the degradation of acidic species (Taylor, 2014).

2.3.1 Organic Acids Production from LAB

Organic acid production from LAB is indeed one of the factors that can increase the shelf life and the safety of food products. Acidification is mainly used to preserve food such as fermented sausage, milk and vegetables. The pathogenic inhibition is also dependant on the production of these organic acids (Ammor & Mayo, 2007). The level and type of organic acids produced depend on different factors such as the species of microorganism, the growing conditions and also the composition of the culture medium (Ozcelik et al., 2016). LAB that yield organic acids as the end products such as *Lactobacillus, Lactococcoccus, Streptococcus, Leuconostoc* and *Pediococcus* are frequently used as the starter cultures for fermentation of milk, vegetable and meat products (Hingsamer and Jungmeier, 2019). Lactic acid is one of the known organic acids produced by LAB that may be present naturally or as a product of *in-situ* microbial fermentation. The inhibition mechanism of lactic acid is probably related to the solubility of the non-dissociated lactic acid and the insolubility of dissociated lactate that causes the acidification within the cytoplasm membrane and the failure of proton motive forces which affects the trans-membrane pH gradient and the amount of available energy for cells to grow will eventually decrease (Oda et al., 2002; Wee et al., 2006). The yield and

productivity of lactic acid production are influenced by temperature (5–45°C), pH (3.5–9.6), nutrients (amino acids, peptides, nucleotides, vitamins, etc.), and the strain of lactic acid bacteria. Commonly used lactic acid strains belong to the genus *Lactobacillus, Leuconostoc, Enterococcus, Pediococcus, Streptococcus, Carnobacterium, Oenococcus, Vagococcus, Aerococcus, Weissella,* and *Tetragenococcus* (Abedi & Hashemi, 2020; Zheng et al., 2020).

Acetic acid also plays some part of inhibiting the growth and reducing the viability of bacteria, yeasts and fungi. The bacteriostatic effect of acetic acid is at 0.2%, while the bactericidal effect is present only above 0.3% (Ray, 2004). The fermentation process of *Lactobacillus delbrueckii* subspecies lactis and *Lactobacillus acidophilus* showed high production of acetic acid (Ozcelik et al., 2016). Benzoic acid that is naturally produced by LAB strains such as *Lactobacillus acidophilus, Lactobacillus casei, Lactobacillus helveticus* and *Streptococcus thermophillus* has commonly been used as a food preservative (Garmiene et al., 2010). Other than that, propionic acid which is produced by heterofermentative LAB such as *Lactobacillus butchneri* and *Lactobacillus diolivorans* are effective in preventing the growth of bacteria and yeasts in food such as cheeses, syrups and bakery products (Ray, 2004).

2.3.2 Hydrogen Peroxide

The production of hydrogen peroxide (H_2O_2) by LAB can also inhibit the growth of foodborne pathogens and be an important aspect in food preservation (Dahiya & Speck, 1968). By oxidizing lactate, most Lactobacilli species are able to produce hydrogen peroxide. Hydrogen peroxide can increase the discoloration and rancidity of the final product where eventually will interfere with the organoleptic properties of fermented meat products (Ammor & Mayo, 2007; Ammor et al., 2005). Depending on certain factors of concentration and environmental such as pH and temperatures, hydrogen peroxide is

considered as bactericidal where it was found to be potent at high temperatures while less sporicidal at room temperature (Juven & Pierson, 1996). Higher temperature and concentration of H_2O_2 cause lysis of dormant bacterial spores by removing the spore coat (Russell, 1982). Further impairment involves germination-like changes (refractility changes in permeabilized spores) or oxidative cortex hydrolysis due to activation of cortex lytic enzymes (Foster & Johnstone, 1987). Germination-like changes of spores that caused by H_2O_2 makes the permeabilized spores unable to germinate thus it will become inactive. At lower concentrations of H_20_2 (less than 1 %) or lower temperatures, H_20_2 killed the spores without lysis or germination-like changes where the dead spores remain intact and fully refractile (Shin et al., 1994). Higher pH (pH 8) of H_2O_2 can also cause lysis of bacteria cells faster comparing to pH lower than pH 6 (Baldry, 1983). Alkaline pH condition will remove the spore coats that composed of an alkali soluble protein as its protective fraction which will ultimately lyse the cells (King & Gould, 1969).

2.3.3 Secondary Metabolites

Production of secondary metabolites through fermentation that usually occurs late in the growth cycle (idiophase) is significantly influenced by various environmental manipulations including exhaustion of a nutrient (phosphorous, nitrogen, and carbon source), metals, enzyme inactivation, growth rate, and feedback control (Bibb, 2005; Sanchez & Demain, 2002). These events initiate signals that can cause a cascade of regulatory events resulting in secondary metabolism and morphogenesis of the microbial metabolite producers. The signal is often an inducer molecule that normally intercepts secondary metabolism and morphogenesis during rapid growth and sufficiency of nutrient by binding to and inactivating its repressor protein (Ohnishi et al., 2005). Secondary metabolites are not essential properties for the growth of cultures but support various survival functions in nature and act as an essential role in health and nutrition for our

societies (Bérdy, 2005; Demain & Fang, 2000). Antibiotics are one of the best known secondary metabolites.

2.3.4 Bacteriocins

Bacteriocins that can also be produced by LAB have the ability to inhibit other bacteria and may act as bacteriostatic or bactericidal agents. Bacteriocins are defined as ribosomally synthesized proteins or protein complexes usually antagonistic to genetically closely related organisms (De Vuyst, 1994). Bacteriocins have two mechanisms of action either by targeting the cell membranes of bacteria which causes the disintegration of the lipid bilayer structure or by disrupting the non-membrane cell where it will target the intracellular components of the cell (Shai, 2002).

Bacteriocins are generally recognized as safe substances (GRAS) that make them very suitable for food preservation. They are non-active and non-toxic on eukaryotic cells; digestive proteases can cause bacteriocins to be inactivated, so they have only a low influence on the gut microbiota. They are generally resistant to high temperatures where they can still maintain their antimicrobial activity after sterilization and pasteurization. They have a relatively wide antimicrobial range against many foodborne pathogenic and spoilage bacteria with the action of bactericidal mode. The bactericidal mode of action usually acts on the cytoplasmic membrane of the bacteria where there is no cross-resistance to antibiotics. Their genetic determinants are usually facilitate genetic manipulation and plasmid-encoded (Ananou et al., 2007).

Bacteriocin class I is known as the post-translational modified bacteriocin and divided into three major classes consisting of small peptides which are less than 5 kDa (19-28 amino acids in length). Class I.a called lantibiotics is further divided into four subclasses with distinct structures or content. The first subclass is made up of unusual amino acids with linear structure (lanthionine) LanB and LanC, transporter LanT and subtilisin-like

sereine proteinases LanP. Nisin is the common example of lantibiotic subclass I. The second subclass has a globular structure and comprises unusual amino acids (lanthionine) large LanM and single multifunctional LanT. Examples of this subclass include Lactocin S and Lacticin 3147 (Rea et al., 2011). The third subclass of bacteriocin class I consists of lantibiotic-like peptides grouped based on related modified enzymes. It has no associated antimicrobial activity for instance, SapB, AmfS and SapT. The fourth subclass is grouped on the basis of related novel class of lanthionine synthetases LanL for example, the lentipeptide. Bacteriocin class I.b known as labyrinthopeptins contains "labyrinthine" structure which is distinguished by the presence of labionin. While bacteriocin class 1.c is called sactibiotics and is characterized with sulphur to α-carbon linkage structure. There are two subclasses of sactibiotics consisting of single and two-peptide bacteriocins. Some examples include subtilosin A and thuricin CD (Rea et al., 2011).

Bacteriocin class II is known as the unmodified bacteriocin which is then divided into four major classes. The bacteriocins from this class consist of standard amino acid residues either linked by disulphide bridges or cyclized at the N and C terminus. They are hence known as a heterogeneous group of peptides that are less than 10 kDa. Class II.a is called as pediocin-like bacteriocin that can be further divided into subclasses I until IV. There are currently 28 different bacteriocins in this class which is based on the differences in specificity in their 3D structures. Examples of bacteriocins under this class are enterocin A and sakacin G (Rea et al., 2011). Bacteriocin class II.b is classified as two-peptide unmodified proteins consisting of mainly LAB origin such as plantaricin S and lactococcin G while another subclass consists of anti-botulinum bacteriocin such as broncochin-C. On the other hand, bacteriocins under that class II.c are known as circular bacteriocins that are ribosomally synthesized which are generally heat-stable and significantly resistant to proteolytic digestion. The two subclasses of circular bacteriocins include enterocin AS-58 and the non-LAB circularin A as well as gassericin A, reutericin

6 and AR10. Another class of bacteriocin,class II.d is the unmodified, linear, non-pediocin-like, one peptide bacteriocins which include lactococcin A. Another class of bacteriocin which is formerly known as bacteriocin class III is now called bacteriolysin. This class of bacteriocins consists of non-bacteriocin lytic proteins (Rea et al., 2011).

Bacteriocin-producing lactic acid bacteria (LAB) have been considered an alternative to chemical preservatives and disinfectants in many food products. Due to their genetics and biosynthetic machinery as well as antimicrobial activity, bacteriocin produced by the LAB such as nisin has been widely recognized as a food additive. The applications of nisin in food products such as vacuum-packed cooked ham, fresh-cut lettuce and starter culture in fermentation of dairy products are approved globally. For example, in the European Union, nisin is identified as E234 in food products it is applied to. In the US and Canada, some bacteriocins and organic acids have been commercially produced in the fermentation of food-grade substrates as in ALTA™ 2431, MICROGARD™, and Bactoferm F-LC (Martínez, 2016).

2.4 Enterococci

Enterococci, genus of Enterococcus belonging to the group of lactic acid bacteria (LAB) were reported to be lacking in the GRAS status, although the application of LAB in the food industry has already been assigned as one. The concerns were raised regarding their use as starter culture due to their relationship with common nosocomial infections. However, the ability of the enteroccoci to produce a wide variety of bacteriocins which often called enterocins makes them a widely studied subject as one of the potential probiotic candidates. The main producers of enterocins are mainly from *E. faecium* and *E. faecalis* and to a lesser extent *E. durans, E. hirae, E. avium* and *E. mundtii.*

2.4.1 Classes of Enterocin

Some of the bacteriocins produced by *Enterococcus* can be grouped together with typical traditional classification of bacteriocins produced by LABs whereas others could not be included (Cotter et al., 2005). Four new classes on grouping enterocins have been suggested by (Franz et al., 2007). Class I is a group for lantibiotic enterocins such as cytolysin (Cox et al., 2005) and enterocin W (Sawa et al., 2012) isolated from from *E. faecalis* which are rarely found in enterococci. The enterocin of this class is considered two-component lantibiotics because it consists of two linear peptides containing lanthionine residues and is structurally different from other linear lantibiotics such as nisin A and Z. Class II is the class of pediocin enterocins which consists of class II.a, II.b and II.c. According to the sequence similarities, Class II.a of pediocin-like bacteriocin is divided into two subgroups (Laukova, 2011). Enterocin A (Aymerich et al., 2000), mundticin which is produced by *E. mundtii* (Kawamoto et al., 2002) and enterocin CRL5 (Saavedra et al., 2004) are in subgroup 1, while subgroup 2 consists of enterocin P (Cintas et al., 1997) and enterocin M which is a variant of enterocin P (Mareková et al., 2007). Enterocins synthesized without a leader peptide refer to Class II.b such as bacteriocin L50A, bacteriocin L50B, enterocin Q (Cintas et al., 2000) and enterocin C (Maldonado-Barragán et al., 2009). Other linear-non-pediocin-type enterocins such as enterocin B are in Class II.c (Casaus et al., 1997). All cyclic antibacterial peptides are regrouped of Class III including enterocin AS-48 produced by *E. faecalis* S-48 (Maqueda et al., 2004). Finally, Class IV includes enterolysin A produced by *E. faecalis* (Nilsen et al., 2003). Enterocins mainly target the cytoplasmic membrane and forming pores that deplete trans-membrane potential and the pH gradient in the cell membrane. This causes a leak out of essential intracellular molecules from cells (Cleveland et al., 2001).

Food pathogenic and spoilage-causing bacteria such as *Staphylococcus spp., Listeria spp. and Bacillus spp*. were efficiently inhibited by enterococci-producing-enterocins in

varying food regimens (Aymerich et al., 2000). Besides inhibiting these food-borne pathogens, enterococci are also responsible for the organoleptic properties development of traditional fermented food products from all regions (Foulquié Moreno et al., 2006).

2.4.2 *Enterococcus faecium* in Dairy Products

Enterococcus faecium is present primarily in raw milk, fermented milk products, and several types of processed foods (Herranz et al., 2001; Saavedra et al., 2003; Wessels, 1988). Enterococci present in raw milk can be used as natural starters because of their ability to survive in different temperatures whether during milk refrigeration or pasteurization because of their psychotropic nature and able to adapt in different substrates and growth conditions (Bhardwaj et al., 2008). Their ability to ferment citrates to form diacetyl and other volatile compounds are important in the maturation of different cheese varieties where they provide a distinct characteristic taste and flavor to the cheese (Foulquié Moreno et al., 2006). It was observed by (Huang et al., 2013) that *E. faecium* RZS C5 produces enterocins with activity against foodborne pathogens in milk and cheese. *E. faecium* EFM01 isolated from cheese produced Enterocin A that is active against many species of Listeria including *L. monocytogenes, L. seeligeri* and *L. innocua* (Ennahar & Deschamps, 2000). *E. faecium F58* was isolated by Achemchem et al. (2005) from goat's cheese made without any addition of starter cultures. The enterocin F-58 *E. faecium* F58 was active against several foodborne pathogenic and spoilage-causing Gram-positive bacteria including *L. monocytogenes, L. innocua, S. aureus, B. cereus, B. subtilis, C. perfringens*, and C. *tyrobutyricum.*

CHAPTER 3: MATERIALS AND METHODS

3.1 Milk Sampling and Bacteria Isolation

Frozen and raw goat milk samples were collected from the store (Salizzi brand) and dairy farms located at Seri Muda, Shah Alam, respectively. The raw milk was collected directly from the goat by the farmer and was kept inside the sterile scott bottle. The collected raw milk was then being pasteurized at 63°C for 30 minutes to eliminate harmful bacteria that may be present in the raw milk and was then left to ferment for 2 days at room temperature. Meanwhile, the frozen milk was left at room temperature until it melted completely and then directly underwent the fermentation process for 2 days at room temperature. For the isolation process, the milk was serially diluted with 10-fold serial dilution and plated in triplicate onto two different specific mediums, de Man Rogosa-Sharpe (MRS) agar and M17 agar for incubation. The plates were incubated at 37°C for 48 to 72 h in anaerobic jars. A variety of bacteria grew on the plates. The total viable count was not determined. Instead, 24 isolates colonies were randomly picked from the plates based on variety of colony morphologies. Each colony was sub-cultured on fresh MRS agar and subculture was done two times to get pure colonies.

3.1.1 Biochemical Test

The selected isolates strains were biochemically identified based on gram staining, oxidase test and the presence of catalase. Briefly, the isolates were sub-cultured onto MRS agar and incubated for overnight at 37ºC. Then single colonies were selected for subsequent biochemical test of gram staining, oxidase test and catalase test. The isolated bacteria was stained following the procedure described by Madigan and Brock (2009). A smear of bacteria was prepared on a glass slide. The smear was heat-fixed before staining with crystal violet. The crystal violet was flooded on the smear for 1 minute and washed with distilled water. The smear was washed with alcohol for 20 seconds and distilled

water again. The smear was counterstained by flooding with safranin for 30 seconds and washed with distilled water. The slide was dried with clean tissue paper. The slide was observed under light microscope using oil immersion lens.

For oxidase test, 1 to 2 drops of 1% NNNN- tetramethyl-p-phenylenediamine dihydrochloride was placed on a filter paper. Then a single colony from the overnight culture was picked using a sterile loop and smeared onto the filter paper already soaked with the solution. The change of colour was observed within 10 to 30 seconds. A positive result is indicated by bluish purple colour (Madigan & Brock, 2009).

The activity of catalase enzyme produced by the isolated bacteria strain was studied according to the method by Rozara (2002). 1 to 3 drops of the 3% hydrogen peroxide solution was placed onto a glass slide and a single colony of bacterial strain was picked and smeared into the solution for the catalase test. The production of bubbles were observed within a few seconds, indicating of a positive catalase activity.

3.1.2 Storage and Maintenance of Bacterial Culture

The storage of bacterial cultures was prepared according to the following procedure. A single colony was inoculated into 5 ml of MRS broth and was incubated at 37ºC overnight. Following incubation, 100 µl of an overnight culture was spread on MRS agar and grown for 24 to 72 hours. The bacterial lawn forming on the plate on the following days was scraped out using inoculating loop and was put inside a cryovial containing 1 ml of MRS broth and 1 ml of 100% sterile glycerol solution to prepare a glycerol stock with final concentration of 50% glycerol. The glycerol stock suspension was stored at -20ºC or -80ºC for long term storage. A loopful of the stock suspension was streaked onto MRS agar plates and incubated overnight at 37ºC. The purity of the isolate was checked. For short term storage, these MRS agar cultures were stored at 4ºC for further use in the experiment as working culture. Throughout the study period, these bacteria cultures were

maintained on agar media at 4ºC up to maximum of two weeks in which sub-culturing was carried out every two weeks to ensure cells viability.

3.2 Screening for Antibacterial Activity

The selected isolates were grown in MRS broth at 37°C for 24 hours. After growing those isolates in MRS broth, the cultures were centrifuged at 10000 x g (9021 rpm) for 20 minutes via the Beckman J2-M1 model of high speed centrifuge to get cell-free culture supernatants (CFCS). The CFCS of each isolate were screened for antimicrobial activity by using agar well diffusion assay. Four pathogenic bacteria that were obtained from the bacterial collection of Microbial Biochemistry Laboratory, Institute Biological of Science, University Malaya were used as indicators for the isolation of antimicrobial compound-producing strains. The four chosen indicators were *Staphylococcus aureus* SA7001, *Shigella boydii* SB1003, *Shigella dysenteriae* SD1007 and *Salmonella thyphimurium* SM4001. Fresh culture of indicator microorganisms were suspended into sterile saline by adjusting the turbidity to be equivalent as McFarland standard 0.5 (OD600 = 0.08 to 0.1). The suspensions were swabbed on Mueller-Hinton agar (Oxoid™, England) plates to make bacterial lawns. The plates were left to dry for five minutes and wells with diameter of 8 mm were made using a sterile pipette tip with the broader end. 100 µl CFCS of test sample was loaded into respective well. Commercial antibiotic has been used as the positive control (ampicillin = 10 µg/ml) and sterile standard saline (0.85%) as negative control. The plates were incubated at 37°C for 24 h. The diameter of the inhibition zone formed on the agar was each measured to indicate the antagonistic activity of selected isolates. Isolates that produce potent antimicrobial activity with high inhibition zone against indicator were selected for further tests.

3.3 Stability Tests

The chosen isolates' cell-free culture supernatants (CFCS) were tested for stability after enzymatic and physiochemical treatment. The parameter tested were temperature, pH and proteolytic enzymes.

To evaluate the effect of heating on antimicrobial activity, the CFCS of the isolate was transferred to different test tubes and were exposed to heat at different temperatures (40°C, 60°C, 80°C and 100°C). The test tubes was incubated in water bath with respective temperature treatment for 20 minutes. The antimicrobial activity was tested after the treatments.

The effect of pH on the antibacterial activity was determined by first adjusting the pH of the CFCS between pH 2 to pH 8 by using sterile 1N NaOH or 1N HCl solution. Then, the samples were assayed for antimicrobial activity after a 2 hour incubation period. Untreated supernatants were used as control for both pH and temperature assays.

Three different enzymes (proteinase K, pepsin and catalase) were used for enzymatic treatment to test the possibility of causing any inactivation of antimicrobial substance. The CFCS of the isolate was treated with respected enzymes with 1 mg/ml final concentration for 1 h and all of the treatments were incubated at 37°C before being tested for antimicrobial activity. The antibacterial activity post-treatment was assayed by the agar-well diffusion assay against indicator strain as described earlier. A sample without enzymes was used as the control of the treatment.

3.4 Effect of CFCS on the Growth of strain *Staphylococcus aureus* SA7001

The mode of action of isolates producing antimicrobial metabolite was explored by adding the cell-free supernatant of selected isolates to the growing broth culture of strain *S. aureus* SA7001. About 20 ml of cell-free supernatant of selected isolates was filter-sterilized and added into 100 ml of early log phase ($OD_{600nm} \approx 0.06$) of indicator strain culture. The incubation took place in a Luria-Bertani (LB) broth at 37°C. The optical density readings were recorded at the wavelength of 600 nm every 2 hours for continous14 hours of incubation. The broth without CFCS of isolate was added to the SA7001 and this was the blank control.

3.5 Bacteria Identification

Polymerase chain reaction (PCR) amplification of 16S rDNA gene and nucleotide sequencing were carried out for molecular identification of selected bacterial strains.

3.5.1 DNA Extraction

To identify the bacterial isolates genetically, the total genomic DNA was extracted from each chosen isolated strain. The DNA was extracted by using heat-shock treatment. One colony of selected isolates was suspended in 30 µl of sterile distilled water and later was boiled at 100°C for 10 minutes. Then, it was immediately cooled in ice for 5 minutes and centrifuged at 13,000 rpm for 10 minutes. The pallet was disposed of while the supernatant (which is also known as lysate) was used.

3.5.2 Polymerase Chain Reaction (PCR) Amplification of 16S rDNA Gene

The extracted DNAs were then amplified using polymerase chain reaction (PCR) with the universal 16S rDNA as primers. The PCR reaction master mix was prepared as stated in Table 3.1 and run under conditions as described in Table 3.2 (Goh & Philip, 2015). The universal primers that were used are as follows:

a) Forward primer (27F): 5'-AGA GTT TGA TCM TGG CTC AG-3'

b) Reserve primer (1492R): 5'-ACG GYT ACC TTG TTA CGA CTT-3'

Table 3.1: PCR master mix preparation for 16S rDNA detection

Reagent	Final conc	50 µl	25 µl
2x MyTaq Red Mix, Bioline	1x	25	12.5
27F	0.4 µM	2	1
1492R	0.4 µM	2	1
Template DNA	~50 ng ++	5	2.5
ddH_20		16	8

Table 3.2: Conditions for 16S rDNA PCR amplification

Step	Temperature ^oC	Time	Number of cycles
Initial denaturation	94	5 min	1
Denaturation	94	1 min	30
Annealing	52	1 min	30
Extension	72	1 min 30 s	30
Final extension	72	10 min	1

The PCR amplicons were analyzed on 1% (w/v) agarose gel with RedSafe nucleic acid staining (iNtRON Biotechnology). The PCR amplicons that matched with the expected size on the gel were sent for purification and sequencing at Apical Scientific Sdn Bhd, Kuala Lumpur. The homology of the partial 16s rRNA sequencing results were compared with NCBI nucleotide sequence databases with a sequence matching program in Basic Local Alignment Search Tool (BLAST) to identify the nucleotide sequence.

3.6 Suitability of Isolates as Probiotic Bacteria

Several tests were carried out to check the suitability of the chosen isolates as probiotic bacteria, such as the survival rate under conditions simulating the human GI tract and the characterization of isolates for their virulence factors.

3.6.1 Survival under Conditions Simulating the Human GI Tract

To test the survival rate of isolate under low pH simulating the human gastrointestinal tract, two conditions were set up: tolerance test towards simulated gastric juice in stomach and bile salt in small intestine. The method was performed as described by Zhang et al. (2011) as illustrared below:

3.6.1.1 Tolerance to Simulated Gastric Juice

An overnight culture of the isolate was harvested at 4ºC for 10 minutes (6000 xg) and was washed twice with PBS solution. Simulated gastric juice was formulated for condition simulating the stomach by suspending pepsin of 0.3 mg/ml in PBS (pH 2.0). Then, 0.2 ml of washed cell suspension of isolate was added into 1 ml of simulated gastric juice with 0.3 ml NaCl (0.5% w/v) then it was mixed and incubated at 37 ºC. Total viable counts were then determined at three different time intervals (0, 1 and 3) hour to assess gastric juice's transit tolerance. The times used were to reflect the duration of food held in the human stomach.

3.6.1.2 Tolerance to Bile Salt Condition in Small Intestine

PBS solution (pH 8.0) containing 0.3% (w/v) of Oxgall bile salt was used to test the tolerance of isolate to the condition in small intestines. 2% of the washed cell suspension isolate was added into this simulated bile salt solution (2 ml of washed cell suspensions was added into 100 ml of simulated bile salt solution) and incubated for 4 hours at 37ºC. Bile tolerance of the isolate was determined by comparing the viable counts on MRS

agars for sample with incubation of 0 and 4 hours with and without the addition of bile salt solution.

3.6.2 Characterization of Isolates for Their Virulence Factors

To test on isolates' virulence factors, several tests were performed such as testing the production of gelatinase, testing the present of hemolysin, and antibiotic susceptibility testing.

3.6.2.1 Detection of Gelatinase and Hemolysin Activity

Gelatinase activity of the strain was determined according to Chajęcka-Wierzchowska et al. (2017). Briefly, the isolate was inoculated on Brain Heart Infusion (BHI) agar containing 3% of gelatin and incubated at 25ºC for 48 hours. The existence of gelatinase activity will be shown by a turbid zone around the inoculated colonies.

The detection of hemolysin activity was performed according to Chajęcka-Wierzchowska et al. (2017). Columbia agar containing 5% sheep blood was used for the bacterial inoculation which was later be incubated at 37ºC for 48 hours. β-hemolysis (positive haemolytic activity) and γ-hemolysis (negative haemolytic activity) were indicated by the presence or absence of clearing zones around the colonies respectively. The α-hemolysis would be indicated by greenish zones around the colonies in which it was regarded as negative result for the assessment of hemolytic activity.

3.6.2.2 Antibiotic Susceptibility Testing

Clinical and Laboratory Standards Institute (CLSI, 2015) has described the standards and criteria to determine the resistance to antibiotic by using a Kirby–Bauer disk diffusion assay. Following this method, seven types of antibiotics were used which includes ampicillin (30 µg/ml), streptomycin (25 µg/disc), chloramphenicol (30 µg/disc), kanamycin (30 µg/disc), tetracyclin (30 µg/disc), ciprofloxacin (5 µg/disc) and

vancomycin (30 µg/disc). An overnight strain culture with saline water (0.5 McFarland turbidity standard) was spread across Mueller-Hinton agar. The antibiotic discs were applied onto the plate and was then incubated at 37ºC. The inhibition zone was measured after 24 hours of incubation period. The diameter of inhibition zones was measured after the incubation and interpreted as per CLSI recommendations (CLSI, 2015) and were classified as resistant, intermediate and sensitive (Bauer, Kirby, Sherris, & Turck, 1966).

3.7 Analysis of Potential Antimicrobial Compounds in Isolates

The analysis of the potential antimicrobial compound from the isolates was carried out by PCR, targeting the known bacteriocin genes and the Q-TOF LC/MS method.

3.7.1 PCR Targeting Known Bacteriocin Genes

The total genomic DNA of an overnight culture of the chosen isolate was extracted using heat shock treatment. This extracted genomic DNA was used as a template for bacteriocin-encoding genes detection by undergoing PCR amplification process. The known bacteriocin genes used in this study are enterocin A and B (Table 3.3). Following a similar study from Huang et al. (2016), PCR reaction condition was described as in Table 3.4. The presence and molecular size of the PCR amplicons were visualized on 1% (w/v) agarose gel pre-stained with RedSafe nucleic acid staining (iNtRON Biotechnology). The PCR amplicons were sent for purification and sequencing at Apical Scientific Sdn Bhd, Kuala Lumpur. The validity of PCR amplicon as the target gene was performed by comparing the sequencing result to the protein database in the GenBank through BLASTX program.

Table 3.3: Primers and conditions for PCR analysis on detection of chosen enterocin

Primers	Sequence	Amplicon size (bp)	Annealing temperature (°C)	References
entA_F	AAA TAT TAT GGA AAT GGA GTG TAT	130	48	du Toit et al. (2000)
entA_R	GCA CTT CCC TGG AAT TGC TC			
entB_F	GAA AAT GAT CAC AGA ATG CCT A	160	48	du Toit et al. (2000)
entB_R	GTT GCA TTT AGA GTA TAC ATT TG			

Table 3.4: PCR master mix preparation for enterocin detection

Reagent	Final conc	50 µl	25 µl
2x MyTaq Red Mix, Bioline	1x	25	12.5
Forward primer	0.5 µM	2.5	1.25
Reverse primer	0.5 µM	2.5	1.25
Template DNA	~50 ng ++	5	2.5
ddH_20		15	7.5

Table 3.5: Conditions for enterocin PCR amplification

Step	Temperature °C	Time	Number of cycles
Initial denaturation	94	1 min	1
Denaturation	94	1 min	35
Annealing	48	1 min	35
Extension	72	1 min	35
Final extension	72	5 min	1

3.7.2 Q-TOF LC/MS Analysis in the Cell-Free Culture Supernatant of Isolate

The isolate was grown in MRS broth at 37°C for 24 h. The secondary metabolites usually produced during late growth phase (idiophase) of isolate (Abdel-Aziz et al., 2017). Therefore, after 24 hours of incubation, the bacterial growth already reached stationary stage and the secondary metabolites were ready to be collected at this stage (Appendix E). Then the incubated isolate was centrifuged at 10000 x g (9021 rpm) for 20 minutes via the Beckman J2-M1 model of high speed centrifuge. The CFCS formed after the centrifugation was transferred into 100 ml sterile scott bottle and the pallet was discarded. 80% (w/w) ammonium sulphates were added to the CFCS solution and was continuously stirring overnight to remove proteins. The solution was centrifuged again under the same condition as previous and the supernatant was filtered through 0.22 µM membrane filter, meanwhile the precipitated products (formed from the addition of ammonium sulphates) were removed. The filtered supernatant was analyzed using Agilent 6500 series Accurate-Mass Q-TOF LC/MS system (Agilent Technologies, Santa Clara, USA) at Centre for Research Services (PPP), INFRA Laboratory, University of Malaya. The injection volume was 3 µl, with a 0.4 ml/min flow rate under a gradient program of two mobile phases. 5 mM ammonium acetate with 0.1% acetic acid (v/v) in water was the composition of mobile phase A, meanwhile, 0.05% acetic acid (v/v) in acetonitrile was the composition of mobile phase B. Mass spectra were acquired with a TOF/Q-TOF mass spectrometer with gas flow 14 L/min, gas temperature 200°C and nebulizer 35 psig. The mass spectrometer with a scanning range of 100–1000 m/z was operated in the positive and negative ion mode. An agilent Technologies Zorbax Eclipse Plus C18 (4.6 × 100 mm, 3.5 µm) was used to perform liquid chromatography separation. The gradient program was applied as described in Table 3.6 (Lau et al., 2015).

Table 3.6: Solvent gradient profile in Q-TOF LC/MS

	Time (min)	A (%)	B (%)
1	0.35	95.00	5.00
2	14.50	34.00	66.00
3	18.00	0.50	99.50
4	33.50	0.50	99.50
5	35.51	0.50	99.50
6	40.00	0.50	99.50

3.8 Statistical Analysis

The statistical analyses were performed using MINITAB version 14 (Minitab Inc., PA, United States). Statistically significant differences of the experiments achieved in the various assays were evaluated by analysis of variance (ANOVA) and Tukey test. The mean values and the standard deviation were calculated from the data obtained through triplicate trials. A probability of $p < 0.05$ was used as the criterion for statistical significance.

CHAPTER 4: RESULTS

4.1 Bacteria Isolation and Biochemical Test

24 bacteria were isolated from both frozen and raw goat milk. 16 bacteria were isolated from frozen goat milk (FG), while 8 other bacteria were isolated from raw goat milk (RG). Out of 16 isolates from frozen goat milk, the first 8 isolates were from MRS plate, labelled with the 3rd capital letter after 'FG', the next 8 were from M17 plate, labelled with numbers after 'FG'. The first 5 isolates from raw goat milk were labelled with 'RG' followed by uppercase letter, while the remaining were followed by numbers as isolated from M17 agar. 24 isolates were tested for their preliminary biochemical test based on catalase, oxidase and gram staining (Table 4.1). The colonies morphologies of these 24 isolates were not recorded.

Table 4.1: The biochemical test of all isolated bacteria based on catalase, oxidase and gram staining test.

Isolates	Catalase test	Oxidase test	Gram Stain
FGa	negative	negative	Gram-positive (Coccus)
FGb	negative	negative	Gram-negative (Coccus)
FGc	negative	negative	Gram-positive (Coccus)
FGd	negative	negative	Gram-positive (Bacillus)
FGe	negative	negative	Gram-positive (Coccus)
FGf	negative	negative	Gram-positive (Coccus)
FGg	negative	negative	Gram-positive (Coccus)
FGh	negative	negative	Gram-positive (Coccus)
RGA	negative	negative	Gram-positive (Coccus)
RGB	negative	negative	Gram-positive (Coccus)
RGC	negative	negative	Gram-positive (Coccus)
RGD	negative	negative	Gram-positive (Coccus)
RGE	negative	negative	Gram-positive (Coccus)

Table 4.1, continued.

Isolates	Catalase test	Oxidase test	Gram Stain
RG1	negative	negative	Gram-positive (Coccus)
RG2	negative	positive	Gram-positive (Coccus)
RG3	negative	positive	Gram-positive (Bacillus)
FG1	negative	negative	Gram-positive (Bacillus)
FG2	negative	negative	Gram-negative (Coccus)
FG3	positive	negative	Gram-negative (Coccus)
FG4	negative	negative	Gram-positive (Bacillus)
FG5	negative	negative	Gram-positive (Bacillus)
FG6	negative	negative	Gram-positive (Bacillus)
FG7	negative	negative	Gram-positive (Bacillus)
FG8	negative	negative	Gram-positive (Bacillus)

Based on Table 4.1, almost all of the isolates gave negative results for catalase test except isolate FG3 and for oxidase test only isolate RG2 and RG3 gave positive results. Gram-staining tests showed that only isolate FGB, FGb, FG2 and FG3 were shown to be gram-negative bacteria while others were gram-positive bacteria. All of these isolates were used for further experiments.

4.2 Screening for Antimicrobial Activity

The cell-free culture supernatant (CFCS) of 24 isolates from the samples have been tested for their antibacterial activity against four (4) different indicators: *Staphylococcus aureus* SA7001, *Shigella boydii* SB1003, *Shigella dysenteriae* SD1007 and *Salmonella typhimurium* SM4001 .

4.2.1 CFCS of isolates against *Staphylococcus aureus* SA7001

Results of inhibition zone of isolates CFCS against *Staphylococcus aureus* SA7001 are shown on Figure 4.1. Only isolate RG3 did not exhibit any zone of inhibition against *S. aureus* SA7001, while others showed quite positive results. Both isolates FGa and RGB presented the highest zone of inhibition at 23.00 mm.

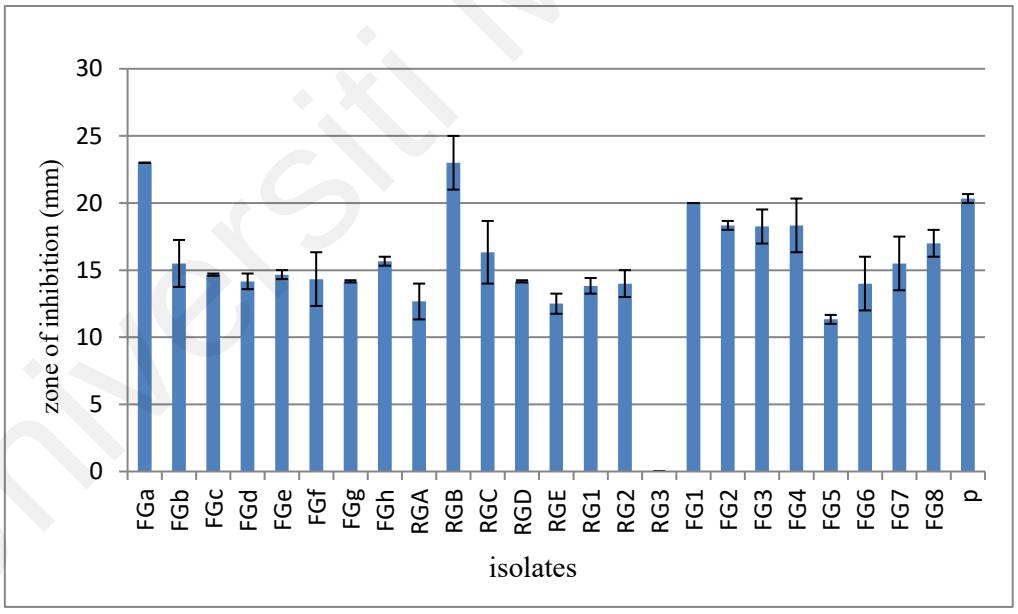

Figure 4.1: Inhibition zone of isolates CFCS against *Staphylococcus aureus* SA7001. The assay displayed the antimicrobial activity of all 24 isolates where p presented as positive control (ampicillin 10 µg/ml).

4.2.2 CFCS of isolates against *Shigella boydii* SB1003

Results of inhibition zone of isolates CFCS against *Shigella boydii* SB1003 are shown on Figure 4.2. Isolate FGb did not showed any antibacterial activity against *Shigella boydii* SB1003. Other isolates showed positive results where RGD showed the highest zone of inhibition with 16.33 mm.

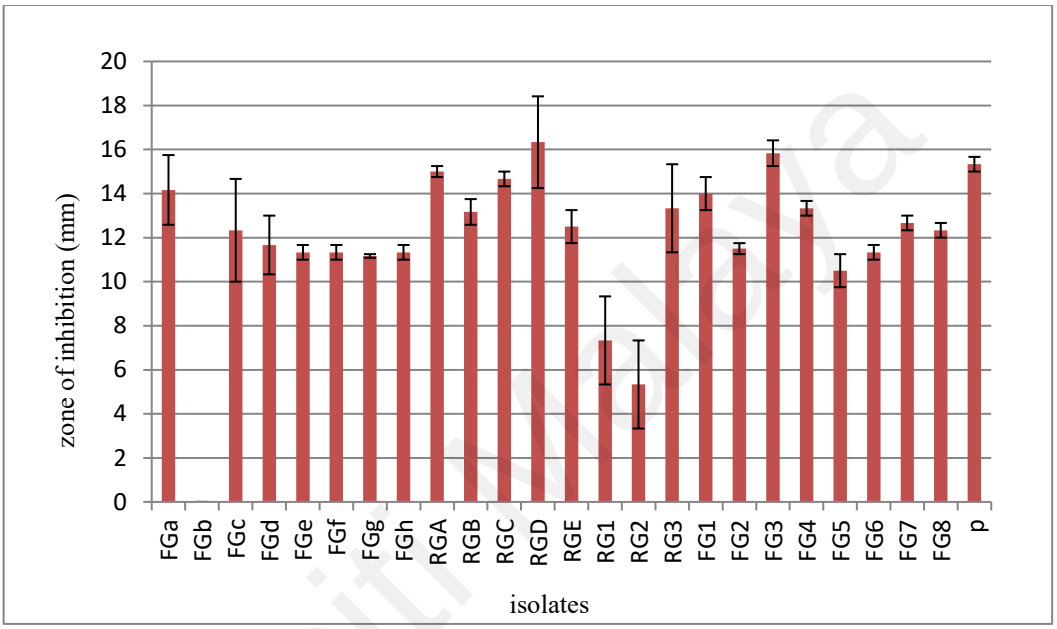

Figure 4.2: Inhibition zone of isolates CFCS against *Shigella boydii* SB1003. The assay displayed the antimicrobial activity of all 24 isolates where p presented as positive control (ampicillin 10 µg/ml).

4.2.3 CFCS of isolates against *Shigella dysenteriae* SD1007

Results of inhibition zone of isolates CFCS against *Shigella dysenteriae* SD1007 are shown on Figure 4.3. Only FG2 did not show any zone of inhibition against *Shigella dysenteriae* SD1007, while FGa gave the highest antibacterial activity against the chosen indicator with 15.33 mm.

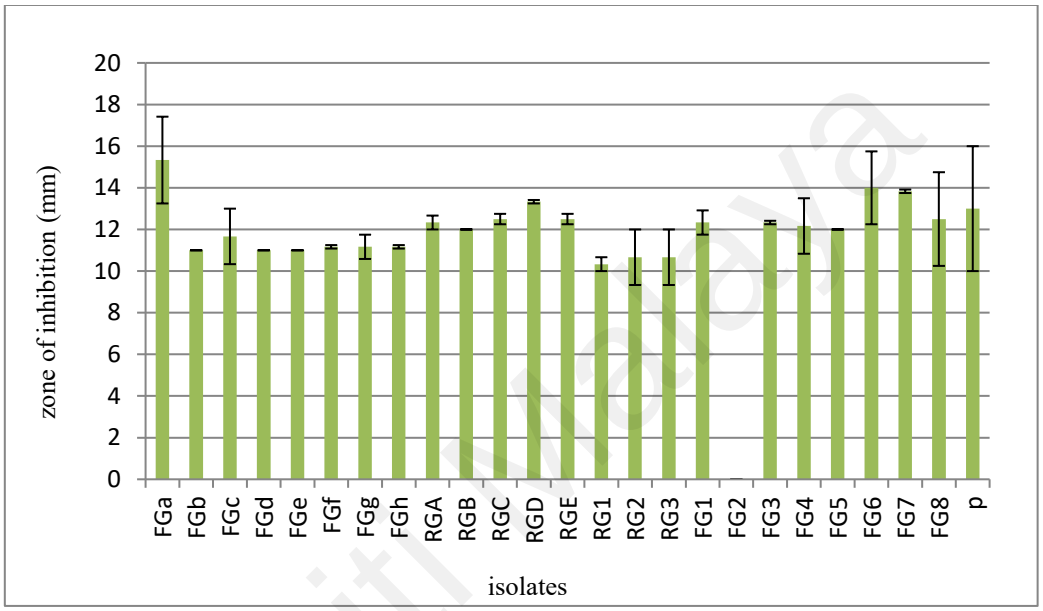

Figure 4.3: Inhibition zone of isolates CFCS against *Shigella dysenteriae* SD1007. The assay displayed the antimicrobial activity of all 24 isolates where p presented as positive control (ampicillin 10 µg/ml).

4.2.4 CFCS of isolates against *Salmonella typhimurium* SM4001

Results of inhibition zone of isolates CFCS against *Salmonella typhimurium* SM4001 are shown on Figure 4.4. Three isolates (FGb, RGF and RGG) did not show any antibacterial activity against *Salmonella typhimurium* SM4001. The highest antibacterial activities are from isolates FGa and FGd where both of them showed 14.67 mm of inhibition zone.

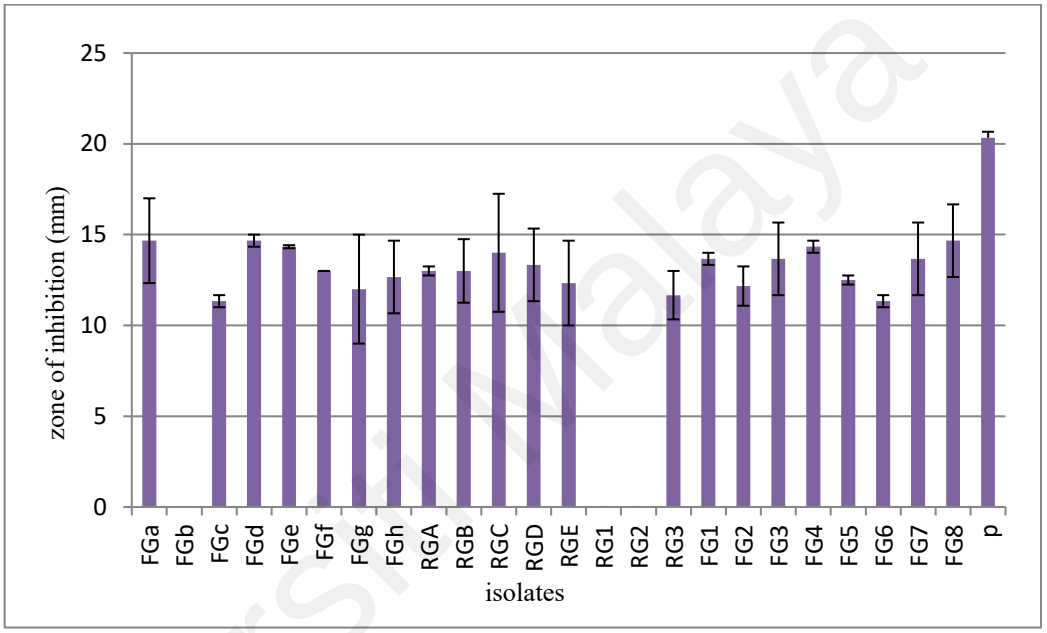

Figure 4.4: Inhibition zone of isolates CFCS against *Salmonella typhimurium* SM4001. The assay displayed the antimicrobial activity of all 24 isolates where p presented as positive control (ampicillin 10 µg/ml).

4.2.5 Selection of Potential Isolates and Sole Indicator

Anova: a single factor was done to check the average of all isolates CFCS against all four chosen indicators. Based on Table 4.2, 4 isolates with the highest average of inhibition zone have been selected for the next experiment. The chosen isolates were FGa, RGB, FG1 and FG3 with 16.79 mm, 15.29 mm, 15.00 mm, and 14.99 mm of inhibition zone respectively. Anova test in Table 4.2 showed significant differences ($p<0.05$) between the isolates against all four tested indicators.

Table 4.2: Summary of anova: single factor for all isolates' CFCS against all indicators

Isolates	*Count*	*Sum*	*Average*	*Variance*
FGa	4	67.17	16.79	17.36
FGb	4	26.50	6.62	61.89
FGc	4	50.00	12.50	2.25
FGd	4	51.50	12.87	3.28
FGe	4	51.33	12.83	3.74
FGf	4	49.83	12.45	2.24
FGg	4	48.50	12.12	2.01
FGh	4	50.83	12.70	4.34
RGA	4	53.00	13.25	1.43
RGB	4	61.16	15.29	26.68
RGC	4	57.50	14.37	2.52
RGD	4	57.16	14.29	2.01
RGE	4	49.83	12.45	0.01
RG1	4	31.50	7.87	34.61
RG2	4	30.00	7.50	37.74
RG3	4	35.67	8.91	36.54
FG1	4	60.00	15.00	11.63
FG2	4	42.00	10.50	58.46
FG3	4	59.99	14.99	6.52
FG4	4	58.16	14.54	7.17
FG5	4	46.33	11.58	0.75
FG6	4	50.67	12.67	2.37
FG7	4	55.67	13.91	1.37
FG8	4	56.50	14.12	4.80
positive	4	69.00	17.25	13.58

Table 4.2, continued.

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Isolates	701.7303	24	29.23876	2.116464	0.007452	1.663338
Within Isolates	1036.118	75	13.81491			
Total	1737.848	99				

SS= Sum-of-squares, df= Degrees of freedom, MS= Mean squares, F= Fisher's ratio, P-value= Probability value, F crit= Fisher's critical

Based on Table 4.3, *Staphylococcus aureus* SA7001 has been chosen as the sole indicator for later experiments because all 4 selected isolates gave the highest antimicrobial activity against it compared to the other three indicators.

Table 4.3: : Inhibition zone of CFCS of isolate FGa, RGB, FG1 and FG3 against all indicators

Isolate	*Inhibition zone (mm)			
	FGa	RGB	FG1	FG3
Staphylococcus aureus SA7001	23.00 ± 0.00^{a}	23.00 ± 2.00^{a}	20.00 ± 0.00^{a}	18.16 ± 1.26^{a}
Shigella boydii SB1003	14.16 ± 1.58^{b}	13.16 ± 0.58^{b}	14.00 ± 0.75^{b}	15.83 ± 0.58^{ab}
Shigella dysenteriae SD1007	15.33 ± 2.08^{b}	12.00 ± 0.00^{b}	12.33 ± 0.58^{b}	12.33 ± 0.08^{b}
Salmonella typhimurium SM4001	14.67 ± 2.33^{b}	13.00 ± 1.75^{b}	13.67 ± 1.33^{b}	13.67 ± 2.00^{b}

Mean of three replicates ± standard deviation measured in millimeter. ($^{a, b}$) = Means that do not share a letter are significantly different ($p < 0.05$).

4.3 Stability Tests of CFCS of isolate FGa, RGB, FG1 and FG3

The CFCS of four chosen isolates (FGa, RGB, FG1 and FG3) were tested for their stability against different temperatures, pH and proteolytic enzymes.

4.3.1 Effect of Temperature

The effect of temperature of isolates CFCS against *Staphylococcus aureus* SA7001 are shown on the data stated in Figure 4.5. All four isolates' CFCS containing antimicrobial compounds still gave positive antimicrobial activity against *Staphylococcus aureus* SA7001 at different temperatures. There is some decrease in their zone of inhibition ($p<0.05$) as the temperature increased.

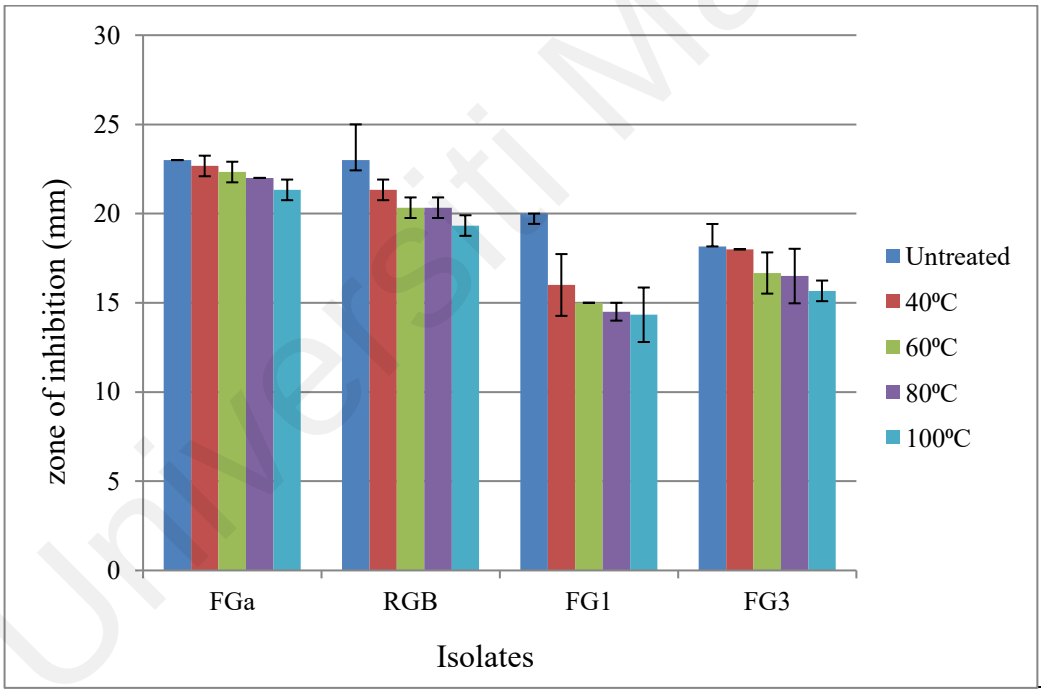

Figure 4.5: The inhibition zone of CFCS of isolate FGa, RGB, FG1 and FG3 against *Staphylococcus aureus* SA7001 at different temperatures.

4.3.2 Effect of pH

The effect of pH of isolates CFCS against *Staphylococcus aureus* SA7001 are shown on Figure 4.6. Most antimicrobial metabolites produced by the chosen isolates showed a very high inhibition zone between the treatments of pH 2-4 where some of them were higher than the untreated control. The inhibition zones were significantly decreased ($p<0.05$) at pH 6 and pH 8 for isolate FGa, RGB and FG1 meanwhile for isolate FG3, it did not showed any activity against *S. aureus* SA7001.

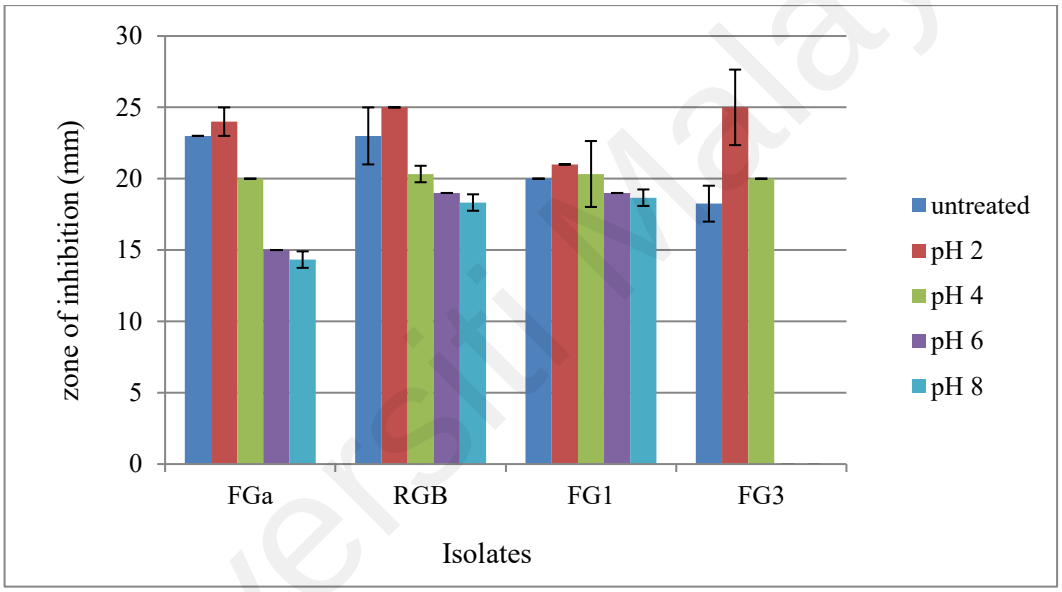

Figure 4.6: Inhibition zone of CFCS of isolate FGa, RGB, FG1 and FG3 against *Staphylococcus aureus* SA7001 at different pH.

4.3.3 Effect of Proteolytic Enzymes

Table 4.4: Enzyme stability tests of CFCS of isolate FGa, RGB, FG1 and FG3 against *Staphylococcus aureus* SA7001

Isolate	Inhibition zone (mm)			
	untreated	Proteinase K	Pepsin	Catalase
FGa	23.00 ± 0.00^{a}	23.00 ± 0.00^{a}	11.83 ± 0.29^{b}	22.67 ± 0.29^{a}
RGB	23.00 ± 2.00^{a}	23.00 ± 0.76^{a}	12.50 ± 1.32^{b}	22.17 ± 0.76^{a}
FG1	20.00 ± 0.00^{a}	19.50 ± 0.50^{a}	19.00 ± 0.58^{a}	20.00 ± 0.50^{a}
FG3	18.25 ± 1.26^{a}	17.50 ± 0.50^{a}	$18..00 \pm 1.00^{a}$	18.17 ± 0.76^{a}

Mean of three replicates ± standard deviation measured in millimeter. ($^{a, b}$) = Means that do not share a letter are significantly different ($p < 0.05$).

The effect of proteolytic enzymes of isolates CFCS against *Staphylococcus aureus* SA7001 are shown on Table 4.4. There was no significant difference ($p>0.05$) of antimicrobial activity against *S. aureus* SA7001 when the CFCS of isolates being treated with proteinase K, pepsin and catalase for both isolate FG1 and FG3. Meanwhile, for isolate FGa and RGB, both showed a stable antimicrobial activity against *S. aureus* SA7001 after being treated with proteinase K and catalase, however, there was a significant reduction of inhibition zones ($p<0.05$) when treated with pepsin.

4.4 Effect of CFCS on the Growth of strain *S. aureus* SA7001

CFCS obtained from overnight cultures of the isolate that was added to cell cultures of *S. aureus* SA7001 in early exponential growth phase (2 h) led to the inhibition of the cells growth of *S. aureus* SA7001. All chosen isolates showed a strong killing effect for the viability of *S. aureus* SA7001 where the CFCS from isolate FGa and RGB inhibit about 71.74% of *S. aureus* SA7001 growth after 14 hours of cultivation. Meanwhile, CFCS from isolate FG1 and FG3 showed about 63.05% inhibition of *S. aureus* SA7001 growth (Figure 4.7).

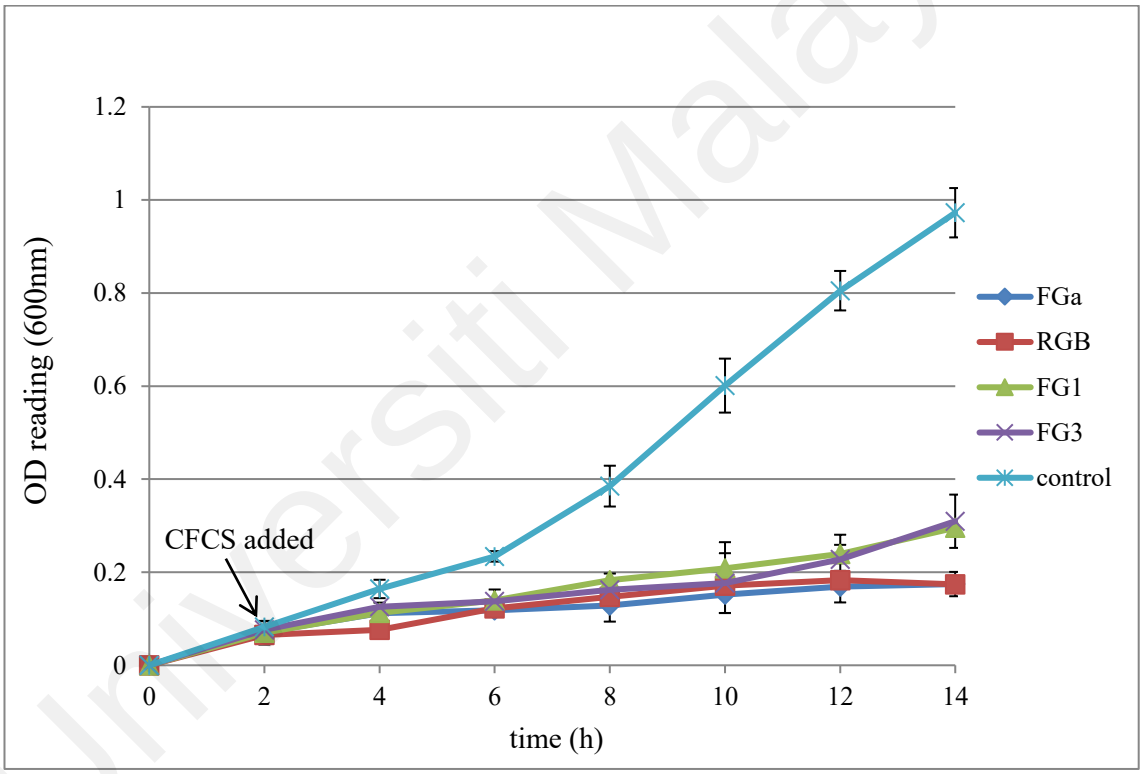

Figure 4.7: The growth of *Staphylococcus aureus* SA7001 with the presence of CFCS during 14 hours of cultivation.

4.5 Identification of Isolate FGa, RGB, FG1 and FG3

The DNA of isolates FGa, RGB, FG1 and FG3 were extracted and amplified by PCR. The PCR products of the chosen isolates were run on gel electrophoresis as shown below. The PCR products of all isolates have the size band of 1500 bp when compared to both ladders shown in Figure 4.8. The BLAST result from the sequencing of the PCR products showed that the sequence information obtained for both isolates of FGa and RGB were belonged to *Enterococcus faecium* species. Meanwhile for FG1 and FG3, they have been identified as *Bacillus cereus* species and *Serratia marcescens* species, respectively. From four isolates, only isolates FGa and RGB have been classified as lactic acid bacteria where both belonged to *Enterococcus faecium* species. Therefore, both of these isolates were used in further tests.

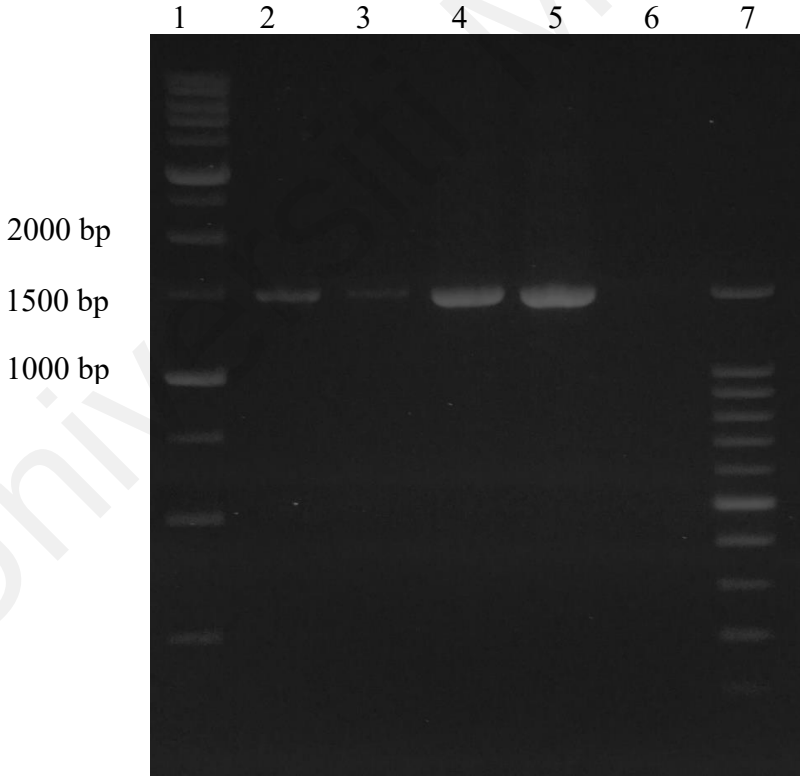

Figure 4.8: Detection of 16S rDNA gene on isolate FGa, RGB, FG1 by gel electrophoresis. The figure displayed the 16S rDNA gene detection of isolates where lane 1 = 1 kb ladder, 2 = isolate FGa, 3 = isolate RGB, 4 = isolate FG1, 5 = isolate FG3, 6 = negative control, 7 = 100 bp ladder.

4.6 Suitability of *E. faecium* FGa and RGB as Probiotic Bacteria

4.6.1 Survival of *E. faecium* FGa and RGB under Condition Simulating the Human GI Tract.

4.6.1.1 Tolerance to Simulated Gastric Juice

Upon adding pepsin with pH 2.0 for simulating gastric juice, the viability of the *E. faecium* FGa and RGB isolates significantly declined ($p<0.05$) with the incubation period of 1 to 3 hours. The viable counts of *E. faecium* FGa and RGB from 0 to 1 hour dropped to 4.47 and 4.37 log CFU/ml (4.09-4.16 log cycle loss) respectively. Meanwhile, from 0 hour to 3 hours, the viable counts of *E. faecium* FGa and RGB decreased more to 4.19 and 4.13 log CFU/ml (4.37-4.40 log cycle loss) respectively (Table 4.5).

Table 4.5: Total viable counts of *E. faecium* FGa and RGB in treatment of simulated gastric juice.

Isolate / Incubation hour	*Total viable counts (log_{10} CFU/ml)		
	0 h	1 h	3 h
Enterococcus faecium FGa	8.56 ± 0.08^{a}	4.47 ± 0.08^{b}	4.19 ± 0.06^{b}
Enterococcus faecium RGB	8.53 ± 0.04^{a}	4.37 ± 0.10^{b}	4.13 ± 0.04^{b}

Bacteria counts are converted to log CFU/ml. Mean of three replicates ± standard deviation. ($^{a, b}$) = Means that do not share a letter are significantly different ($p < 0.05$).

4.6.1.2 Tolerance to Simulated Bile Salt Condition in Small Intestine

For tolerance towards bile salt condition, both isolates *E. faecium* FGa and RGB could survive well in the presence of 0.3% bile salts after 4 hours of incubation. Their viability was retained with less than 1 log cycle loss as shown in Table 4.6. For *E. faecium* FGa, there was no significant difference ($p>0.05$) on the total viable counts after being treated with 0.3% bile salts; meanwhile, the total viable counts for *E. faecium* RGB were significantly lower ($p<0.05$) after the addition of 0.3% bile salts (Figure 4.9).

Table 4.6: Total viable counts of *E. faecium* FGa and RGB with and without the presence of 0.3% bile salts.

Isolate / Condition	Total viable counts (log_{10} CFU/ml)	
	Without bile salts	With 0.3% bile salts
Enterococcus faecium FGa	8.817 ± 0.07^{a}	8.755 ± 0.07^{a}
Enterococcus faecium RGB	8.863 ± 0.02^{a}	8.781 ± 0.00^{b}

Bacteria counts are converted to log CFU/ml. Mean of three replicates ± standard deviation. ($^{a, b}$) = Means that do not share a letter are significantly different ($p < 0.05$).

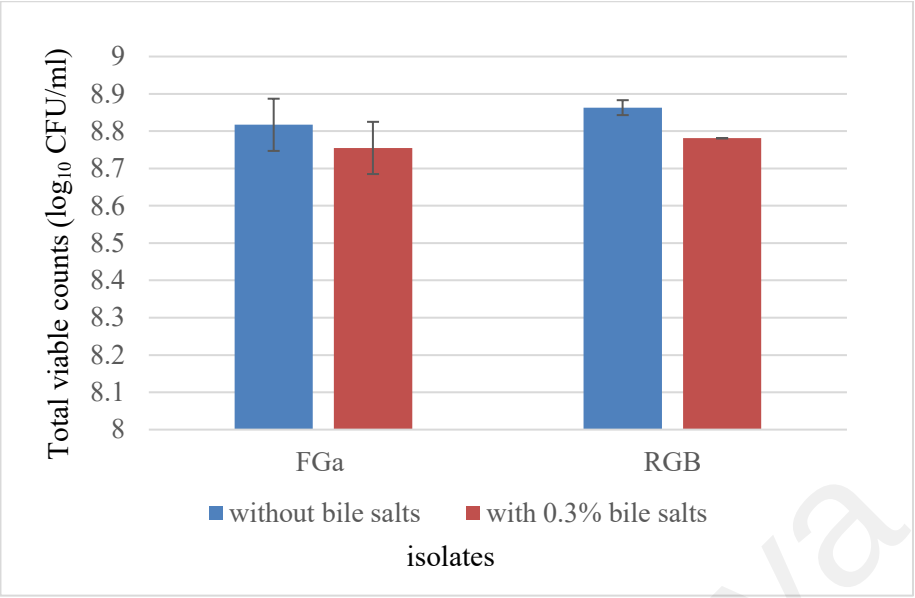

Figure 4.9: Total viable counts of *E. faecium* FGa and RGB with and without the presence of 0.3% bile salts.

4.6.2 Characterization of *E. faecium* FGa and RGB for Their Virulence Factors

4.6.2.1 Detection of Gelatinase and Hemolysin Activity

In gelatinase activity detection, both isolates *E. faecium* FGa and RGB did not showed any turbid zones around the colonies. Both of these bacteria strains do not produced gelatinase. Hemolysin detection test also showed negative haemolytic activity where both isolates of *E. faecium* FGa and RGB do not have clearing zone around their colonies which means they are γ-hemolysis (Table 4.7).

Table 4.7: Detection of gelatinase and hemolysin activity in *E. faecium* FGa and RGB

Isolates	Gelatinase test	Hemolysin test	
	Formation of turbid zone		Activity
Enterococcus faecium FGa	None	None	γ-hemolysis
Enterococcus faecium RGB	None	None	γ-hemolysis

4.6.2.2 Antibiotic Susceptibility Testing

Seven common antibiotics have been chosen for antibiotic susceptibility testing: ampicillin, streptomycin, chloramphenicol, kanamycin, tetracyclin, ciprofloxacin, and vancomycin. The results of antibiotic susceptibility testing are shown on Figure 4.10. The antibiotics still gave inhibition zones for both strains of *E. faecium* FGa and RGB, indicating that both strains are sensitive to all selected antibiotics.

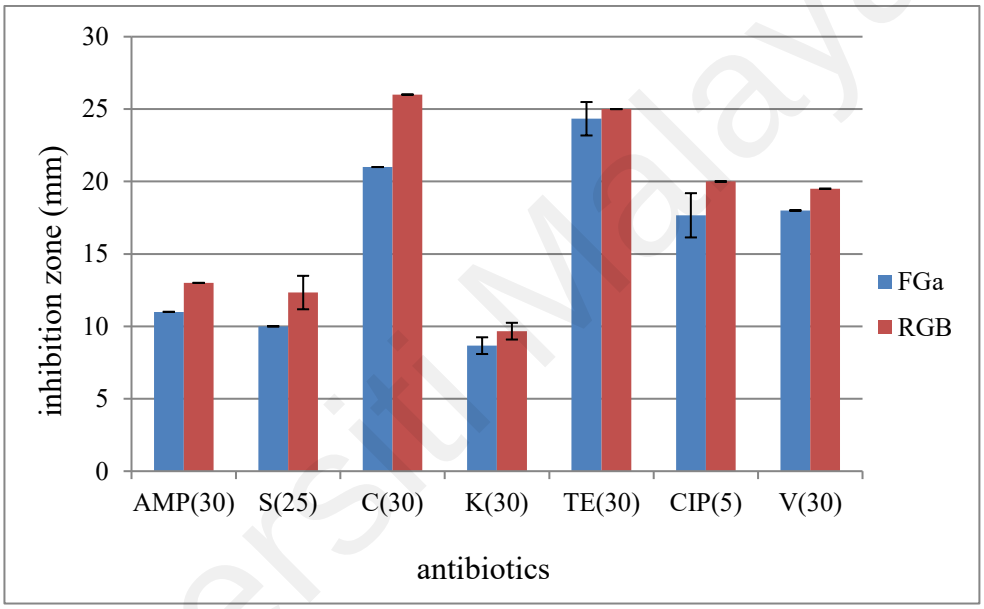

Figure 4.10: The inhibition zone *E. faecium* FGa and RGB by chosen antibiotics

4.7 Analysis of Potential Antimicrobial Compounds in of *E. faecium* FGa and RGB

4.7.1 PCR targeting Enterocin A and Enterocin B gene

From two enterocin structural genes used in this study, only enterocin A was detected in isolate *E. faecium* FGa with one visible band around 130 bp. As for isolate *E. faecium* RGB none was detected (Figure 4.11). The PCR product of target gene was purified and sequenced to confirm the validity of the target gene. This was to make sure that the PCR product formed at particular size band size was the expected target gene. The sequencing result obtained was BLAST in BLASTX from NCBI. The BLASTX result showed that the sequence information obtained has 100% similarity to class IIa bacteriocin, enterocin A that present in *Enterococcus faecium* in the GenBank (Appendix F). Since only isolate *E. faecium* FGa contains an enterocin with possible antimicrobial activity, this isolate was used for the next experiment.

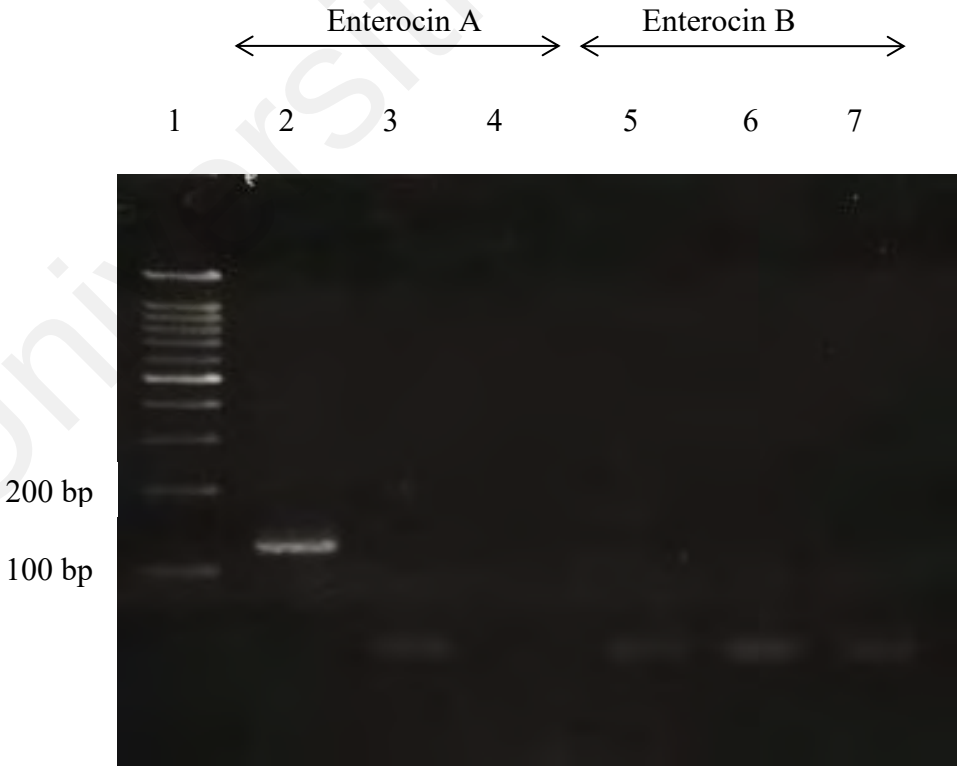

Figure 4.11: Detection of enterocin A and enterocin B on *E. faecium* FGa and RGB by gel electrophoresis. The figure displayed the detection of enterocins on *E. faecium* isolates where lane 1 = 100 bp ladder, 2 & 5 = isolate FGa, 3 & 6 = isolate RGB, 4 & 7 = negative control

4.7.2 Q-TOF LC/MS Analysis in The Cell-Free Culture Supernatant of *E. faecium* FGa

Isolate *E. faecium* FGa has been sent for Q-TOF LC/MS analysis to further check for compounds that contribute to antimicrobial activity based on their CFCS components. Numerous compounds were found in CFCS of isolate *E. faecium* FGa from positive and negative ion mass spectrometry. The compounds found from this analysis that may contribute to antimicrobial activity have been summarized in Table 4.10.

There are almost 200 compounds found from both positive and negative ion of Q-TOF LC/MS analysis, there are some small peptides, secondary metabolites and also organic acids. Three of the compounds were selected as the potential compounds that may contribute to antibacterial activity in this study based on the previous intensive studies of these compounds by other researchers' studies (Table 4.8). Based on those studies, these compounds proven to have significant antibacterial effects against food-borne pathogenic bacteria.

Meanwhile, most of other compounds detected were previously studied by other researchers only for their antiviral, antioxidant and anticonvulsant treatment, where some of other compounds only act as a precursor for antibiotic which is mostly non-active (Appendix H). Other than that, small peptides with short amino acid sequences were also detected (Appendix I). A BLAST search http://web.expasy.org/blast/) were conducted for these small peptides to identify similar sequences of known protein that responsible for antibacterial activity, however no record of matching sequences was found. Therefore, these compounds were not selected as the potential compounds that might contribute to antibacterial activity in this study.

Table 4.8: Possible antimicrobial compounds produced by CFCS of isolate *E. faecium* FGa

Compound	Type	Ionization mode	Retention time (min)	m/z	mass
Netilmicin	Secondary metabolite	positive	7.568	476.3062	475.2990
Maleic acid	Organic acid	negative	2.749	115.0038	134.0219
2-furoic acid	Organic acid	negative	2.912	111.0088	112.0161

CHAPTER 5: DISCUSSION

LAB are widely known for their ability to inhibit the growth of pathogenic bacteria making them generally recognized as safe (GRAS) and can act as potential protective cultures among other microorganisms. Foodborne pathogens colonization can easily cause problems in the food matrix, hence antimicrobial compounds that LAB naturally produces can be one of the potential solutions.

The four isolates from 24 bacteria that were isolated from goat milk and identified as *Enterococcus faecium* FGa, *Enterococcus faecium* RGB, *Bacillus cereus* FG1 and *Serratia marcescens* FG3 shows good inhibition zones against varying types of foodborne pathogens such as *Staphylococcus aureus* SA7001*, Shigella boydii* SB1003*, Shigella dysenteriae* SD1007 and *Salmonella typhimirium* SM4001. Previous studies showed similar spectra of inhibitory activity by antimicrobial compound-producing *E. faecium* against *S. aureus* (Aspri et al., 2017; Belgacem et al., 2010), *Shigella* and *Salmonella* species (Karimaei et al., 2016). In the present study, all four isolates were observed to be more efficient at inhibiting *Staphylococcus aureus* (Gram-positive) than other tested indicators (mostly Gram-negative bacteria). This may be correlated to the fact that Gram-positive bacteria typically lacks the presence of the outer membrane which acts as the permeability barrier for Gram-negative, regulating the uptake or entry of certain antimicrobial drugs and antibiotics (Exner et al., 2017). This result is also in concurrence with previous studies for *Enterococcus* species in which concluded that most of the enterococcal bacteriocins (produced by LAB which is Gram-positive in nature) always narrow scope of antimicrobial activity and commonly inhibits the growth of closely related bacteria such as Gram-positive bacteria (Ahmadova et al., 2013; Hadji-Sfaxi et al., 2011). *B. cereus* also has been previously reported to exhibit significant antibacterial activity against *S. aureus* (Basit et al., 2018; Nasser & Qaddoumi, 2016). A previous study from Kadouri & Shanks (2013) also reported that *Serratia marcescens* was able to

inhibit the growth of nine different methicillin-resistant *Staphylococcus aureus* (MRSA) isolates.

The antimicrobial activity in the CFCS of all isolates was found to be slightly decreasing after being exposed to different temperature treatments. Well diffusion assay showed that the size of inhibitory zone was slightly affected as the temperature increases for all isolates. It is suspected that the antibacterial compounds were proteinous and their protein structures might be slighty degraded when exposed to the temperature. However, the antimicrobial activity still remained active and gave more than 90% residual activity in compared to control for isolate *Enterococcus faecium* FGa. This showed that the antibacterial proteinous compound can be heat stable. Similar results of heat-stability bacteriocin were reported from *E. faecium* isolated from donkey milk and raw camel milk by studies from Aspri et al. (2017) and Rahmeh et al. (2018) respectively. *B. cereus* ATCC 14579 were also heat-stable where they still retained their activity after incubation at high temperatures (Risøen et al., 2004).

The antimicrobial activity for all isolates were found to be stable in acidic condition (at pH 2 to 4) and the antimicrobial activity were slightly decreased when treated towards more neutral pH condition to more alkaline condition for *E. faecium* FGa, *E. faecium* RGB and *B. cereus* FG1. Meanwhile, there is no activity shown for *S. marcescens* FG3 when exposed to pH 6 to 8. Similar to many bacteriocins reported previously where antimicrobial activity was highest at lower pH (Devi Avaiyarasi et al., 2016). This finding indicates that organic acids (could potentially or may also) contribute to antimicrobial activity. According to Freitas's and a few other research groups, *E. faecium* do produced several organic acids that have strong antimicrobial activity (Freitas et al., 1999). Organic acids that were produced by *E. faecium* EK13 present an efficient barrier inhibiting adherence of pathogens to the intestinal mucosa (Strompfová et al., 2006). Previous

studies reported that the production of organic acids by *S. marcescens* and *B. cereus* can contribute to antimicrobial activity in lower pH (Chen et al., 2006; Mumtaz et al., 2019).

The results in this study showed that the antimicrobial activity of *B. cereus* FG1 and *S. marcescens* FG3 remains unaffected after being treated with all tested proteolytic enzymes. Meanwhile, both *E. faecium* FGa and *E. faecium* RGB maintained their activity when treated with catalase and proteinase K, but the antimicrobial activity was significantly reduced when exposed to pepsin. Similarly, the antimicrobial activity of enterocin A produced by *Enterococcus faecium* CTC492 also showed to be highly affected with the presence of pepsin (Hu et al., 2014). Enterocin A is sensitive to digestive protease pepsin due to the high content of basic and aromatic amino acids in its structure (Herranz et al., 2001; Rodríguez et al., 2002). However, on the other hand, the antimicrobial activity of bacteriocin produced by *E. faecium* from Aspri et al. (2017) showed that the bacteriocin activity was not inactivated by the present of pepsin. It was reported that, the strain contained both enterocin A and enterocin B which may be the reason for a different response of antibacterial activity when it was exposed to pepsin comparing to this study. Other than that, *E. faecium* from Rahmeh et al. (2018) that contain enterocin A did not tested the strain with pepsin as to be used for the supporting statement for this study. The difference of activity for *E. faecium* FGa and *E. faecium* RGB when exposed to proteinase K and pepsin indicates that each bacteriocin-like substance may be sensitive to one or more proteases depends on their amino acid structures (Schittler et al., 2019). There was insufficient support from other published research for both *B. cereus* FG1 and *S. marcescens* FG3 as they still can withstand unaffected after being treated with proteolytic enzymes. The antimicrobial activity of all isolates was suggested to be not related to H_2O_2 production due to insensitivity towards the catalase.

The addition of the cell-free supernatants of these isolates into 2 h culture of *S. aureus* SA7001 has induced unbalanced growth of its cell cycle after 14-h of incubation compared to untreated control. This suggests a bactericidal mode of activity of all of these strains which can lyse the cells of *S. aureus* SA7001. The same mode of bactericidal action of CFCS of *E. faecium* species was studied previously by Hadji-Sfaxi et al. (2011) where the viabilility loss was lower than 1.5 log units after 24 hours of incubation after addition of CFCS of *E. faecium* PC4.1. In other study, the addition of CFCS of *E. faecium* AQ71 showed some decrease of OD reading of the indicator bacteria (Ahmadova et al., 2013).

Upon investigating the probiotic value of isolates, this study further proceeds with two isolates that have been classified as lactic acid bacteria which are *E. faecium* FGa and RGB. These two isolates also gave us the highest antimicrobial activity against *S. aureus* SA7001 compared to *Bacillus cereus* FG1 and *Serratia marcescens* FG3. Although *Enterococcus* strain is already categorized as lactic acid bacteria, this strain still did not obtain the GRAS status due to safety concerns and lack of safety information (Franz et al., 2011). However, some strains of *E. faecium* have been proven to be safe and effective that they already have been used as food supplements in several probiotic preparations (Serio et al., 2010). Therefore, several tests were carried out in this study, to evaluate the potential probiotic properties and safety of the *E. faecium* strains.

The ability of the strain to grow in simulated GI tract conditions *in vitro* were tested which may predict the actual survival of the strains *in vivo* when consumed in a non-protected way. These findings showed that both strains lost more than 4 log cycle of their growth ability after 3 h of incubation in the presence of pepsin (pH 2). Previous literatures also stated that most lactic acid strains lost their viability when exposed pepsin at pH 2. So our findings are in agreement with the data reported (Barbosa et al., 2014; Fernández et al., 2003; Zhang et al., 2011). Although the strain might lose some viability at pH 2 *in*

vitro, it still has the ability to work as starters or adjuncts in a matrix of fermented milk. Since most probiotic bacteria consume milk proteins, these bacteria have a protective effect on the starters (Fernández et al., 2003).

0.3% is the usual intestinal bile concentration suitable for bacteria survival (Status, 1999). Upon exposure to bile acids, lipid bilayer and integral protein of bacterial cell membranes dissociate due to cellular homeostasis disruptions resulting in the leakage of bacterial content and ultimately causing cell death (Mandal et al., 2006). However in this study, both *E. faecium* FGa and RGB could survive well in this condition and the growth pattern was normal. Therefore, there is a high possibility that growing these strains in bile salts concentrations found in human guts would not be a problem. Our finding agrees with the data reported by another research group on the viability of *E. faecium* with the presence of bile salt from Ankaiah et al. (2017).

To evaluate the safety of *E. faecium* FGa and RGB in food applications, the production of gelatinase, hemolysin, and antibiotic susceptibility were screened which can characterize the presence or absence of virulence factors in the strains. Both *E. faecium* FGa and RGB were negative for gelatinase production and demonstrated γ-haemolytic activity where there was a lack of hemolysis in the area around the bacterial colonies when grown on sheep blood agar. Both enzymes are considered as potential virulence factors where gelatinase is an extracellular metalloproteinase containing zinc which is capable of hydrolysing insulin, haemoglobin, gelatin, collagen, as well as various bioactive peptides (Chajęcka-Wierzchowska et al., 2017). On the other hand, hemolysin is a cytolytic protein capable of lysing red blood cells and haemolysins producing strains of Enterococci that have been shown to be virulent in animal models and human infections (Chow et al., 1993; Ike et al., 1987). Schittler et al. (2019) have stated in their study that hemolysin production may also increase the possibility of enterococcal infection. Similar results were reported for several *E. faecium* strains from the previous

studies. The strains were also observed to be negative-gelatinase and do not exhibit haemolytic activity (Chakchouk-Mtibaa et al., 2018; Furlaneto-Maia et al., 2020). Gelatinase activity was also reported previously showing low occurrence among non-faecalis isolates (Barbosa et al., 2010).

The ability of enterococci to exchange genetic elements as plasmids and transposons cause controversial issues on whether bacteria in food contribute to the distribution of antibiotic resistance (Rice & Carias, 1998). Both *E. faecium* FGa and RGB are susceptible to ampicillin, streptomycin, chloramphenicol, kanamycin, tetracycline, ciprofloxacin and vancomycin. A similar profile of susceptibility to antibiotics was observed in the study of Ahmadova et al. (2013) and Belgacem et al. (2010). However, the food-derived *E. faecium* isolates investigated in both studies were resistant to kanamycin and ciprofloxacin respectively while our strains are susceptible to both of these antibiotics. The characteristic of antibiotic susceptibility of both *E. faecium* FGa and RGB in antibiotic resistance are favourable and may provide their applications with safety property for consumption . In addition, vancomycin-resistant enterococci (VRE) is still of great concern due to its increasing numbers each day and is not only restricted to clinical isolates but also can be found in food products from animal origins. Therefore, the safety evaluation of enterococci on its behaviour towards vancomycin is really important (Franz et al., 2001; Messi et al., 2006). Our study showed that both *E. faecium* FGa and RGB are highly susceptible towards vancomycin. Hadji-Sfaxi et al. (2011) also found that the *E. faecium* isolated from Mongol yoghurt was also sensitive to vancomycin.

The detection of known enterocin structural genes showed that *E. faecium* FGa carries genes encoding enterocin A but does not possess enterocin B, meanwhile *E. faecium* RGB does not possess any of them. Enterocin A consists of 47 amino acids with a molecular weight of 4839 Da which is a thermostable bacteriocin that belongs to class II.a (Fimland et al., 2005). Enterocin A mainly targets the bacterial cell membrane by attaching and

subsequently permeabilizing it causing the indispensable intracellular molecules to leak out hence killing the cell (Drider et al., 2006). Therefore, the antimicrobial activity of *E. faecium* due to the presence of enterocin A was proved to be efficient against food-borne and spoilage-causing Gram-positive bacteria including *S. aureus, L. monocytogenes, C. botulinum,* and *C. perfringens* (Ahmadova et al., 2013; Herranz et al., 2001). *E. faecium* species that possessed gene encoding enterocin A have also been reported in previous studies from Huang et al. (2016) and Rahmeh et al. (2018).

Other factors may also contribute to the inhibition, such as secondary metabolites and also organic acid. Therefore, in the present study, the components of CFCS of *E. faecium* FGa were also investigated via Q-TOF LC/MS analysis. Since only *E. faecium* FGa contains an enterocin with antimicrobial activity, this isolate was further selected to be used for this analysis.

Secondary metabolites from microbial have low molecular mass products and are usually produced during the late growth phase of producing microorganisms (Bibb, 2005). The secondary metabolite that was found in the CFCS of *E. faecium* FGa is netilmicin with molecular weight of 475.29 Da. Netilmicin is classified as a water-soluble antibiotic of the aminoglycoside group and is usually formed during fermentation. Netilmicin can inhibit the bacteria from synthesizing proteins vital to their growth by binding to the bacterial 30S subunit which will obstruct the assembly of initiation complex between mRNA and the bacterial ribosome (Awad et al., 2012). In addition, netilmicin can also cause the translational frameshift by inducing the misreading of mRNA template, resulting in premature termination that eventually leads to bacterial cell death (NCBI, 2021, February 7). According to Campoli-Richards in 1989, netilmicin is active at low concentrations against various pathogenic bacteria, including *Escherichia coli, Salmonella* sp., *Shigella* sp*, Hemophilus influenzae* and against penicillinase and non-penicillinase-producing *Staphylococcus* including methicillin-resistant strains.

Therefore, netilmicin has been proven by other studies that it has the ability to inhibit a wide range of bacterial growth that may support the antimicrobial activity from the CFCS of *E. faecium* FGa.

Organic acids possess comprehensive antimicrobial potential. They are mostly common intermediates in living organisms. Organic acids that are found in the CFCS of *E. faecium* FGa are maleic acid and 2-furoic acid with the molecular weight of 116.01 Da and 112.02 Da respectively. Maleic acid has shown antimicrobial activity against *E. faecalis* biofilm and also against *Candida albicans* and *Staphylococcus aureus* (Ballal et al., 2011; Ferrer-Luque et al., 2010). 2-furoic acid has been used as a pharmaceutical intermediate, anti-inflammatory agents' fungicide and preservative hypolipidimic (Chamoulaud et al., 2001). It can act as a potent biofilm inhibitor against Staphylococcal species. The 2-furoic acid possesses a good antibacterial activity where it can inhibit the proliferation of *S. bacteria* and *B. subtilis* (Chai et al., 2013). Hence, both maleic acid and 2-furoic acid are potential contributors to antimicrobial activity in the CFCS of *E. faecium* FGa.

In this study, several compounds were found that may or may not contribute to antibacterial activity. The inhibitory activity produced by the strain could be contributed by the compound individually or by the mixed combination of these compounds found (enterocin A, netilmicin, maleic acid and 2-furoic acid). Therefore, further study should be done to investigate which compounds were actually contributed to the antibacterial activity in this study. To prove this effect for enterocin A activity, organic acid or any other contaminants should be separated, removed or degraded either mechanically or enzymatically before proceeding with the antibacterial test. For example, purification of enterocin A by gel chromatography and RP-HPLC can be done to rule out any other component present (Goh & Philip, 2015). Meanwhile, extraction through different solvent–extractant mixtures for netilmicin, maleic acid and 2-furoic acid can also be done

(Hasret et al., 2018). Further experiments such as time-kill assay and biofilm susceptibility test using the extracted purified compound can be used to prove that these compounds were the compound involved in the antibacterial activities (Ballal et al., 2011; Ferrer-Luque et al., 2010).

In this study, small peptides with short amino acid sequences were also detected from the Q-TOF LC/MS analysis. From the result, there is no matching sequence of these peptides with the known peptides that have antibacterial activity, however, these peptides can also be a novel peptides that could contribute to this activity. Therefore, in future study, these peptides can be further extracted or chemically synthesized and determining their inhibitory activities to prove their responsibility activity.

Other than that, further study on procurements of virulence genes ability should also be evaluated before this strain can be applied in the food industries. Furthermore, our potential non-LAB isolates, *Bacillus cereus* FG1 and *Serratia marcescens* FG3 could also be studied more for their possible antimicrobial compounds.

CHAPTER 6: CONCLUSION

In conclusion, the screening of antimicrobial activity of bacteria from goat milk led to the isolation of several active bacterial strains that are not only LAB such as *Enterococcus faecium* FGa and *Enterococcus faecium* RGB but also non-LAB such as *Bacillus cereus* FG1 and *Serratia marcescens* FG3. The antimicrobial activity that was observed in the supernatants from these four cultures against both Gram-positive and Gram-negative bacteria that plays an important role in the pathogenicity and contamination of food makes these isolates promising candidates in our food industry and/or in the pharmaceutical context. From these four isolates, *E. faecium* FGa was shown to be the most promising strain where it can produce several antimicrobial compounds such as class II bacteriocin, secondary metabolite and organic acids. Furthermore, *E. faecium* FGa strain appears to possess potential probiotic properties where it can survive well in simulated gastrointestinal conditions and sensitive towards several antibiotics. This strain with such beneficial prospects can play as more fascinating roles in future such as in anti-quorum sensing strategies and site-specific drug delivery. Anti-quorum sensing strategies is a mechanism of disrupting cell-to-cell communication which leads to attenuation of microbial virulence (Finch et al., 1998). The ideal anti-quorum sensing strategies have been defined to exhibit high degree of specificity for the quorum sensing regulator without toxic side effects on the bacteria. This strain with anti-quorum sensing strategies may create the development of new, nontoxic and broad-spectrum drugs from the strain. In future, this strain with the combination of site-specific drug delivery may delivers the antibacterial compounds to the specific targeted site without harming any unrelated site. Therefore, this strain can be a potential candidate for bio-preservative and/or protective culture.

REFERENCES

Abdel-Aziz, S. M., Abo Elsoud, M. M., & Anise, A. A. H. (2017). Microbial Biosynthesis: A Repertory of Vital Natural Products. *Food Biosynthesis*, *2*, 25–54.

Abedi, E., & Hashemi, S. (2020). Lactic acid production - producing microorganisms and substrates sources-state of art. *Heliyon*, *6*(10), Article#e04974

Achemchem, F., Martínez-Bueno, M., Abrini, J., Valdivia, E., & Maqueda, M. (2005). *Enterococcus faecium* F58, a bacteriocinogenic strain naturally occurring in Jben, a soft, farmhouse goat's cheese made in Morocco. *Journal of Applied Microbiology*, *99*(1), 141-150.

Ahmadova, A., Todorov, S. D., Choiset, Y., Rabesona, H., Mirhadi Zadi, T., Kuliyev, A., Franco, B. D. G. d. M., Chobert, J.-M., & Haertlé, T. (2013). Evaluation of antimicrobial activity, probiotic properties and safety of wild strain *Enterococcus faecium* AQ71 isolated from Azerbaijani Motal cheese. *Food Control*, *30*(2), 631-641.

Álvarez, M., Rodríguez, A., Peromingo, B., Núñez, F., & Rodríguez, M. (2020). *Enterococcus faecium*: a promising protective culture to control growth of ochratoxigenic moulds and mycotoxin production in dry-fermented sausages. *Mycotoxin Research*, *36*(2), 137-145.

Ammor, M. S., & Mayo, B. (2007). Selection criteria for lactic acid bacteria to be used as functional starter cultures in dry sausage production: An update. *Meat Science*, *76*(1), 138-146.

Ammor, S., Dufour, E., Zagorec, M., Chaillou, S., & Chevallier, I. (2005). Characterization and selection of *Lactobacillus sakei* strains isolated from traditional dry sausage for their potential use as starter cultures. *Food Microbiology*, *22*(6), 529-538.

Ananou, S., Maqueda, M., Martínez-Bueno, M., & Valdivia, E. (2007). Biopreservation, an ecological approach to improve the safety and shelf-life of foods.

Ankaiah, D., Esakkiraj, P., Perumal, V., Ayyanna, R., & Venkatesan, A. (2017). Probiotic characterization of *Enterococcus faecium* por1: Cloning, over expression of Enterocin-A and evaluation of antibacterial, anti-cancer properties. *Journal of Functional Foods*, *38*, 280-292.

Aspri, M., O'Connor, P. M., Field, D., Cotter, P. D., Ross, P., Hill, C., & Papademas, P. (2017). Application of bacteriocin-producing *Enterococcus faecium* isolated from donkey milk, in the bio-control of *Listeria monocytogenes* in fresh whey cheese. *International Dairy Journal*, *73*, 1-9.

Awad, H., El–Shahed, K., Aziz, R., Sarmidi, M., & El Enshasy, H. (2012). Antibiotics as Microbial Secondary Metabolites: Production and Application. *Jurnal Tekologi*, *59*, 101-111.

Aymerich, T., Artigas, M. G., Garriga, M., Monfort, J. M., & Hugas, M. (2000). Effect of sausage ingredients and additives on the production of enterocin A and B by *Enterococcus faecium* CTC492. Optimization of in vitro production and anti-listerial effect in dry fermented sausages. *Journal of Applied Microbiology*, *88*(4), 686-694.

Badis, A., Guetarni, D., Moussa Boudjema, B., Henni, D. E., & Kihal, M. (2004). Identification and technological properties of lactic acid bacteria isolated from raw goat milk of four Algerian races. *Food Microbiology*, *21*(5), 579-588.

Baldry, M. G. C. 1983. The bactericidal, fungicidal and sporicidal properties of hydrogen peroxide and peracetic acid. *Journal of Applied Bacteriology*. *54*, 417-423.

Ballal, N. V., Yegneswaran, P. P., Mala, K., & Bhat, K. S. (2011). In vitro antimicrobial activity of maleic acid and ethylenediaminetetraacetic acid on endodontic pathogens. *Journal of Oral Surgery, Oral Medicine, Oral Pathology, and Oral Radiology*, *112*(5), 696-700.

Barbosa, J., Borges, S., & Teixeira, P. (2014). Selection of potential probiotic *Enterococcus faecium* isolated from Portuguese fermented food. International *Journal of Food Microbiology*, *191*, 144-148.

Barbosa, J., Gibbs, P. A., & Teixeira, P. (2010). Virulence factors among enterococci isolated from traditional fermented meat products produced in the North of Portugal. *Food Control*, *21*(5), 651-656.

Basit, M., Rasool, M. H., Naqvi, S. A. R., Waseem, M., & Aslam, B. (2018). Biosurfactants production potential of native strains of *Bacillus cereus* and their antimicrobial, cytotoxic and antioxidant activities. *Pakistan Journal of Pharmaceutical Sciences*, *31*(1(Suppl.)), 251-256.

Bauer, A., Kirby, W., Sherris, J. C., & Turck, M. (1966). Antibiotic susceptibility testing by a standardized single disk method. *American Journal of Clinical Pathology*, *45*(4), 493.

Beirão, B. C. B., Ingberman, M., Fávaro, C., Jr., Mesa, D., Bittencourt, L. C., Fascina, V. B., & Caron, L. F. (2018). Effect of an *Enterococcus faecium* probiotic on specific IgA following live *Salmonella Enteritidis* vaccination of layer chickens. *Avian Pathology*, *47*(3), 325-333.

Belgacem, Z. B., Abriouel, H., Omar, N. B., Lucas, R., Martínez-Canamero, M., Gálvez, A., & Manai, M. (2010). Antimicrobial activity, safety aspects, and some technological properties of bacteriocinogenic *Enterococcus faecium* from artisanal Tunisian fermented meat. *Food Control*, *21*(4), 462-470.

Bérdy, J. (2005). Bioactive Microbial Metabolites. *The Journal of Antibiotics*, *58*(1), 1-26.

Bhardwaj, A., Malik, R. K., & Chauhan, P. (2008). Functional and safety aspects of enterococci in dairy foods. *Indian Journal of Microbiology*, *48*(3), 317-325.

Bibb, M. J. (2005). Regulation of secondary metabolism in streptomycetes. *Current Opinion in Microbiology*, *8*(2), 208-215.

Callon, C., Duthoit, F., Delbes, C., Ferrand, M., Le Frileux, Y., De Cremoux, R. & Montel, M. C. (2007) Stability of microbial communities in goat milk during a lactation year: molecular approaches. *Systematic and Applied Microbiology*, *30*, 547–560.

Campoli-Richards, D. M., Chaplin, S., Sayce, R. H., & Goa, K. L. (1989). Netilmicin. A review of its antibacterial activity, pharmacokinetic properties and therapeutic use. *Drugs*, *38*(5), 703-756.

Caplice, E., & Fitzgerald, G. F. (1999). Food fermentations: role of microorganisms in food production and preservation. *International Journal of Food Microbiology*, *50*(1-2), 131-149.

Casaus, P., Nilsen, T., Cintas, L. M., Nes, I. F., Hernández, P. E., & Holo, H. (1997). Enterocin B, a new bacteriocin from *Enterococcus faecium* T136 which can act synergistically with enterocin A. *Microbiology (Reading)*, *143* (Pt 7), 2287-2294.

Centers for Disease Control and Prevention (2015). Antibiotic/Antimicrobial Resistance. *What Exactly is Antibiotic Resistance?* Retrieved on 13 December 2021 from https://www.cdc.gov/drugresistance/about.html

Chai, W. M., Liu, X., Hu, Y. H., Feng, H. L., Jia, Y. L., Guo, Y. J., Zhou, H. T., & Chen, Q. X. (2013). Antityrosinase and antimicrobial activities of furfuryl alcohol, furfural and furoic acid. *Intermational Journal Biological Macromolecules*, *57*, 151-155.

Chajęcka-Wierzchowska, W., Zadernowska, A., & Łaniewska-Trokenheim, Ł. (2017). Virulence factors of *Enterococcus* spp. presented in food. *LWT – Food Science and Technology*, *75*, 670-676.

Chakchouk-Mtibaa, A., Sellem, I., Kamoun, Y., Smaoui, S., Karray-Rebai, I., & Mellouli, L. (2018). Safety Aspect of *Enterococcus faecium* FL31 Strain and Antibacterial Mechanism of Its Hydroxylated Bacteriocin BacFL31 against *Listeria monocytogenes*. *Biomed Research International*, *2018*, Article#5308464.

Chamoulaud, G., Floner, D., Moinet, C., Lamy, C., & Belgsir, E. M. (2001). Biomass conversion II: simultaneous electrosyntheses of furoic acid and furfuryl alcohol on modified graphite felt electrodes. *Electrochimica Acta*, *46*(18), 2757-2760.

Chen, Y. P., Rekha, P. D., Arun, A. B., Shen, F. T., Lai, W. A., & Young, C. C. (2006). Phosphate solubilizing bacteria from subtropical soil and their tricalcium phosphate solubilizing abilities. *Applied Soil Ecology*, *34*(1), 33-41.

Chow, J. W., Thal, L. A., Perri, M. B., Vazquez, J. A., Donabedian, S. M., Clewell, D. B., & Zervos, M. J. (1993). Plasmid-associated hemolysin and aggregation substance production contribute to virulence in experimental enterococcal endocarditis. *Antimicrobial Agents and Chemotherapy*, *37*(11), 2474-2477.

Cintas, L. M., Casaus, P., Håvarstein, L. S., Hernández, P. E., & Nes, I. F. (1997). Biochemical and genetic characterization of enterocin P, a novel sec-dependent bacteriocin from *Enterococcus faecium* P13 with a broad antimicrobial spectrum. *Applied and Environmental Microbiology, 63*(11), 4321-4330.

Cintas, L. M., Casaus, P., Herranz, C., Hâvarstein, L. S., Holo, H., Hernández, P. E., & Nes, I. F. (2000). Biochemical and genetic evidence that *Enterococcus faecium* L50 produces enterocins L50A and L50B, the sec-dependent enterocin P, and a novel bacteriocin secreted without an N-terminal extension termed enterocin Q. *Journal of Bacteriology, 182*(23), 6806-6814.

Cleveland, J., Montville, T. J., Nes, I. F., & Chikindas, M. L. (2001). Bacteriocins: safe, natural antimicrobials for food preservation. *International Journal of Food Microbiology*, *71*(1), 1-20.

Cotter, P. D., Hill, C., & Ross, R. P. (2005). Bacteriocins: developing innate immunity for food. *Nature Reviews Microbiology*, *3*(10), 777-788.

Cole, K. R., & Castellino, F. J. (1984). The binding of antifibrinolytic amino acids to kringle-4-containing fragments of plasminogen. *Archives of Biochemistry and Biophysics*, *229*(2), 568–575.

Cox, C. R., Coburn, P. S., & Gilmore, M. S. (2005). Enterococcal cytolysin: a novel two component peptide system that serves as a bacterial defense against eukaryotic and prokaryotic cells. *Current Protein and Peptide Science*, *6*(1), 77-84.

Dahiya, R. S., & Speck, M. L. (1968). Hydrogen peroxide formation by lactobacilli and its effect on *Staphylococcus aureus*. *Journal of Dairy Science*, *51*(10), 1568-1572.

De Vuyst, L., and Vandamme, E. (1994). Lactic Acid Bacteria and Bacteriocins: Their Practical Importance. Springer US.

Del Rio, B., Sánchez-Llana, E., Redruello, B., Magadan, A. H., Fernández, M., Martin, M. C., Ladero, V., & Alvarez, M. A. (2019). *Enterococcus faecalis* Bacteriophage 156 Is an Effective Biotechnological Tool for Reducing the Presence of Tyramine and Putrescine in an Experimental Cheese Model. *Frontiers in Microbiology*, *10*, Article#566.

Demain, A. L., & Fang, A. (2000). The natural functions of secondary metabolites. *Advances in Biochemical Engineering/Biotechnology*, *69*, 1-39.

Devi Avaiyarasi, N., David Ravindran, A., Venkatesh, P., & Arul, V. (2016). In vitro selection, characterization and cytotoxic effect of bacteriocin of *Lactobacillus sakei* GM3 isolated from goat milk. *Food Control*, *69*, 124-133.

Drider, D., Fimland, G., Héchard, Y., McMullen, L. M., & Prévost, H. (2006). The continuing story of class IIa bacteriocins. *Microbiology and Molecular Biology Reviews*, *70*(2), 564-582.

du Toit, M., Franz, C. M., Dicks, L. M., & Holzapfel, W. H. (2000). Preliminary characterization of bacteriocins produced by *Enterococcus faecium* and

Enterococcus faecalis isolated from pig faeces. *Journal of Applied Microbiology*, *88*(3), 482-494.

Ennahar, S., & Deschamps, N. (2000). Anti-Listeria effect of enterocin A, produced by cheese-isolated *Enterococcus faecium* EFM01, relative to other bacteriocins from lactic acid bacteria. *Journal of Applied Microbiology*, *88*(3), 449-457.

Exner, M., Bhattacharya, S., Christiansen, B., Gebel, J., Goroncy-Bermes, P., Hartemann, P., Trautmann, M. (2017). Antibiotic resistance: What is so special about multidrug-resistant Gram-negative bacteria?. *GMS Hygiene and Infection Control*, *12*, Article#E05.

Fernández, M. F., Boris, S., & Barbés, C. (2003). Probiotic properties of human lactobacilli strains to be used in the gastrointestinal tract. *Journal of Applied Microbiology*, *94*(3), 449-455.

Ferrer-Luque, C. M., Arias-Moliz, M. T., González-Rodríguez, M. P., & Baca, P. (2010). Antimicrobial activity of maleic acid and combinations of cetrimide with chelating agents against *Enterococcus faecalis* biofilm. *Journal of Endodontics*, *36*(10), 1673-1675.

Fimland, G., Johnsen, L., Dalhus, B., & Nissen-Meyer, J. (2005). Pediocin-like antimicrobial peptides (class IIa bacteriocins) and their immunity proteins: biosynthesis, structure, and mode of action. *Journal of Peptide Science*, *11*(11), 688-696.

Finch, R., Pritchard, D., Bycroft, B., Williams, P., Stewart, G. (1998). Quorum sensing – A novel target for anti-infective therapy. *Journal of Antimicrobial Chemotherapy*, *42*, 569–71.

Foulquié Moreno, M. R., Sarantinopoulos, P., Tsakalidou, E., & De Vuyst, L. (2006). The role and application of enterococci in food and health. *International Journal of Food Microbiology*, *106*(1), 1-24.

Foster, S. J., and K. Johnstone. 1987. Purification and properties of a germination-specific cortex-lytic enzyme from spores of *Bacillus megaterium* KM. *Biochemical Journal*, *242*, 573-579.

Franz, C. M., Huch, M., Abriouel, H., Holzapfel, W., & Gálvez, A. (2011). Enterococci as probiotics and their implications in food safety. *International Journal of Food Microbiology*, *151*(2), 125-140.

Franz, C. M., Muscholl-Silberhorn, A. B., Yousif, N. M., Vancanneyt, M., Swings, J., & Holzapfel, W. H. (2001). Incidence of virulence factors and antibiotic resistance among Enterococci isolated from food. *Applied and Environmental Microbiology*, *67*(9), 4385-4389.

Franz, C. M., van Belkum, M. J., Holzapfel, W. H., Abriouel, H., & Gálvez, A. (2007). Diversity of enterococcal bacteriocins and their grouping in a new classification scheme. *FEMS Microbiology Reviews*, *31*(3), 293-310.

Freitas, A. C., Pintado, A. E., Pintado, M. E., & Malcata, F. X. (1999). Organic acids produced by lactobacilli, enterococci and yeasts isolated from Picante cheese. *European Food Research and Technology*, *209*(6), 434-438.

Furlaneto-Maia, L., Ramalho, R., Rocha, K. R., & Furlaneto, M. C. (2020). Antimicrobial activity of enterocins against *Listeria* sp. and other food spoilage bacteria. *Biotechnology Letters*, *42*(5), 797-806.

Goetsch, A. L., Zeng, S. S. & Gipson, T. A. (2011) Factors affecting goat milk production and quality. *Small Ruminant Research, 101*, 55–63.

Gao, W., Howden, B. P., & Stinear, T. P. (2018). Evolution of virulence in *Enterococcus faecium*, a hospital-adapted opportunistic pathogen. *Current Opinion in Microbiology*, *41*, 76-82.

Garmiene, G., Šalomskienė, J., Jasutiene, I., Mačionienė, I., & Miliauskienė, I. (2010). Production of benzoic acid by lactic acid bacteria from *Lactobacillus, Lactococcus* and *Streptococcus* genera in milk. *Milchwissenschaft – Milk Science International*, *65*, 295-298.

Goh, H. F., & Philip, K. (2015). Purification and Characterization of Bacteriocin Produced by *Weissella confusa* A3 of Dairy Origin. *PLoS One*, *10*(10), Article#e0140434.

Gooding, J. M., & Corssen, G. (1976). Etomidate: an ultrashort-acting nonbarbiturate agent for anesthesia induction. *Anesthesia and Analgesia*, *55*(2), 286–289.

Hadji-Sfaxi, I., El-Ghaish, S., Ahmadova, A., Batdorj, B., Le Blay-Laliberté, G., Barbier, G., Haertlé, T., & Chobert, J.-M. (2011). Antimicrobial activity and safety of use of *Enterococcus faecium* PC4.1 isolated from Mongol yogurt. *Food Control*, *22*(12), 2020-2027.

Hanchi, H., Mottawea, W., Sebei, K., & Hammami, R. (2018). The Genus Enterococcus: Between Probiotic Potential and Safety Concerns-An Update. *Frontiers in Microbiology*, *9*, 1791.

Hasret, E., Kırbaşlar, Ş. İ., & Uslu, H. (2018). Extraction of Citric Acid and Maleic Acid from Their Aqueous Solutions Using a Phosphorus-Bonded Extractant, Tri-n-octylphosphineoxide, and a Secondary Amine, Dioctylamine. *Journal of Chemical & Engineering Data*, *63(*1), 39-48.

Herranz, C., Casaus, P., Mukhopadhyay, S., Martínez, J. M., Rodríguez, J. M., Nes, I. F., Hernández, P. E., & Cintas, L. M. (2001). *Enterococcus faecium* P21: a strain occurring naturally in dry-fermented sausages producing the class II bacteriocins enterocin A and enterocin B. *Food Microbiology*, *18*(2), 115-131.

Holzapfel, W., Arini, A., Aeschbacher, M., Coppolecchia, R., & Pot, B. (2018). *Enterococcus faecium* SF68 as a model for efficacy and safety evaluation of pharmaceutical probiotics. *Beneficial Microbes*, *9*(3), 375-388.

Hu, X., Mao, R., Zhang, Y., Teng, D., Wang, X., Xi, D., Huang, J., & Wang, J. (2014). Biotechnical paving of recombinant enterocin A as the candidate of anti-Listeriaagent. *BMC Microbiology*, *14*(1), 220.

Huang, E., Zhang, L., Chung, Y. K., Zheng, Z., & Yousef, A. E. (2013). Characterization and application of enterocin RM6, a bacteriocin from *Enterococcus faecalis*. *Biomed Research International*, *2013*, Article#206917.

Huang, Y., Ye, K., Yu, K., Wang, K., & Zhou, G. (2016). The potential influence of two *Enterococcus faecium* on the growth of *Listeria monocytogenes*. *Food Control*, *67*, 18-24.

Himmelmann, A., Bergbrant, A., Svensson, A., Hansson, L., Aurell, M. (1996). Remikiren (Ro 42-5892)—An Orally Active Renin Inhibitor in Essential Hypertension: Effects on Blood Pressure and the Renin-Angiotensin-Aldosterone System, *American Journal of Hypertension*, *9*(6), 517–522.

Hingsamer, M., & Jungmeier, G. (2019). Biorefineries. In The Role of Bioenergy in the Bioeconomy (pp. 179–222). Academic Press.

Hirai, S., Endo, S., Saito, R., Hirose, M., Ueno, T., Suzuki, H., Yamato, K., Abei, M., & Hyodo, I. (2014). Antitumor effects of a sirtuin inhibitor, tenovin-6, against gastric cancer cells via death receptor 5 up-regulation. *PloS one*, 9(7), Article#e102831.

Ike, Y., Hashimoto, H., & Clewell, D. B. (1987). High incidence of hemolysin production by *Enterococcus (Streptococcus) faecalis* strains associated with human parenteral infections. *Journal of Clinical Microbiology*, *25*(8), 1524-1528.

Ito, T., Tokoro, M., Hori, R., Hemmi, H., & Yoshimura, T. (2018). Production of Ophthalmic Acid Using Engineered *Escherichia coli*. *Applied and Environmental Microbiology*, *84*(7), Article#e02806-17.

Juven, B. J., & Pierson, M. D. (1996). Antibacterial Effects of Hydrogen Peroxide and Methods for Its Detection and Quantitation (†). *Journal of Food Protection*, *59*(11), 1233-1241.

Kadouri, D. E., & Shanks, R. M. (2013). Identification of a methicillin-resistant *Staphylococcus aureus* inhibitory compound isolated from *Serratia marcescens. Research in Microbiology*, *164*(8), 821-826.

Karimaei, S., Sadeghi, J., Asadian, M., Esghaei, M., Pourshafie, M. R., & Talebi, M. (2016). Antibacterial potential and genetic profile of *Enterococcus faecium* strains isolated from human normal flora. *Microbial Pathogenesis*, *96*, 67-71.

Kawamoto, S., Shima, J., Sato, R., Eguchi, T., Ohmomo, S., Shibato, J., Horikoshi, N., Takeshita, K., & Sameshima, T. (2002). Biochemical and genetic characterization of mundticin KS, an antilisterial peptide produced by *Enterococcus mundtii* NFRI 7393. *Applied and Environmental Microbiology*, *68*(8), 3830-3840.

King, W. L., & Gould, G. W. (1969). Lysis of Bacterial Spores with Hydrogen Peroxide. *Journal of Applied Bacteriology*, *32*(4), 481–490.

Kohlpaintner, C., Schulte, M., Falbe, J., Lappe, P., Weber, J., & Frey, G. D. (2013). Aldehydes, Araliphatic. *Ullmann's Encyclopedia of Industrial Chemistry*.

Lau, S. K., Lam, C. W., Curreem, S. O., Lee, K. C., Lau, C. C., Chow, W. N., Ngan, A. H., To, K. K., Chan, J. F., Hung, I. F., Yam, W. C., Yuen, K. Y., & Woo, P. C. (2015). Identification of specific metabolites in culture supernatant of *Mycobacterium tuberculosis* using metabolomics: exploration of potential biomarkers. *Emerging Microbes & Infections*, *4*(1), Article#e6.

Laukova, A. (2011). Potential Applications of Probiotic, Bacteriocin-Producing Enterococci and Their Bacteriocins. *Lactic Acid Bacteria - Microbiological and Functional Aspects*, 39-62.

Madigan, M. T., & Brock, T. D. (2009). Brock biology of microorganisms (12th ed.). Boston: Pearson.

Maldonado-Barragán, A., Caballero-Guerrero, B., Jiménez, E., Jiménez-Díaz, R., Ruiz-Barba, J. L., & Rodríguez, J. M. (2009). Enterocin C, a class IIb bacteriocin produced by *E. faecalis* C901, a strain isolated from human colostrum. *International Journal of Food Microbiology*, *133*(1-2), 105-112.

Mandal, S., Puniya, A. K., & Singh, K. (2006). Effect of alginate concentrations on survival of microencapsulated *Lactobacillus casei* NCDC-298. *International Dairy Journal*, *16*(10), 1190-1195.

Maqueda, M., Gálvez, A., Bueno, M. M., Sanchez-Barrena, M. J., González, C., Albert, A., Rico, M., & Valdivia, E. (2004). Peptide AS-48: prototype of a new class of cyclic bacteriocins. *Current Protein & Peptide Science*, *5*(5), 399-416.

Mareková, M., Lauková, A., Skaugen, M., & Nes, I. (2007). Isolation and characterization of a new bacteriocin, termed enterocin M, produced by environmental isolate *Enterococcus faecium* AL41. *Journal of Industrial Microbiology and Biotechnology*, *34*(8), 533-537.

Martínez, B., Rodríguez, A., and Suárez, E. . (2016). Antimicrobial Peptides Produced by Bacteria: The Bacteriocins (Vol. 2). Springer International Publishing.

Mayo, B., Aleksandrzak-Piekarczyk, T., Fernández, M., Kowalczyk, M., Álvarez-Martín, P., & Bardowski, J. (2010). *Updates in the Metabolism of Lactic Acid Bacteria. In Biotechnology of Lactic Acid Bacteria* (pp. 3-33).

Messi, P., Guerrieri, E., de Niederhäusern, S., Sabia, C., & Bondi, M. (2006). Vancomycin-resistant enterococci (VRE) in meat and environmental samples. *International Journal of Food Microbiology*, *107*(2), 218-222.

Mumtaz, M. Z., Barry, K. M., Baker, A. L., Nichols, D. S., Ahmad, M., Zahir, Z. A., & Britz, M. L. (2019). Production of lactic and acetic acids by *Bacillus* sp. ZM20 and *Bacillus cereus* following exposure to zinc oxide: A possible mechanism for Zn solubilization. *Rhizosphere*, *12*, Article#100170.

NA (1999). Fasoracetam. *Drugs in R & D*, *2*(2), 135–136.

Nasser, E., & Qaddoumi, S. Q. (2016). Antimicrobial activity of *Bacillus cereus*: Isolation, identification and the effect of carbon and nitrogen source on its antagonistic activity. *Journal of Microbiology and Antimicrobials*, *8*, 7-13.

National Center for Biotechnology Information (2021). *PubChem Compound Summary for CID 441306, Netilmicin*. Retrieved on 7 February 2021 from https://pubchem.ncbi.nlm.nih.gov/compound/Netilmicin.

National Center for Biotechnology Information (2022). P*ubChem Compound Summary for CID 6211, Barbituric acid*. Retrieved on 18 April 2022 from https://pubchem.ncbi.nlm.nih.gov/compound/Barbituric-acid.

National Center for Biotechnology Information (2022). *PubChem Compound Summary for CID 2294, Barbital*. Retrieved on 18 April 2022 from https://pubchem.ncbi.nlm.nih.gov/compound/Barbital.

National Center for Biotechnology Information (2022). *PubChem Compound Summary for CID 1052, Pyridoxamine*. Retrieved on 18 April 2022 from https://pubchem.ncbi.nlm.nih.gov/compound/Pyridoxamine.

National Center for Biotechnology Information (2022). *PubChem Compound Summary for CID 21704, Vidarabine.* Retrieved on 18 April 2022 from https://pubchem.ncbi.nlm.nih.gov/compound/Vidarabine.

National Center for Biotechnology Information (2022). *PubChem Compound Summary for CID 34359, Carbidopa*. Retrieved on 20 April 2022 from https://pubchem.ncbi.nlm.nih.gov/compound/Carbidopa.

National Center for Biotechnology Information (2022). *PubChem Compound Summary for CID 6613, Pantothenic acid*. Retrieved on 20 April 2022 from https://pubchem.ncbi.nlm.nih.gov/compound/Pantothenic-acid.

National Center for Biotechnology Information (2022*). PubChem Compound Summary for CID 3292, Ethotoin*. Retrieved on 20 April 2022 from https://pubchem.ncbi.nlm.nih.gov/compound/Ethotoin.

Nesher, M., Shpolansky, U., Viola, N., Dvela, M., Buzaglo, N., Cohen Ben-Ami, H., Rosen, H., & Lichtstein, D. (2010). Ouabain attenuates cardiotoxicity induced by other cardiac steroids. *British Journal of Pharmacology*, *160*(2), 346–354.

Nilsen, T., Nes, I. F., & Holo, H. (2003). Enterolysin A, a cell wall-degrading bacteriocin from *Enterococcus faecalis* LMG 2333. *Applied and Environmental Microbiology*, *69*(5), 2975-2984.

Oda, Y., Saito, K., Yamauchi, H., & Mori, M. (2002). Lactic acid fermentation of potato pulp by the fungus *Rhizopus oryzae*. *Current Microbiology*, *45*(1), 1-4.

Ohnishi, Y., Yamazaki, H., Kato, J., Tomono, A. & Horinouchi, S. AdpA, (2005). a central transcriptional regulator in the A-factor regulatory cascade that leads to morphological development and secondary metabolism in *Streptomyces griseus*. *Bioscience, Biotechnology, and Biochemistry*, *69*, 431–439

Ozcelik, S., Kuley, E., & Ozogul, F. (2016). Formation of lactic, acetic, succinic, propionic, formic and butyric acid by lactic acid bacteria. *Lebensmittel-Wissenschaft & Technologie*, *73*, 536–542

Paudel, B., Maharjan, R., Rajbhandari, P., Aryal, N., Aziz, S., Bhattarai, K., Baral, B., Malla, R. & Bhattarai, H. D. (2021) Maculosin, a non-toxic antioxidant compound isolated from *Streptomyces sp*. KTM18, *Pharmaceutical Biology*, *59*(1), 931-934.

Psoni, L., Kotzamanidis, C., Yiangou, M., Tzanetakis, N., & Litopoulou-Tzanetaki, E. (2007). Genotypic and phenotypic diversity of *Lactococcus lactis* isolates from Batzos, a Greek PDO raw goat milk cheese. *International Journal of Food Microbiology*, *114*(2), 211-220.

Puchter, L., Chaberny, I. F., Schwab, F., Vonberg, R. P., Bange, F. C., & Ebadi, E. (2018). Economic burden of nosocomial infections caused by vancomycin-resistant enterococci. *Antimicrobial Resistance & Infection Control*, *7*, 1.

Qiao, X., Du, R., Wang, Y., Han, Y., & Zhou, Z. (2020). Purification, characterization and mode of action of enterocin, a novel bacteriocin produced by *Enterococcus faecium* TJUQ1. *International Journal of Biological Macromolecules, 144*, 151-159.

Quigley, L., O'Sullivan, O., Stanton, C., Beresford, T. P., Ross, R. P., Fitzgerald, G. F., & Cotter, P. D. (2013). The complex microbiota of raw milk. *FEMS Microbiological Reviews*, *37*(5), 664-698.

Rahmeh, R., Akbar, A., Kishk, M., Al Onaizi, T., Al-Shatti, A., Shajan, A., Akbar, B., Al-Mutairi, S., & Yateem, A. (2018). Characterization of semipurified enterocins produced by *Enterococcus faecium* strains isolated from raw camel milk. *Journal of Dairy Science*, *101*(6), 4944-4952.

Ray, B. (2004). Fundamental food microbiology. CRC Press LLC.

Rea, M. C., Ross, R. P., Cotter, P. D., & Hill, C. (2011). Classification of Bacteriocins from Gram-Positive Bacteria. In D. Drider & S. Rebuffat (Eds.), *Prokaryotic Antimicrobial Peptides: From Genes to Application*s (pp. 29-53). Springer New York.

Reis, J. A., Paula, A. T., Casarotti, S. N., & Penna, A. L. B. (2012). Lactic Acid Bacteria Antimicrobial Compounds: Characteristics and Applications. *Food Engineering Reviews*, *4*(2), 124-140.

Rice, L. B., & Carias, L. L. (1998). Transfer of Tn5385, a composite, multiresistance chromosomal element from *Enterococcus faecalis. Journal of Bacteriology*, *180*(3), 714-721.

Riddler, S. A., Anderson, R. E., & Mellors, J. W. (1995). Antiretroviral activity of stavudine (2′,3′-didehydro-3′-deoxythymidine, D4T). *Antiviral Research*, *27*(3), 189–203.

Risøen, P. A., Rønning, P., Hegna, I. K., & Kolstø, A. B. (2004). Characterization of a broad range antimicrobial substance from *Bacillus cereus. Journal of Applied Microbiology*, *96*(4), 648-655.

Russell, A. D. 1982. The destruction of bacterial spores, p. 211. Academic Press, London.

Rodríguez, J. M., Martínez, M. I., & Kok, J. (2002). Pediocin PA-1, a wide-spectrum bacteriocin from lactic acid bacteria. *Critical Reviews in Food Science and Nutrition*, *42*(2), 91-121.

Rozara, A. (2002). Practical Methods for Environmental Microbiology and Biotechnology (1 ed.). Meerut, India: Krishna Prakashan Media.

Saavedra, L., Minahk, C., de Ruiz Holgado, A. P., & Sesma, F. (2004). Enhancement of the enterocin CRL35 activity by a synthetic peptide derived from the NH2-terminal sequence. *Antimicrobial Agents and Chemotherapy*, *48*(7), 2778-2781.

Saavedra, L., Taranto, M. P., Sesma, F., & de Valdez, G. F. (2003). Homemade traditional cheeses for the isolation of probiotic *Enterococcus faecium* strains. *International Journal and Food Microbiology*, *88*(2-3), 241-245.

Saga, T., & Yamaguchi, K. (2009). History of Antimicrobial Agents and Resistant. *Japan Medical Association Journal*, *52*, 103-108.

Sanchez, S., & Demain, A. L. (2002). Metabolic regulation of fermentation processes. *Enzyme and Microbial Technology*, *31*(7), 895-906.

Sawa, N., Wilaipun, P., Kinoshita, S., Zendo, T., Leelawatcharamas, V., Nakayama, J., & Sonomoto, K. (2012). Isolation and characterization of enterocin W, a novel two-peptide lantibiotic produced by *Enterococcus faecalis* NKR-4-1. *Applied and Environmental Microbiology*, *78*(3), 900-903.

Schirru, S., Todorov, S. D., Favaro, L., Mangia, N. P., Basaglia, M., Casella, S., Comunian, R., Franco, B. D. G. d. M., & Deiana, P. (2012). Sardinian goat's milk as source of bacteriocinogenic potential protective cultures. *Food Control*, *25*(1), 309-320.

Schittler, L., Perin, L. M., de Lima Marques, J., Lando, V., Todorov, S. D., Nero, L. A., & da Silva, W. P. (2019). Isolation of *Enterococcus faecium*, characterization of its antimicrobial metabolites and viability in probiotic Minas Frescal cheese. *Journal of Food Science and Technology*, *56*(11), 5128-5137.

Serio, A., Chaves-López, C., Paparella, A., & Suzzi, G. (2010). Evaluation of metabolic activities of enterococci isolated from Pecorino Abruzzese cheese. *International Dairy Journal*, *20*(7), 459-464.

Shai, Y. (2002). Mode of action of membrane active antimicrobial peptides. *Biopolymers*, *66*(4), 236-248.

Shin, S.-Y., Calvisi, E. G., Beaman, T. C., Pankratz, H. S., Gerhardt, P., and Marquis, R. E. (1994). Microscopic and Thermal Characterization of Hydrogen Peroxide

Killing and Lysis of Spores and Protection by Transition Metal Ions, Chelators, and Antioxidants. *Applied and Environmental Microbiology*, *60*(9), 3192–3197.

Status, T. S. (1999). Scientifics status summary (Vol. 53).

Strompfová, V., Marciňáková, M., Simonová, M., Gancarčíková, S., Jonecová, Z., Sciranková, Ľ., Koščová, J., Buleca, V., Čobanová, K., & Lauková, A. (2006). *Enterococcus faecium* EK13—an enterocin A-producing strain with probiotic character and its effect in piglets. *Anaerobe*, *12*(5), 242-248.

Taylor, M. (2014). Handbook of Natural Antimicrobials for Food Safety and Quality. Elsevier Science.

The Human Metabolome Database (2022). The Metabolomics Innovation Centre. *Ascorbic acid 2-sulfate (HMDB0060649)*. Retrieved on 19 April 2022 from https://hmdb.ca/metabolites/HMDB0060649

The Human Metabolome Database (2022). The Metabolomics Innovation Centre. *2-Propylglutaric acid (HMDB0060684)*. Retrieved on 20 April 2022 from https://hmdb.ca/metabolites/HMDB0060684

The Human Metabolome Database (2022). The Metabolomics Innovation Centre. *Amobarbital (HMDB0015440)*. Retrieved on 20 April 2022 from https://hmdb.ca/metabolites/HMDB0015440

Wee, Y.-J., Kim, J.-N., & Ryu, H.-W. (2006). Biotechnological production of lactic acid and its recent applications. *Food Technology and Biotechnology*, *44*(2), 163-172.

Wessels, D. J., P.J.; Mostert, J.F. (1988). Occurrence and significance of Enterobacteriaceae isolates in milk and dairy products. *South African Journal of Dairy Science*, *20*, 23-28.

Whitby, K., Pierson, T. C., Geiss, B., Lane, K., Engle, M., Zhou, Y., Doms, R. W., & Diamond, M. S. (2005). Castanospermine, a potent inhibitor of dengue virus infection in vitro and in vivo. *Journal of Virology*, *79*(14), 8698–8706.

Wlodarska, M., Luo, C., Kolde, R., d'Hennezel, E., Annand, J. W., Heim, C. E., Krastel, P., Schmitt, E. K., Omar, A. S., Creasey, E. A., Garner, A. L., Mohammadi, S., O'Connell, D. J., Abubucker, S., Arthur, T. D., Franzosa, E. A., Huttenhower, C., Murphy, L. O., Haiser, H. J., Vlamakis, H., … Xavier, R. J. (2017). Indoleacrylic Acid Produced by Commensal *Peptostreptococcus* Species Suppresses Inflammation. *Cell Host & Microbe*, *22*(1), 25–37. Article#e6.

Zhang, Y., Zhang, L., Du, M., Yi, H., Guo, C., Tuo, Y., Han, X., Li, J., Zhang, L., & Yang, L. (2011). Antimicrobial activity against *Shigella sonnei* and probiotic properties of wild lactobacilli from fermented food. *Microbiological Research*, *167*(1), 27-31.