2014

The Effect of Probiotics on Bacterial Human Skin Pathogens

Al-Attwani, Jasim Hussein

The Effect of Probiotics on Bacterial Human Skin Pathogens

By

Jasim Hussein Al-Attwani

A thesis submitted to the Plymouth University in partial fulfilment of the requirement for the degree of

DOCTOR OF PHILOSOPHY

School of Biological Sciences

Faculty of Science and Technology

Plymouth University

United Kingdom

This project funded by Ministry of Higher Education and Scientific Research

Baghdad, Iraq

July 2014

The effect of probiotics on bacterial human skin pathogens

Abstract

Probiotic bacteria have been investigated in the prevention and treatment of various diseases and allergies. The current study was undertaken to determine the effect of eight probiotic *Lactobacillus* species against bacterial human skin pathogens using several techniques. Antimicrobial activity of lactobacilli against *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Pseudomonas aeruginosa* and *Propionibacterium acnes* was evaluated using lactobacilli broth cultures (BCB) and cell free supernatant (CFS). Antimicrobial activity was significantly greater with BCB compared with CFS especially for *Lactobacillus plantarum* and *Lactobacillus acidophilus*. Lactobacilli and pathogen aggregation, biofilm formation and adhesion to keratin were assessed. *L. casei* and *L. plantarum* were selected for further study as they showed the greatest co-aggregation (18.02 ± 1.34% with *L. casei* and 14.92 ± 1.45% with *L. plantarum*) with the pathogens (16.63 ± 1.65% with *S. aureus* 3761 and 14.58 ± 1.68% with *P. aeruginosa*) and prevention of biofilm formation by the pathogens. The antimicrobial activity of human beta defensin-2 (hBD-2) alone or with *L. plantarum* against pathogens was assessed. The results with hBD-2 showed that hBD-2 (10 µg / ml for 5 h) and *L. plantarum* together were significantly more inhibitory against *S. aureus* than hBD-2 alone. The presence of NaCl reduced the effectiveness of hBD-2 alone and with *L. plantarum*. In the presence of *L. plantarum*, inactivation of *mprF* and *dlt* genes led to increased binding of hBD-2 by the bacterial cell wall, and then inhibition growth of bacterial cell wall. Studies investigated the effect of exposure of methicillin resistant *Staphylococcus aureus* (MRSA) to the supernatant of *L. plantarum* the susceptibility of MRSA to β-lactams. MRSA became sensitive to β-lactams when treated with culture supernatant of *L. plantarum*. Gene expression studies demonstrated that the *mec*R1-*mec*I-*mecA*-PBP2 signalling pathway was impeded by exposure to culture supernatant of *L. plantarum* and β-lactams. The studies reported here demonstrate a possible alternative approach to dealing with skin pathogens, which may have clinical implications especially with regard to MRSA infections, and continued research is advised.

List of contents

List of Figures

List of Tables

Acknowledgements

Above all, I would like to thank and praise to my God, and I owe it all to Almighty God for granting me the wisdom, health, and strength to undertake this research task and enabling me to its completion.

This dissertation would not have been possible and completed without the help of so many people in so many ways. I would like to express my sincere deepest gratitude and thankfulness to all of them.

It is my pleasure to express my sincere thanks to supervisors, Dr. Jane Beal, Dr. Paul Waines and Dr. Graham Bradley for their assistance, encouragement, guidance, advice, criticism and support at all stages of my research project.

I would like to express my deep thanks to my sponsor 'The Ministry of Higher Education and Scientific Research in Baghdad / Iraq (MHESR) for financing the scholarship that has enabled me to complete this thesis.

In addition, I would like to thanks for excellent technical assistance staff in laboratory for support and helpful suggestions. My thanks also to members and colleagues of the faculty of Science and Technology at Plymouth University who have helped me at various times during this project.

I'm very grateful, and all the love and affection to my family members one by one, especially my mother, the cuddles warm and the permanent tenderness (her all the love and gratitude). I would like to thank my sister (Alia), and all my brothers (Alaa, Alwan and Kadhum) for the encouragement and their endless support in all of my endeavours and for always believing in me through my study.

Always and never forget, and I really appreciate all the tiredness that has been made by my wife, who gave me the time and always encouragement to achieve my work. She was always supportive. I ask Almighty God to reward her for her good deeds. I could never forget my lovely children, who always make me happy: my son Mohammed Baqer, and lovely boy Al-Hassan, and I would like to dedicate sincere thanks and gratitude to them to carry around with me alienation and the difficult conditions, and supported me in every possible way to see the completion of this work. I supplicate to God to keep them safe and support them in all stages of their life. Thank you all!

Jassim Al-Quraishi

Abbreviations and acronyms

Abbreviation used	Glossary of terms used
ΔC_T	Delta cycle threshold
AA	Acetic acid
ACDP	Advisory Committee on Dangerous Pathogens
AD	Atopic dermatitis
AMN	Ampicillin
AMPs	antimicrobial peptides
ANOVA	Analysis of variance
ATCC	American Type Culture Collection
AZM	Azithromycin
BCB	Broth culture bacteria
BF	Biofilm formation
BHIB	Brain Heart Infusion broth
CHP	Chloramphenicol
CA	Citric acid
CA-MRSA	Community-associated MRSA
cDNA	Complementary deoxyribonucleic acid
CFS	Cell free supernatant
CFU	Colony forming unit
CIP	Ciprofloxacin
CLFB	Clumping factor B
Co.N.S.	Coagulase negative *Staphylococcus*
Co.P.S.	Coagulase positive *Staphylococcus*
Ct	Cycle threshold

CTX	Cefotaxime
DMS	Deutsche Sammlung von Mikroorganismen und Zellkulturen
dNTPs	Deoxynucleotide triphosphates
DSMZ	Deutsche Sammlung von Mikroorganismen und Zellkulturen
dlt	D-alanyl-lipoteichoic acid
EDTA	Ethylene diamine tetraacetic acid
EM	Electron Microscope
Esp	Epidermin serine protease
ET	Exofoliative toxines
g	Gravity
FA	Fatty acid
GN	Gentamicin
GIT	Gastro-Intestinal tract
h	Hour
H_2O_2	Hydrogen peroxide
hBD	Human beta defensin
HA-MRSA	Healthcare-associated MRSA
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
kDa	kilo Dalton
K	Keratin
L	*Lactobacillus*

LA	Lactic acid
LAB	Lactic acid bacteria
LPS	Lipopolysaccharide
LSD	Least significant differences
LTA	Lipoteichoic acid
MHB	Mueller Hinton broth
MIC	Minimum inhibitory concentration
mprF	Multiple peptide resistance factor
MRS	de Man Rogosa and Sharpe
MRSB	de Man Rogosa and Sharpe broth
MRSA	Methicillin resistant *Staphylococcus aureus*
MY	Lincomycin
NA	Nutrient agar
NB	Nutrient broth
NCIB	National Collection of Industrial Bacteria
NCO	National Collection Office
NCTC	National Collection of Type Cultures
NK	Natural killer
OD	Optical Density
PA	Propionic acid
PAMPS	Pathogen-associated molecular patterns
PBPs	Penicillin binding proteins
PBS	Phosphate buffer saline
PG	Peptidoglycan
PIA	*Pseudomonas* isolation agar

PSIA	Polysaccharide intracellular adhesion
PYK	Pyruvate kinase
qRT-PCR	Quantitative real time-polymerase chain reaction
RNA	Ribonucleic acid
rpm	Rotation per minute
RQ	Relative quantification
RT	Real time
RTs	Reverse transcription
S	Streptomycin
SCC*mec*	Staphylococcal cassette chromosome *mec*
SCFA	Short chain fatty acids
SE	Standard Error
SEM	Scanning Electron Microscope
SPB	Sodium phosphate buffer
SPSS	Statistical Package Social Sciences
SSSS	Staphylococcal scalded skin syndrome
SXT	Sulphatrimethoprim
TA	Techoic acid
TAE	Tris-Acetate ethylene diamine tetraacetic acid
TET	Tetracycline
Th	T helper
TLRs	Toll-like receptors
TNF-α	Tumour necrosis factor-alfa
TSS-1	Staphylococcal shock syndrome
UDP	Uridine-diphosphate

UGT	Urogenital tract
v / v	Volume / Volume
W	Weak
w / v	Weight / Volume

Dedication

This thesis is dedicated to the memory of my deceased father (27th October 1999), and my martyr brother 'Ali' (29th April 2006).

Author's Declaration

At no time during the registration for the degree of Ph.D has the author been registered for any other University award, without prior agreement of the Graduate Committee. This study was financed with the aid of the Ministry of the Higher Education and Scientific Research, Baghdad, Iraq. Relevant scientific seminars and conferences were attended at which work was presented and papers have been prepared for publication:

Peer Reviewed Publication

1. Al-Attwani, J.H., Waines, P. L., Raid, N., and Beal, J. (2014). Restoration of the susceptibility of methicillin-resistant *Staphylococcus aureus* (MRSA) to β-lactam antibiotics by probiotic *Lactobacillus plantarum* (*L. plantarum*). In: A. Méndez-Vilas, An Industrial, medical and environmental applications of microorganisms: current status and trends. Wageningen Academic Publishers. ISBN: 978-90-8686-243-6, ISBN E-book: 978-90-8686-795-0, pp. 585-589.

2. Al-Attwani, J.H., Waines, P. L., and Beal, J. (2014). Antimicrobial activity and assessment of biofilm formation to bacterial human skin pathogens and probiotic *Lactobacillus* species. *African Journal of Microbiology Research*. (Accepted, subject to corrections).

Total word count of the main body of the thesis: 52,555

Signed……..…….

Date……..

Chapter 1

General introduction and literature review

Chapter 1: General introduction and literature review

1.1 Introduction

The skin is a uniquely engineered organ that permits terrestrial life by regulating heat and water loss form the body whilst preventing the ingress of noxious chemicals or different microorganisms (Figure 1.1). It is also the largest organ of the human body, providing around 15% of the body mass of an average person, and it covers an average area of 1.5 - 2 m^2 (Richardson, 2002). The main role of the skin is to serve as a natural (physical) barrier, to protect from ultraviolet radiation, to restrict the inward and outward passage of water, and to protect bodies from potential attack by harmful microorganisms or dangerous substances (Zaidi and Lanigan, 2010). The skin is also an interface with the external environment and is colonized by several groups of microbes: bacteria, fungi, viruses and mites (Cogen *et al.*, 2008). The skin is the principal barrier against various microbial invasions; it continuously interacts with the external environment and is colonized with a large population of microorganisms. The vast majority of colonizing microflora consists of bacteria, whether Gram positive or Gram negative bacteria (Harder *et al.*, 1997). Skin and soft tissue infections (SSTIs) are a prevalent problem confronted in clinical practice and range from mild infections, e.g. pyoderma, to serious life threatening ones such as necrotizing fasciitis. Many genera of bacteria infect the skin, such as *Staphylococcus*, *Pseudomonas*, *Propionibacterium* and others (Swartz, 2004; Hersh *et al.*, 2008).

Species of *Staphylococcus* bacteria are identified as one of the most important causes of acute disease in humans in many areas of the world. *Staphylococcus aureus* causes a wide range of infectious diseases, whether

localised to the skin or systemic diseases, in humans and animals (Vincze *et al.*, 2013). These include atopic dermatitis (AD), also known as eczema, boils (furuncles), carbuncles, impetigo, folliculitis, endocarditis, osteomalitis, pneumonia, meningitis, food poisoning, mastitis and urinary tract infections (Ogston and Witte, 1984).

Furthermore, *Pseudomonas aeruginosa* causes several diseases, such as cutaneous diseases, especially folliculitis and dermatitis. In addition, *P. aeruginosa* causes systemic diseases, for example cystic fibrosis, or is associated with acquired immune deficiency syndrome patients (Shanson, 1990; Elkin and Geddes, 2003). In the past two decades, *P. aeruginosa* has emerged as a significant pathogen, which causes between 10 - 20% of infections in hospitals. *P. aeruginosa* causes numerous infectious diseases to humans and animals, e.g. septicaemia, leg ulcers and burn wound infections (Buivydas *et al.*, 2013).

As for the *Propionibacterium* species, *Propionibacterium acnes* is the primary cause of irritant dermatitis of the wall sebaceous follicles (Bek-Thomsen *et al.*, 2008). Liu and Huang (2012) observed that *Pr. acnes* play an important role in the development of inflammatory acne when *Pr. acnes* over-grows in pilosebaceous units.

The spread of multiple drug resistant bacteria indicates a growing need for new antimicrobial agents. Researchers are attempting to find successful solutions to overcome microbial infections, such as methicillin resistant *Staphylococcus aureus* (MRSA), which is an example of a multiple drug resistant bacterial species. One potential area of investigation is the use of probiotics. Probiotics are beneficial microorganisms, such as some bacterial

genera, e.g. *Bifidobacterium*, *Lactobacillus, Micrococcus, Propionibacterium, Pediococcus, Enterococcus*, *Bacillus* and others, and some yeast species, such as *Saccharomyces boulardii* and *Saccharomyces cerevisiae*. Scientists have found a range of potentially useful medical applications, e.g. reducing inflammation (Reid *et al.*, 2003), improving immune function and preventing infections (Cremonini *et al.*, 2002; Reid *et al.*, 2003), reducing the occurrence of colon cancer (Wollowski *et al.*, 2001), lowering blood pressure, lowering cholesterol and alleviating lactose intolerance (Sanders, 2000).

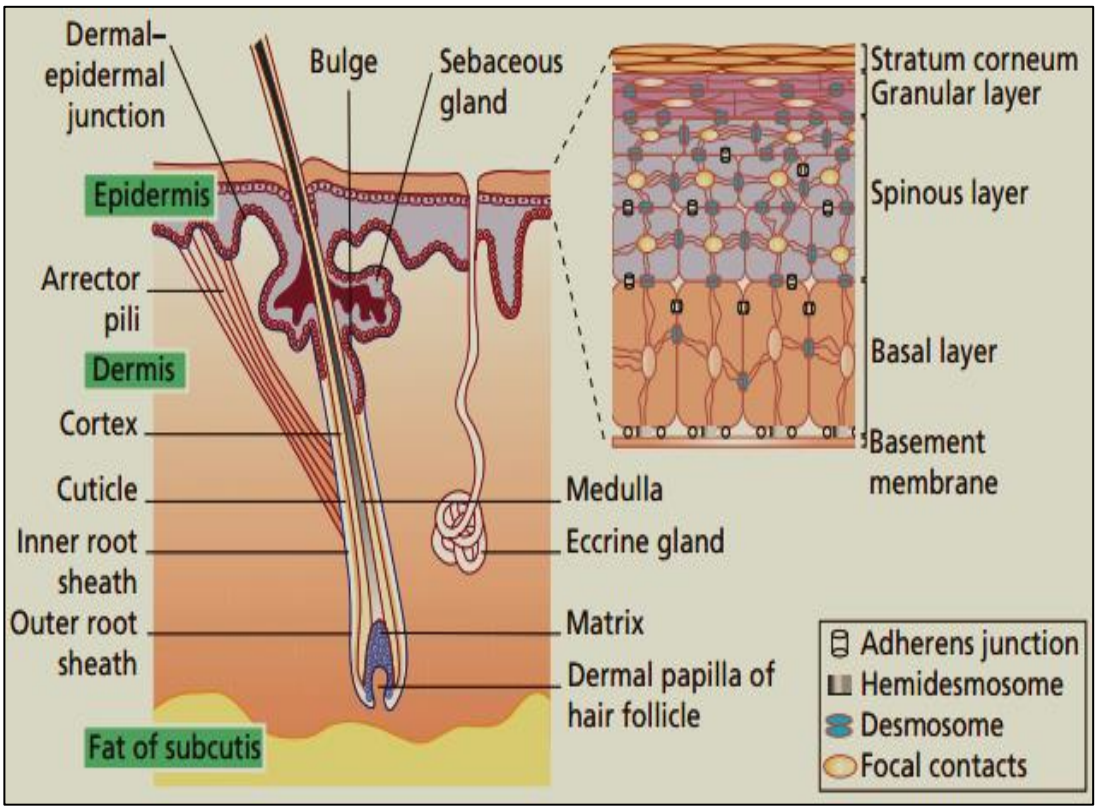


Figure 1.1 Illustration of the human skin depicting three main layers; epidermis, dermis and subcutis. Each layer has its specific structures and functions, taken from McGrath *et al.* (2010).

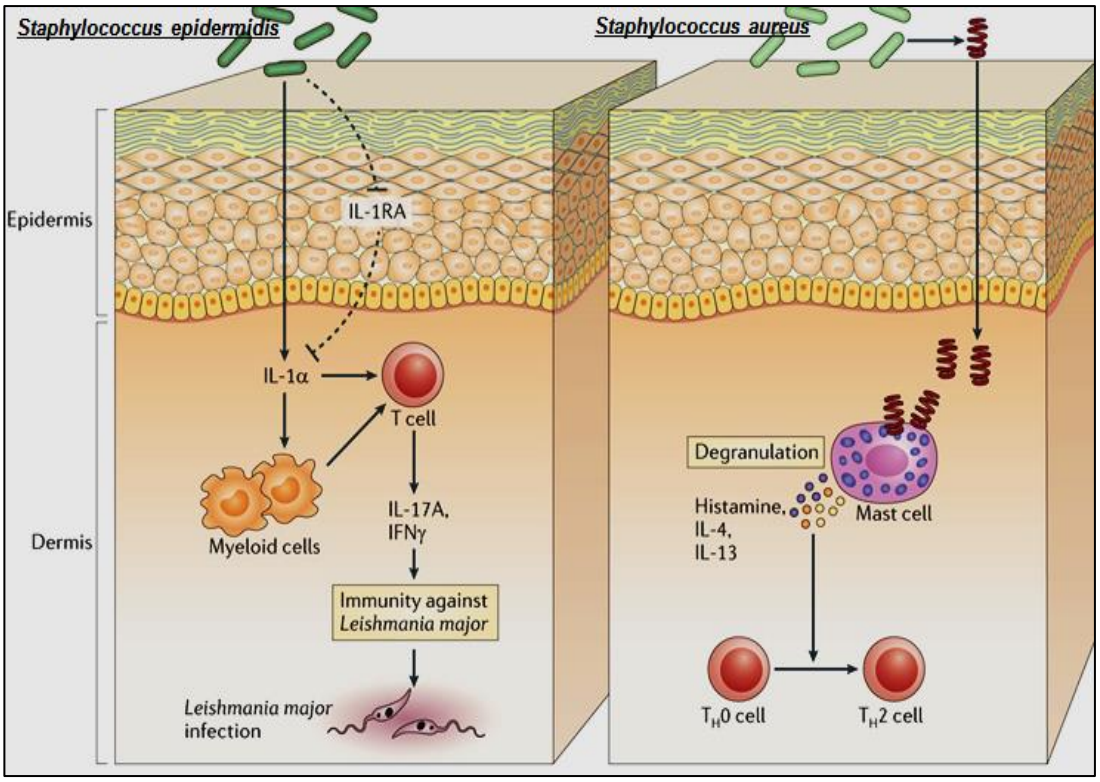


Figure 1.2 Cell function and protective immunity to cutaneous microorganisms (A) Skin commensals control skin-resident T cell. Colonization of the skin with the commensal *Staphylococcus epidermidis* was shown to be required for the production of interleukin-17A (IL-17A) by skin-resident T cells136. (B) *S. aureus* induce mast cell degranulation and allergic skin inflammation. Colonization of the skin with *S. aureus*, a pathogenic bacterium that is often found on the skin of patients with atopic dermatitis, triggers local allergic responses, which directly induces degranulation of dermal mast cells, promoting innate and adaptive T helper 2(TH2)-type responses, taken from Pasparakis *et al.* (2014).

1.2 Literature review

1.2.1 *Staphylococcus* bacterium and taxonomy

Staphylococcus is a genus of Gram positive bacteria of the Staphylococcaceae family (Table 1.1), which appears as cocci (approximately 1 - 1.5 µm in diameter) under a microscope and forms grape-like clusters (Figure 1.3). Staphylococci are usually commensal with the host's body, whether human or animal, and cause superficial skin lesions and localized abscesses in other sites, and also cause deep-seated systemic

infections (Chiller *et al.*, 2001; Sung *et al.*, 2008). The most important pathogenic species are *S. aureus* and *Staphylococcus epidermidis*. Species of the *Staphylococcus* genus can be classified into two groups, coagulase positive *Staphylococcus* (CoPS) and coagulase negative *Staphylococcus* (CoNS) (Foster, 1996).

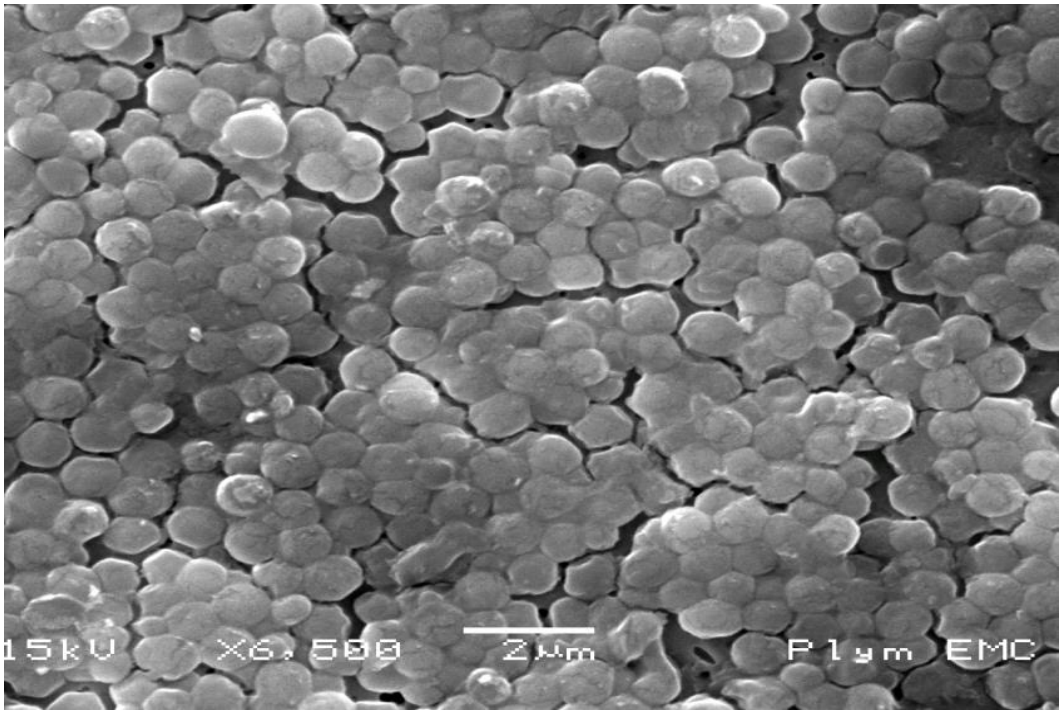


Figure 1.3 SEM micrograph of *S. aureus* cells. Observe the grape-like gathering common to *Staphylococcus* species.

Table 1.1 Scientific classification of Staphylococcus bacterium.

Kingdom: Bacteria
Phylum: Firmicutes
Class: Bacilli
Order: Bacillales
Family: Staphylococcaceae
Genus: *Staphylococcus*

1.2.1.1 Coagulase positive *Staphylococcus*

The Coagulase positive *Staphylococcus* (CoPS) includes seven species*: S. intermedius, S. schleiferi* subsp. *coagulans*, *S. hyicus, S. lutrae, S. delphini, and S. pseudintermedius*, and also *S. aureus* (Freney *et al.*, 1999; Devriese *et al.*, 2005), as outlined in Table 1.2. *S. aureus* is distinguished by round, golden-yellow colonies on nutrient agar, and beta-haemolysis on blood agar, and CoPS are most frequently connected with pathogenicity in the host. Clinical diseases caused by *S. aureus* range from minor and limited cutaneous infections to invasive and life-threatening diseases (Iwatsuki *et al.*, 2006). In addition, *S. aureus*, often referred to simply as ''staph'', is an important species compared to others, and is commonly found on the skin of healthy people (Abudu *et al.*, 2001). *S. aureus* causes many diseases in humans and animals, especially cutaneous diseases, such as wound infections, abscesses, furuncles, carbuncles, impetigo and others (Brown *et al.*, 2003; Iwatsuki *et al.*, 2006) and is a major cause of cellulitis. In addition, it causes other deep-seated infections, such as osteomyelitis, endocarditis, pneumonia, and septicaemia. Toxigenic bacterial strains can cause toxic shock syndrome-1 (TSS-1), staphylococcal scalded skin syndrome (SSSS) and staphylococcal food poisoning (Ogston and Witte, 1984; Projan and Novick, 1997).

Table 1.2 The taxonomy of coagulase positive *Staphylococcus* (Co.P.S) using the 16S rRNA gene sequence analysis, adapted from Takahashi *et al.* (1999).

Group	CoPS
S. aureus	*S. aureus*
S. hyicus-intermedius	*S. delphin*i, *S. hyicus*, *S. intermedius*, *S. lutrae*, *S. pseudintermedius*, *S. schleiferi* subsp. *coagulans*

1.2.1.2 Coagulase negative *Staphylococcus*

The Coagulase negative *Staphylococcus* (CoNS) includes *S. epidermidis, S. saprophyticus* and others (Table 1.3). CoNS is common in infections associated with hospitals (nosocomial infections) and can cause blood stream infections. These infections are often reported in infants, as well as in immune-compromised patients with diseases such as lymphoma and leukaemia. Furthermore, they are important in burns, indwelling intravascular devices, peritoneal catheters and ventricular shunts (Krediet *et al.*, 2001).

Table 1.3 The taxonomy of coagulase negative *Staphylococcus* (CoNS) using the 16S rRNA gene sequence analysis, adapted from Takahashi *et al*. (1999).

Group	CoNS
S. aureus	*S. simiae*
S. auricularis	*S auricularis*
S. carnosus	*S. carnosus S. carnosus*, *S. condimenti*, *S. massiliensis*, *S. piscifermentans*, *S. Simulans*
S. hyicus-intermedius	*S. chromogenes*, *S. felis*, *S. microti*, *S. muscae*, *S. rostri*,
S. epidermidis	*S. capitis*, *S. caprae*, *S. epidermidis*, *S. saccharolyticus*
S. haemolyticus	*S. devriesei*, *S. haemolyticus*, *S. hominis*
S. lugdunensis	*S. lugdunensis*
S. saprophyticus	*S. arlettae*, *S. cohnii*, *S. equorum*, *S. gallinarum*, *S. kloosii*, *S. leei*, *S. nepalensis*, *S. saprophyticus*, *S. succinus*, *S. xylosus*
S. sciuri	*S. fleurettii*, *S. lentus*, *S. sciuri*, *S. stepanovicii*, *S. vitulinus*
S. simulans	*S. simulans*
S. warneri	*S. pasteuri*, *S. warneri*

1.2.3 Skin diseases caused by staphylococci

1.2.3.1 Folliculitis

Folliculitis is an inflammation of one or more hair follicles or an infection of the external part of the pilosebaceous unit. There are several causative agents, but *S. aureus* has become the main reason for infection, localizing on the skin and then spreading to several parts of the host's body, such as the perineum, nares, vagina and axilla. The symptoms of folliculitis are eruptions on hair follicles, which is surrounded by erythema centred on the hair follicle (Wickboldt and Fenske, 1986; Sanford *et al.*, 1994).

1.2.3.2 Boils (furuncles) and carbuncles

S. aureus is the most common cause of boils, carbuncles, and skin abscesses (Iwatsuki *et al.*, 2006; McCaig *et al.*, 2006). A boil or furuncle is an infection of the hair follicle, which is almost always caused by MRSA, resulting in a painful swollen region on the skin caused by an accumulation of purulent and necrotic tissue (Ibler and Kromann, 2014). Singular boils gathered to each other are named carbuncles. Carbuncles are an accumulation of infected hair follicles that produce broad, bloated, erythematous, deep, and painful masses that usually open and drain via multiple tracts (Stulberg *et al.*, 2002). Signs and symptoms of boils include bumpy, red, purulent-filled masses around a hair follicle, which are fragile, warm, and very painful. A yellow or white point at the centre of the mass can be observed when the boil is ready to drain purulence. In a serious infection, an individual may experience fever, swollen lymph nodes and fatigue.

1.2.3.3 Impetigo

Impetigo is a common superficial bacterial infection of the skin's surface, characterized by an inflamed and infected epidermis. Usually, impetigo is caused by one of two bacteria: *S. aureus*, or sometimes *Streptococcus pyogenes* group A, or both (Dagan and Bar-David, 1992). Impetigo commonly infects children aged 2 - 5 years (Adams, 2002). The causative agent enters broken skin, such as scratches, which cause red bumps to form, mostly on the face, (mainly around the nose and mouth), and the legs, though these bumps may appear on other parts of the host's body.

1.2.3.4 Toxic shock syndrome-1

Todd *et al.* (1978) were the first to describe toxic shock syndrome-1 (TSS-1), when they described the staphylococcal illness in three boys and four girls aged 8 - 17 years. Toxigenic staphylococci cause a severe illness called toxic shock syndrome, especially during menstruation and also in the postpartum period. The causative agent secretes many serotoxins, such as TSS-1 toxin (Kotzin *et al.*, 1993). *S. aureus* can enter via cuts to the skin, surgery or burns.

1.2.3.5 Staphylococcal scalded skin syndrome (SSSS)

S. aureus secretes exofoliative toxins (ET), also known as epidermolytic toxins, which induce SSSS (Melish and Glasgow, 1970). This syndrome affects neonates, children and occasionally adults. Li *et al.* (2014) demonstrated that mortality in children with SSSS is approximately 4%. The site of infection depends on age. In infants, the lesion is often present on two

regions, either the perineum or periumbilicus, or both. SSSS consists of many stages, starting with erythema that soon rupture, leaving large regions of bare, infected skin (Melish, 1982; Cribier *et al.*, 1994).

1.2.4 Methicillin resistant *Staphylococcus aureus*

Methicillin resistant *Staphylococcus aureus* (MRSA) is one of the most common etiological and opportunistic bacterial pathogens, and is responsible for healthcare-associated infections (Plata *et al.*, 2009).

1.2.4.1 History of antibiotic resistance

MRSA has acquired the *mecA* gene giving resistance to methicillin and other β- lactam antibiotics. The emergence of *S. aureus* resistance to β-lactam antibiotics was first observed in the 1940s, particularly with penicillin. MRSA was first recognized in the early 1960s and became an increasingly important pathogen (Rolinson, 1961). However, Barrett *et al.* (1968) reported the first documented outbreak of disorders in the United States, in Boston City Hospital. By the late 1970s and early 1980s, MRSA was responsible for outbreaks in several hospitals in the United States (File, 2008). Nowadays, diseases caused by MRSA have become a major epidemiological and clinical problem in health centres. In 2003, Naimi *et al.* (2003) proposed the term 'community-associated MRSA' (CA-MRSA) in response to its appearance in the wider community.

In the United Kingdom, resistance to methicillin among *S. aureus* isolates recovered from cerebrospinal fluid or blood, was stable at about 1.5% of isolates from 1989 - 1991, but increased afterwards to 13.2% in 1995

(Speller *et al.*, 1997). Mayor (1999) demonstrated that in health centres in the UK, the predominance of MRSA has reached epidemic levels, has increased twelve fold since 1991 and was responsible for 37% of all *S. aureus* infections in 1999, in comparison to 3% in 1991. Furthermore, in the U.K., the proportion of *S. aureus* infections which were due to MRSA increased to 25 - 50% between 2006 - 2009 (Stefani *et al.*, 2012). A predominance of more than 25 - 30% was also observed in other European countries, e.g. Spain, France and Italy, as outlined in Figure 1.4 (Voss *et al.*, 1994).

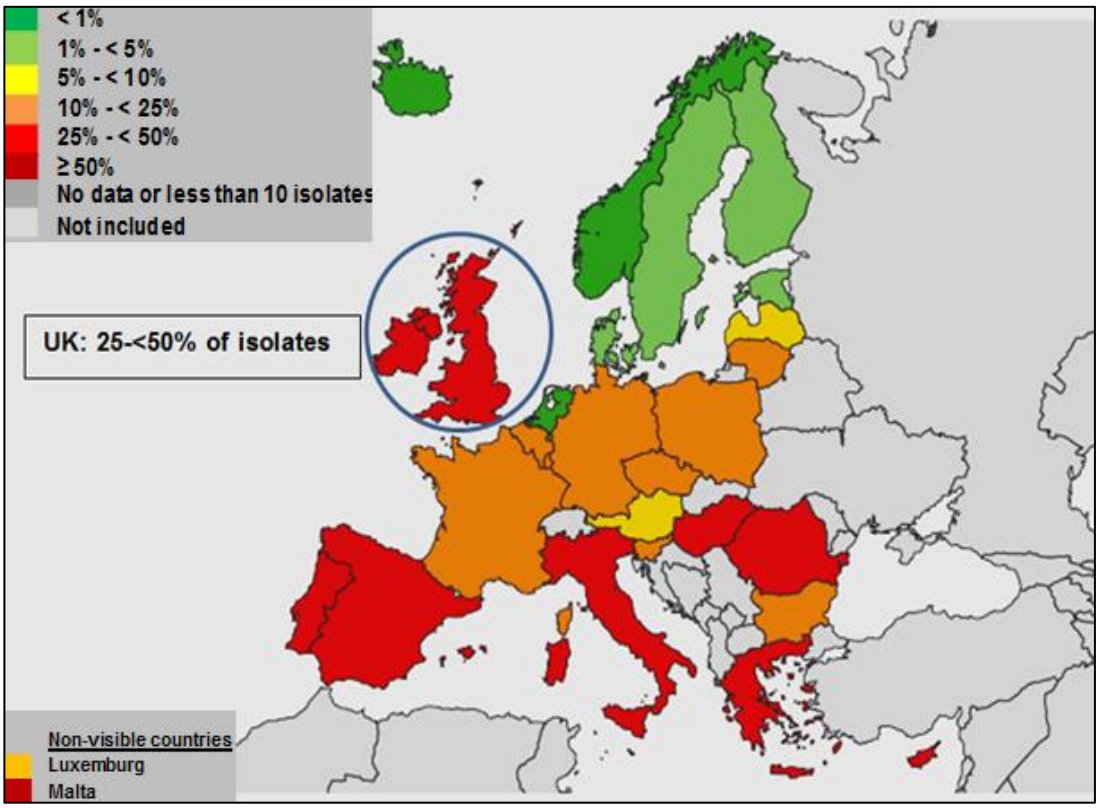


Figure 1.4 Distribution of methicillin resistant *Staphylococcus aureus* in European countries, 2006 - 2009, adapted from Stefani *et al.* (2012).

1.2.4.2 Methicillin resistant *Staphylococcus aureus* cell wall structure and biosynthesis

The *Staphylococcus* cell wall is a semi rigid structure, composed of three components: peptidoglycan (PG), teichoic acids (TAs), and surface proteins (Figure 1.5). PG is the major component of the bacterial cell wall, determining its shape and integrity (Labischinski, 1992). There are several steps in PG synthesis; the first step takes place in the cytoplasm, which leads to the synthesis of nucleotide sugar-linked precursors. The second stage occurs in the cytoplasmic membrane. In this step, MRSA produces lipid I and II (Scheffers and Pinho, 2005; Bouhss *et al.*, 2007). The third stage takes place outside the cytoplasmic membrane and includes incorporation of the recently synthesized disaccharide peptide units into the PG. The fourth stage of PG biosynthesis is carried out by penicillin binding proteins (PBPs), which catalyse the trans-glycosylation and trans-peptidation reactions, e.g. formation of the glycosidic and peptide bonds, respectively (Barrett *et al.*, 2005). The second major component of the *S. aureus* cell wall are TAs, which are polymers of ribitol residues or polymers of glycerol phosphate, and account for up to half of the bacterial cell wall dry weight. Chatterjee (1969) observed that TA contributes to the negative charge present on the cell surface, which plays an important role in acquisition of ions. The third component of the *S. aureus* cell wall consists of surface proteins, including microbial surface components, recognizing adhesive molecules. There are several surface proteins, such as protein A, collagen binding protein, fibronectin binding protein and clumping factor A (Foster and Höök, 1998).

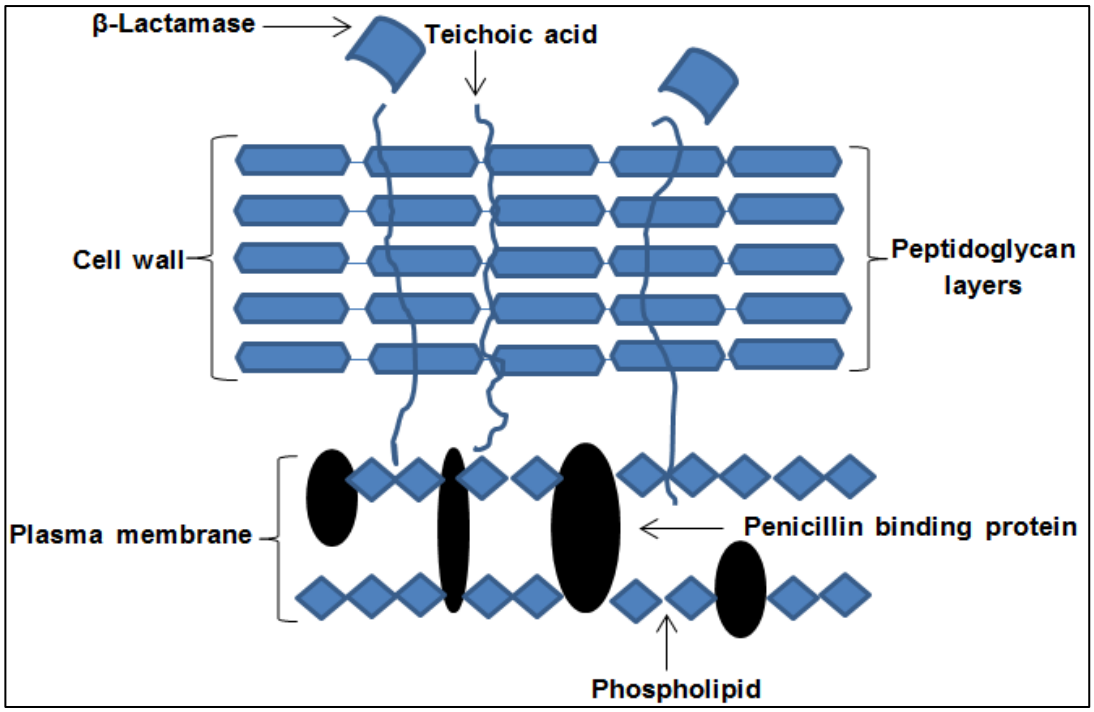


Figure 1.5 Illustration of the basic structure of MRSA cell wall, adapted from (http://www.cehs.siu.edu/fix/medmicro/genmicr.htm).

1.2.4.3 Mechanism of β-lactam action and resistance

Several antibiotics belong to β-lactams, including penicillins, cephalosporins, methicillin and oxacillin, which are considered bactericidal. The cell wall is the main target of β-lactams, especially the transpeptidation step of the PG synthesis, which is achieved by binding or inactivating the transpeptidase domain of PBPs in the bacterial cell wall. Moreover, β-lactams are structural analogs of the natural substrate of PBPs (D-alanyl-D-alanine) of the PG (Chambers, 2004). The reaction between the substrate of PBPs and β-lactams begins with a non-covalent association between these two molecules. This intermediary can either dissociate or undergo an irreversible reaction of acylation process when the PBPs covalently bind with β-lactams at its active site, cleaving the cyclic amide bond in the β-lactams. In the case of resistance, the natural substrate for PBP, D-alanyl-D-alanine, undergoes a

rapid de-acylation by hydrolysis, which liberates the PBP for the next around of transpeptidation. Conversely, in the case of sensitivity, the de-acylation process is very slow and the PBPs are effectively inactivated. When the PBPs ceases to function, the cell wall synthesis is inhibited and death of the bacterial cell occurs (Chambers, 2003).

1.2.4.4 Mechanism of resistance to methicillin

Several β-lactam antibiotics, such as penicillins and cephalosporins, damage the bacterial cell wall by inactivating PBPs and enzymes, which are essential in the assembly of the bacterial cell wall. The cell wall becomes osmotically fragile and easily lysed when treated with antibiotics (Pinho *et al.*, 2001; Weese *et al.*, 2005).

Resistant *S. aureus* has developed resistance to β-lactam antibiotics through acquisition of the *mecA* gene, which is carried on the staphylococcal cassette chromosome *mec* (SCC*mec*) elements (Berger-Bächi and Tschierske, 1998; Chambers, 2003). *MecA* encodes an alternative PBP2a, which has a low affinity to β-lactam antibiotics. Goffin and Ghuysen (1998) observed that PBP2a belongs to a group in the family of PBPs possessing a high molecular mass (78 kDa), and consists of a transpeptidase domain and a non-penicillin binding domain of unknown function. PBP2a possesses a low affinity for β-lactam antibiotics, which allows MRSA to develop and grow in methicillin concentrations that inactivate all native types of PBPs (Gaisford and Reynolds, 1989). The serine in the active site of the transpeptidase domain in PBP2a is responsible for nucleophilic attack on both the β-lactam antibiotic ring and the D-alanyl-D-alanine substrate, and is located in an extended

narrow groove. The groove mediates non-covalent interactions with the β-lactam that place the β-lactam in an un-favourable position for interaction with the serine in the active site. Consequently, the acylation between the β-lactam antibiotic and the active site does not occur (Pinho *et al.*, 2001). PBP2a successfully balances the critical transpeptidase activity with a decreased affinity toward β-lactams. Therefore, PBP2a is able to synthesize the bacterial cell wall at otherwise lethal concentrations of β-lactams (Lim and Strynadka, 2002).

1.2.5 *Pseudomonas* bacterium

The *Pseudomonas* bacteria are Gram negative rods. Nearly all strains are motile by means of a single polar flagellum, and are free-living bacteria. Generally, *Pseudomonas* is found in soil, water and other places which contain moisture. This bacterium is a member of the genus gamma proteobacteria belonging to the bacterial family pseudomonadaceae (Kreig *et al.*, 1984). A *Pseudomonas* disease refers to a disease caused by one of the species of the *Pseudomonas*, which is considered to be an opportunistic microorganism (Christensen *et al.*, 2013).

1.2.5.1 *Pseudomonas aeruginosa*

Pseudomonas aeruginosa is found throughout the environment and inhabits miscellaneous environments, including humans, animals, soil, water and plants (Green *et al.*, 1974; Lyczak *et al.*, 2000). It is a metabolically diverse microorganism which is tolerant of poor nutritional conditions and capable of tolerating miscellaneous hostile environmental conditions, for example, high

salt concentrations, as well as a wide range of temperatures (15 - 42 °C), in weak antiseptic solutions, and in the presence of several antimicrobial agents (Ernst *et al.*, 2006). These features have contributed to the success of *P. aeruginosa* as an opportunistic pathogen to humans and animals (Bodey *et al.*, 1983; Lyczak *et al.*, 2000). Since *P. aeruginosa* is an opportunistic bacterium, it most commonly affects immune-compromised patients, such as those with cystic fibrosis, or AIDS patients (Elkin and Geddes, 2003).

McGowan (2006) observed that *Pseudomonas* infections can affect several parts of the host's body, especially the respiratory tract, particularly in cystic fibrosis sufferers. Moreover, *P. aeruginosa* is a major cause of skin diseases, particularly folliculitis and dermatitis, and such diseases may be spread either by direct contact or via contaminated water, with bacteria attaching to the skin and entering hair follicles. The clinical signs of such infections may manifest themselves as a red and itchy rash or resemble a burn (Levy *et al.*, 1998). In addition, Christensen *et al.* (2013) observed that *P. aeruginosa* is able to form a biofilm in numerous conditions.

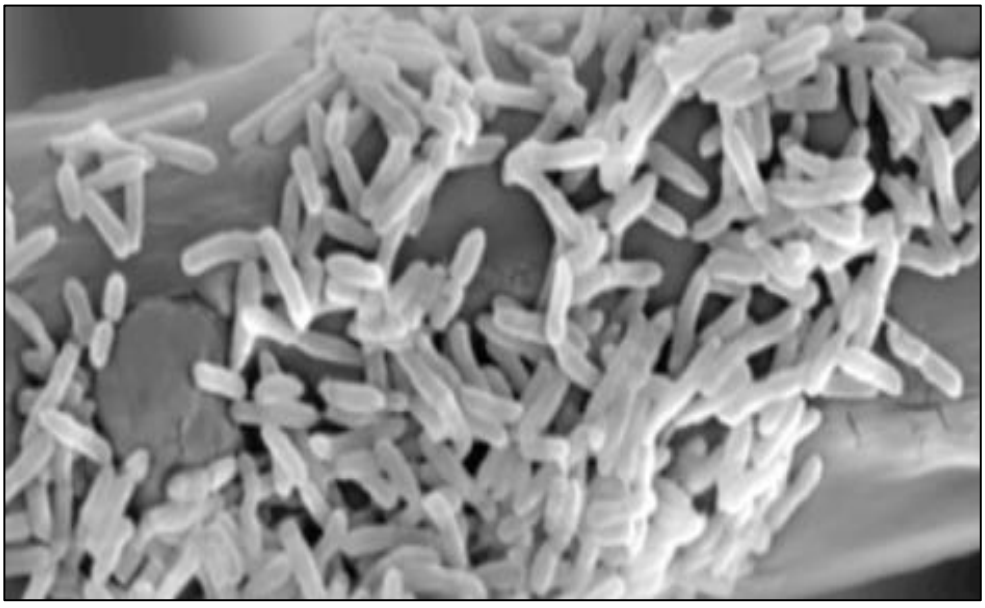


Figure 1.6 SEM micrograph of *P. aeruginosa* cells, taken from Walker *et al*. (2003).

1.2.5.2 *Pseudomonas* and skin burn infections

P. aeruginosa has emerged as a significant pathogen during the past two decades. It causes between 10 - 20% of infections in most hospitals. *Pseudomonas* disorders are particularly widespread among patients with burn wound infections (Bodey *et al.*, 1983). This bacterium causes suppression of the immune system as a result of a break in the protective skin barrier by burn infection, after which it rapidly colonizes and infects the burn wound site (DeBoer and O'Connor, 2004). The denatured protein of the burn wound provides nutrition for microorganisms. Church *et al.* (2006) observed that rapid colonization of *P. aeruginosa* in burns, followed by distribution into distant organs via the bloodstream leads to bacteraemia, endotoxic shock and sepsis. Moreover, oral administration of antibiotics is usually ineffective against the most dangerous SSTIs by *P. aeruginosa* (McManus *et al.*, 1985).

1.2.6 *Propionibacterium*

Propionibacterium species are Gram positive, non-motile, non-spore bearing bacilli, generally diphtheroid or club-shaped with one end rounded and the other end tapered. Bacterial cells may be coccoid, elongated or even branched. Ray and Kellum (1970) observed that the growth of *Propionibacterium* species are anaerobic or aero-tolerant, growing deep inside skin pores and hair follicles, where it feeds on the sebum that is produced by the sebaceous glands that surround the base of the hair shaft. The genus *Propionibacterium* contains eight species. *Pr. acnes* is a major member of the skin's normal flora, and probably the predominant bacterium

of sebaceous areas. This bacterium inhabits the surface of the skin and is commonly non-pathogenic Levy *et al.*,(2008).

1.2.6.1 *Propionibacterium acnes*

Propionibacterium acnes is a Gram positive anaerobic or aero-tolerant rod (Figure 1.7) and inhabits the human skin as flora in conjunctiva (Doyle *et al.*, 1995). *Pr. acnes* is associated with acne vulgaris, and believed usually to have a low pathogenicity (Eady and Ingham, 1994). Lee *et al*. (1978) observed that more than 40% of *Pr. acnes* infections are resistant to antibiotics. Kellum *et al.* (1970) revealed that the important role of *Pr. acne* in diseases is that it releases fatty acids by hydrolysis of triglycerides and these acids produce a primary irritant dermatitis of the wall of sebaceous follicles.

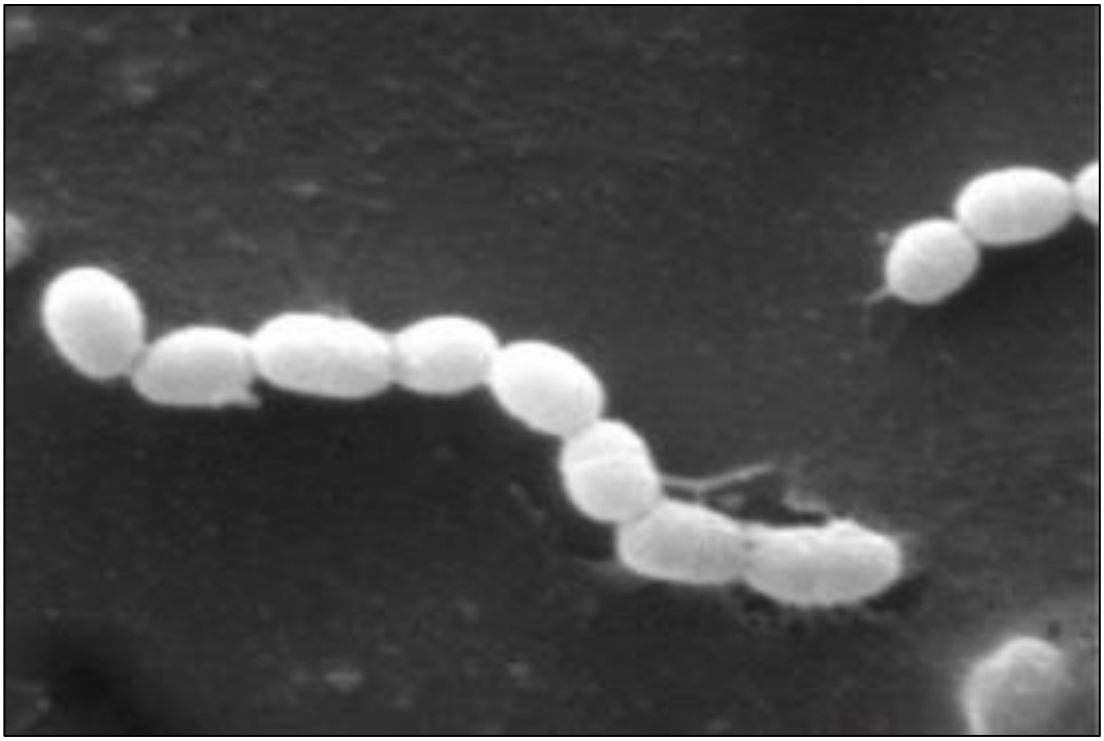


Figure 1.7 SEM micrograph of *Pr. acnes* cells, taken from Pan *et al*. (2009).

1.2.7 Probiotics

1.2.7.1 Introduction

Probiotics are defined as live microorganism that, when administrated in sufficient amounts, confer a health benefit on the host (Salminen *et al.*, 1998); or a varied group of useful bacteria that are functionally defined by their capability to alleviate inflammation when introduced into the inflamed gastrointestinal tract (GIT) (Boirivant and Strober, 2007). The most frequently used probiotic genera in humans and terrestrial animals are *Lactobacillus*, *Bifidobacterium* and *Enterococcus*, which are normal residents in the GIT. There are some evidences to suggest that probiotics can prevent intestinal infection, which is caused by several microorganisms. Granto *et al.* (1999) tested 47 different strains of lactic acid bacteria (LAB) and found that they can discourage the growth of pathogenic bacteria, most frequently those from stool samples. The vast majority of these appear effective against *S. aureus* and *Salmonella enterica* serovar *typhimurium* (Simmering and Blaut, 2001). At the beginning of the 21st century, research indicated that the effect of probiotics was useful in improving the microbial balance in the intestine of the host, the final outcome being the prevention or inhibition causative agents of diseases involving the toxin-producing bacteria. The Russian researcher and Nobel laureate Metchnikoff was the first to propose and use alternative therapy (probiotics), especially LAB, for the treatment of bacterial infections. LAB have properties such as treatment of infections (Morelli, 2000; Mercenier *et al.*, 2003). Several studies have used probiotics for the treatment of AD disease in infants and have observed significant improvement to the infections (Rosenfeldt *et al.*, 2003; Weston *et al.*, 2005). The spread of AD in

children could be reduced by giving probiotics to the mother before and after birth for six months (Kalliomäki *et al.*, 2001). Probiotics are available to consumers, especially in the form of dietary supplements and food (Fuller, 1989).

Kopp-Hoolihan (2001) stated that the first clinical trials were done in the 1930s on the beneficial effects of probiotic microorganisms on constipation. Research on probiotics has steadily increased since then, much of it taking place in Europe and Asia. Since then, there has been a rapid increase in the search for new formulations of probiotics, as well as in the consumption of probiotics in commercially available products.

1.2.7.2 Historical perspectives on probiotics

The potential benefits of probiotic microorganisms were first shown in the early 1900s. For example, Tissier (1907) demonstrated their potential benefits in the context of the relief of gastrointestinal diseases. Also in 1907, Nobel prize-winner Metchnikoff advocated that the consumption of *Lactobacillus* helps in controlling endogenous intoxication caused by an imbalance within the intestinal microflora. He noted the positive effect of LAB, which is present in fermented milk products, on longevity in humans. The works of Tissier and Metchnikoff were the first to make scientific suggestions about the use of bacteria in this way (Kopp-Hoolihan, 2001). Orrhage *et al.* (1994) reported that the first clinical experiments on the effect of probiotics microorganisms on constipation were performed in the 1930s. In the 1950s, probiotic microorganism products were authorized in the United States of America. Despite inconclusive evidence of the health effects of yogurt

bacteria, research interest intensified in subsequent years. Ferdinand Vergin (1954) was the first to introduce the term “probiotic,” mainly to differentiate it from antibiotics. Kollath (1954) used the term to designate “active substances that are essential for healthy development of life”. However, Lilly and Stillwell (1965) were the first to introduce the term “probiotic” to describe growth-promoting factors produced by microorganisms. The word probiotic is derived from the Greek and means “pro”: for and “bio”: life (hence ‘for life’). This is the converse of “antibiotic” which means “against” life (Ghadban, 2002). Moreover, Parker (1974) defined probiotics as microorganisms or substances that contribute to intestinal microbial balance. Furthermore, Crawford (1979) defined a probiotic as a culture of specific living microorganisms, mainly *Lactobacillus* species, which is established in the host animal and ensures the rapid and effective establishment of a population of beneficial organisms in the intestines. Having considered the definition given by Parker (1974), Fuller (1989) narrowed the definition of “probiotics,” wanting to exclude antibiotic preparations, and re-defined “probiotic” as a live microbial feed supplement that has a beneficial effect on the host animal by improving its intestinal microbial balance. Vanbelle *et al.* (1990) mentioned that the majority of researchers considered probiotics as selected and concentrated viable counts of LAB. In 1995 Gunther broadened the term even more and defined probiotics as ‘organisms, live or dead, or as products of microbial fermentation, nucleotides and their metabolisable products, metabolites of the proteins and derived substances, organic acids, in addition to enzymes of a hydrolytic type, that beneficially affect the host’. However, Curbelo *et al.* (2005) observed that the majority of authors agree in defining probiotics as

feed additives, formed by live microbes that beneficially affect the health of the host (Schrezenmeir and de Vrese, 2001).

1.2.7.3 Criteria for the selection of probiotics

The criteria for probiotic selection are application-based and depends on the specific probiotic effects preferred and the site of action (Saarela *et al.*, 2000). Several criteria have been defined for the choice of probiotic strains. The most significant is that the chosen strains have to be safe for use in the host and for the environment, as briefly outlined in Figure 1.8. There is a wide range of requirements for applications of probiotics, e.g. the capacity of adhesion and colonization of the human body, immunomodulation, production of antimicrobial substances and competition with pathogens for nutrition and sites (Fooks and Gibson, 2002). Furthermore, there are other probiotic properties such as resistance to, and survival of natural human defence mechanisms during the GIT transit, prolonged residence period in the GIT (Lee, 2009). Safety aspects of probiotic bacteria include being of human origin and isolation from healthy human GIT. In addition, probiotics must be non-pathogenic, conferring clinically established physiological benefits and must maintain their activity. Moreover, probiotic strains must maintain viability throughout product processing and manufacture; they must be able to survive during passage via the host's upper GIT, and be able to tolerate the conditions predominant in the GIT, e.g. a high acidity level and the presence of bile acids, pancreatic and other digestive enzymes (Dunne *et al.*, 1999; Ouwehand *et al.*, 1999; Reid *et al.*, 2003).

Figure1.8 The theoretical and necessary basic rules for choice of probiotic microorganisms include several characteristics, such as safety, functional and technological aspects, such as antimicrobial substances production, survival, adherence, colonisation immune stimulation, antigenotoxic activity , prevention of pathogens sensory properties stability, phage resistance and viability in processes, adapted from Saarela *et al.*(2000).

1.2.7.4 Normal flora and the skin

Limited numbers of particular species of microbes are present in the normal flora on the human skin. In general, two types of microbes exist on the skin, resident and transient. The resident organisms belong to a relatively stable group of microorganisms that are routinely found in the skin and that re-

establish themselves after perturbation. Resident microbes are often considered to be commensal, meaning that these microbes are not harmful and may provide benefit to the host (Somerville and Noble, 1974). The transient organisms do not establish themselves permanently on the surface of skin, but rather arise from the environment and persist for hours to days. Transient and resident organisms are not pathogenic under normal circumstances if proper hygiene is maintained and if the normal resident flora, immune responses, and skin barrier function are intact. Nevertheless, if the skin barrier is disrupted in some way, resident and / or transient microbial communities can colonize, proliferate, and cause disease. For example, *Staphylococcus epidermidis* is a commensal skin associated species but can be an opportunistic pathogen in immune-compromised hosts (Otto, 2009). Adult skin comprises an average area of 1.7 m^2, and microbial diversity varies across the various niches found on it (Zaidi and Lanigan, 2010). For instance, hairy, moist underarms lie a short distance from smooth dry forearms, but these two niches are ecologically distinct as are their resident microbial populations (Grice *et al.*, 2009). Johnson *et al.* (2002) and Kong and Segre (2012) demonstrated that essential factors including age, genotype, and immune reactivity also influence the composition of cutaneous microorganism communities. Extrinsic factors, such as hygiene and environmental factors, such as climate may also have profound effects on microbial communities. Distinct habitats are characterized by differences in skin thickness and folds as well as the density of hair follicles and sweat glands. Cutaneous invaginations and appendages, including sebaceous

glands, sweat glands, and hair follicles, are likely to be associated with their own unique microbiota (Figure 1.9).

Sebaceous glands secrete lipid-rich sebum, a hydrophobic coating that protects and lubricates hair and skin. Although sebum generally serves as an antimicrobial coating, *Propionibacterium acnes* hydrolyses tri-glycerides present in sebum, releases free fatty acids (FA) that promote bacterial adherence, and then colonizes sebaceous units (Marples, 1969). Several genera and species of bacteria live on the skin, such as *Pr. acnes*, *Propionibacterium granulosum*, *S. epidermidis*, *Micrococcus* species, *Corynebacterium* species and *Acitobacter* species (Grice *et al.*, 2008). *Propionibacterium* species are important in certain medical conditions, such as acne and inflammation of the hair follicles (Leyden *et al.*, 1998). However, sometimes these organisms may become dangerous to the host when the latter is exposed to shock, injury or a change in the immune defences, where they become opportunistic pathogens and may cause diseases. There are physical and chemical agents which determine the quality and quantity of bacterial species that reside on the skin (Leyden *et al.*, 1987; Bojar and Holland, 2002). The physical factors involve the number and type of glands, gland functions, number and size of hair follicles, safety and functions of the skin barrier, osmotic pressure and pH of the skin. Chemical factors involve several compounds, e.g. fat, amino acids, vitamins and lactate. Moreover, biochemical molecules formed by microorganisms as a result of metabolic activity to the skin will be important for colonization on the skin (Leyden *et al.*, 1987).

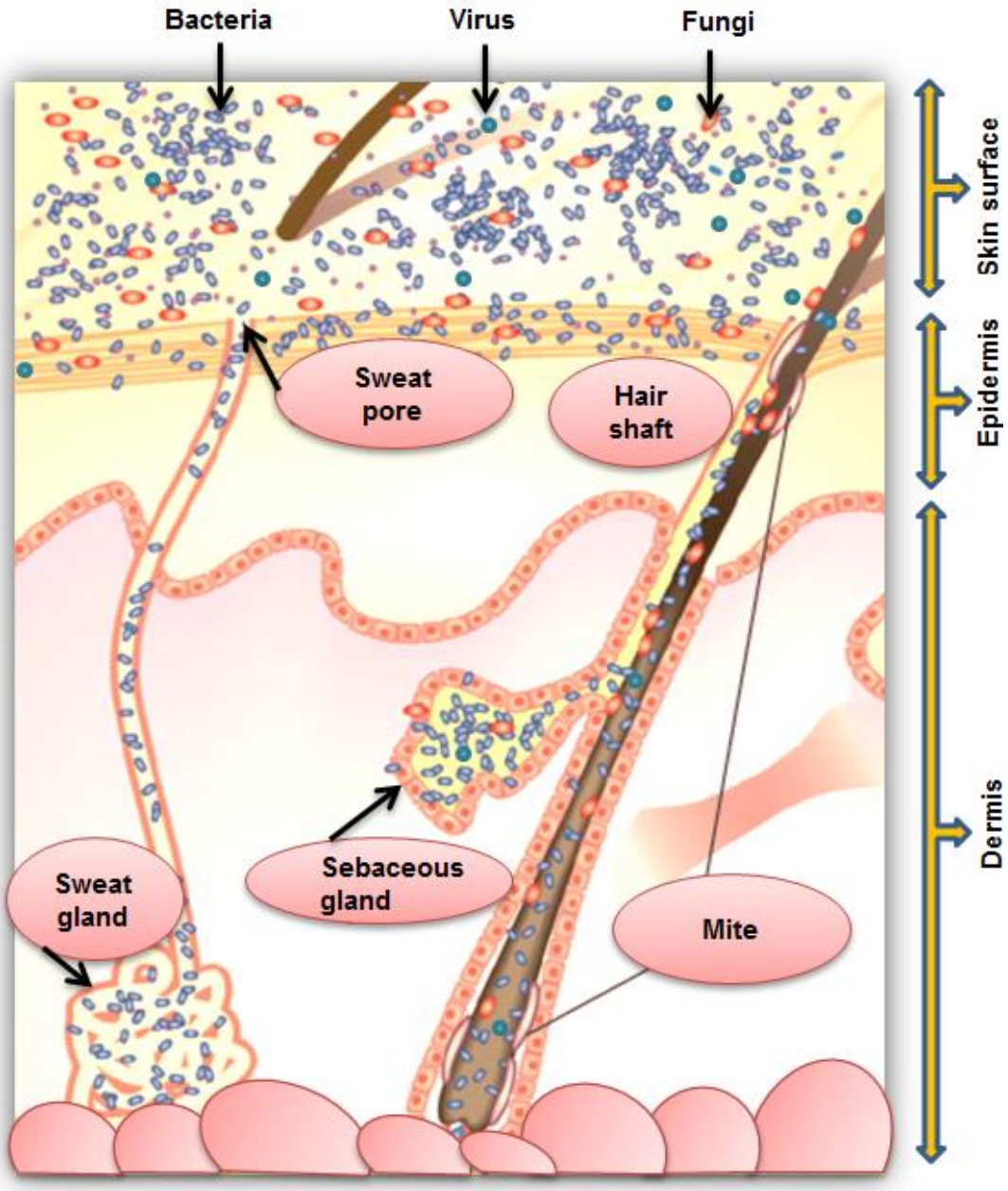


Figure 1.9 Illustration of skin histology viewed in cross section with different microbial communities and skin appendages. Microbial communities (bacteria, viruses, fungi, and mites) cover the surface of the skin and reside deep within glands and hair follicles, adapted from Kong and Segre (2012).

1.2.7.5 Role of probiotics in skin diseases

The studies investigating the role of probiotics in cosmetics and skin disease are very few compared to other studies dealing with the importance of probiotics in other locations. Currently, it is thought that probiotics can

positively affect the normal flora in a number of ways, for example by reducing the number of potential pathogens, in turn positively affecting GIT structure and function. It is now generally believed that probiotic microorganisms exert positive effects by improving the properties of the GIT's normal flora (Ouwehand *et al.*, 2002b). Kaila *et al.* (1992) and Saavedra *et al.* (1994), reported that probiotic LAB have been linked to several effects involving improving rates of recovery from inflammation of the GIT and diarrhoea of bacterial and viral origins. Foolad *et al.* (2013) demonstrated that certain types of nutrient supplementation and the probiotic *Lactobacillus rhamnosus* GG are useful in preventing AD development and reducing its severity**.** Additionally, probiotic microorganisms administered prior and postpartum for 6 months may be able to reduce the incidence of eczema in children at high risk from atopic diseases as compared to placebo treatment (Kalliomäki *et al.*, 2001). Moreover, Guéniche *et al.* (2006a) observed that nutritional supplementation of hairless mice with *Lactobacillus johnsonii* provided protection of the cutaneous immune system against ultraviolet B radiation Ouwehand *et al*. (2003) revealed the difficulty in recognizing advantageous bacteria in view of the severe environmental circumstances that may prevent colonization of cutaneous surfaces by probiotic strains. Two studies suggest that topical application of *Vitreoscilla filiformis* exerts positive effects in patients with atopic eczema and seborrheic dermatitis (Guéniche *et al.*, 2006b; Gueniche *et al.*, 2008). In fact, the exact mechanisms through which cutaneous symptoms are improved in seborrheic and atopic dermatitis are not yet known. This hypothesis is supported by very recent research demonstrating that *V. filiformis* has a profound effect on the skin immune

system response, such as improving skin defence mechanisms, balancing cutaneous immune-homeostasis, and the direct immunomodulation of the skin-associated immune response (Guéniche *et al.*, 2006b; Gueniche *et al.*, 2008).

1.2.7.6 Probiotics, the skin and Immune system

The skin is an immunogenic organ that works as the first defence line and biologic sensor against pathogens. Contact with skin allergens can favour the beginning of allergic disorders, and atopic response can be secondary to the skin barrier disruption (Gallo and Nakatsuji, 2011). The keratinocytes secrete a large number of immune mediators: several interleukins (IL), such as 1, 3, 6, 7, 8, 10, 12, 15 and 18, interferon (IFN) α, β and γ, tumour necrosis factor (TNF) α and β, granulocyte macrophage colony-stimulating factor, macrophage colony-stimulating factor, chemokines and growth factors (Pivarcsi and Homey, 2005; Strid and Strobel, 2005). Steinhoff *et al.* (2001) mentioned that a large numbers of cells in the dermal and perivascular regions play an important role in immune activities such as macrophages, mast cells, monocytes and T cells. Recently, Wollenberg *et al.* (2002) reported that epidermal dendritic cells were found in infected cutaneous, e.g. Langerhans cells, and identified populations of inflammatory dendritic epidermal cells. Cutaneous dendritic cells serve as a main part within the infiltrated portion of AD. As antigen-presenting cells, they are capable of adjusting the amount and property of T lymphocyte responses, and thus they probably play an important role in the pathogenesis of T lymphocyte skin disorders like AD (Strid *et al.*, 2004; Strid and Strobel, 2005). Strid and

Strobel (2005) observed that lymph node cells are activated with notable secretion of IL-4, restricted IFN-γ secretion, a large amount of production of B cells, immunoglobulin (Ig) E and G1.

1.2.7.7 Mechanism action of probiotics

Probiotics are considered helpful and play an important role in the health of the host (Tuohy *et al.*, 2003). There are numerous studies on the subject of how probiotic microorganisms work. Therefore, several mechanisms from these studies attempt to describe how probiotics could protect the host from disease (Table 1.4). The precise way in which probiotic microorganisms achieve their effects is not fully understood. Nevertheless, some mechanisms of the action of probiotics have been observed, such as competition for adhesion receptors, competition for nutrients, immune clearance and others (Fooks and Gibson, 2002). The mechanism of action for probiotics can be divided, based on specific effects of probiotics on the microbial environment, epithelium, immune system response e.g. epithelial cells, dendritic cells, natural killer cells and others (Neurath, 2007; Zhang *et al.*, 2007). Several mechanisms of the action of probiotics are briefly listed below.

1.2.7.7.1 Competition of adhesion (blocking of adhesion sites)

Alaner *et al.* (1997) mentioned that binding to the epithelium is one of the determinants in establishing the ability of a probiotic, whereby probiotic bacteria compete with pathogens for a limited number of receptors present on the surface of the epithelium. There are several examples, such as *L. rhamnosus* GG and *L. plantarum*, which competitively impede the attachment

of entero-haemorrhagic *Escherichia coli* (Mack *et al.*, 1999). Moreover, certain strains of probiotic *Lactobacillus* species are able to block receptor sites, preventing the invasion of pathogens (Bernet *et al.*, 1994). In addition, adhesion and growth of pathogenic bacteria in the host is inhibited by lowering pH, producing lactic acid (LA) with other bactericidal metabolites (De Keersmaecker *et al.*, 2006).

1.2.7.7.2 Release of antimicrobial substances

The production of antimicrobial substances, such as organic acids, hydrogen peroxide (H_2O_2) and bacteriocins are inhibitory to both Gram positive and Gram negative bacteria (Silva *et al.*, 1987; Meurman *et al.*, 1995). Furthermore, antimicrobial substances may induce an antagonistic action to pathogens (Balcázar *et al.*, 2007). Moreover, the accumulation of such metabolites, e.g. short chain fatty acids (SCFA), can reduce the pH of the surrounding environment, which may directly impede the growth of pathogens. LAB also release other antimicrobial factors, for example bacteriocins and reuterin (Vandenbergh, 1993; O'Hara *et al.*, 2007).

1.2.7.7.3 Competition for nutrients

Wilson and Perini (1988) observed that probiotics may also compete for nutrients otherwise consumed by pathogens. Consumption of monosaccharides by probiotics may reduce the growth of *Clostridium difficile*, which is dependent on monosaccharides for growth.

1.2.7.7.4 Modify toxin receptors

Probiotics may also modify toxin receptors and block toxin mediated pathology via an enzymatic mechanism, which has been seen with *Saccharomyces boulardii* via its effect on the *Clostridium difficile* toxin A receptor (Pothoulakis *et al.*, 1993). Cebra (1999) observed that the effect of *Saccharomyces boulardii* on *Clostridium difficile* toxins was supposed when investigators witnessed clinical improvement without a change in the concentration of *Clostridium difficile* in the stools.

1.2.7.7.5 Modification of microflora

Probiotics modulate the composition of the human GIT microbiota. The useful impacts may result from inhibition of pathogens or stimulation of organisms, which contribute in a positive way to the nutrition and health of the host (Fuller and Gibson, 1997). Several studies propose the ingestion of certain probiotic *Lactobacillus* and *Bifidobacterium* species. These probiotics decrease the faecal concentrations of *Escherichia coli*, *Clostridium* and *Bacteroides* and increase the endogenous levels of *Lactobacillus* and *Bifidobacterium*, and in addition significantly affect the metabolic activities of microflora by reducing the production of cancer-causing materials such as nitroreductase, faecal azoreductase and β-glucoronidase (Cebra, 1999).

1.2.7.7.6 Stimulation of immune response

Many probiotics, such as some *Lactobacillus* spp., produce substances that have detrimental effects on pathogens. When probiotic agents are ingested by the host, antimicrobial substances can be secreted by the bacteria

themselves at the site of the infected region in the target organ (Steidler *et al.*, 2000). In addition, the maturation the immune system may be stimulated by probiotics (Viljanen *et al.*, 2005).

The immune system response to particular pathogens must induce a suitable set of effector functions that can eradicate the disease cause or its toxic products from the host. The Th-1 subgroup is responsible for several cell-mediated functions and the secretion of IgA, IgG, IgM and IgE. Some interleukins, for example IL-4, are necessary for the increase of a Th-2 response. IL-12, IL-18 and IFN-γ are essential to the physiological development of Th-1 lymphocytes. In addition, IFN-γ is generated by stimulation of T lymphocytes from the activation of natural killer (NK) cells. IFN-γ induces the up-regulation of IL-12 receptors on activated T lymphocytes. Moreover, Th-2 cells produce a profile of interleukins, such as IL-10 and IL-13, that synthesize of IgE and promote eosinophil activation. Probiotics exert immune-enhancing effects by increasing both specific and non-specific immune system responses (Ouwehand *et al.*, 2002b; Rook and Brunet, 2005). Lymphocytes can be stimulated by probiotics to produce some cytokine, like IFN-γ, and stimulate nonspecific lymphocytic and phagocytic activity. Moreover, introduction of antigens via the oral route induces secretion of Immunoglobulins like IgA and IgM (Maassen *et al*., 2000; Rook and Brunet, 2005). Haller *et al*. (2000) explained that probiotics have also been found to improve defective immune system function through stimulating the cytokines IL-10, IL-12, IFN-γ, all of which have a supposed suppressive effect on antigen specific immune system responses. Some probiotic *Lactobacillus* species, like the *L. casei and L. johnsonii* have been reported

as stimulating some cytokines like IL-10 and IFN-γ secretion (Miettinen *et al.*, 1996). There is research demonstrating that oral administration of *L. casei* to laboratory-bred strain of the house mice leads to improvement of the innate immune system response and reduces dermatitis (Sheil *et al.*, 2004; Galdeano and Perdigon, 2006). Intra-peritoneal injection of *L. casei* induced an IL-12 response in the transgenic mice serum. Dieleman *et al.* (2003) observed the effectiveness of *L. rhamnosus* GG in the prevention of colon inflammation by reducing IL-1 and TNF-α levels and increasing cecal IL-10 content. Galdeano and Perdigon (2006) reported that both subcutaneous and oral administration of probiotic microorganisms promotes this effect. The effectiveness of probiotic *Lactobacillus* through the subcutaneous route protects not only against colon inflammation, but also against collagen joints inflammation.

Table 1.4 Mechanisms of action of probiotic, adapted from Ng *et al.* (2009).

Immunomodulation	Antimicrobial activity	Enhancement of the barrier function
-Effects on epithelial cells (recognition molecules or TLR-2 and TLR - 4).	-Decrease luminal pH	-Increase mucus production
-Effect on dendritic cells (regulatory T cells and IgA-producing B cells through production of cytokines, such as IL - 10).	-Secrete antimicrobial peptides	-Enhance barrier integrity
-Effects on monocytes / macrophages (promoted the production of IFNγ, IL - 12 and IL - 18).	-Inhibit bacterial invasion	-Improve mucus quality
-Effects on lymphocytes : • B lymphocytes (antibody production, such as IgG, IgA and IgM). • Natural killer cells (increased production of IL - 10). • T cells (induced IL - 2, IL - 4 and IL - 10). • T cells redistribution (improving the competence of lymphatic cells to trap T lymphocytes)	-Block bacterial adhesion to epithelial cells	

1.2.8 Types of probiotic microorganisms

Numerous genera of microorganisms are considered as probiotic, both LAB and non-LAB. LAB includes *Lactobacillus* species, *Bifidobacterium* species and others. There are other LAB for example, *Enterococcus faecium, Lactococcus lactis, Leuconstoc mesenteroides, Pediococcus acidilactici* and *Streptococcus thermophilus* (Klein *et al.*, 1998). Moreover, there are non-LAB such as *Bacillus subtilis* (Spano and Massa, 2006), *E. coli* strain Nissle 1917 (Altenhoefer *et al.*, 2004), *Saccharomyces boulardii* (Czerucka *et al.*, 2007) and *Saccharomyces cerevisiae* (Shetty and Jespersen, 2006).

Lactobacillus bacterium is a Gram-positive, facultative anaerobes, non-spore forming, non-flagellated and rod or coccobacilli. So far, there are more than 56 species of *Lactobacillus* probiotics (Gupta and Garg, 2009). *Lactobacillus* is a normal resident of the human GIT and vagina, and significant for public health. The various beneficial of *Lactobacillus* include: reducing GIT permeability and maintain of the mucosal barrier (Rohde *et al.*, 2007; Grice *et al.*, 2008); inhibiting the growth of pathogens by producing antimicrobial substances, e.g. organic acids, H_2O_2 and others (Bezkorovainy, 2001); modulation of immunity, such as by effect on the function of immune response (Tahmourespour and Kermanshahi, 2011); and inhibiting some species of harmful yeast e.g. *Candida albicans* (Lehrer *et al.*, 1993). There are several important lactobacilli species which are probiotic, such as *L. casei*, *L. plantarum*, *L. salivarius* and *L. acidophilus*. *L. casei* is one of the many species of bacteria belonging in the genus *Lactobacillus*. *L. casei* is a mesophilic that is Gram positive, non-motile, rod shaped and non-sporing (Holzapfel *et al.*, 2001). Cai *et al.* (2007) demonstrated that *L. casei* can be

found in different environments, such as raw and fermented dairy products, intestinal tracts, reproductive systems of humans and animals, fresh and fermented plant products. Moreover, *L. casei* can produce LA via fermentation and reduce cholesterol levels, enhance immune response, control diarrhoea, alleviate lactose intolerance, inhibit intestinal pathogens and serve as a probiotic (Mishra and Prasad, 2005). Furthermore, *L. casei* has demonstrated effectiveness in raising immunoglobulin, especially IgA in new-borns infected with rotavirus, and in shortening the duration of diarrhoea (Ouwehand *et al.*, 2002a). Moreover, *L. casei* has induced production of IL-10 and IL-12 from human blood mononuclear cells, or monocytes (Hessle *et al.*, 1999).

With regards to *L. plantarum*, this is the most prevalent species in most naturally fermented foods and has the capacity to block receptors for Gram negative bacteria, as well as having an important antimicrobial effect. Furthermore, this bacterium is very resistant to harsh conditions involving high temperature, ethanol and acidic pH (Zarazaga *et al.*, 2004). As for *L. salivarius*, it is considered non-pathogenic and is exploited as a probiotic. *L. salivarius* produces large amounts of antimicrobial substances such as LA, which inhibit the growth of pathogenic bacteria. Bacteriocin was the first natural substance which was isolated from *L. salivarius* (Flynn *et al.*, 2002). *L. salivarius* possesses several features, e.g. it is a moderately heat tolerant microorganism and loses viability after storage under non-refrigerated temperatures. With reference to *L. acidophilus*, this bacterium stimulates the immune system, increasing levels of cytokines, especially IL-1 α and TNF-α, which suppresses cancerous tumour growth, and produces biosurfactant

(Rangavajhyala *et al.*, 1997). *L. acidophilus* possesses a range of health benefits which include: providing immune system support, reducing diarrhoea in humans, lowering cholesterol and improving the symptoms of lactose intolerance (Tahmourespour *et al.*, 2011).

1.2.9 Metabolic products of lactic acid bacteria

The most important role of LAB is the fermentation of products and production of antimicrobial substances. Antimicrobial substances have an important role in disrupting the growth of harmful bacteria (Lasagno *et al.*, 2002). Ennahar *et al.* (2000) and Lasagno *et al.* (2002) demonstrated that inhibition may be due to the production of metabolites, such as organic acids, H_2O_2, diacetyl, carbon dioxide (CO_2) and bacteriocins. Antimicrobial substances produced by LAB can be divided into two major groups: high molecular mass substances (> 1000 Da) e.g. bacteriocins, and low molecular mass substances (< 1000 Da), such as phenyllactic acid. All non-bacteriocin antimicrobial substances from LAB are of low molecular mass (Collins *et al.*, 2009). These metabolites are discussed in more detail in subsequent sections.

1.2.9.1 Organic acids

The majority of products of LAB are organic acids, which are significant and the best antimicrobial products. These acids, produced through fermentation, affect the consequent microbial activity in fermented substances. Acetic acid (AA) is considered to have the most antagonistic effect against fungi, especially yeasts, compared to other organic acids, such as lactic acid (LA)

(Daeschel *et al.*, 1987). The types and levels of organic acids are dependent on the probiotic species and growth conditions. Homo-fermentative LAB use the glycolytic pathway to produce 2 pyruvates, which are further converted to lactate. In addition, to the pH effect, the un-dissociated acid collapses the electrochemical proton gradient, causing bacteriostasis, followed by the death of pathogens. Furthermore, LA has been observed to permeabilize the external membrane of Gram negative bacteria by releasing of lipopolysaccharides (LPS), leading to loss of viability (Alakomi *et al.*, 2000). Caplice and Fitzgerald (1999) demonstrated that the action of LA may help the action of other antimicrobial substances. The effect of AA and LA against different pathogenic bacteria, moulds and yeasts is well documented.

1.2.9.2 Hydrogen peroxide

The antimicrobial activity of hydrogen peroxide (H_2O_2) is due to its strong oxidising effect on the microbial cells, and damage to the basic molecular structures of cell proteins (Lindgren and Dobrogosz, 1990). Reid (2008) mentioned that production of H_2O_2 has been considered as the chief metabolite of LAB that could protect against urogenital tract (UGT) disorders.

1.2.9.3 Bacteriocins

Bacteriocins are small antimicrobial substances produced by several bacteria (Hassan *et al.*, 2012), and are proteinaceous materials with bactericidal activity. Bacteriocins are produced by LAB (Table 1.5), and are small antimicrobial peptides or proteins that have activity closely related to Gram positive bacteria (Klaenhammer, 1988; Chen and Hoover, 2003). Hassan *et*

al. (2012) observed that the mechanism of action of bacteriocins are represented in the destruction of target cells by pore formation (efflux of cell substances) and / or inhibition of cell wall synthesis. Numerous researches have revealed that bacteriocins display a large potential in the medical sector as bacteriocinogenic probiotics, and in the clinic as therapeutic agents (De Vuyst and Leroy, 2007). Meanwhile, Kirkup (2006) observed that numerous types of bacteriocins have been proposed for application in GIT microbiology, and reduce dental caries.

Table 1.5 Major bacteriocins produced by probiotic LAB, adapted from Soomro *et al.* (2002) and Zacharof and Lovitt (2012).

Bacteriocin	Producer microorganism
Lactacin F	*Lactobacillus johnsonii*
Lactocin 705	*Lactobacillus casei*
Lactoccin G	*Lactobacillus lactis*
Lactococcin MN	*Lactococcus lactis*
Nisin, Lacticin (3147 and 481)	*Lactococcus lactis* subsp. *lactis*
Leucocin H.	*Leuconostoc* spp.
Plantaricin EF	*Lactobacillus plantarum*
Plantaricin W	*Lactobacillus plantarum*
Plantaricin JK	*Lactobacillus plantarum*
Plantaricin S	*Lactobacillus plantarum*
Leucocin	*Leuconostoc gelidum* UAL 187
Helveticin J	*Lactobacillus helveticus*
Carnobacteriocin	*Carnobacterium piscicola* LV17
Pediocin A, and AcH	*Pediococcus pentosaceus* FBB61
Enterocin A	*Enterococcus* species
Carnocin U149 and Piscicolin 126	*Carnobacterium* species

1.2.9.4 Carbon dioxide

Carbon dioxide (CO_2) is significant as an antimicrobial agent, and has an influence on product preservation. CO_2 is mainly produced from hetero-lactic fermentation and is directly generated in anaerobic conditions. In addition, CO_2 is toxic to certain aerobic microorganisms through its action on the membranes of bacterial cells (Šušković *et al.*, 2010). The antimicrobial action of the CO_2 molecule is thought to be because of the inhibition of enzymatic de-carboxylation and the accumulation of CO_2 in the membrane lipid bi-layers causing defective function in membrane permeability (Lindgren and Dobrogosz, 1990).

1.2.9.5 Diacetyl, acetaldehyde and acetoin

Decarboxylation of pyruvate is a process that produces active acetaldehyde by hetero-fermentative LAB, after which this product condenses together with pyruvate, making a-acetolactate, and is then converted to diacetyl by a-acetolactate synthases. Acetoin is considered the product of decarboxylation of acetolactate and reduction of diacetyl (Jyoti *et al.*, 2003). These metabolites are very important as a preservative of food from harmful bacteria, and are considered essential for the inhibition of pathogens and also very important for controlling the growth of pathogens in collaboration with other antimicrobial metabolites (Vandenbergh, 1993).

1.2.9.6 Low molecular mass antimicrobials

There are several low molecular mass compounds that have antimicrobial activity against pathogens. These antimicrobials include antifungal cyclic

dipeptides, 4-hydroxyphenyllactic 3-hydroxy fatty acids and phenyllactic acid (Strom *et al.*, 2002; Valerio *et al.*, 2004). Moreover, there are new types of antimicrobial compounds created by *L. plantarum* like benzoic acid, which are effective against fungi and some Gram-negative bacteria (Niku Paavola *et al.*, 1999).

1.2.10 Antimicrobial peptides

Antimicrobial peptides (AMPs) are an abundant and diverse group of small cationic polypeptides (less than 100 amino acids) that are produced by several tissues and cell types in a variety of invertebrate, plant and animal species (Brogden, 2005).

1.2.10.1 Antimicrobial peptides in the skin; biological and clinical relevance

AMPs were first observed in mammalian skin tissues when cathelicidins were discovered in porcine wound fluid (Gallo *et al.*, 1994). In the early 1980s, Steiner *et al.* (1981) reported that investigators injected pupae of the cecropia moth with *Escherichia coli* and several other Gram negative bacteria, and later purified a newly synthesized 37 amino acid cationic peptide from the hemolymph. The induced cecropin peptides demonstrated potent antibacterial activity against multiple Gram negative bacteria including, *E. coli* and *P. aeruginosa*. AMPs are considered bacteriocidal against Gram positive and Gram negative bacteria, fungi and some envelope viruses (Lehrer *et al.*, 1985). Moreover, β-defensins are abundant in psoriatic scale (Harder *et al.*, 1997). In human skin, there is a wide range of peptides, including enzyme

inhibitors and some enzymes and neuropeptides, which possess inherent antimicrobial activity (Izadpanah and Gallo, 2005). The cathelicidine and defensins were the first to be discovered and possess the ability to kill pathogens (Cole and Ganz, 2000). A cathelicidin could function in the skin to kill both Gram positive and Gram negative bacteria and to aid wound healing (Agerberth *et al.*, 1991; Storici and Zanetti, 1993). In humans, cathelicidin was initially detected directly in the human skin keratinocyte only at the site of inflammation (Izadpanah and Gallo, 2005). Frohm *et al.* (1997) reported that the LL-37 (two leucine residues and is 37 amino acids long) is the first cathelicidin and peptide isolated from humans. The mature LL-37 peptide has a different spectrum of antimicrobial activity against different pathogens, and can also directly modify the host's immune system response (Murakami *et al.*, 2002). In the skin, the LL-37 is made by several cells e.g. mast cells, neutrophils and keratinocytes, and activated by inflammatory processes (Zanetti *et al.*, 1991).

Moreover, defensins are a broadly dispersed family of AMPs, which are expressed by keratinocytes and mucosal epithelial cells such as the GIT, UGT and pulmonary epithelia (Raj and Dentino, 2002). Ganz (2003) demonstrated that defensins contain 6 - 8 cystine residues that form characteristic disulphide bridges, which are divided into three distinct subfamilies, α-defensin, β-defensin and θ-defensin. Defensins display high levels of activity against Gram positive and Gram negative bacteria, fungi and certain enveloped viruses. Defensins are isolated from immune cells, such as neutrophils, macrophages and some epithelial cells in GIT (Kagan *et al.*, 1994). α-defensins contains 1 - 6 types. Human neutrophils express 4 distinct

α-defensins (1 - 4) that are also referred to as human neutrophil peptides- 1 - 4 (White *et al.*, 1995). Other human defensins (5 and 6) are expressed in Paneth cells of GIT and in epithelial cells of the female UGT (Jones and Bevins, 1993).

In addition, human beta-defensins (hBD) contain four types (1 - 4). HBD-1 contains 36 amino acids, which is constitutively produced by epithelial tissue in the respiratory system and UGT. Moreover, hBD 2 - 4 have been isolated from extracts of lesional scales of psoriatic cutaneous tissues (Harder *et al.* 1997). Furthermore, hBD-2 (41 amino acid) and hBD-3 (45 amino acid) expression is inducible by several agents such as lFN-1β, lFN-γ, TNF-α, Gram negative and Gram positive bacteria (Yang *et al.*, 2001).

Concerning hBD-2, a study was conducted by Schröder and Harder (1999), who observed that hBD-2 is a cysteine rich cationic peptide of the innate immune system and has a low molecular weight. hBD-2 was first discovered in the human skin in 1997. Butmarc *et al.* (2004) showed that expression of hBD-2 is widely observed in epithelial cells of the skin tissue. The induction of hBD-2 is by mean of a probiotic cocktail; four *Lactobacillus* species (*L. acidophilus*, *L. paracasei* , *L. delbrueckii* ssp. *bulgaricus* and *L. plantarum*), three *Bifidobacterium* species (*B. longum*, *B. infantis* and *B. breve*) and *Streptococcus salivarius* ssp. *thermophilus* (Schlee *et al.*, 2008). Moreover, pathogen-associated molecular patterns (PAMPS), for example bacterial flagella antigen, LPS, PG, and DNA are capable of inducing hBD-2 expression in epithelial cells (Takahashi, 2001, Platz *et al.* 2004).

1.2.10.2 Mechanism of action of AMPs

The activity of AMPs against microorganisms is related to their positive charge and their structure. The positive charge allows AMPs to be attracted to negative components on the surface lipid membranes of different microorganisms, such as bacteria, fungi, viruses and protozoa (Kagan *et al*., 1994). This amphipathic structure then drives entry or penetration of the AMPs via the cell membrane, and disruption of the cell membrane can occur by several mechanisms, including pore formation or a detergent-like solubilisation. Because of this change in membrane permeability, ion gradients and energy are dissipated, leading to cell lysis (Gutsmann *et al.*, 2001). The bacterial cell is exposed to AMPs and protein synthesis decreases before cell lysis. It appears that these AMPs penetrate the cell membrane to enter the target cell, with subsequent disruption of protein synthesis (Boman *et al.*, 1993; Chan and Gallo, 1998). In fact, several observations suggest that translocated AMPs can change cytoplasmic membrane septum formation, for example by inhibiting cell wall synthesis, inhibiting protein synthesis, inhibiting nucleic acid synthesis, and / or inhibiting enzymatic activity (Brodgen, 2005).

In *S. aureus*, there are several pathways to regulate the mechanism of action of AMPs. Essentially, the mechanism of action against AMPs depends on regulation of multiple peptide resistant factor (*mprF*) and D-alanyl-lipoteichoic acid (*dlt*) genes. White and Frerman (1967) demonstrated that *S. aureus* cells have three major phospholipids; phosphatidylglycerol, lysylphosphotidylglycerol (LPG) and cardiolipin. Generally, the *mprF* gene in *S. aureus* encodes LPG synthesis (Oku *et al.*, 2004). *MprF* encodes LPG

synthase which transfers positively charged L-lysine molecules from lysyl-tRNA and adds them to negatively charged phosphatidylglycerol within the *S. aureus* cell membrane.

S. aureus achieves AMPs resistance by modifying negatively charged phosphatidylglycerel with positively charged L-lysine, resulting in repulsion of the AMPs (Staubitz *et al.*, 2004). This unusual lipid is identified not only in *Staphylococcus*, but also in other important bacteria, e.g. *Enterococcus faecalis* (Houtsmuller and Deenen, 1965) and *P. aeruginosa* (Kenward et al., 1979). Lately, Peschel *et al*. (2001) and Krisian *et al*. (2003) reported that an *S. aureus mprF* gene mutant, in which LPG is absent, and is sensitive to cationic AMPs of the innate immune system. Interestingly, Ruzin *et al*. (2003) observed that *mprF* gene mutation also sensitized the *S. aureus* cells to some antibiotics, such as vancomycin, bacitracin and β-lactams, and suggesting that LPG has an important role in the multi-drug resistance in a series of MRSA strains.

As for *dlt*, staphylococcal species, such as *S. aureus* and *S. xylosus,* which tolerate high concentrations of several AMPs, were mutagenized to identify genes responsible for this insensitivity. Several mutants with increased sensitivity were obtained, which displayed an altered structure of TAs, major components of the Gram positive cell wall. The mutant TAs lacked positively charged D-alanine, as a result of which the cells carried an increased negative surface charge (Peschel *et al.*, 1999). The mutated genes shared sequence similarity with the *dlt* genes involved in the transfer of positively charged D-alanine into negatively charged techoic acids from other Gram positive bacteria. Wild type bacterial strains bearing additional copies of

the *dlt* operon produced TAs with higher amounts of D-alanine esters and increase net positive surface charge by covalently attaching D-alanine to cell wall TA, bound positively charged proteins less effectively and were less sensitive to AMPs (Weidenmaier *et al.*, 2005).

Since both the *mprF* gene and *dlt* operon participate in maintaining overall staphylococcal surface cationic charge (Peschel *et al.*, 2001; Oku *et al.*, 2004; Staubitz *et al.*, 2004).

1.2.11 Advantages and disadvantages of probiotics

There are certain strains of probiotics that have been observed to usefully affect the structure and / or metabolic vitality of endogenous microbiota, as outlined in Figure 1.10. Some of these have also been observed to reduce the growth of a wide range of enteropathogenic bacteria and create antimicrobial metabolites (Coconnier *et al.*, 1998). There are several advantages, including lactose production, alleviation of symptoms of lactose intolerance and malabsorption, lowering serum cholesterol concentration and reducing blood pressure in hypertension (Sanders, 2000), and prevention and reduction of certain colon cancers (Wollowski *et al.*, 2001). Moreover, probiotics have effects on the immune system, e.g. improving immune function and prevention of infections, inhibition of pathogen growth, stimulation of GIT immunity, reduced chance of infection from common pathogens (Hooper and Gordon, 2001) and reducing inflammation (Braat *et al.*, 2004). In addition, probiotics promote recovery from diarrhoea, decrease constipation, treat colitis (Whorwell *et al.*, 2006) and improve UGT health (Reid, 2001).

Regarding the disadvantages of probiotics, in some specific cases, particularly in persons infected with dangerous diseases, it has been shown that administration of a combination of probiotic bacteria may be harmful (Reyed, 2007). Moreover, an increased mortality rate was observed in patients with predicted severe acute pancreatitis who had received a probiotic prophylactic treatment, in comparison to patients receiving a placebo, whilst no reduction in infectious complications was observed in either group (Besselink *et al.*, 2008). Uchida et al. (2011) reported that administration of probiotic bacteria such as *Lactococcus lactis* may cause septicaemia in neonates, which is a potentially dangerous disease, especially when the patient has a lowered immune system.

Figure 1.10 Prophylactic properties and beneficial effects of probiotics on human health, adapted from Parvez *et al.* (2006).

1.2.12 Summary

Some *Staphylococcus* spp., *Pseudomonas* spp. and *Propionibacterium* spp. are important bacteria in skin infections. Some strains of *S. aureus* are resistant to antibiotics, and are called MRSA, whilst *P. aeruginosa* is very important in burn infections. For this reason, conventional treatments are becoming less effective, and therefore researchers have to find alternative ways to treat diseases, including skin diseases, such as employing probiotics and AMPs. Probiotics have selective criteria, including antimicrobial substances, modulation of immune response and others. Probiotics were first used in veterinary medicine to treat digestive diseases, especially diarrhoea, and then more recently applied to other diseases. Probiotic treatment has already made its way into the treatment of a number of conditions. However, before bringing probiotics into routine usage, proper assessment of these products is necessary. The important point is careful selection of the probiotic agent, its dose standardization, and a thorough knowledge of its beneficial effects over and above the toxic effects, so that this conventional treatment proves to be an effective tool for medical treatment.

1.2.13 Aims and objectives

The current study aimed to investigate the role of selective probiotics on some bacterial human skin pathogens.

The specific objectives of this study were to:

1. Study and evaluate the antimicrobial activity of selected *Lactobacillus* species against some human bacterial skin pathogens.
2. Investigate the aggregation, biofilm formation, and adhesion to keratin between selected probiotic *Lactobacillus* species and some human bacterial skin pathogens.
3. Evaluate and determine the antimicrobial activity of the probiotic *L. plantarum*, both alone and in synergy with the recombinant hBD-2, against human bacterial skin pathogens.
4. Appraise the antimicrobial activity of some probiotic *Lactobacillus* species against pathogenic human skin MRSA strains, as well as to investigate the possible role of probiotics in the modulation of methicillin resistance in MRSA, using culture supernatants derived from selected *Lactobacillus* species.

Chapter 2
Evaluation of antimicrobial activity of probiotic *Lactobacillus* species against bacterial human skin pathogens

The results from this chapter have been presented at the XVth International Congress on Animal Hygiene 2011, 3th - 7th July 2011, Vienna, Austria Congress and the abstract published in the conference proceedings. In addition, the results have also been presented at the Plymouth University Postgraduate Society Conference Series, Wednesday 29th June 2011 and the abstract published in the conference proceedings.

Chapter 2: Evaluation of antimicrobial activity of probiotic *Lactobacillus* species against bacterial human skin pathogens

2.1 Introduction

Probiotics are living microorganisms which, upon ingestion in certain numbers, exert useful health effects beyond inherent general nutrition (Guarner and Schaafsma, 1998). LAB have an important role in the inhibition of pathogenic microorganisms with antimicrobial substances, including: organic acids (lactic, acetic and propionic acids), H_2O_2, polypeptides and others (Cálix-Lara *et al.*, 2014). Ouwehand *et al.* (2002b) reported that probiotics have been widely used for the treatment / prevention of GIT diseases, but a growing number of clinical studies have reported that probiotic strategies induce systemic effects which extend beyond the GIT and may even affect selected functions of the skin (Gatesoupe, 1999; Bodera and Chcialowski, 2009; Gueniche *et al.*, 2010). In addition, Kalliomäki *et al.* (2001) and Guéniche *et al.* (2006a) demonstrated that modulation of the GIT's microflora, via probiotics, appears to cause beneficial effects in healthy as well as diseased human skin. Modulation via GIT is probably due to interaction with the immune system, but also systematically and the latter property may be of importance for human skin (Link-Amster *et al.*, 1994). Most probiotics are included in food supplements and are limited at functioning in the GIT (Simmering and Blaut, 2001). Reid (1999) reported that non-GIT application of probiotics are few and focus on the UGT. The skin also has its own normal microflora, albeit less complex than the GIT microbiota. However, the probiotic principle is likely to be applicable to any

environment where a normal microbiota exists. The normal microflora of the skin is likely to be involved in competitive exclusion of pathogens, a function that could possibly be developed with the use of probiotics (Ouwehand *et al.*, 2003). Because the skin is a very different environment from the GIT, some different selection criteria for probiotics in this environment would apply. Bile and acid resistance are main selection criteria for GIT probiotics, obviously these are not relevant for application to the skin. Nevertheless, the ability of probiotics to adhere at the required site (be it the GIT or the skin) remains an important characteristic, in order to facilitate their colonization, be it temporary or permanent, and subsequent beneficial effects against harmful pathogens. Therefore, adhesion to and inhibition of pathogen adhesion to human keratin were assessed. In addition, the production of antimicrobial substances is important for an application on the skin, together with inhibition of pathogen adhesion (Ouwehand *et al.*, 2003). Several methods of measuring antimicrobial activity were used, namely the agar well diffusion and agar spot assay. The agar well diffusion method has long been used for testing antimicrobial activity, especially for biologically derived compounds. Fleming first used it in 1924 and includes; agar well diffusion and spot methods. The results with these tests are generally semi-quantitative (Parish and Davidson, 1993). The method requires that the indicator organisms must grow rapidly, uniformly, and aerobically. Since highly hydrophobic antimicrobial compounds cannot diffuse in agar, they are not appropriate for testing by this method (Piddock, 1990). The assay used for the determination of the antimicrobial activity of different species of LAB was slightly different with respect to the sizes of the well and the samples, and the incubation

conditions were dependent on the indicator organisms used. Several modified procedures based on the agar well diffusion method have also been used to test the antimicrobial activity of LAB, including the agar spot test (Daeschel and Klaenhammer, 1985). Christiansen *et al.* (2005) used the agar well diffusion assay to evaluate the antimicrobial activity of *L. paracasei* strains against each other and against other *Lactobacillus* species.

Probiotic lactobacilli can be grown in a broad range of temperatures (facultative bacterium) and different conditions (Anas *et al*., 2008). In addition, lactobacilli can be grown in modified and unmodified MRS, but this bacterium has a great potential in unmodified MRS to produce antimicrobial substances that inhibit and control pathogens. Unmodified de Man Rogosa and Sharpe (MRS) is important as it contains a large amount of glucose (2%) which acts as a precursor for organic acid production (Soleimani *et al*., 2010).

The objective of the current study was the evaluation of the antimicrobial activity of *Lactobacillus* species against some bacterial human skin pathogens and to determine the antimicrobial activity of *Lactobacillus* species as a probiotic.

2.2 Material and methods

2.2.1 Reagents and media

Unless otherwise stated, all laboratory materials, chemicals and reagents were purchased from Fisher Scientific Ltd. (Loughborough, Leicestershire, UK), Qiagen (West Sussex, UK) or Oxoid, UK. Different media and equipment requiring sterilization were sterilized by autoclaving (121 °C, 15 minutes, pressure 15 lbs / in^2). Cultures were mixed with sterile glycerol (30% *v / v)*, and stored at - 20 °C. Agar cultures were stored at 4 °C and sub-cultured every 3 - 4 weeks.

2.2.2 Preparation of standard calibration curve for estimation of probiotic *Lactobacillus* and pathogen species numbers

Based on a specific absorbance or optical density (OD) reading, a standard calibration curve of OD versus CFU numbers was produced for probiotic and pathogen species.

Sterile de Man Rogosa and Sharpe broth (MRSB) and nutrient broth (NB) broths were placed in five sterile 2 ml Eppendorf tubes (one ml in each tube). One ml of exponentially growing bacteria broth culture was then added to the first tube and mixed well. From the first tube, one ml was then added to a second tube and so on to obtain a twofold dilution series down to $^1/_{32}$. The spectrophotometer (Unicam, UK) was blanked using sterile MRSB and NB. One ml from each dilution and the undiluted stock was placed in a cuvette and OD taken at wavelength of 595 nanometres (nm) using the spectrophotometer. In addition, tenfold serial dilution series (10^{-1} to 10^{-7}) of exponentially growing bacteria (lactobacilli and pathogen species) were

prepared using sterile phosphate buffered saline (PBS). From each dilution, 100 µl was inoculated onto MRS and nutrient agars, and then incubated at 37 ºC for 24 h in triplicate. The mean number of CFUs was counted, and expressed as CFU ml^{-1} (Morris and Nicholls, 1978; Koch, 1994; Sutton, 2011).

2.2.3 Microorganisms

All microorganisms used in this study were categorized 1 or 2 according to the Advisory Committee on Dangerous Pathogens (ACDP). A total of 13 microbial species were used in this experiment, these are listed in Table 2.1. Microorganisms were obtained from the School of Biological Sciences, Plymouth University culture collection which were stored in liquid nitrogen. All microorganisms were grown according to the optimum oxygen requirement, temperature and incubation time for each species.

Table 2.1 Bacterial strains used in this study and their optimum growth conditions.

Bacteria	O_2 requirement	Grow th temperature (°C)	Grow th medium	Isolated from
Probiotics				
Lactobacillus casei	Microaerophlic	37	de Man Rogosa Sharpe medium (MRS) broth and agar	PUCC*
Lactobacillus rhamnosus NCTC 10302	Microaerophlic	37	"	"
Lactobacillus plantarum NCIMB 41605	Microaerophlic	37	"	Not disclosed
Lactobacillus reuteri DSMZ 20016	Microaerophlic	37	"	Intestine
Lactobacillus salivarius DSMZ 20492	Microaerophlic	37	"	Saliva
Lactobacillus vaginalis DSMZ 5837	Microaerophlic	37	"	Vagina
Lactobacillus jensenii DSMZ 20557	Microaerophlic	37	"	"
Lactobacillus acidophilus DSMZ 20079	Microaerophlic	37	"	Human
Pathogens and opportunistic pathogens				
Staphylococcus aureus NCO 3750	Aerobic	37	Nutrient (N) broth and agar, Staph 110	Skin
Staphylococcus aureus NCO 3761	Aerobic	37	"	"
Staphylococcus aureus NCO 4137	Aerobic	37	"	"
Staphylococcus epidermidis NCO 6513	Aerobic	37	"	"
Staphylococcus epidermidis NCO 11047	Aerobic	37	"	"
Pseudomonas aeruginosa NCIB 8626	Aerobic	37	Nutrient and *Pseudomonas* isolation agar (PIA)	"
Propionibacterium acnes NCTC 737	Anaerobic	37	Brain and Heart Infusion (BHI) broth and agar	Hair follicle
Methicillin resistant *S. aureus* ATCC 33591 (MRSA 33591)	Aerobic	37	Mueller Hinton broth (MHB) and N	Lung
Methicillin resistant *S. aureus* NCTC 12493 (MRSA 12493)	Aerobic	37	MHB and N	Skin abscess
Methicillin resistant *S. aureus* DSMZ 25691 (MRSA 25691)	Aerobic	37	"	Skin

* Probiotic strains / species were obtained from the Plymouth University Culture Collection.
NCO: National Collection Office, Inc. Horsham, PA, UK.
DSMZ: Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Germany.
NCTC: National Collection of Type Cultures.
NCIMB: National Collection of Industrial, Marine, and Food Bacteria.

2.2.4 Agar well diffusion method

2.2.4.1 Preparation of broth culture bacteria (BCB)

The antimicrobial activity of probiotic *Lactobacillus* species and *S. epidermidis* as a probiotic against *S. aureus* was assessed. Several species were used in this experiment: *L. casei*, *L. rhamnosus*, *L. jensenii*, *L. reuteri*, *L. salivarius*, *L. vaginalis*, *L. acidophilus*, *L. plantarum S. epidermidis* 6513 and *S. epidermidis* 11047 were tested as the probiotics. The bacterial human skin pathogens used in this experiment were *S. aureus* 3750, *S. aureus* 3761 and *S. aureus* 4137. Each probiotic *Lactobacillus* species was cultured in MRSB (10^7 CFU / ml) at 37 °C with 5% CO_2 for 18 - 20 h, and was used as the broth culture bacteria (BCB). In addition, *Staphylococcus* strains were grown on NB and incubated at 37 °C for 18 - 20 h.

2.2.4.2 Preparation of CFS

Cell free supernatant (CFS) for each probiotic *Lactobacillus* species were produced using MRSB (10^7 CFU / ml) incubated for 18 - 20 h at 37 °C and 5% CO_2. CFS was obtained by centrifuging the culture at 2772 × *g* (Harrier 18 / 80, MSE, UK) for 10 minutes, followed by filtration of the supernatant through a 0.22 µm pore size filter (MILLEX-HA MF Millipore MCE Member, Corrighwohill, Co. Cork, Ireland), according to Nowroozi *et al*. (2004). CFS was neutralised to pH 7.0 ± 0.2 by adding 1M NaOH, and afterwards filtered, as described previously.

2.2.4.3 Procedure for agar well diffusion method

Petri dishes containing 20 ml of nutrient agar were prepared, and inoculated with 100 µl of 20 h broth culture of *S. aureus* strains (10^7 CFU / ml) using the spread plating method, and left for 1 h at room temperature. Then four wells were cut into the agar of each plate. Each well was filled with 50 µl of: BCB, non-neutralised and neutralised CFS according to Kalalou *et al.* (2004). Aliquots of fresh NB and MRSB were used as controls for *S. epidermidis* and *Lactobacillus* species respectively, according to Anas *et al.* (2008). Agar plates were incubated at 37 °C for 24 h. After incubation, the diameter of the inhibition zone was measured (in mm) with Vernier callipers (Figure 2.1). All tests were replicated three times under the same identical experimental conditions.

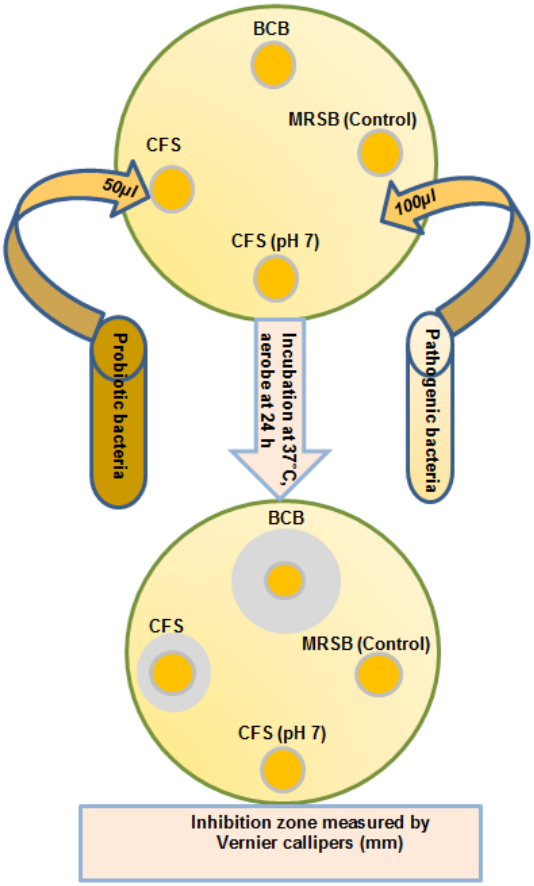


Figure 2.1 Procedure for agar well diffusion method, which shows the antimicrobial activity of probiotic *Lactobacillus* species against bacterial human skin pathogens.

2.2.5 Agar spot method

To confirm the results with the agar well diffusion method, an agar spot method was used. In this method, the *Lactobacillus* species were tested against *S. aureus* and *S. epidermidis*. Strains were those that demonstrated significantly greater inhibition zones than the other species of *Lactobacillus* in the agar well diffusion method. The materials used were according to the previous assay (agar well diffusion method). The method was a modification of that described by Schillinger and Lücke (1989), and four probiotic *Lactobacillus* species (*L. casei*, *L. salivarius*, *L. acidophilus* and *L. plantarum*) were used. Three µl of each test probiotic (10^7 CFU / ml) were spotted on the surface of modified MRS and unmodified MRS agar. Modified MRS and unmodified MRS agar, containing 0.2% and 2% glucose respectively, to observe the importance of the role of organic acids, which are produced from fermented glucose, against pathogens. Plates were incubated anaerobically at 37 °C with 5% CO_2 (GasPak system; BBL Microbiology Systems, Cockeysville, Md.) for 24 h, and aerobically at 37 °C and 30 °C for 24 h to develop the colony spots. Sterile MRSB was used as a negative control on each plate. After the probiotic had grown, a 100 µl volume of an overnight broth culture of each *Staphylococcus* broth (18 - 20 h), was mixed with 10 ml of soft nutrient agar (0.7% *w* / *v* bacteriological agar in nutrient broth). Then the mixture was poured over the plate with the probiotic spotted onto the surface, and left for 1 h to solidify. The plates were incubated aerobically at 37 °C for 24 h. After incubation, the diameter of the inhibition zone was measured in mm with Vernier callipers (Figure 2.2). A clear zone of more than 1 mm around a spot was considered as an indicator of antimicrobial

effect (Tahara *et al.*, 1996). All tests were replicated three times under the same identical experimental conditions.

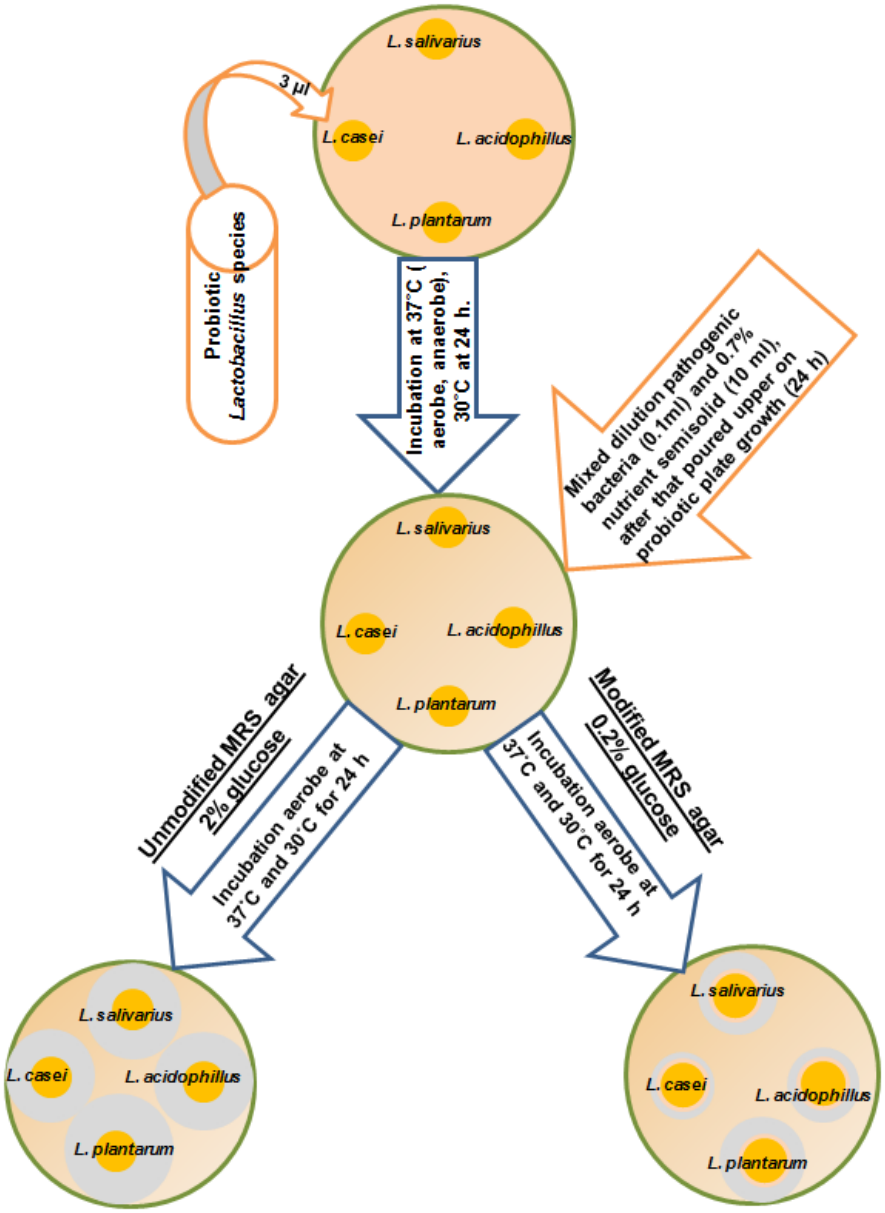


Figure 2.2 Procedure for agar spot method (modified and unmodified MRS), which is show the antimicrobial activity of probiotic *Lactobacillus* species against bacterial human skin pathogens.

2.2.6 Statistical analysis

Data were subjected to balance analysis of variance (ANOVA) followed by Tukeys' HSD multiple range *post hoc* testing by using Minitab v.16. Data are shown as mean ± SD, and $P < 0.05$ was considered as significant.

2.3 Results

2.3.1 Agar well diffusion method

For the results with BCB, only five species of *Lactobacillus* demonstrated a significant inhibition zone with staphylococci species. Overall, the results with probiotic *Lactobacillus* species displayed the greatest inhibition ($P < 0.05$) against *S. aureus* strains. The results were as follows: *L. plantarum* with *S. aureus* 3761 (10.83 ± 0.28 mm) and *S. aureus* 4137 (7.50 ± 0.5 mm) and *L. acidophilus* with *S. aureus* 3750 (9.83 ± 0.28 mm) respectively, as outlined in Table 2.2.

For the results with CFS, the overall main antimicrobial activities of the probiotics were as follows: *L. plantarum* with *S. aureus* 3761 (6.83 ± 0.28 mm), *S. aureus* 3750 (6.16 ± 0.28), *S. aureus* 4137 (3.50 ± 0.5 mm) and *L. acidophilus* with *S. aureus* 3750 (6.16 ± 0.28) respectively, as outlined in Table 2.2. These results displayed highly significant differences ($P < 0.05$) against *S. aureus* strains respectively, while pH 7.0 CFS and controls were negative and there were no significant differences ($P > 0.05$).

The statistical analyses observed high significant differences ($P < 0.05$) with pathogens, probiotics, and treatments (BCB and CFS).

Table 2.2 Zones of inhibition (mm; mean ± SD) for *S. aureus* strains with a broth culture (BCB) and cell free supernatant (CFS) of probiotic *Lactobacillus* species.

Probiotics	*S. aureus* 3750		*S. aureus* 3761		*S. aureus* 4137	
	BCB	CFS	BCB	CFS	BCB	CFS
L. casei	$7.00 \pm 1.00^{c, 1, 2}$	$3.83 \pm 0.28^{b, 2}$	$7.66 \pm 0.57^{b, c, 1}$	$4.83 \pm 0.28^{b, c, 1}$	$6.00 \pm 0.86^{b, 2}$	$3.33 \pm 0.57^{a, b, 2}$
L. rhamnosus	$6.33 \pm 0.57^{c, d, 1, 2}$	$3.83 \pm 0.28^{b, 1, 2}$	$7.00 \pm 1.00^{c, d, 1}$	$4.16 \pm 0.76^{c, d, 2}$	$5.83 \pm 0.28^{b, 2}$	$3.50 \pm 0.50^{a, b, 2}$
L. jensenii	$6.00 \pm 1.00^{c, d, 1, 2}$	$3.50 \pm 0.50^{b, 1, 2}$	$6.66 \pm 0.57^{c, d, 1}$	$3.83 \pm 0.76^{d, 1}$	$5.33 \pm 0.57^{b, c, 2}$	$2.66 \pm 0.57^{b, 2}$
L. reuteri	$3.33 \pm 1.15^{e, 1, 2}$	$1.66 \pm 0.57^{c, 1}$	$3.66 \pm 0.57^{e, 1}$	$1.66 \pm 0.57^{e, 1}$	$2.50 \pm 0.50^{e, 2}$	$1.33 \pm 0.57^{c, 1}$
L. salivarius	$9.00 \pm 1.00^{b, 1}$	$5.83 \pm 0.28^{a, 1}$	$8.66 \pm 0.57^{b, 1}$	$5.66 \pm 0.57^{b, 1}$	$5.83 \pm 0.28^{b, 2}$	$3.83 \pm 0.76^{a, 2}$
L. vaginalis	$5.66 \pm 0.57^{d, 1}$	$3.33 \pm 0.57^{b, 1}$	$6.33 \pm 0.57^{d, 1}$	$3.66 \pm 0.57^{d, 1}$	$4.50 \pm 0.50^{c, 2}$	$3.00 \pm 1.00^{a, b, 1}$
L. acidophilus	$10.50 \pm 0.50^{a, 1}$	$6.16 \pm 0.28^{a, 1}$	$10.83 \pm 0.28^{a, 1}$	$6.50 \pm 0.86^{a, b, 1}$	$6.16 \pm 0.28^{b, 2}$	$3.50 \pm 0.50^{a, b, 2}$
L. plantarum	$9.83 \pm 0.28^{a, b, 1}$	$6.16 \pm 0.28^{a, 1}$	$10.50 \pm 0.50^{a, 1}$	$6.83 \pm 0.28^{a, 1}$	$7.50 \pm 0.50^{a, 2}$	$3.33 \pm 0.57^{a, b, 2}$

Values with the same superscript letters in each column are not significantly different, while mean values with the same superscript numbers in each row are not significantly different.

In addition, *S. epidermidis* strains were tested as probiotics against *S. aureus* strains. The results were negative (no inhibition zone) with all tests, whether BCB, filtered neutralised and non-neutralised CFS.

Species of *Lactobacillus* were also tested for antimicrobial activity as probiotics against *S. epidermidis* strains. The results were similar to the results for *S. aureus* strains, with some minor differences in inhibition zone widths.

The overall mean zone widths with BCB were as follows: *L. plantarum* (10.00 ± 1 mm) and *S. epidermidis* 11047 (8.66 ± 1.15 mm) respectively (Table 2.3). Furthermore, the important results of *S. epidermidis* with non-neutralised CFS were as follows: *L. plantarum* with *S. epidermidis* 6513 (6.16 ± 0.28 mm) and *S. epidermidis* 11047 (4.33 ± 0.57 mm) respectively (Table 2.3).

The statistical analyses found highly significant differences ($P < 0.05$) with pathogens, probiotics and treatments (BCB and CFS).

Table 2.3 Zones of inhibition (mm; mean ± SD) for *S. epidermidis* strains with a broth culture and cell free supernatant of probiotic *Lactobacillus* species.

Probiotics	*S. epidermidis* 6513		*S. epidermidis* 11047	
	BCB	CFS	BCB	CFS
L. casei	7.66 ± 0.57$^{b, 1}$	3.66 ± 0.57$^{c, 1}$	7.00 ± 1.00$^{b, 1}$	3.33 ± 0.57$^{b, c, 1}$
L. rhamnosus	6.33 ± 0.57$^{b, c, 1}$	3.50 ± 0.50$^{c, 1}$	5.50 ± 0.50$^{c, 1}$	2.83 ± 0.28$^{c, 1}$
L. jensenii	5.66 ± 0.57$^{c, d, 1}$	3.33 ± 0.57$^{c, d, 1}$	4.83 ± 0.28$^{c, d, 1}$	2.50 ± 0.50$^{c, d, 1}$
L. reuteri	3.66 ± 0.66$^{e, 1}$	1.66 ± 0.57$^{e, 1}$	3.16 ± 0.28$^{e, 1}$	1.33 ± 0.57$^{e, 1}$
L. salivarius	10.00 ± 1.00$^{a, 1}$	4.83 ± 0.28$^{b, 1}$	7.66 ± 0.57$^{a, b, 2}$	3.60 ± 1.15$^{a, b, c, 2}$
L. vaginalis	4.33 ± 0.57$^{d, e, 1}$	2.50 ± 0.50$^{d, e, 1}$	3.66 ± 0.57$^{d, e, 1}$	1.83 ± 0.28$^{d, e, 1}$
L. acidophilus	9.33 ± 1.52$^{a, 1}$	6.16 ± 0.28$^{a, 1}$	8.33 ± 0.57$^{a, b, 1}$	4.33 ± 0.57$^{a, 2}$
L. plantarum	10.00 ± 1.00$^{a, 1}$	5.83 ± 0.57$^{a, 1}$	8.66 ± 1.15$^{a, 2}$	4.16 ± 0.28$^{a, b, 2}$

Values with the same superscript letters in each column are not significantly different, while mean values with the same superscript numbers in each row is not significantly different.

2.3.2 Agar spot method

The results with the agar spot method were different compared with the agar well diffusion method. In addition, there were also differences between modified and unmodified MRS results.

The factorial analysis showed that in modified MRS, the most inhibitory probiotic species was *L. plantarum* with an overall mean inhibition zone of 0.99 ± 0.23 mm.

The mean of the inhibition zones in modified MRS were as follows: *L. plantarum* (0.99 ± 0.23 mm) with 30 °C incubation, *L. acidophilus* (1.09 ± 0.25 mm) with aerobic 37 °C incubation and *L. plantarum* (1.26 ± 0.22 mm) with anaerobic 37 °C incubation respectively (Tables 2.4 - 2.6). The inhibition zones ranged between 0.33 - 1.33 mm.

The inhibition zones produced in the case of pathogens varied according to incubation temperatures: there were specific significant differences between certain individual pathogen species and certain individual probiotic species ($P < 0.05$) for example at 30 ºC, a significant difference was observed between inhibition zones of *S. aureus* 4137 and *S. epidermidis* 6513 in the presence of *L. acidophilus*. However, as a general rule, there were no overall significant differences between probiotics and pathogens at 30 °C, aerobic 37 °C, and anaerobic 37 °C, respectively.

Inhibition zones were as follows: *S. epidermidis* 6513 (0.99 ± 0.3 mm) with 30 °C incubation, *S. aureus* 3761 (1.03 ± 0.28 mm) and *S. epidermidis* 6513 (1.03 ± 0.15 mm) with aerobic 37 °C incubation and *S. aureus* 3761 (1.07 ± 0.16 mm) with anaerobic 37 °C incubation, respectively (Tables 2.4 - 2.6).

Table 2.4 Zones of inhibition (mm; mean ± SD) on modified MRS 0.2% glucose for *Staphylococcus* species with broth culture of probiotic *Lactobacillus* species incubated at 30 °C aerobic for 24 h.

Staphylococcus spp.	*L. casei*	*L. salivarius*	*L. acidophilus*	*L. plantarum*
S. aureus 3750	$0.50 \pm 0.50^{a, 2}$	$0.66 \pm 0.28^{a, 2}$	$0.83 \pm 0.28^{a, b, 1, 2}$	$1.33 \pm 0.57^{a, 1}$
S. aureus 3761	$0.83 \pm 0.28^{a, 1}$	$0.66 \pm 0.28^{a, 1}$	$0.83 \pm 0.28^{a, b, 1}$	$0.83 \pm 0.28^{a, 1}$
S. aureus 4137	$0.33 \pm 0.28^{a, 1}$	$0.33 \pm 0.28^{a, 1}$	$0.66 \pm 0.28^{b, 1}$	$0.83 \pm 0.28^{a, 1}$
S. epidermidis 6513	$0.66 \pm 0.57^{a, 2}$	$0.83 \pm 0.28^{a, 1, 2}$	$1.33 \pm 0.57^{a, 1}$	$1.16 \pm 0.28^{a, 1, 2}$
S. epidermidis 11047	$0.50 \pm 0.50^{a, 1}$	$0.66 \pm 0.28^{a, 1}$	$1.00 \pm 0.5^{a, b, 1}$	$0.83 \pm 0.28^{a, 1}$

Values with the same superscript letters in each column are not significantly different, while mean values with the same superscript numbers in each row are not significantly different.

Table 2.5 Zones of inhibition (mm; mean ± SD) on modified MRS 0.2% glucose for *Staphylococcus* species with broth culture of probiotic *Lactobacillus* species incubated at 37 °C aerobic for 24 h.

Staphylococcus spp.	*L. casei*	*L. salivarius*	*L. acidophilus*	*L. plantarum*
S. aureus 3750	$0.50 \pm 0.5^{a, 2}$	$0.66 \pm 0.28^{a, b, 1, 2}$	$0.83 \pm 0.28^{a, 1, 2}$	$1.16 \pm 0.28^{a, 1}$
S. aureus 3761	$0.66 \pm 0.28^{a, 2}$	$1.16 \pm 0.28^{a, 1, 2}$	$1.33 \pm 0.57^{a, 1}$	$1.00 \pm 0.50^{a, 1, 2}$
S. aureus 4137	$0.33 \pm 0.28^{a, 1}$	$0.33 \pm 0.28^{b, 1}$	$0.83 \pm 0.28^{a, 1}$	$0.83 \pm 0.28^{a, 1}$
S. epidermidis 6513	$0.83 \pm 0.28^{a, 1}$	$1.16 \pm 0.28^{a, 1}$	$1.16 \pm 0.28^{a, 1}$	$1.00 \pm 0.50^{a, 1}$
S. epidermidis 11047	$0.50 \pm 0.50^{a, 2}$	$0.83 \pm 0.28^{a, b, 1, 2}$	$1.33 \pm 0.57^{a, 1}$	$1.33 \pm 0.28^{a, 1}$

Values with the same superscript letters in each column are not significantly different, while mean values with the same superscript numbers in each row are not significantly different.

Table 2.6 Zones of inhibition (mm; mean ± SD) on modified MRS 0.2% glucose for *Staphylococcus* species with broth culture of probiotic *Lactobacillus* species incubated at 37 °C anaerobic for 24 h.

Staphylococcus spp.	*L. casei*	*L. salivarius*	*L. acidophilus*	*L. plantarum*
S. aureus 3750	$0.66 \pm 0.28^{a, 3}$	$0.83 \pm 0.28^{a, b, 2, 3}$	$1.33 \pm 0.57^{a, 1, 2}$	$1.66 \pm 0.57^{a, 1}$
S. aureus 3761	$0.83 \pm 0.28^{a, 1}$	$1.16 \pm 0.28^{a, 1}$	$1.16 \pm 0.28^{a, 1}$	$1.16 \pm 0.28^{a, 1}$
S. aureus 4137	$0.33 \pm 0.28^{a, 3}$	$0.33 \pm 0.28^{b, 2, 3}$	$1.00 \pm 0.50^{a, 1}$	$1.16 \pm 0.28^{a, 1}$
S. epidermidis 6513	$0.83 \pm 0.28^{a, 1}$	$0.83 \pm 0.28^{a, b, 1}$	$1.33 \pm 0.57^{a, 1}$	$1.16 \pm 0.28^{a, 1}$
S. epidermidis 11047	$0.50 \pm 0.50^{a, 2}$	$0.66 \pm 0.28^{b, 1, 2}$	$0.83 \pm 0.28^{a, 1, 2}$	$1.16 \pm 0.28^{a, 1}$

Values with the same superscript letters in each column are not significantly different, while mean values with the same superscript numbers in each row are not significantly different.

For the unmodified MRS agar (2% glucose), inhibition zones were large compared to modified MRS agar. In the case of unmodified MRS agar, different results were revealed, but there was a significantly large inhibition zone with *L. plantarum* for all tests (Tables 2.7 - 2.9), ranging between 15.33 - 27.66 mm.

The results were as follows: *L. plantarum* (25.06 ± 2.68 mm) with 30 °C incubation, (25.33 ± 3.74 mm) with aerobic 37 °C incubation and (25.33 ± 1.2 mm) with anaerobic 37 °C incubation.

As for pathogens, the results were higher significant on the following, according to temperatures: *S. epidermidis* 6513 (23.58 ± 4.34 mm) with 30 °C incubation, *S. aureus* 3750 (23.75 ± 3.63 mm) with aerobic 37 °C incubation and *S. aureus* 3750 also (23.50 ± 3.31 mm) with anaerobic 37 °C incubation (Tables 2.7 - 2.9).

Overall, no significant differences were observed between probiotics and pathogens at 30 °C and 37 °C, as well as at 37 °C under anaerobic conditions ($P > 0.05$, Tables 2.7 - 2.9). However, some significant differences were observed. For example, generally speaking, *S. aureus* 4137 was in many cases significantly less susceptible to probiotics than the other pathogens used.

Table 2.7 Zones of inhibition (mm; mean ± SD) on unmodified MRS (2% glucose) for *Staphylococcus* species with probiotic *Lactobacillus* species incubated at 30 °C aerobic for 24 h.

Staphylococcus spp.	*L. casei*	*L. salivarius*	*L. acidophilus*
S. aureus 3750	$20.00 \pm 1.00^{a, 2, 3}$	$18.33 \pm 3.51^{a, 3}$	$23.33 \pm 1.15^{b, 1, 2}$
S. aureus 3761	$20.33 \pm 0.57^{a, 2, 3}$	$18.60 \pm 2.08^{a, 3}$	$22.60 \pm 2.51^{b, 1, 2}$
S. aureus 4137	$16.00 \pm 2.00^{b, 2, 3}$	$15.66 \pm 3.51^{b, 3}$	$19.33 \pm 1.15^{c, 1, 2}$
S. epidermidis 6513	$20.00 \pm 2.00^{a, 2}$	$19.66 \pm 2.88^{a, 2}$	$27.00 \pm 2.64^{a, 1}$
S. epidermidis 11047	$21.00 \pm 1.00^{a, 2}$	$18.00 \pm 2.64^{a, 2}$	$27.33 \pm 0.57^{a, 1}$

Values with the same superscript in each column are not significantly different, while mean values with the same superscript numbers in each row are not significantly different.

Table 2.8 Zones of inhibition (mm; mean ± SD) on unmodified MRS (2% glucose) for *Staphylococcus* species with probiotic *Lactobacillus* species incubated at 37 °C aerobic for 24 h.

Staphylococcus spp.	*L. casei*	*L. salivarius*	*L. acidophilus*	*L. plantarum*
S. aureus 3750	$21.00 \pm 1.73^{a, 2}$	$20.33 \pm 1.52^{a, 2}$	$26.00 \pm 1.00^{a, 1}$	$27.66 \pm 0.57^{a, 1}$
S. aureus 3761	$20.33 \pm 0.57^{a, b, 2}$	$18.00 \pm 2.00^{a, b, 2}$	$25.66 \pm 1.15^{a, 1}$	$27.00 \pm 1.00^{a, 1}$
S. aureus 4137	$16.00 \pm 2.64^{d, 2}$	$18.00 \pm 1.00^{b, 1, 2}$	$19.66 \pm 0.57^{c, 1}$	$18.66 \pm 1.52^{b, 1}$
S. epidermidis 6513	$18.33 \pm 1.52^{b, c, 3}$	$17.66 \pm 1.15^{b, 3}$	$22.00 \pm 1.00^{b, 2}$	$26.66 \pm 1.50^{a, 1}$
S. epidermidis 11047	$18.00 \pm 2.00^{c, d, 3}$	$19.66 \pm 0.57^{a, b, 3}$	$23.00 \pm 1.00^{b, 2}$	$26.66 \pm 0.57^{a, 1}$

Values with the same superscript in each column are not significantly different, while mean values with the same superscript numbers in each row are not significantly different.

Table 2.9 Zones of inhibition (mm; mean ± SD) on unmodified MRS (2% glucose) for *Staphylococcus* species with probiotic *Lactobacillus* species incubated at 37 °C anaerobic for 24 h.

Staphylococcus spp.	*L. casei*	*L. salivarius*	*L. acidophilus*	*L. plantarum*
S. aureus 3750	$20.66 \pm 1.15^{a, 3}$	$20.66 \pm 2.08^{a, 3}$	$25.66 \pm 0.57^{a, 2}$	$27.00 \pm 1.00^{a, 1}$
S. aureus 3761	$20.00 \pm 1.00^{a, 4}$	$18.00 \pm 2.00^{b, c, 3}$	$23.33 \pm 1.52^{b, 2}$	$26.66 \pm 0.57^{a, 1}$
S. aureus 4137	$17.00 \pm 1.73^{b, 3}$	$18.33 \pm 1.15^{b, c, 2}$	$20.00 \pm 1.00^{c, 1}$	$20.66 \pm 2.08^{b, 1}$
S. epidermidis 6513	$15.00 \pm 1.00^{b, 4}$	$17.00 \pm 2.00^{c, 3}$	$24.33 \pm 2.10^{a, b, 2}$	$25.33 \pm 1.15^{a, 1}$
S. epidermidis 11047	$17.00 \pm 1.73^{b, 4}$	$19.66 \pm 0.57^{a, b, 3}$	$23.66 \pm 0.57^{a, b, 2}$	$27.00 \pm 1.00^{a, 1}$

Values with the same superscript in each column are not significantly different, while mean values with the same superscript numbers in each row are not significantly different.

There were major differences between inhibition zones in the two assays, depending on whether modified or unmodified MRS agar were used. With respect to the modified MRS assay, several of the *Lactobacillus* species showed weak inhibition (*L. plantarum* and *L. acidophilus*) or no inhibition on some skin pathogenic strains. By contrast, the unmodified MRS assay demonstrated significant differences in the inhibition zone and a large inhibition zones around different bacterial skin colonies were observed.

2.4 Discussion

Based on susceptibility tests the effectiveness of *Lactobacillus* species isolates against bacterial human skin pathogens was observed. The majority of *S. aureus* strains appeared sensitive to *Lactobacillus* species. Five probiotic *Lactobacillus* species indicated significantly greater ($P < 0.05$) inhibition zones against *S. aureus* strains compared with other species. These results may be explained by the antimicrobial activity of different *Lactobacillus* species via production of numerous antibacterial substances, such as organic acids, bacteriocins, CO_2, H_2O_2 or other substances (Vallor *et al.*, 2001; Noordiana *et al.*, 2013). Pathogenic bacteria were found to be sensitive to most *Lactobacillus* species, especially to non-neutralised CFS, but with a lesser inhibition zone compared to BCB results. This may be due to presence of greater levels of inhibitory substances in the BCB, such as those associated with whole bacteria and antimicrobial substances. In contrast, CFS was likely to only contain antimicrobial substances without whole bacteria. *Lactobacillus* species possess a high ability to inhibit *Staphylococcus* species growth and proliferation via competition with other pathogenic microorganisms for nutritional requirements (Vallor *et al.*, 2001; Cadieux *et al.*, 2002), and have been observed to have stronger antimicrobial properties against Gram positive bacteria such as *S. aureus* (Gilliland and Speck, 1977).

The role of *S. epidermidis* as a probiotic is not clear from the current study. All strains of *S. aureus* showed strong resistance (no inhibition zone) to *S. epidermidis* and neutralised CFS. The reason may be due to the lack of production of organic acids or other products that can impede or inhibit

growth of *S. aureus* strains. These factors may be the main reason for the effectiveness of competition for nutrition as well as competition for adhesion sites (Malago and Koninkx, 2011).

The application of probiotic bacteria to the cutaneous region has up to now not been well studied. Nevertheless, the probiotic principle may be supposed to function there as well. Verschuere (2000) observed that some probiotic bacteria can be applied to diseased skin. It was shown that the *Lactobacillus* species were strong and effective probiotics and produced a range of antimicrobial agents. According to Ryan *et al.* (1999), using non antibiotic (probiotic) formulations to prevent udder disorders can reduce the need for the use of antibiotics in treatment of these disorders. Therefore, the problems of the emergence of antibiotic resistance in bacterial pathogens can be reduced.

The results of the current study are compatible with other studies, for example a study conducted by Tejero-Sarinena *et al.* (2012), used an agar spot method to show that most of the selected strains of probiotic *Lactobacillus*, *Bifidobacterium*, *Lactococcus*, *Streptococcus* and *Bacillus* genera were able to produce active compounds against pathogens such as *S. aureus*. Wang *et al.* (2012) showed that several *Lactobacillus* species, such as *L. crispatus* and *L. jensenii* demonstrated the ability to inhibit *S. aureus* growth and block *S. aureus* adherence to HeLa cells *in vitro*.

In unmodified MRS, all tested probiotic *Lactobacillus* strains were found to disrupt the growth of pathogen strains in the agar spot assay, but in modified MRS, the effect on pathogens by probiotic *Lactobacillus* species was slight. The reason may be due to amount of glucose in MRS agar. In general, the

largest zones of inhibition of pathogens was found in *L. plantarum*, whether in modified or unmodified MRS. The current study found that there were no significant differences between different temperatures, because these bacteria (*Lactobacillus* species) can be grown in a broad range of temperatures and different conditions. These results were in agreement with the finding of Anas *et al.* (2008), who revealed that probiotics have a proteinic nature causing the growth inhibition of *S. aureus*. In the current study, the results observed were that the inhibitory effect of the unmodified MRS was stronger than the modified MRS, which may be due to the amount of glucose (a precursor for organic acid production). On the contrary, in unmodified MRS, the results demonstrated a large inhibition zone. This signifies that the action is due to organic acids and / or other substances. This study agrees with the findings of Soleimani *et al.* (2010), who observed that probiotic *Lactobacillus* species have a great potential to produce antimicrobial substances that inhibit and control pathogens such as *S. aureus*, with some differences as to conditions and bacterial regions. Generally, the agar spot method was a very effective method compared to agar well diffusion method. *L. plantarum* was the most effective of the *Lactobacillus* species studied.

2.5 Conclusions

The results of the current study revealed that *Lactobacillus* species were able to inhibit growth of *Staphylococcus* species. Moreover, BCB were more effective in inhibiting the growth of *Staphylococcus* species in comparison with CFS. The results demonstrated that there was no antimicrobial activity of *S. epidermidis* isolates against *S. aureus* strains. In addition, the results with unmodified MRS agar spot assay were more effective compared to modified MRS agar spot and agar well diffusion assays. This suggests the antimicrobial activity of the *Lactobacillus* was due to LA production and / or other substances.

Generally, probiotic *Lactobacillus* species can be applied as successful solutions to bacterial antibiotic resistance. What is very interesting, as observed by the current study, is that one can select mixtures of different probiotic microorganisms to better adapt their common action and obtain remarkable results.

Chapter 3
Assessment of aggregation, biofilm formation, and adhesion to keratin in probiotics and human skin pathogens

The results from this chapter have been presented at NEMO Keele Conference, Keele University, Keele, UK, "Prebiotics and Probiotics in Medicine, Veterinary Sciences and Aquaculture" 9th - 11th September 2012, and the abstract published in the conference proceedings. In addition, the results have also been presented in Annual Conference, in the Centre for Agricultural and Rural Sustainability, 19th June 2013, Plymouth, UK and the abstract published in the conference proceedings.

Chapter 3: Assessment of probiotic aggregation, biofilm formation, and adhesion to keratin in probiotics and human skin pathogens

3.1 Introduction

Adhesion to epithelial tissue is a prerequisite for temporary colonization of probiotic species. Several bacterial components, such as carbohydrates, cell wall proteins and lipo-teichoic acid (LTA) play an important role in the adhesion of bacteria to epithelial tissue (Gusils *et al.*, 2002). Collado *et al.* (2008) observed that co-aggregation is a process by which genetically distinct bacteria become attached to one another via specific lectin-like adhesions and receptor molecules. A relationship between auto-aggregation and adhesion ability has been reported for some *Bifidobacterium* species (Vlková *et al.*, 2008). In addition, connection between adhesive ability and hydrophobicity has been observed in certain lactobacilli (Del Re *et al.*, 2000). Furthermore, Collado *et al.* (2007), indicated that co-aggregation of a bacteriocin producing LAB with harmful bacteria may represent an important host defence mechanism.

Biofilms are structured microbial populations attached to a surface. Individual microorganisms in biofilms are implanted inside a matrix of often slimy extracellular polymers (Douglas, 2003). There are three main stages to the formation of a biofilm: (1) the reversible and irreversible attachment stage, (2) the micro-colony stage and (3) the detachment stage. In the first stage, Postollec *et al.* (2006) showed that the bacteria surface and interface are important in biofilm formation due to facilitation of the acquisition of nutrients. There is always an initial attachment of a forerunner bacterium on the surface. Wolcott *et al.* (2008) observed that there are two types of attachment

during biofilm formation, the reversible and the irreversible. In the second stage, the matrix assists the microorganism to self-attach to the surface and protects the colony of microorganisms from harsh ecological and host stress. Stoodley *et al.* (2002) observed that at the third stage a micro-colony attains a high density of microbial population. Several studies have shown that microorganisms living in biofilm have a high resistance to antibiotics compared to planktonic cells (Stewart and William Costerton, 2001). Several mechanisms of drug resistance have been suggested: (a) penetration of drugs is restricted through the biofilm substance, (b) restriction of nutrients, decreased growth rate for microorganisms and altered microenvironment (c) adaptive responses and (d) genetic modification to persister cells (Mah and O'Toole, 2001).

With regard to adhesion to keratin, Reid (1999) indicated that other regions for application of probiotic bacteria are few compared to the GIT, and especially when compared with the UGT. The main purpose of this experiment was therefore to research the likelihood of applying probiotic *Lactobacillus* species to the skin. Ouwehand *et al.* (2003) showed that because the human skin is a very different environment from the GIT, and therefore different selection measures for probiotic *Lactobacillus* would apply in this environment. In addition, adhesion is important for the skin as well, to improve temporary settlement and colonization resistance towards latent harmful bacteria. Therefore, inhibition of adhesion of bacterial human skin pathogens was assessed in the current study. Several antimicrobial substances can be produced by probiotic LAB, such as *Propionibacterium*

freudenreichii subsp*. freudenreichii* and *L. rhamnosus*, for application to the skin (Fuller, 1989; Ouwehand *et al.*, 2003).

The aims of this study were to investigate aggregation (auto-aggregation and co-aggregation), biofilm formation, and adhesion to keratin between probiotics and human skin pathogens.

3.2 Materials and methods

3.2.1 Aggregation assay

The auto-aggregation assay was achieved according to Del Re *et al.* (2000), and is represented in Figure 3.1. Some modification was applied to these methods. *L. casei*, *L. salivarius*, *L. acidophilus* and *L. plantarum* were selected in this study as they showed the greatest antimicrobial activity against selected bacterial human skin pathogens, as reported in Chapter 2.

Probiotic bacteria *L. casei*, *L. salivarius*, *L. acidophilus* and *L. plantarum* (Table 2.1) were grown for 18 - 20 h at 37 °C in MRSB. Pathogen strains, e.g. *S. aureus* strains and *P. aeruginosa* (Table 2.1) were grown in NB for 18 h at 37 °C, as outlined in Figure 3.1 (1). The bacterial cells (probiotics and pathogens) were harvested by centrifugation at 4300 x *g* (Harrier 18 / 80, MSE, UK) at 4 °C for 15 minutes (2). Bacterial pellets were washed with PBS three times (3). Then pellets were re-suspended in the same buffer to give an OD of 0.5 ($OD_{600\ nm}$) (4). Five ml (10^7 CFU / ml) of each bacterial suspension in PBS were centrifuged at 4300 x *g* at 4 °C for 15 minutes (5). The pellets were re-suspended in 4 ml of their own filtered (0.22 µm) sterilized culture supernatant fluid, mixed for 10 seconds, then the absorbance at 600_{nm} was measured (OD1, 6), and incubated for 4 h at room temperature. The auto-aggregation was determined by taking 1 ml of the upper suspension into a cuvette and measuring absorbance at 600_{nm} (OD2, 7). The equation used to calculate the percentage auto-aggregation was:

Auto-aggregation (%) = $[(OD_1 - OD_2) / (OD_1) \times 100]$ (8) (Vandevoorde *et al*., 1992).

Where OD1 and OD2 represent first optical density and second optical density after 4 h, respectively.

All tests were replicated three times under identical experimental conditions.

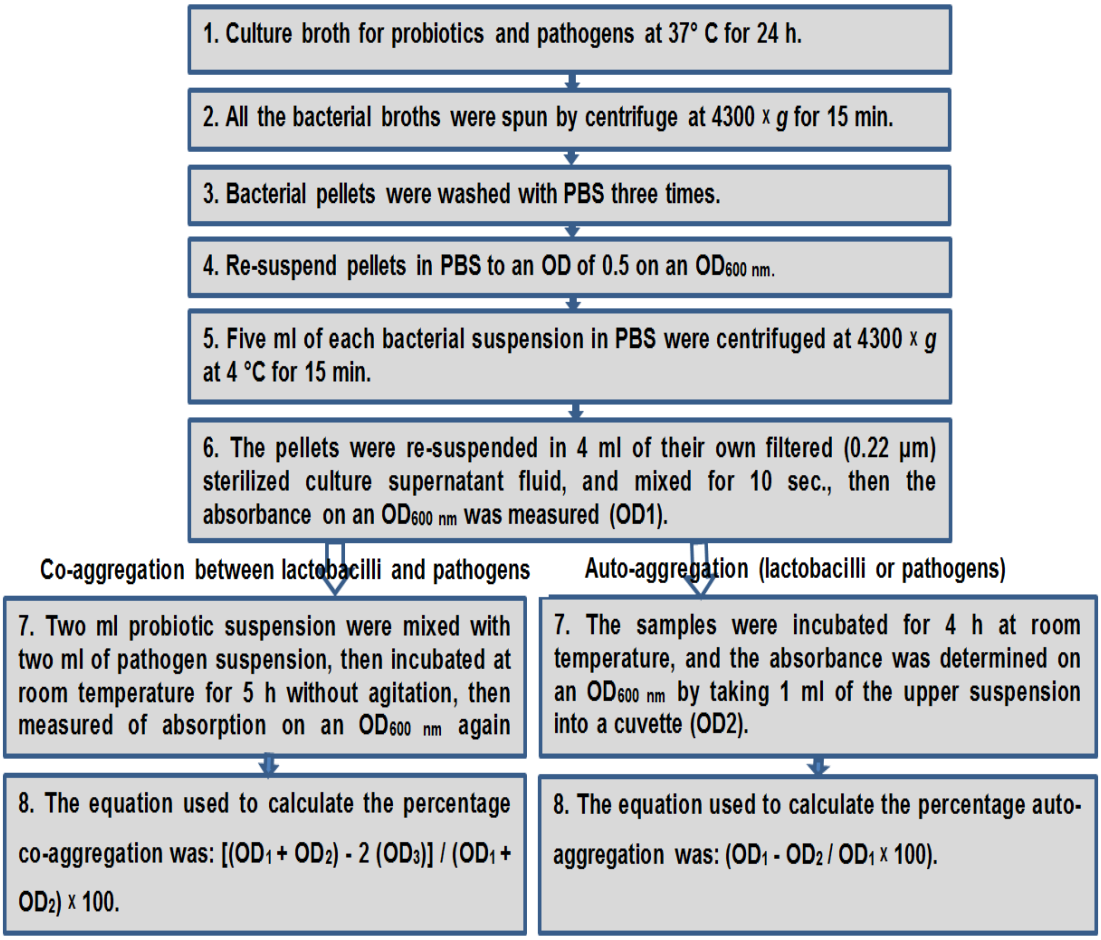


Figure 3.1 Procedure of aggregation (auto-aggregation and co-aggregation) method with some probiotic *Lactobacillus* species and bacterial human skin pathogens.

Co-aggregation of pathogens with probiotic strains, as described in Figure 3.1. Steps 1 - 6 were the same in the auto-aggregation. Equal volumes of cells (2 ml) for both probiotic and pathogens were mixed and incubated at room temperature at 5 h without agitation. The absorbance on an $OD_{600\ nm}$ of the suspensions was measured after mixing and after 5 h of incubation at room temperature (OD3) (7). The equation used to calculate the percentage co-aggregation was:

Co-aggregation (%) = $[(OD_1 + OD_2) - 2 (OD_3) / (OD_1 + OD_2) \times 100]$ (8) (Vandevoorde *et al.*, 1992).

Where; OD1: optical density of *Lactobacillus* strain, OD2: optical density of pathogen strain and OD3: optical density of mixture bacteria after 5 h.

All tests were replicated three times under identical experimental conditions.

3.2.2 Preparing aggregation samples for SEM examination

Scanning electron microscopy (SEM) was used to visualize bacteria (auto-aggregation) and their interaction with each other (co-aggregation). Ten µl of each suspension was placed on a slide, then left to air dry. Samples were then fixed with one ml 2.5% glutaraldehyde (*w/v* in 0.2 M sodium cacodylate buffer; 1:1 vol., pH 7.2, Sigma, Chemical Company, St. Louis, MO, USA) for 2 h at 25 °C. Fixative removal from samples was carried out by rinsing 3 times with 0.1 M sodium cacodylate buffer for 15 minutes at pH 7. The samples were dehydrated via a graded series of acetone and ethanol mixtures, and samples were critical point drier (EMITECH K850, Ashford, Kent, UK) to remove all ethanol (at 35 °C and 1250 psi for 15 minutes). Dried samples are then mounted on aluminium stubs and sputter coated (EMITECH / K550, UK) with gold, and examined with a Jeol JSM 5600 LV electron microscope at 15 kV accelerating voltage (Jeol, Tokyo, Japan).

3.2.3 Crystal violet assay for assessment of biofilm formation

In this study, several strains of human skin pathogens and probiotic *Lactobacillus* species were tested: pathogen *S. aureus* (3750, 3761 and 4137), *S. epidermidis* (6513 and 11047), *P. aeruginosa*, *Pr. acnes* and probiotic *Lactobacillus* species (*L. casei* and *L. plantarum*) (Table 2.1). All pathogens were cultured in 10 ml nutrient broth at 37 °C aerobically for 24 h, except *Pr. acnes* which was incubated in strict anaerobic condition at 37 °C for 3 - 5 days (GasPak Anaerobic System, OXOID Ltd. Company, UK). *Lactobacillus* species were cultivated in 10 ml unmodified MRS 2% (20 g / l) glucose or modified MRS 0.2% (2 g / l) glucose, which were incubated in 5% CO_2 at 37 °C aerobe for 24 h. The OD (570 nm) were determined by a Versa Max Plate Reader (Versa Max Plate Reader [Molecular devices, UK] Ltd., UK South).

For individual bacteria strains (pathogen or probiotic bacteria), microtitre plates were inoculated with 250 µl broth culture per well (10^7 CFU / ml). Sterile nutrient and MRS broth were inoculated as controls. Plates were covered and incubated at 37 °C for 24 h. After incubation, the bacterial culture broth (BCB) was removed from each well, each well was washed three times with 300 µl sterile PBS to remove bacteria that were not adhered to the wells and vigorously shaken. Biofilms adhering to the wells were fixed with 250 µl of 96% ethanol per well for 15 minutes. The ethanol was then removed and the plate was left to dry. Each well was stained with 0.2 ml crystal violet solution (2% *w* / *v*) for five minutes, and excess removed by washing with molecular grade water. The quantitative examination of biofilm production was achieved by adding 200 µl of 33% glacial acetic acid (*v* / *v*)

per well, incubating for 15 minutes, and measuring absorbance at 570_{nm} using a Versa Max Plate Reader, as described previously by Christensen *et al.* (1985). All tests were replicated three times under the same experimental conditions, as according to the method of Tahmourespour and Kermanshahi (2011). The strains were classified as follows, according to Christensen *et al*. (1985):

If the optical densities were less than or equal to 0.120, the microorganism was classified as non-adherent.

-If the optical densities were more than 0.120 and less than 0.240 they were classified as weakly adherent.

-If the optical densities in either medium exceeded 0.240, the strain was classified as strongly adherent.

For the mixed strains (probiotic and pathogen), to evaluate the effectiveness of probiotics *Lactobacillus* species on biofilm formation, the pathogenic strains and *Lactobacillus* species were grown in nutrient broth and MRSB, respectively for 18 - 20 h at 37 °C aerobically.

There were two procedures used to evaluate the effects of probiotic *Lactobacillus* species on biofilm formation in human pathogenic strains.

(1), *Lactobacillus* species (0.125 ml) were incubated at the same time as pathogens (0.125 ml), (2) incubation of *Lactobacillus* species (0.125 ml) for 30 minutes prior to addition of pathogens (0.125 ml). The control wells contained PBS instead of broth, as mentioned above. Staining with crystal violet was done, as described previously. The absorbance was determined by using a Versa Max Plate Reader, as described formerly by Christensen *et al.* (1985). All tests were replicated three times under the same conditions.

3.2.4 Detection of bacterial adhesion to keratin with the crystal violet assay

In this technique, human skin pathogenic strains (*S. aureus* 3750, *P. aeruginosa*, *Pr. acnes*), two probiotic *Lactobacillus* species; *L. casei* and *L. plantarum* (10^7 CFU / ml) were used (Table 2.1). Keratin from human hair [Meta-Keratins surface coating I (K1), II (K2), III (K3) and IV (K4)] (KeraFAST Company, Winston-Salem City, NC state, USA) were used. A Versa Max Plate Reader was used to determine optical density and the absorbance values were 570 nm (Versa Max Plate Reader [Molecular devices, UK] Ltd., UK South).

The method consisted of preparing the keratin surface coating on the day of the experiment. A stock solution of keratin was prepared with sterile water at the concentration of 1 mg / ml. The vial was incubated for thirty minutes at 37 °C to dissolve the keratin completely. This stock solution was diluted 1:4 to a working solution by using sterile water, according to the manufacturing company's instructions. The working solution (0.15 ml) was added to each well of a 96 well microtitre plate, and then the microtitre plates were incubated for 2 h at 37 °C. After that, the working solution was carefully aspirated from each well. Broth cultures of bacteria for pathogens and *Lactobacillus* species (10^7 CFU / ml) were added to wells and incubated at 37 °C for 1 h, and then the wells were washed with PBS three times. NB and MRSB were used as a control for pathogens and *Lactobacillus* species, respectively.

The crystal violet assay was modified after Vesterlund *et al.* (2005). In short, bacterial broths were added as a volume of 0.1 ml from each bacterium into

96 microtitre plate wells coated with 0.15 ml of working solution. A large volume of working solution compared to the volume of added bacteria was used to reduce contact of the crystal violet stain with the wall of the plate. The plates were incubated for 1 h at 37 °C. Non-adherent bacterial cells were removed by washing three times with 0.25 ml of PBS. The adherent bacterial cells were fixed at 60 °C for 20 minutes, and then stained with 0.1 ml (0.1% solution) crystal violet for 45 minutes at room temperature. The wells were washed five times with PBS to remove excess crystal violet stain. The crystal violet stain bound to the bacterial cell was released by adding 0.1 ml of citrate buffer (pH 4.3) for 45 minutes at room temperature. The absorbance was determined by using a Versa Max Plate Reader (Molecular devices, UK. Ltd.). Stained Meta-keratin without added bacteria cells was used as a control. All tests were replicated three times under the same identical experimental conditions.

3.2.5 Statistical analysis

Data (mean ± SD OD) were subjected to balanced ANOVA using Minitab v.16 and least significant differences (LSD) *post cost* testing. A *P* value of less than 0.05 was used to indicate a significant difference.

3.3 Results

3.3.1 Aggregation assay

The results of OD for aggregation (auto-aggregation and co-aggregation) methods at 600 nm are seen in Tables 3.1 - 3.2. The auto-aggregation for different probiotic *Lactobacillus* species was measured after 4 h. The results showed that *L. casei* displayed a stronger auto-aggregation (mean ± SD OD_{600nm}) (11.48 ± 1.15%) than other *Lactobacillus* species, while the results with other species were as follows: *L. salivarius* (7.47 ± 1.2%), *L. plantarum* (7.4 ± 0.36%) and *L. acidophilus* (6.52 ± 0.94%) respectively, as outlined in Table 3.1.

Table 3.1 The optical density (600nm) percentages ([$(OD_1 - OD_2) / (OD_1)$ x 100]) of auto-aggregated *Lactobacillus* and pathogen species.

Lactobacillus and pathogen spp.	Mean of auto-aggregation (%)
L. casei	11.48 ± 1.15^{b}
L. salivarius	7.47 ± 1.20^{d}
L. acidophilus	6.52 ± 0.94^{d}
L. plantarum	7.40 ± 0.36^{d}
S. aureus 4137	14.40 ± 0.14^{a}
P. aeruginosa 8626	12.60 ± 0.65^{b}
S. aureus 3761	10.07 ± 0.02^{c}
S. aureus 3750	9.48 ± 1.38^{c}

Values with the same superscript letters in each column are not significantly different.

The results with auto-aggregation for pathogen strains (mean ± SD OD_{600nm}), showed that *S. aureus* 4137 demonstrated significantly higher auto-aggregation (14.4 ± 0.14%) compared with other strains of pathogen, *P. aeruginosa* (12.6 ± 0.65%) or *S. aureus* 3761 (10.07 ± 0.02%) and *S. aureus* 3750 (9.48 ± 1.38%), respectively (Table 3.1).

The results for the co-aggregation assay varied among bacterial species. The results were expressed as the percentage reduction after 5 h in the absorbance after 5 h of a mixed suspension compared with the individual suspension. All experiments displayed co-aggregation characteristics in different degrees (Table 3.2). The majority of bacterial strains demonstrated weak co-aggregation abilities in comparison with auto-aggregation, but this depended on specific strains and the duration of incubation. The results were analysed as a factorial design with the factors being the pathogens and the probiotics. In the factorial analysis, the highest effective probiotic was *L. casei* (11.48 ± 1.15%). The most susceptible pathogen was *S. aureus* 3761 (19.03 ± 0.71%). In the current experiment, *L. casei* demonstrated the greatest co-aggregation with all pathogen strains, and the results (mean ± SD OD_{600nm}) were as follows: *S. aureus* 3761 (19.03 ± 0.71%), *S. aureus* 4137 (18.58 ± 0.46%), *S. aureus* 3750 (18.45 ± 0.93%) and (16.04 ± 0.98%) with *P. aeruginosa* respectively (Table 3.2). *S. aureus* 3761 was greatest overall.

Table 3.2 The optical density (600nm) percentages ([$(OD_1 + OD_2) - 2 (OD_3) / (OD_1 + OD_2) \times 100$]) of co-aggregated *Lactobacillus* species and bacterial human skin pathogens.

Pathogens	*L. casei*	*L. salivarius*	*L. acidophilus*	*L. plantarum*
S. aureus 3761	$19.03 \pm 0.71^{a, 1}$	$15.84 \pm 0.84^{a, 2}$	$15.29 \pm 0.25^{a, 2}$	$16.36 \pm 0.11^{a, 2}$
S. aureus 4137	$18.58 \pm 0.46^{a, 1}$	$12.75 \pm 0.84^{b, 3}$	$11.85 \pm 0.52^{b, 3}$	$14.69 \pm 1.61^{b, 2}$
S. aureus 3750	$18.45 \pm 0.93^{a, 1}$	$10.07 \pm 0.59^{c, 3}$	$13.17 \pm 0.05^{b, 2}$	$12.88 \pm 0.19^{c, 2}$
P. aeruginosa 8626	$16.04 \pm 0.98^{b, 1}$	$12.38 \pm 1.21^{b, 3}$	$14.13 \pm 0.19^{a, 2}$	$15.75 \pm 1.20^{a, b, 1}$

Values with the same superscript letters in each column are not significantly different, while mean values with the same superscript numbers in each row are not significantly different.

In cases where auto-aggregation did not display significant differences, scanning electron microscope examination was conducted to determine the susceptibility of strains to auto-aggregation (Figures 3.2 - 3.3).

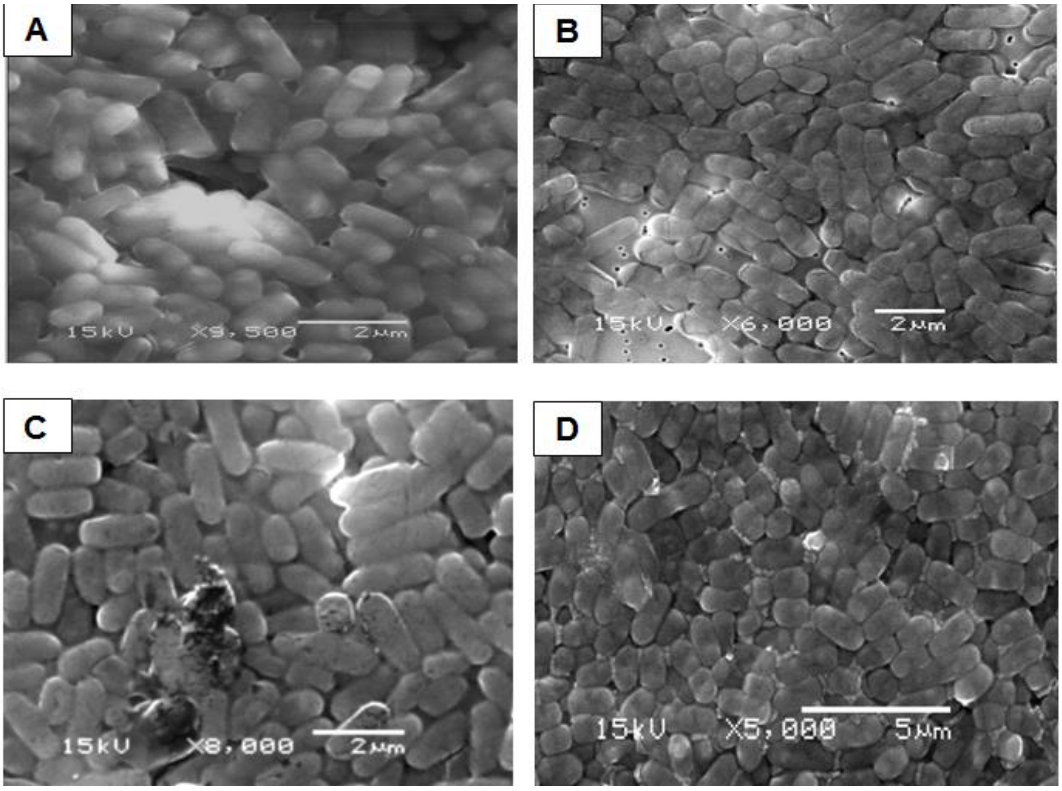


Figure 3.2 Auto-aggregation reactions for some *Lactobacillus* species under SEM, (A) *L. casei*, (B) *L. acidophilus*, (C) *L. salivarius* and (D) *L. plantarum*.

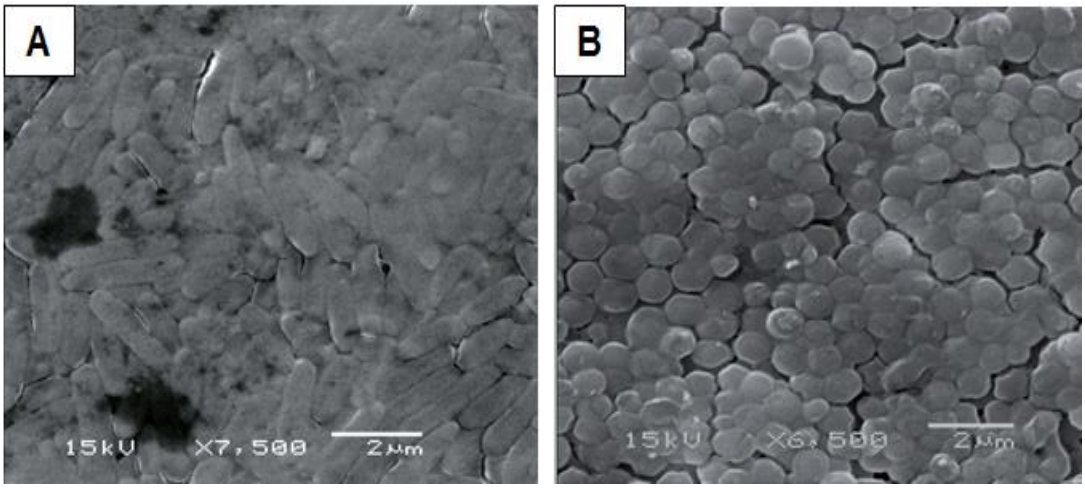


Figure 3.3 Auto-aggregation reactions for some bacterial human skin pathogens under SEM, (A) *P. aeruginosa* and (B) *S. aureus* 3750.

In the majority of co-aggregates, the scanning electron micrograph demonstrated the presence of large contact regions between probiotic lactobacilli and bacterial human skin pathogens (Figure 3.4).

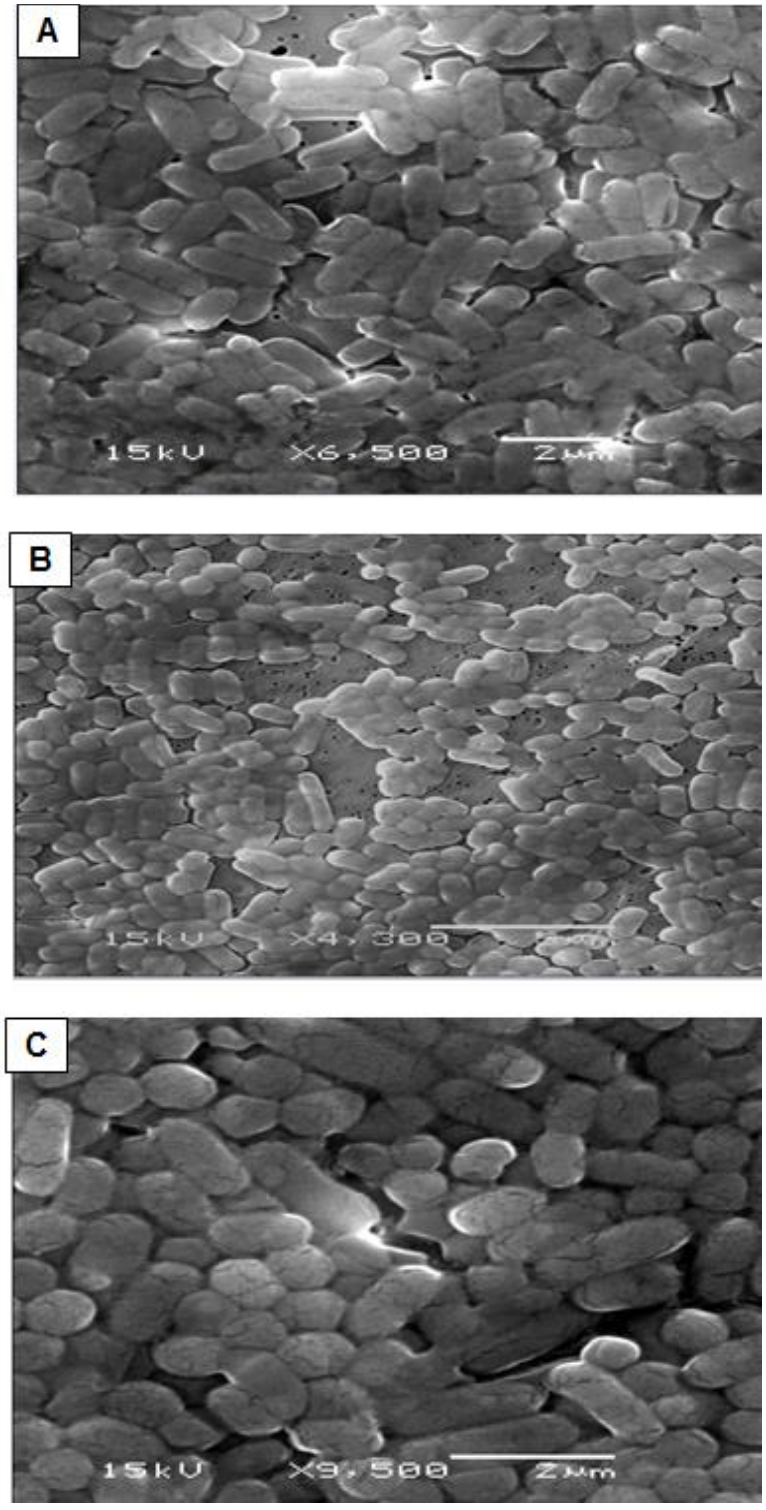


Figure 3.4 Co-aggregation reactions for *Lactobacillus* species and bacterial human skin pathogens under SEM, (A) *L. plantarum* and *P. aeruginosa* 8626, (B) *L. casei* and *S. aureus* 4137 and (C) *L. salivarius* and *S. aureus* 3750.

3.3.2 Crystal violet assay for assessment of biofilm formation

For individual bacteria, the ability of bacterial species (*Lactobacillus* and pathogens) to form a biofilm varied. The results indicated biofilm production by all tested pathogen strains. The pathogenic strains showed strong and significant differences by increase of OD_{570nm} from 0.2 - 1.5 (Table 3.3). *P. aeruginosa* produced the highest OD_{570nm} (1.45 ± 0.03).

Table 3.3 Biofilm formation (expressed as OD_{570nm} mean ± SD) of bacterial human skin pathogens.

Pathogens	OD_{570nm}	BF
P. aeruginosa 8626	1.45 ± 0.00^{a}	S
Pr. acnes 737	1.34 ± 0.00^{b}	S
S. aureus 4137	0.89 ± 0.01^{c}	S
S. epidermidis 11047	0.67 ± 0.00^{d}	S
S. aureus 3750	0.44 ± 0.01^{e}	S
S. aureus 3761	0.24 ± 0.01^{f}	W
S. epidermidis 6513	0.23 ± 0.03^{g}	W
Control (N.B)	0.00 ± 0.00^{h}	NA

Values with the same superscript letters in each column are not significantly different, BF: biofilm formation, S: strong, W: weak and NA: no adherence.

In addition, the results showed no adherence or no significant differences between *Lactobacillus* species in unmodified and modified MRS (Table 3.4).

Table 3.4 Biofilm formation (expressed as OD_{570nm} mean ± SD) of *Lactobacillus* species in unmodified MRS (2% glucose) and modified MRS (0.2% glucose).

Probiotics	unmodified (OD)	modified (OD)	BF
L. plantarum	$0.08 \pm 0.00^{a, 1}$	$0.07 \pm 0.00^{a, 2}$	NA
L. casei	$0.07 \pm 0.01^{a, 1}$	$0.07 \pm 0.00^{a, 1}$	NA
Control (MRS)	$0.00 \pm 0.00^{b, 1}$	$0.00 \pm 0.00^{b, 1}$	NA

Values with the same superscript letters in each column are not significantly different**,** while mean values with the same superscript numbers in each row are not significantly different, BF: biofilm formation, NA: no adherence.

For results with mixed bacteria, all results demonstrated a large significant reduction in adherence the presence of probiotic species, whether in unmodified or modified MRS (Tables 3.5 - 3.8). The results demonstrated highly significant differences ($P < 0.05$), but no significant differences between unmodified and modified MRS assays, except for changes in the rates of the scores between them.

Table 3.5 Biofilm formation (expressed as OD_{570nm} mean ± SD) of *Lactobacillus* species with bacterial human skin pathogens (unmodified MRS glucose; probiotics added at the same time with pathogens).

Pathogens	Individual pathogen	*L. casei*	*L. plantarum*	BF
P. aeruginosa 8626	1.45 ± 0.00^{a}	$0.14 \pm 0.01^{a, 1}$	$0.13 \pm 0.00^{a, b, 2}$	W
Pr. acnes 737	1.34 ± 0.04^{b}	$0.13 \pm 0.01^{a, 1}$	$0.13 \pm 0.00^{a, 1}$	W
S. aureus 4137	0.89 ± 0.01^{c}	$0.11 \pm 0.01^{b, 2}$	$0.12 \pm 0.00^{b, 1}$	NA
S. epidermidis 11047	0.67 ± 0.00^{d}	$0.09 \pm 0.01^{c, 2}$	$0.12 \pm 0.00^{b, 1}$	NA
S. epidermidis 6513	0.44 ± 0.01^{e}	$0.08 \pm 0.00^{c, d, 1}$	$0.08 \pm 0.00^{c, 1}$	NA
S. aureus 3750	0.24 ± 0.01^{g}	$0.08 \pm 0.00^{d, 1}$	$0.08 \pm 0.00^{c, 1}$	NA
S. aureus 3761	0.23 ± 0.00^{f}	$0.08 \pm 0.01^{d, 1}$	$0.08 \pm 0.01^{c, 1}$	NA
Control (PBS)	0.00 ± 0.00^{h}	$0.00 \pm 0.00^{e, 1}$	$0.00 \pm 0.00^{d, 1}$	NA

Values with the same superscript letters in each column are not significantly different, while mean values with the same superscript numbers in each row are not significantly different, BF: biofilm formation, NA: no adherence, W: weak and S: strong.

Table 3.6 Biofilm formation (expressed as OD_{570nm} mean ± SD) of *Lactobacillus* species with bacterial human skin pathogens (unmodified MRS glucose; probiotics added 30 minutes before pathogens).

Pathogens	Individual pathogen	*L. casei*	*L. plantarum*	BF
P. aeruginosa 8626	1.45 ± 0.03^{a}	$0.14 \pm 0.01^{a, 1}$	$0.13 \pm 0.01^{a, 1}$	W
Pr. acnes 737	1.34 ± 0.04^{b}	$0.13 \pm 0.01^{a, 1}$	$0.14 \pm 0.01^{a, 1}$	W
S. aureus 4137	0.89 ± 0.05^{c}	$0.12 \pm 0.00^{b, 1}$	$0.13 \pm 0.01^{a, 1}$	W
S. epidermidis 11047	0.67 ± 0.00^{d}	$0.12 \pm 0.01^{b, 2}$	$0.13 \pm 0.01^{a, 1}$	W
S. epidermidis 6513	0.44 ± 0.01^{e}	$0.09 \pm 0.01^{c, 1}$	$0.08 \pm 0.00^{b, 1}$	NA
S. aureus 3750	0.24 ± 0.01^{g}	$0.08 \pm 0.00^{c, d, 1}$	$0.09 \pm 0.00^{b, 1}$	NA
S. aureus 3761	0.23 ± 0.03^{f}	$0.08 \pm 0.00^{d, 1}$	$0.08 \pm 0.00^{b, 1}$	NA
Control (PBS)	0.00 ± 0.00^{h}	$0.00 \pm 0.00^{e, 1}$	$0.00 \pm 0.00^{c, 1}$	NA

Values with the same superscript letters in each column are not significantly different, while mean values with the same superscript numbers in each row are not significantly different, BF: biofilm formation, NA: no adherence, W: weak and S: strong.

Table 3.7 Biofilm formation (expressed as OD_{570nm} mean ± SD) of *Lactobacillus* species with bacterial human skin pathogens (modified MRS 0.2%; added at the same time).

Pathogens	Individual pathogen	*L. casei*	*L. plantarum*	BF
P. aeruginosa 8626	1.45 ± 0.03^{a}	$0.13 \pm 0.00^{a, 1}$	$0.13 \pm 0.00^{a, 1}$	W
Pr. acnes 737	1.34 ± 0.04^{b}	$0.13 \pm 0.00^{a, 1}$	$0.13 \pm 0.00^{a, 1}$	W
S. aureus 4137	0.89 ± 0.01^{c}	$0.12 \pm 0.00^{b, 1}$	$0.12 \pm 0.00^{b, 1}$	W
S. epidermidis 11047	0.67 ± 0.00^{d}	$0.12 \pm 0.00^{b, 1}$	$0.12 \pm 0.00^{b, 1}$	NA
S. epidermidis 6513	0.44 ± 0.01^{e}	$0.09 \pm 0.01^{c, 1}$	$0.09 \pm 0.00^{c, 1}$	NA
S. aureus 3750	0.24 ± 0.01^{g}	$0.08 \pm 0.00^{d, 2}$	$0.09 \pm 0.00^{c, d, 1}$	NA
S. aureus 3761	0.23 ± 0.03^{f}	$0.08 \pm 0.00^{d, 2}$	$0.09 \pm 0.00^{d, 1}$	NA
Control (PBS)	0.00 ± 0.00^{h}	$0.00 \pm 0.00^{e, 1}$	$0.00 \pm 0.00^{e, 1}$	NA

Values with the same superscript letters in each column are not significantly different, while mean values with the same superscript numbers in each row are not significantly different, BF: biofilm formation, NA: no adherence, W: weak and S: strong.

Table 3.8 Biofilm formation (expressed as OD_{570nm} mean ± SD) of *Lactobacillus* species with bacterial human skin pathogens (modified MRS 0.2%; probiotics added 30 minutes before pathogens).

Pathogens	Individual pathogen	*L. casei*	*L. plantarum*	BF
Pr. acnes 737	1.45 ± 0.03^{a}	$0.14 \pm 0.00^{a, 1}$	$0.13 \pm 0.00^{a, 1}$	W
P. aeruginosa 8626	1.34 ± 0.04^{b}	$0.13 \pm 0.00^{a, 1}$	$0.13 \pm 0.01^{a, 1}$	W
S. aureus 4137	0.89 ± 0.05^{c}	$0.13 \pm 0.00^{b, 2}$	$0.13 \pm 0.00^{b, 1}$	W
S. epidermidis 11047	0.67 ± 0.00^{d}	$0.12 \pm 0.01^{c, 1}$	$0.12 \pm 0.00^{c, 1}$	NA
S. epidermidis 6513	0.44 ± 0.01^{e}	$0.09 \pm 0.00^{d, 1}$	$0.09 \pm 0.00^{d, 1}$	NA
S. aureus 3750	0.24 ± 0.01^{g}	$0.08 \pm 0.01^{e, 1}$	$0.09 \pm 0.01^{d, 1}$	NA
S. aureus 3761	0.23 ± 0.03^{f}	$0.08 \pm 0.01^{e, 1}$	$0.09 \pm 0.00^{d, 1}$	NA
Control (PBS)	0.00 ± 0.00^{h}	$0.00 \pm 0.00^{f, 1}$	$0.00 \pm 0.00^{e, 1}$	NA

Values with the same superscript letters in each column are not significantly different, while mean values with the same superscript numbers in each row are not significantly different, BF: biofilm formation, NA: no adherence, W: weak and S: strong.

3.3.3 Adhesion to keratin

The bacterial human skin pathogens were found to adhere to keratin and *Lactobacillus* species, and to adhere to keratin from 0.055 - 0.113 OD_{570nm}. The results with different keratin (mean ± SD OD_{570nm}) were as follows: keratin III (0.085 ± 0.029), keratin IV (0.083 ± 0.038), keratin II (0.061 ± 0.014) and keratin I (0.059 ± 0.021), respectively (Table 3.9). All results with pathogens exhibited adhesion to keratin, but in different degrees. There were no significant differences ($P > 0.05$) between them. *P. aeruginosa* (0.113 ± 0.029) had shown significant differences ($P < 0.05$) with other strains.

Table 3.9 Adhesion ability (expressed as OD_{570nm} mean ± SD) between probiotics, bacterial human skin pathogens and four types of keratin.

Bacteria	Keratin 1	Keratin 2	Keratin 3	Keratin 4
P. aeruginosa 8626	$0.09 \pm 0.01^{a, 1}$	$0.08 \pm 0.00^{a, 1}$	$0.13 \pm 0.02^{a, 1}$	$0.15 \pm 0.02^{a, 1}$
Pr. acnes 737	$0.07 \pm 0.01^{a, b, 1}$	$0.07 \pm 0.08^{a, 1}$	$0.10 \pm 0.08^{a, b, 1}$	$0.09 \pm 0.01^{a, b, 1}$
S. aureus 3750	$0.06 \pm 0.00^{a, b, 1}$	$0.07 \pm 0.01^{a, 1}$	$0.09 \pm 0.00^{a, b, 1}$	$0.10 \pm 0.02^{a, b, 1}$
L. casei	$0.01 \pm 0.00^{b, 1}$	$0.05 \pm 0.01^{a, 1}$	$0.07 \pm 0.01^{a, b, 1}$	$0.06 \pm 0.00^{b, 2}$
L. plantarum	$0.04 \pm 0.00^{b, 1}$	$0.07 \pm 0.01^{a, 1}$	$0.07 \pm 0.01^{a, b, 1}$	$0.07 \pm 0.02^{b, 1}$
Control (PBS)	$0.00 \pm 0.00^{c,1}$	$0.00 \pm 0.00^{b, 1}$	$0.00 \pm 0.00^{c,1}$	$0.00 \pm 0.00^{c, 1}$

Values with the same superscript letters in each column are not significantly different, while mean values with the same superscript numbers in each row are not significantly different.

3.4 Discussion

In this study, the highest auto-aggregation was shown with *L. casei* after incubation at 25 °C for 4 h. Boris *et al.* (1997) showed that most of the auto-aggregation was attributable to adherence properties. The observed auto-aggregation of the potential probiotic strain *L. casei* could be related to cell surface components, because it was not lost after washing and suspension of the cells in PBS. The current study was compatible with the findings of Kos *et al.* (2003), and Woo and Ahn (2013), which showed the ability of some *Lactobacillus* strains, e.g. *L. acidophilus*, *L. casei*, *L. paracasei* and *L. plantarum* to adhere and aggregate with some pathogenic bacteria, such as *Enterococcus faecium* (19.46%), *E. coli* (15.7%) and *Salmonella typhimurium* (15.11%) respectively. On the other hand, the current study was incompatible with the findings of Zakaria Gomaa (2013), which observed the high ability of some probiotic *Lactobacillus* species, e.g. *L. casei*, *L. paracasei*, *L. plantarum* and others to adhere and aggregate with some pathogenic microorganisms, such as *S. aureus* (22.10 - 48.88%), *Proteus vulgaris* (22.19 - 45.76%) and *Candida albicans* (27.55 - 59.37%), respectively.

Several surface proteins, such as S-layer proteins, are found in *Lactobacillus* species. These 45 KDa have been shown to encourage binding to ecological surfaces, such as the surface of other bacteria, and play an important role in aggregation (Pouwels *et al.*, 1998; Sara and Sleytr, 2000). This structure forms a crystalline layer around many bacterial species, reaching 15 - 20% of the total cellular protein content, with apparent molecular weights of 40 - 200 kDa. These structures are believed to play an important role in cell protection,

surface recognition, and potential mediators in the initial steps included in adhesion.

In addition, carbohydrate and basic protein material on the cell surface are responsible for adhesion (Greene and Klaenhammer, 1994). The ability to adhere to epithelial cells and mucous surfaces has been proposed as a significant characteristic of several bacterial isolates used as probiotics. Many studies have indicated that structures, forces and composition of interaction are correlated with the ability of bacteria to adhere to epithelial cells (Del Re *et al.*, 2000) and mucus (Collado *et al.*, 2005). Tareb *et al.* (2013) observed that bacterial aggregation and / or adhesion are generally key factors for colonization of the target environment and the ability of probiotic strains to exclude pathogens.

The results of the co-aggregation assay in the current study observed overlap between probiotic *Lactobacillus* species and bacterial human skin pathogens. *Lactobacillus* species exerted some co-aggregation than individual strains alone. In the present study, the majority of probiotic *Lactobacillus* species showed some co-aggregation with *S. aureus* strains or *P. aeruginosa*. The reason may be due to conditions of study, such as temperature, osmolality, acidic conditions, adhesion factors, type of strain, source of strain, growth of culture, technique and / or others.

The current study was incompatible with the findings of Zakaria Gomaa (2013), which observed the highest ability of some probiotic *Lactobacillus* species, e.g. *L. casei*, *L. paracasei*, *L. plantarum* and others to adhere and aggregate with some pathogenic microorganisms, such as *S. aureus* (22.10 -

48.88%), *Proteus vulgaris* (22.19 - 45.76%) and *Candida albicans* (27.55 - 59.37%), respectively.

In addition, the current study examined the ability of probiotic *Lactobacillus* species to inhibit adherence of human skin pathogens *in vitro*. LAB produce several substances, e.g. organic acids, bacteriocins, diacetyl and H_2O_2 (Millette *et al.*, 2008; Lengkey and Adriani, 2009). Modified MRS limits acid or other bio-products production of these products by LAB. Therefore, the reduction of adherence of pathogens by probiotics in modified MRS is probably due to interaction between the bacteria rather than the effect of the LA. *S. epidermidis* and *S. aureus* have the ability to produce polysaccharide intracellular adhesion (PSIA) molecules on the surfaces (Stepanovil *et al.*, 2007), which allowed them to form biofilms. This structure is very important in contributing to the virulence of *Staphylococcus* (Satorius *et al.*, 2013). Moreover, *P. aeruginosa* also demonstrated strong adherence and biofilm formation compared to other bacteria. *P. aeruginosa* produces a mucoid exopolysaccharide matrix with alginate, as well as other structures, such as lipopolysaccharide (LPS) (Gupta *et al.*, 1994), and filament surface appendages, pili and flagella (Drake and Montie, 1988). These surface-associated structures play an important role in their adherence, and are called adhesion factors. Gupta and Garg (2009) reported that probiotic *Lactobacillus* species can produce low molecular weight antimicrobial agents, adhesion inhibitors, such as bio-surfactants and, as well as several antimicrobial substances, e.g. organic acids, bacteriocins, H_2O_2, CO_2, diacetyl. Many bio-surfactants, e.g. surlactin, have a glycoproteonaceous character (Gołek *et al*., 2007). Van Hoogmoed *et al.* (2004) revealed that bio-

surfactants are very important because they decrease the colonisation sites of pathogens and overlap with the microbial adhesion. Bio-surfactants play an important role in the regulation of the adhesion of probiotic lactobacilli to host cells. Bio-surfactants are important in reducing adhesion and biofilm formation by *Staphylococcus* strains (Walencka *et al.*, 2008). Moreover, Pascual *et al*. (2008) demonstrated that some probiotic *Lactobacillus* species play this protective role by producing antimicrobial substances, such as H_2O_2, bacteriocins, and organic acid (LA and AA), and bio-surfactants, which inhibit the growth of pathogens.

With regard to *Pr. acnes*, Burkhart and Burkhart (2003) elucidated that *Pr. acnes* live in a population of various bacteria that coat themselves inside an extracellular polysaccharide lining, which they secrete after adherence to the surface. This layer (glycocalyx polymer) acts as a protective exo-skeleton and helps as a natural barrier and in addition, it limits effective antimicrobial concentrations in the biofilm micro-environment. In the current study, *Pr. acnes* exhibited high biofilm formation. The reason may be due to the presence of a glycocalyx polymer layer.

In the case of biofilm formation with mixed cultures of bacterial strains, inoculation of probiotic *Lactobacillus* species at the same time as bacterial human skin pathogen strains had more effect on adherence reduction, but without any significant differences ($P > 0.05$) compared to inoculation of probiotic *Lactobacillus* species with pathogens thirty minutes before. Comelli *et al.* (2002) and Tahmourespour *et al.* (2011) demonstrated that the reduction of adherence may be due to interaction of bacteria and the colonisation of the adhesion site with probiotic *Lactobacillus* species.

Furthermore, *Lactobacillus* species were able to modify the proportion of pathogens within the biofilm. Comelli *et al.* (2002) indicated that the reduction of pathogens can be due either to competition for adhesion sites or to other growth factors. In another study, Meurman *et al.* (1995) observed the inhibitory activity of some *Lactobacillus* species against pathogens in low pH. Finally, results with modified MRS were generally the same as the results with unmodified MRS, although there were some minor differences in the rates of results.

As regards adhesion to keratin, *P. aeruginosa* displayed significant differences ($P < 0.05$) in comparison with other bacterial strains, such as *S. aureus*, *Pr. acnes*, *L. casei* and *L. plantarum*. The reason may be the nature of these bacterial cells or other determinants. The cell wall structure of *P. aeruginosa* contains several components, e.g. flagella and pili, which play an important role in the adhesion (Drake and Montie, 1988). These factors are very important for the pathogenesis of bacteria in adhering to and damaging mucosal epithelia, traumatized tissue and burn infections (Johanson Jr *et al.*, 1980). Some *Staphylococcus* strains demonstrated adherence with keratin because these strains contain many substances, such as PSIA and proteinaceous factors, which allowed them to form biofilms (Stepanovil *et al*., 2007). These substances are very important for *Staphylococcus* adherence in human skin (Fitzpatrick *et al.*, 2005; Toledo-Arana *et al.*, 2005). In addition, O'Brien *et al.* (2002) showed that *Staphylococcus*, especially *S. aureus*, has a surface-expressed protein, clumping factor B (CLFB), which promotes adherence to immobilized epidermal cyto-keratins *in vitro*. These factors are

very important for the severity and pathogenicity of bacteria in the incidence of infections (Gotz, 2004).

Pr. acnes revealed adherence with keratin because this bacterium contains an important structure, the so-called glycocalyx polymer (glue). Burkhart and Burkhart (2007) indicated that *Pr. acnes* contained a glue which allows adherence to follicular walls. This glue is secreted by *Pr. acnes* and presents a sebum composition.

In confirmation, Tareb *et al.* (2013) mentioned that the adhesive capacities of probiotic *Lactobacillus* species were also displayed at significant levels in GIT cells, mucin and extra-cellular matrix material (*in vivo*).

3.5 Conclusions

There was significant auto-aggregation among all bacterial groups. *L. casei* was exhibited the weakest co-aggregation with all of the human bacterial skin pathogens tested. Species of different bacteria were more adherent alone than when mixed with other species, suggesting that they form a more robust biofilm. When mixing probiotic *Lactobacillus* species with pathogens biofilm formation and adhesion of pathogenic bacteria were reduced. Strains that are adherent to human keratin, the main protein component found in skin, can be readily identified, but this does not necessarily mean that they can be classified as a successful probiotic. Therefore, further studies are needed which focus on the identification and assessment of strains that exhibit activity against potential skin pathogens, and will also persist on the skin *in vivo* and be active there.

Chapter 4

Antimicrobial activity of probiotic *Lactobacillus plantarum* and human beta-defensin-2 (hBD-2) against bacterial human skin pathogens

The results have been presented at SFAM conference, 1st - 4th July, Cardiff, UK and the abstract published in the conference proceedings. In addition, the results have also been presented at V International Conference on Environmental, Industrial, and Applied Microbiology, Medicine College, Madrid University, Madrid, Spain, 2nd - 4th October 2013 and and the abstract published in the conference proceedings.

Chapter 4: Antimicrobial activity of probiotic *Lactobacillus plantarum* and human β-defensin-2 (hBD-2) against bacterial human skin pathogens

4.1 Introduction

L. plantarum is the most prevalent species in most naturally fermented foods and has the ability to block receptors for Gram negative bacteria, as well as having an important antimicrobial effect (Zarazaga *et al.*, 2004). Antimicrobial peptides (AMPs) are a group of predominantly small cationic polypeptides (less than 100 amino acids) that are classified together because of their ability to impede the growth of microorganisms. AMPs have been known for more than twenty years (Ganz, 2003; Bardan *et al.*, 2004). AMPs, especially defensins, have a broad range of antimicrobial activity against different bacteria, fungi and viruses (Lehrer *et al.*, 1993). Defensins and cathelicidins are the two major groups of epidermal AMPs that possess inherent antimicrobial activity (Zaiou *et al.*, 2003). AMPs were the first observed in mammalian cutaneous tissues when cathelicidins were discovered in porcine wound fluid (Gallo *et al.*, 1994). Defensins are small cationic, cysteine-rich peptides with a wide range of antimicrobial activities. Defensins are divided into α-, β- and θ- subfamilies (Ganz, 2003) and exhibit a high level of activity against both Gram positive and Gram negative bacteria, fungi and certain enveloped viruses, as well as immune cells, such as neutrophils, macrophages and some epithelial cells in the small intestine (Kagan *et al.*, 1994). Jones and Bevins (1992) observed that six α-defensins have been identified. Human α-defensins 1 - 4 are known as human neutrophil peptides,

and are associated with human neutrophils. Human α-defensins 5 and 6 are expressed in Paneth cells of the GIT and epithelial cells of the female UGT.

HBD have been identified in numerous cells, including neutrophils and epithelial cells (Liu *et al.*, 2002). Valore *et al.* (1998) clarified that hBD 1 - 4 have been identified in humans. HBD-1 (36 amino acid) is constitutively produced by epithelial tissue in the respiratory system and UGT. HBD 2 - 4 have been isolated from lesional scales of psoriatic cutaneous tissues (Harder *et al*., 1997). Yand *et al.* (2001) reported that hBD-2 (41 amino acid) and hBD-3 (45 amino acid) expression is inducible (stimuli) by external agents, e.g. IFN-1β, IFN-γ, TNF-α, Gram negative and Gram positive bacteria.

HBD-2 is a cysteine-rich cationic peptide of the innate immune system that serves as an AMP. HBD-2 contains forty one amino acids with low molecular weight, and was first discovered in human skin in the 1990s (Schröder and Harder, 1999). The expression of hBD-2 is prevalently observed in epithelial cells of cutaneous tissue (Butmarc *et al.*, 2004), the GIT (Wagner, 2000) and respiratory system (Bals *et al.*, 1998). Wehkamp *et al.* (2004) mentioned that there are specific probiotic bacteria, such as *E. coli* Nissle strain 17 which potentiate and up regulate expression of hBD-2 in Caco-2 cells. Moreover, Schlee *et al.* (2008) showed that the induction of hBD-2 by a probiotic cocktail of four *Lactobacillus* species (*L. acidophilus*, *L. paracasei* , *L. delbrueckii* spp. *bulgaricus* and *L. plantarum*), three *Bifidobacterium* species (*B. longum*, *B. infantis* and *B. breve*) and one *Streptococcus salivarius* subsp. *thermophilus*. In addition, pathogen-associated molecular patterns (PAMPS) molecules, e.g. bacterial flagella antigens, LPS, PG and

DNA are capable of inducing hBD-2 expression in epithelial cells (Takahashi *et al.*, 2001; Platz *et al.*, 2004; Vora *et al.*, 2004).

Pathogens (Gram positive and Gram negative) contain several genes, such as *mprF*, *dlt*, *pmr*, *pagp* and others to protect themselves from hBD-2 and other host defense peptides (Nizet, 2006). *Dlt* types catalyse the introduction of D-alanine into TA, staphylococcal cell wall polymers, whereas the *mprF* gene is involved in modification of membrane phosphatidylglycerol with L-lysine (Peschel *et al*., 1999; Peschel *et al*., 2001). Furthermore, Peschel *et al*. (1999) and Peschel *et al*. (2001) demonstrated that esterification of cell envelope components with amino acids leads to a decrease in the net negative surface charge of the bacteria, and consequently to the repulsion of positively charged AMPs.

Interestingly, sodium chloride (NaCl) may be responsible for the decrease in mucosal defenses. NaCl reduces killing of bacteria by a number of defensins, including hBD (Bals *et al.*, 1998; Singh *et al.*, 1998). Interestingly, Krishnakumari *et al.* (2006) reported that the inhibitory effect on the activity of hBD depends on the concentration of NaCl and the concentration of the peptide. The antimicrobial activity of most hBD-2 are decreased in the presence of NaCl concentrations (Bals *et al.*, 1998). Therefore, hBD2 may exhibit antimicrobial activity at physiological salt concentrations if its concentration is sufficiently high.

The aims of the current study were to determine the antimicrobial activity of probiotic *L. plantarum*, the effectiveness of a mixture of probiotic *L. plantarum* with hBD-2 and different concentrations NaCl, and synergism between the

probiotic *L. plantarum* and hBD-2 against pathogens, such as *S. aureus*, MRSA and *P. aeruginosa* strains.

4.2 Materials and methods

4.2.1 Materials

L. plantarum was selected in the current experiment as it showed the greatest antimicrobial activity, aggregation and biofilm formation against bacterial human skin pathogens, as reported in Chapters 2 and 3. Recombinant hBD-2 (100 µg / ml) was supplied by Pepro Tech, (Pepro Tech Inc., UK) and tested against different bacteria, such as *P. aeruginosa*, *S. aureus* strains (3761 and 4137), MRSA 12493 strain and *L. plantarum* 41605 (Table 2.1).

Staph 110, nutrient agar and broth (NA and NB), *Pseudomonas* isolation agar (PIA), MRS agar and broth, sodium phosphate buffer (SPB; 8.2 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.4) and 2 M NaCl, Oxoid (Oxoid, UK) were used. In addition, Lysostaphin (1 mg / ml) was supplied by Sigma Aldrich, (UK). Molecular grade water was used (Fisher Scientific, UK Ltd.).

4.2.2 Methods

Recombinant hBD-2 was dissolved in molecular biology grade water at a concentration 100 µg / ml and stored at - 20 °C. A single colony of MRSA, *P. aeruginosa* and *S. aureus* strains was used to inoculate 5 ml of NB. In addition, a single colony of *L. plantarum* was used individually to inoculate 5 ml of MRS broth. After that, broth cultures were incubated at 37 °C for 18 - 20 h. Broths were centrifuged at 4300 × *g* (Harrier 18 / 80, MSE, UK.) at 4 °C for

10 minutes. The bacterial cell pellet was re-suspended in SPB. This procedure was modified, as is described previously by Huang *et al.* (2007). In short, the optical density of the suspension was adjusted to 0.2 at 620 nm (10^7 CFU / ml) by adding an appropriate volume of SPB. Reaction mixtures (a total volume 20 µl) from overnight broth culture bacteria from each bacteria and hBD-2 (2.5, 5, 10, 20 and 30 µg / ml) were incubated at 37 °C for 2 and 5 h. Following incubation, the samples were diluted 5000-fold in SPB, and 10 µl of this dilution was plated onto both NA and Staph 110 agar (for MRSA and *S. aureus* strains), NA and PIA (for *P. aeruginosa*), and MRS agar (for *L. plantarum*). After incubation overnight at 37 °C, colonies were counted. Using the same method and procedure as above, hBD-2 and *L. plantarum* were used together (synergistically) against pathogens. In addition, hBD-2 (5 µg / ml) was mixed with different NaCl concentrations (0, 25, 50, 100 and 150 mM), and applied on: *P. aeruginosa* and *S. aureus* 4137, using the same procedure as above. After incubation 18 - 20 h at 37 °C, the microbial colonies were counted. HBD-2 and *L. plantarum* were also used, and mixed with different NaCl concentrations, as described above. All tests were replicated three times under the same identical experimental conditions. The percentage viability following exposure to hBD-2 and/ or *L. plantarum* was calculated as follows:

[(mean number of colonies observed on treated plates/ number of colonies observed on non-treated control plates) x 100].

4.2.3 Effect of *L. plantarum* and hBD-2 on *mprF* and *dlt* genes mediating hBD-2 resistance in MRSA 33591

hBD-2 and *L. plantarum* were used to measure the effect of hBD-2 and *L. plantarum* on *mprF* and *dlt* gene expression because when used together in a culture-based study, MRSA viability was reduced to 71% of control levels, as reported in section 4.3.1 (Figure 4.3, A).

4.2.4 Modulation of *mprF* gene by hBD-2 and *L. plantarum*

4.2.4.1 RNA extraction from bacterial cells

MRSA 33591 cells (10^7 CFU / ml) were cultured at 37 °C for 18 - 20 h in 20 ml nutrient broth. Cells were harvested by centrifuging at 2772 × *g* (Harrier 18 / 80, MSE, UK) for 10 minutes, and washed twice using SPB. Pellets were re-suspended in 1 ml SPB. Recombinant hBD-2 (10 µg / ml) was mixed with a suspension of MRSA 33591 (1 ml a total volume), and incubated at 37 °C for 2 h. Total RNA was extracted using the Rneasy Protect Bacteria Mini Kit (Qiagen, UK). A control set was prepared and incubated at 37 °C for 2 h. The cells were centrifuged at 13000 x *g* (Heraeus Fresco 21 Microcentrifuge, USA) for 3 minutes, and washed twice using SPB. Cells were lysed by the addition of 1 mg / ml lysostaphin (30 µl), and incubation at 37 °C for 15 minutes. RNA was extracted and purified according to the manufacturer's recommendations for bacteria RNA isolation protocol, using the optional step of using the RNase-free DNase Kits (Qiagen, UK), as outlined in Figure 4.1.

Finally, RNA concentration and quality were evaluated using a NanoDrop spectrophotometer, as presented in Appendix 2 (Figure 2.1). RNA samples with low purity were discarded, and the extractions were repeated if required.

Four microliters of each RNA sample was electrophoresed in a 1 g / 50 ml agarose gel to appraise RNA integrity. Aliquots of RNA were stored in sterile 1.5 ml micro-centrifuge tubes at - 80 °C until further analysis. *L. plantarum* and hBD-2 were also used against MRSA, according to the same procedure as described above.

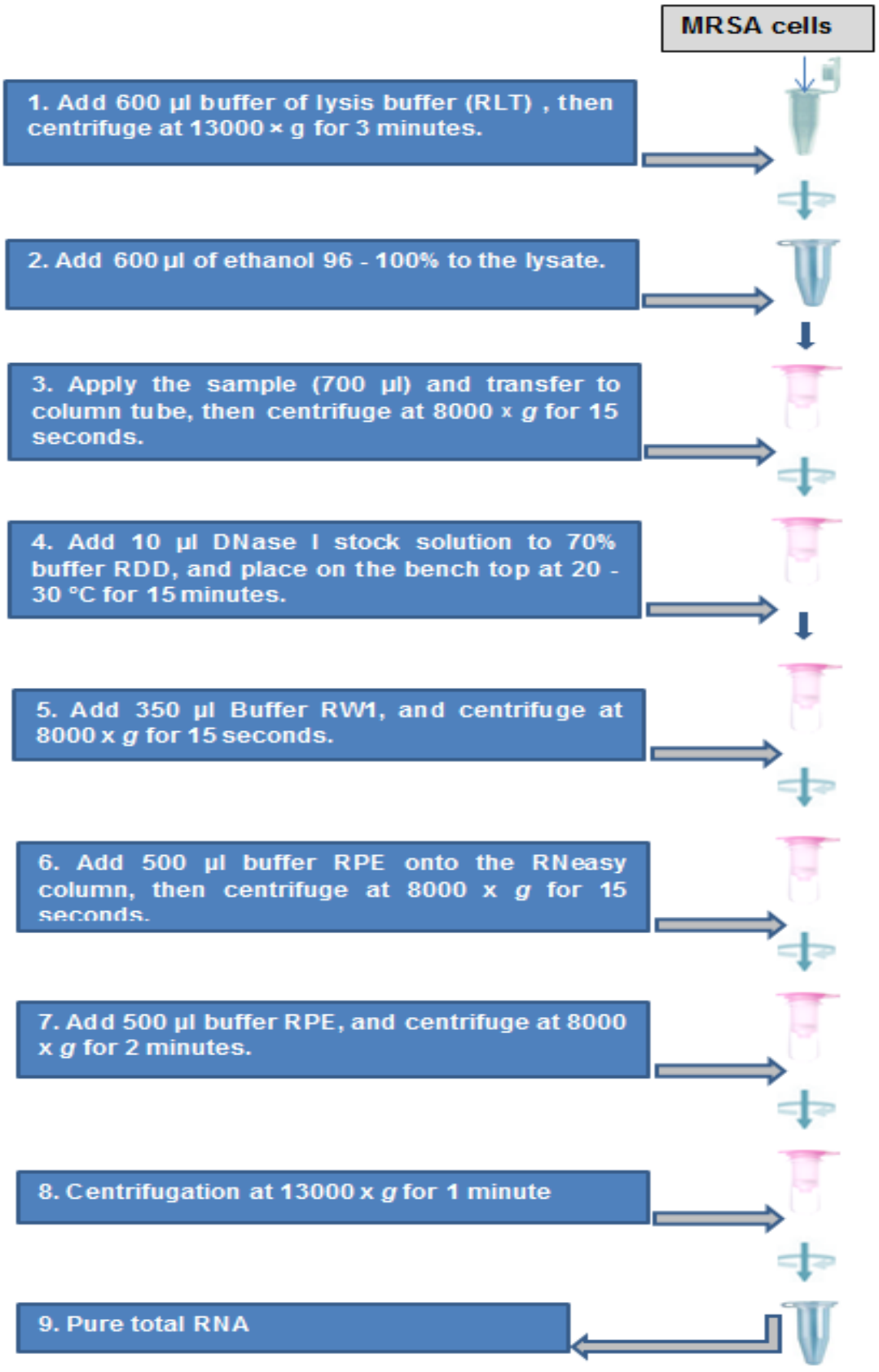


Figure 4.1 Protocol for the extraction of total RNA from bacterial human skin pathogens, according to the manufacturer's Rneasy Protect Bacteria Mini Kit (Qiagen, UK) recommendations.

4.2.4.2 Agarose gel electrophoresis

In order to determine the quality of RNA, agarose gel electrophoresis was used. The gel was prepared by dissolving 1 g agarose into 50 ml Tris-acetate ethylene diamine tetraacetic acid (EDTA) buffer (1 x TAE) in a conical flask using a microwave oven on medium power for 2 - 3 minutes, until the agarose was completely dissolved. The molten agarose was poured into casting tray with a comb inserted. RNA samples (4 µl) were prepared by mixing them with RNA loading buffer (4 µl). The comb was removed when the gel was left to set. Hyper Ladder IV (3µl; Bioline, UK) was used. The ladder and samples were loaded into the gel's wells. The gel was run at 90 V for 1 h. The RNA bands were visualized in a gel documentation system (Chemi Doc HR, UVP, UK) using ethidium bromide and UV light and images were captured.

4.2.4.3 Reverse transcription and cDNA synthesis

Reverse transcription (RTs) was performed to create a complementary deoxyribonucleic acid (cDNA). This was carried out using a High Capacity RNA-to-cDNA 50 x Kit (Fisher Scientific, UK Ltd.), according to the manufacturer's instructions. The cDNA was prepared in 0.2 ml RNase- / DNase-free micro-centrifuge tubes The RNA (1 µg) was mixed with 1 µl RT (200 U / µl) and 10 µl of mixed deoxynucleotide triphosphates (dNTPs). Molecular grade water was added to a final volume of 20 µl. The contents were mixed gently and briefly centrifuged at 13000 x *g* for 30 seconds and tubes put in a Thermal Cycler System (Applied Biosystem, UK) at 37 °C for 1

h, followed immediately by 85 °C for 5 minutes. The reaction mixture was then stored at - 20 °C until use for qPCR.

4.2.4.4 Quantitative RT - PCR (qRT - PCR)

qPCR was carried out to semi-quantitatively to analyse gene expression levels in the experiment relative to the reference gene (the control). PCR amplification was performed with primers, with pyruvate kinase (*Pyk*) as a housekeeping gene, and multiple peptide resistance factor (*mprF*) and D-alanyl-lipoteichoic acid (*dlt*) as target genes (Table 4.2). As stated previously, these genes were targeted as they are responsible for bacterial resistance to hBD-2, and specific gene-targeting primers were designed using the mRNA sequences, which were obtained from a primer blast in the NCBI (National Centre for Biotechnology Information). The criteria for primer selection included the GC ratio, temperature and product length (http://ecom.mwgdna.com/services/home.tcl). Primers were obtained from Eurofins MWG Operon (MWG, Germany). The reaction mixture consisted of 5 µl cDNA, 12.5 µl SYBR® green master mix (Qiagen, UK), 0.3 µl reference dye, 0.8 µl primer mix (10 pmol) and 6.4 µl molecular grade water to give a final volume of 25 µl for each reaction. Reactions were run in triplicate in three independent experiments. The geometric mean of the housekeeping gene was used as an internal control to normalize the variability in expression levels. PCR reactions were carried out using the following conditions: denaturant at 95 °C for 10 minutes, followed by 40 cycles, single cycle 95 °C for 20 seconds, annealing 52 °C for 30 seconds and amplification (extension step) at 72 °C for 1 minute. This was immediately followed with a

melting cycle of 95 °C for 15 seconds and 55 °C for 1 minute and of 95 °C for 15 seconds. The PCR was run using an Applied Biosystems StepOne Plus real time PCR cycler equipped with StepOne software v2.1.

Table 4.1 Primers sequences (housekeeping and target genes) used for qRT-PCR reactions.

Primer	Primer sequence (5´…3´)	Product size (bp)	Primer concentration (pmol)	Accession No.
mprF, F	5´-AGA CCA CCC GAT AAA AAC AAT C-3´	114	10	HM140975.1
mprF, R	5´-AGC GTC AAC AAT TAC ACC AC-3´	114	"	"
Pyk, F	5´-GCA TCT GTA CTC TTA CGT CC-3´	90	"	Theis *et al.* (2007)
Pyk, R	5´-GGT GAC TCC AAG TGA AGA-3´	90	"	"
dlt, F	5´-CAAG TGC GAC GAT TTA CA A C-3´	129	"	D86240.2
dlt, R	5´-GTT GAA AGAC TAG GCG CAA-3´	129	"	"

4.2.4.5 Normalization of qRT - PCR data

In most cases, the data were normalized using the instrument software with auto-normalization. However, the data were also normalized manually producing the relative quantification (RQ) values. The data was first normalized against the housekeeping gene (*Pyk*). This was achieved by subtracting the cycle threshold (C_T) value of the housekeeping gene from the C_T value target genes (*mprF* and *dlt*) to produce the delta cycle threshold (ΔC_T). The $\Delta\Delta C_T$ values of each sample were then produced by subtracting the ΔC_T of the control (vehicle control) from the ΔC_T value for each treatment. The RQ of individual samples was then produced using the following equation: ($RQ= 2^{-\Delta\Delta CT}$).

4.2.5 Statistical analysis

Data were subjected to balanced analysis of variance (ANOVA) and *post hoc* LSD tests using Minitab v.16 and Statistical Package for the Social Sciences (SPSS). In addition, the numerical data for gene expression were analyzed using SPSS v.20 statistical software, using two-way ANOVA and univariate comparison *post hoc* tests (Fisher's LSD). Data were shown as mean ± Standard Deviation (mean ± SD), and $P < 0.05$ was considered significant.

4.3 Results

4.3.1 Results of hBD-2 alone, and hBD-2 mix with *L. plantarum* against some bacterial human skin pathogens

Recombinant hBD-2 was observed to display a large amount of antimicrobial activity against the bacterial human skin pathogens used in the current study. Incubation with 10 µg / ml hBD-2 alone / with *L. plantarum* for 2 h was shown to kill a portion of the bacterial population. The percentage viabilities [(mean number of colonies/ number of colonies observed on non-treated control) x 100)] with hBD-2 alone were as follows: *S. aureus* 4137 (26.33 ± 3.4%), *aureus* 3761 (23.87 ± 2.1%) and *P*. *aeruginosa* (23.28 ± 2.26%), as outlined in Figures 4.2 (A - C).

With regards to the investigation of synergy between hBD-2 and *L. plantarum*, the percentage viabilities were demonstrated to be lower compared to hBD-2 alone. The results were significantly different to those observed when only hBD-2 was used ($P < 0.05$), and were as follows: *S. aureus* 3761 (4.34 ± 1.17%), *S. aureus* 4137 (9.46 ± 3.4%) *P. aeruginosa* 8626 (8.54 ± 2%), as outlined in Figures 4.2 (A - C).

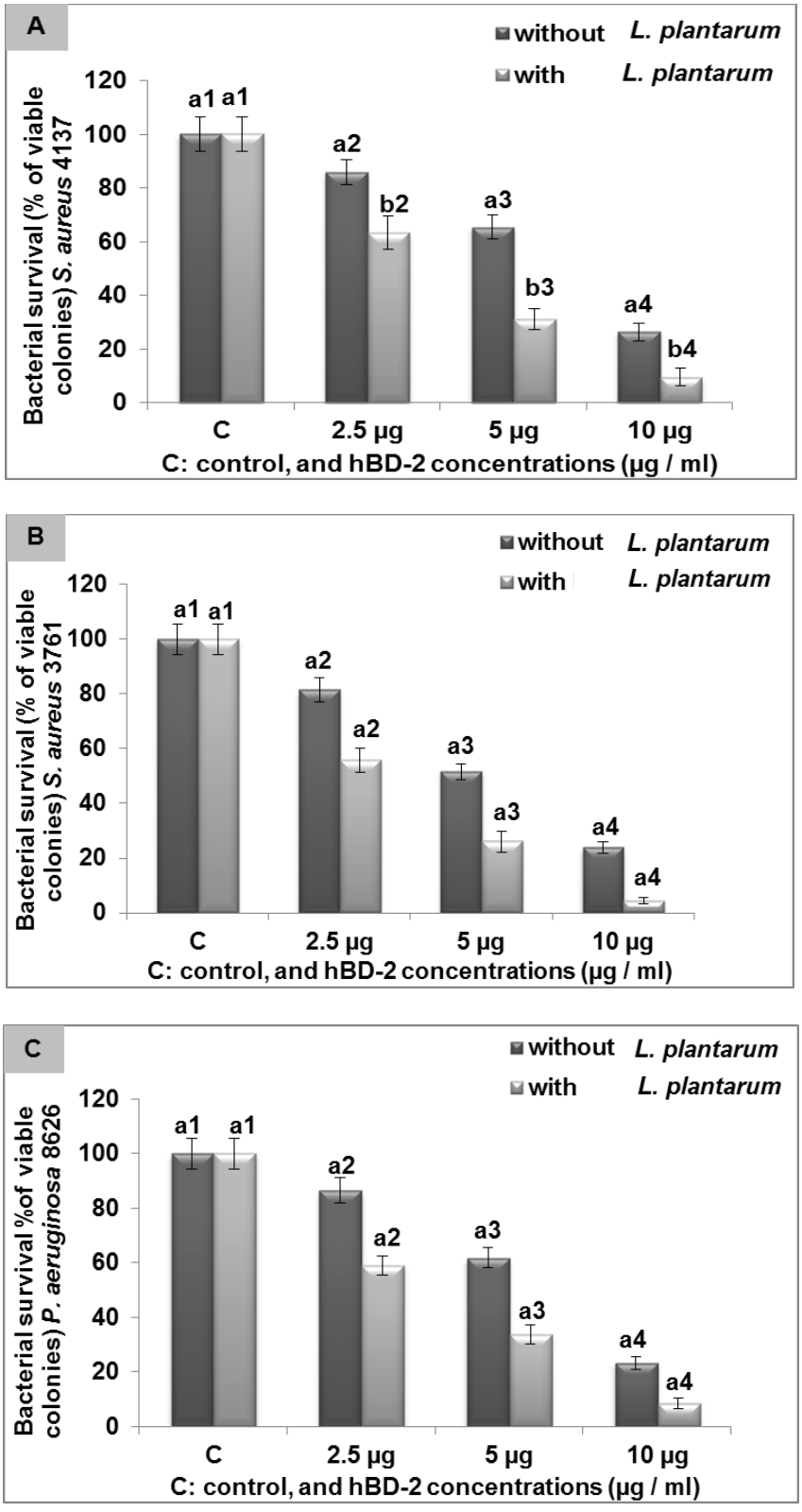


Figure 4.2 Antimicrobial activity of different hBD-2 concentrations and *L. plantarum* against: (A) *S. aureus* 4137, (B) *S. aureus* 3761, and (C) *P. aeruginosa* 8626 when used without / with *L. plantarum* after 2 h. Values are presented as mean ± SD. Values with different superscript letters within experimental groups (i.e. with or without *L. plantarum* at each concentration) are significantly different, while mean values with different superscript numbers across different hBD-2 concentrations (either with/ without *L. plantarum*) are significantly different.

With regards to the investigation of hBD-2 (30 µg / ml; without *L. plantarum*) for 2 h, percentage viabilities of MRSA strains were as follows: MRSA 33591 (35.53 ± 3.4%) and MRSA 12493 (39.56 ± 2.7%), as outlined in Figures 4.3 (A - B).

With regards to the investigation of synergy between hBD-2 and *L. plantarum*, the percentage viabilities were demonstrated to be significantly lower compared to hBD-2 alone ($P < 0.05$): MRSA 12493 (15.28 ± 1.9%) and MRSA 33591 (17.76 ± 3.4%), as outlined in Figures 4.3 (A - B).

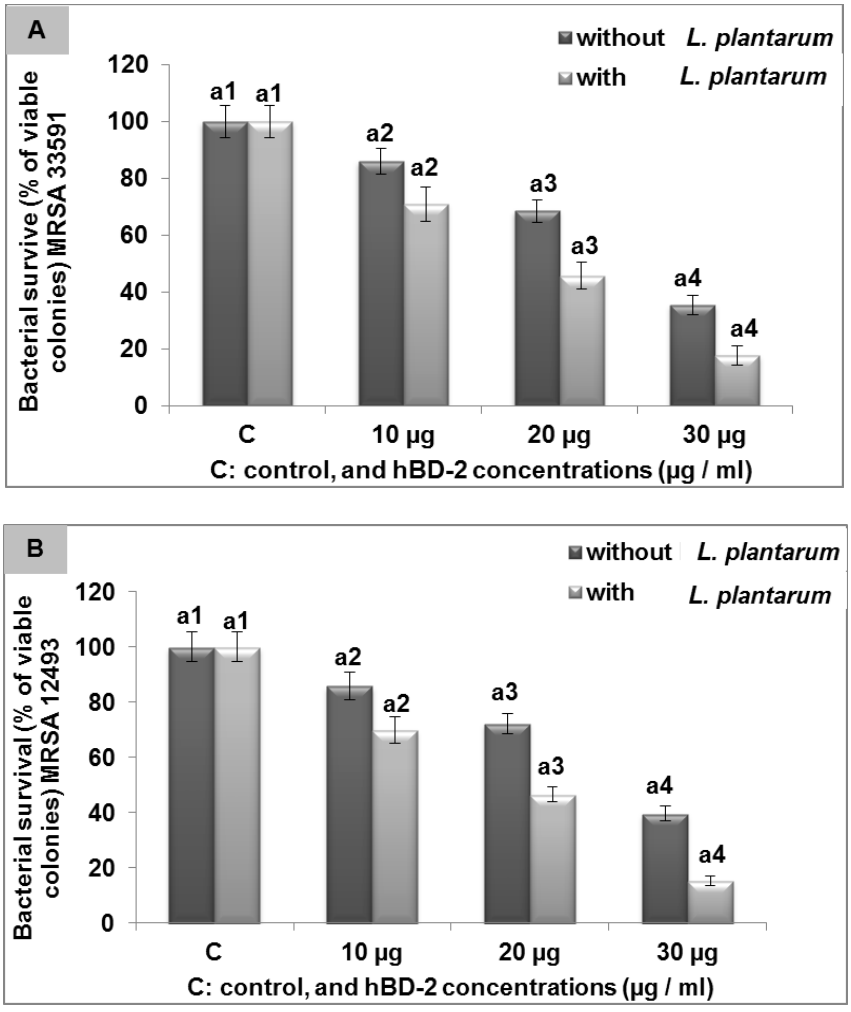


Figure 4.3 Antimicrobial activity of different hBD-2 concentrations and *L. plantarum* against: (A) MRSA 33591, MRSA and (B) 12493 when used without / with *L. plantarum* after 2 h. Values are presented as mean ± SD. Values with different superscript letters within experimental groups (i.e. with or without *L. plantarum* at each concentration) are significantly different, while mean values with different superscript numbers across different hBD-2 concentrations (either with / without *L. plantarum*) are significantly different.

Increasing the incubation period to 5 h incubation time (with / without *L. plantarum*) decreased the survival rate of the bacterial population. With regards to the investigation of hBD-2 (10 µg / ml; without *L. plantarum*) for 5 h, percentage viabilities of *S. aureus* strains/ *P. aeruginosa* were as follows: *S. aureus* 3761 (8.37± 2.59%), *S. aureus* 4137 (11.4 ± 2.6%), *P. aeruginosa* (12.56± 3.53%) as outlined in Figures 4.4 (A - C).

With regards to the investigation of synergy between hBD-2 and *L. plantarum*, the percentage viabilities were demonstrated to be significantly lower compared to hBD-2 alone ($P < 0.05$): S*. aureus* 3761 (1.28 ± 1%), *P. aeruginosa* (1.76 ± 0.8%), *S. aureus* 4137 (2.37 ± 0.74%), as outlined in Figures 4.4 (A - C).

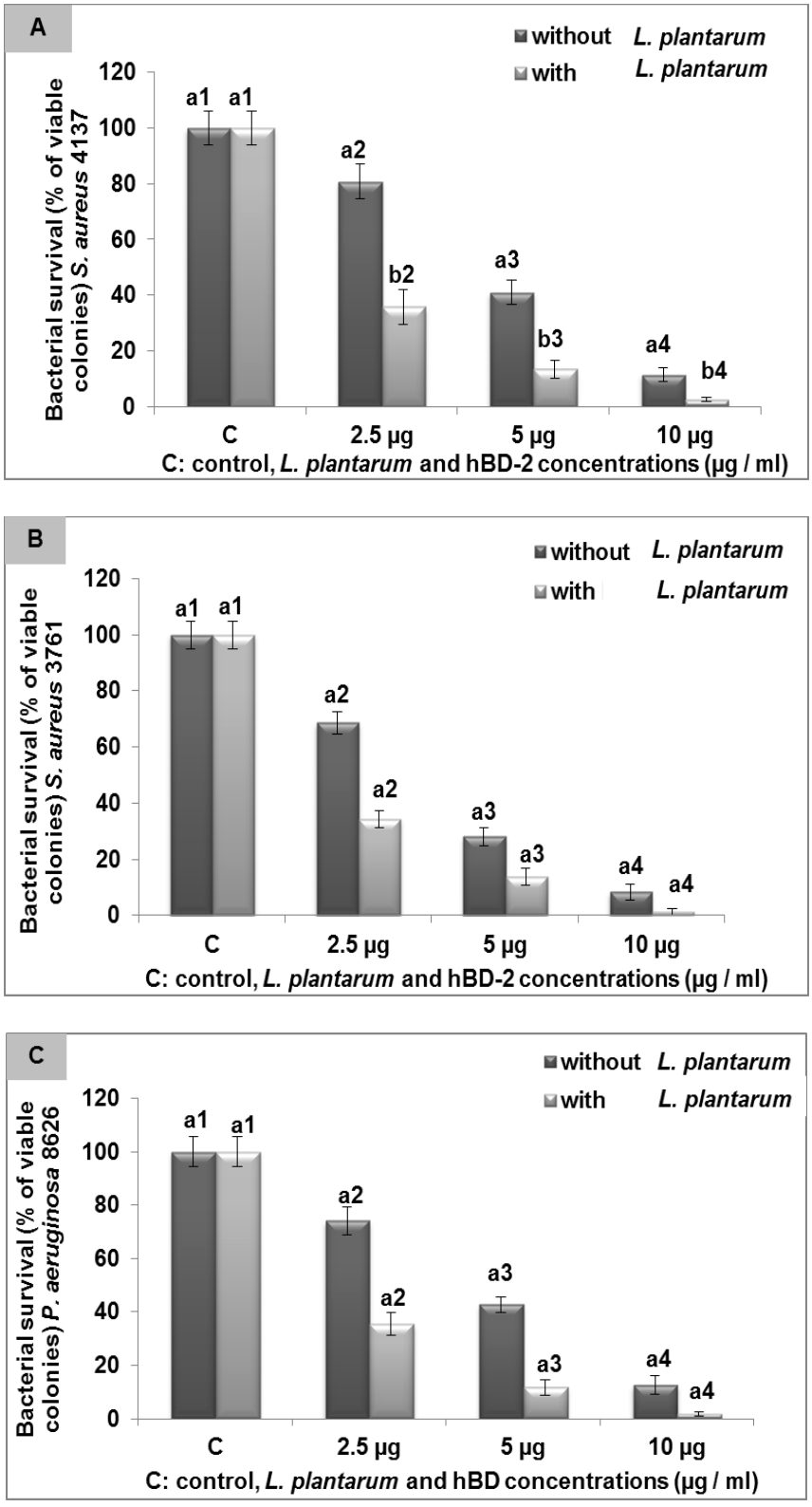


Figure 4.4 Antimicrobial activity of different hBD-2 concentrations and *L. plantarum* against: (A) *S. aureus* 4137, (B) *S. aureus* 3761, and (C) *P. aeruginosa* 8626 when used without / with *L. plantarum* after 5 h. Values are presented as mean ± SD. Values with different superscript letters within experimental groups (i.e. with or without *L. plantarum* at each concentration) are significantly different, while mean values with different superscript numbers across different hBD-2 concentrations (either with / without *L. plantarum*) are significantly different.

With regards to the investigation of hBD-2 (30 µg / ml; without *L. plantarum*) for 5 h, percentage viabilities of MRSA were as follows: MRSA 33591 (16.16 ± 4.2%) and MRSA 12493 (13.66 ± 3.16%), as outlined in Figures 4.5 (A - B). With regards to the investigation of synergy between hBD-2 and *L. plantarum*, the percentage viabilities were demonstrated to be significantly lower compared to hBD-2 alone ($P < 0.05$): MRSA 12493 (2.84 ± 0.98%) and MRSA 33591 (4.79 ± 1.97%), as outlined in Figures 4.5 (A - B).

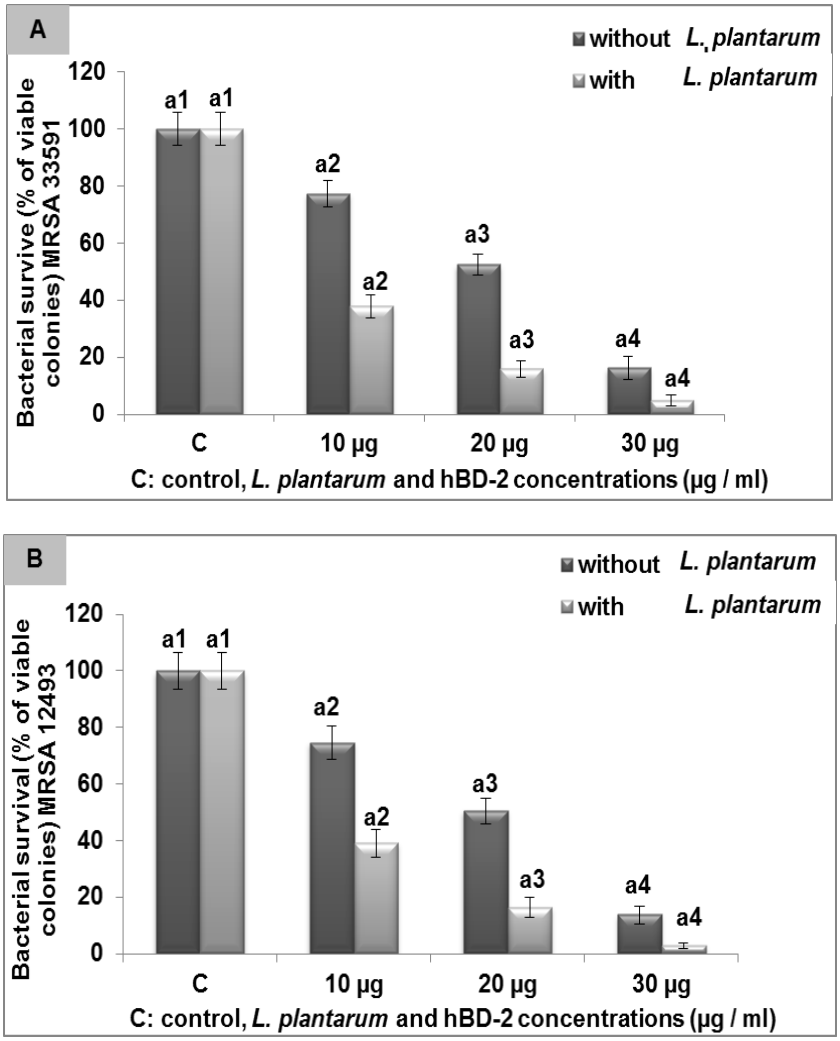


Figure 4.5 Antimicrobial activity of different hBD-2 concentrations and *L. plantarum* against: (A) MRSA 33591, and (B) MRSA 12493 when used without / with *L. plantarum* after 5 h. Values are presented as mean ± SD. Values with different superscript letters within experimental groups (i.e. with or without *L. plantarum* at each concentration) are significantly different, while mean values with different superscript numbers across different hBD-2 concentrations (either with / without *L. plantarum*) are significantly different.

With regards to the investigation of hBD-2 (10 µg / ml maximum concentration) for 2 and 5 h, percentage viabilities of *L. plantarum* were as follows: 63.5 ± 4.37% and 52.87 ± 4% for 2 and 5 h, respectively, as outlined in Figures 4.6 (A - B).

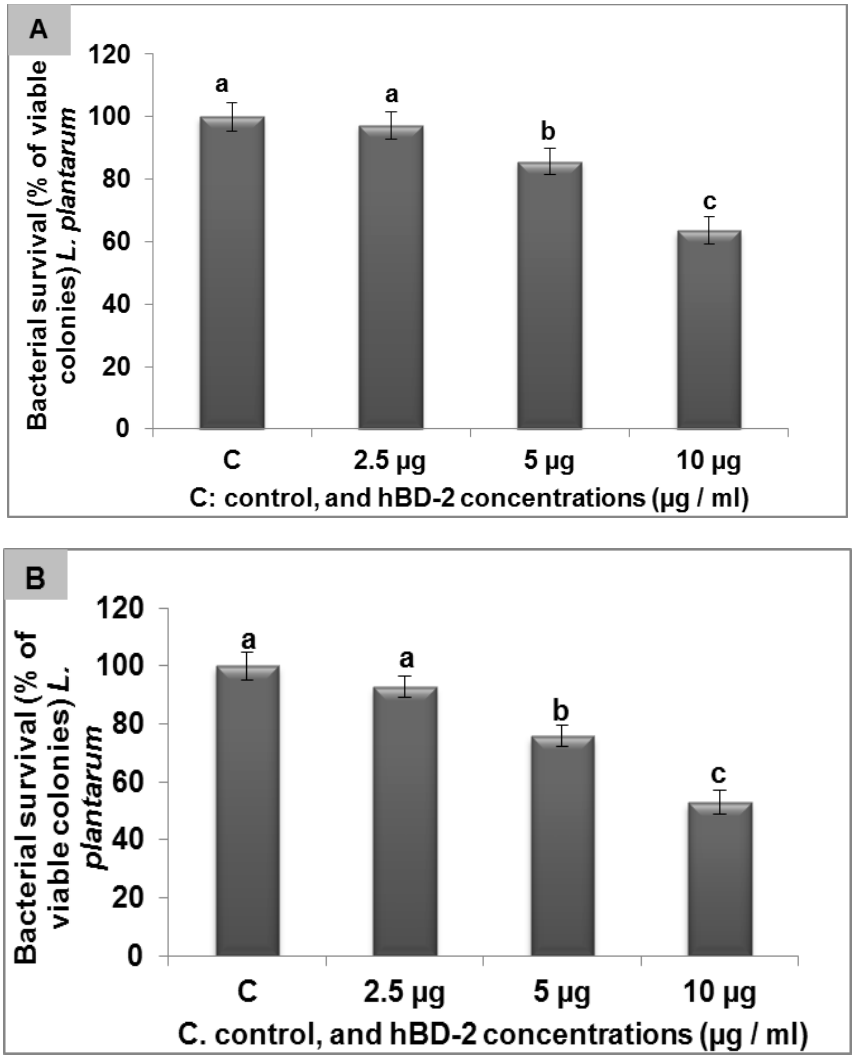


Figure 4.6 Antimicrobial activity of different hBD-2 concentrations against *L. plantarum* after (A) 2 h, and (B) 5 h. Values are presented as mean ± SD. Values with different superscript letters between hBD-2 concentrations are significantly different.

The statistical analyses observed high significant differences ($P < 0.05$) with all pathogens, incubation periods (2 h and 5 h), and treatments (hBD-2 and *L. plantarum*).

4.3.2 Influence of different sodium chloride concentrations on the bacterial activity of hBD-2

To investigate the influence of different sodium chloride concentrations on the bacterial activity of hBD-2, different strains of bacterial human skin pathogens were incubated with hBD-2 (5 µg / ml) for 2 h incubation times with different NaCl concentrations, increasing from 25 to 150 mM. It was found that the survival of bacteria (mean ± SD) (numbers of viable colonies) gradually increased (25 - 150 mM) significantly ($P < 0.05$), as follows: from 13.14 - 90.73% with *S. aureus* 4137 and 9.32 - 83.6% with *P. aeruginosa*, as described in Figure 4.7.

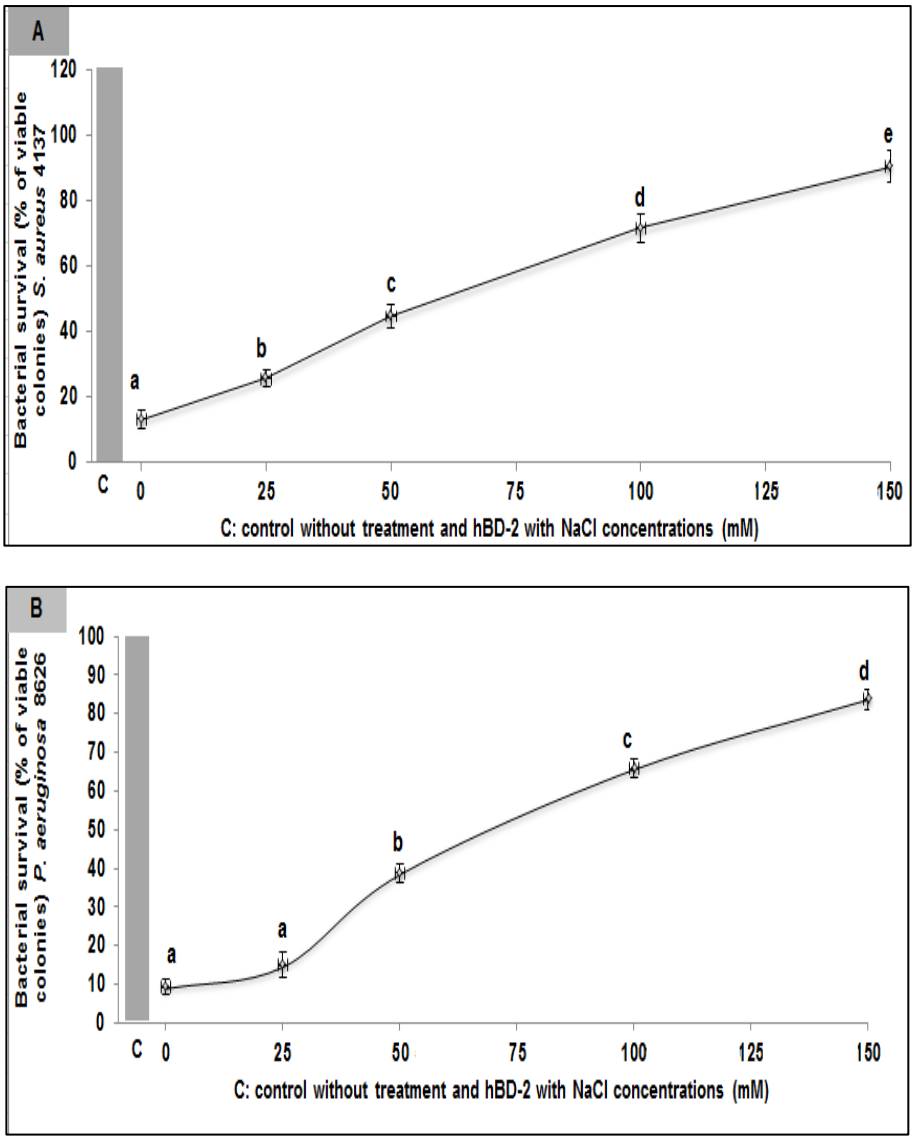


Figure 4.7 Effect of NaCl concentrations on the antimicrobial activity (number of viable colonies) for hBD-2 (5 µg / ml) against pathogens; (A) *S. aureus* 4137, and (B) *P. aeruginosa* 8626. The data presented as mean ± SD from (three independent experiments). Mean values with the same superscript letters within each hBD-2 concentrations are not significantly different.

4.3.3 Influence of different sodium chloride concentrations on the bacterial activity of hBD-2 and *L. plantarum*

The results of *L. plantarum* and hBD-2 with NaCl against pathogens were significant compared to the non-treated control samples ($P < 0.05$), and reduced the viable counts (mean ± SD) compared with the results when using hBD-2 alone. The numbers of viable colonies was gradually increased

(25 - 150 mM), as follows: from 25.68 - 62.84% with *S. aureus* 4137 and 28.26 - 67.9% with *P. aeruginosa*, as described in Figure 4.8.

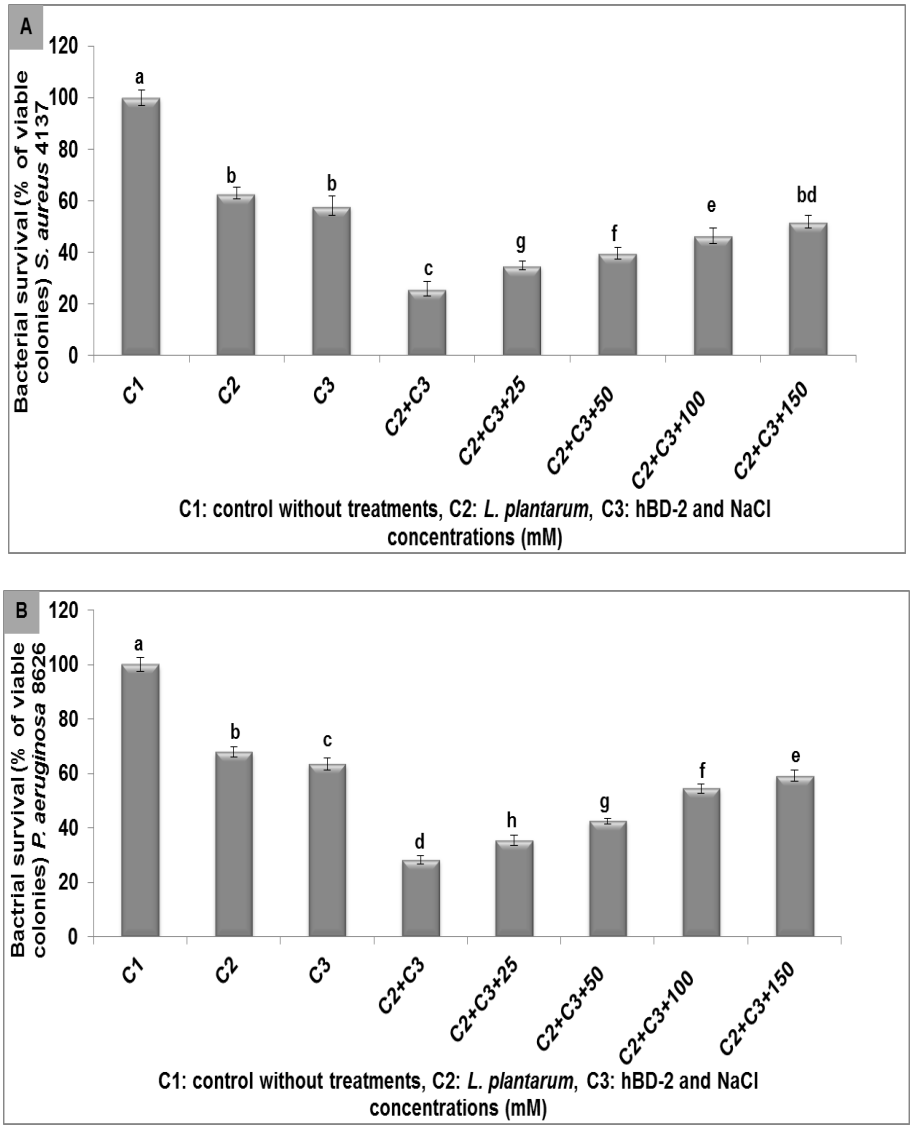


Figure 4.8 Effect of NaCl concentrations on the antimicrobial activity (percentage of viable colonies) for hBD-2 (5 µg / ml) and *L. plantarum* against pathogens; (A) *S. aureus* 4137, and (B) *P. aeruginosa* 8626. The data presented as mean ± SD from (three independent experiments). Mean values with the same superscript letters within each the following: control, hBD-2, *L. plantarum*, hBD-2 and *L. plantarum* with different NaCl concentrations are not significantly different.

4.3.4 Measurement of *mprF* and *dlt* gene expression using qRT-PCR

The results with *mprF* and *dlt* gene expression using qRT - PCR, hBD-2 alone and hBD-2 with *L. plantarum* together showed an up-regulation of the expression of the *mprF* and *dlt* genes in MRSA. The RNA of MRSA was successfully extracted, as outlined in Figures 4.9 - 4.10 and Appendix 2: Figure 2.2. cDNA was synthesized using conventional PCR assay and qRT - PCR. The observed results were that the MRSA cultured in the presence of hBD-2 alone and hBD-2 with *L. plantarum* displayed significant differences of *mprF* and *dlt* gene expression compared to controls.

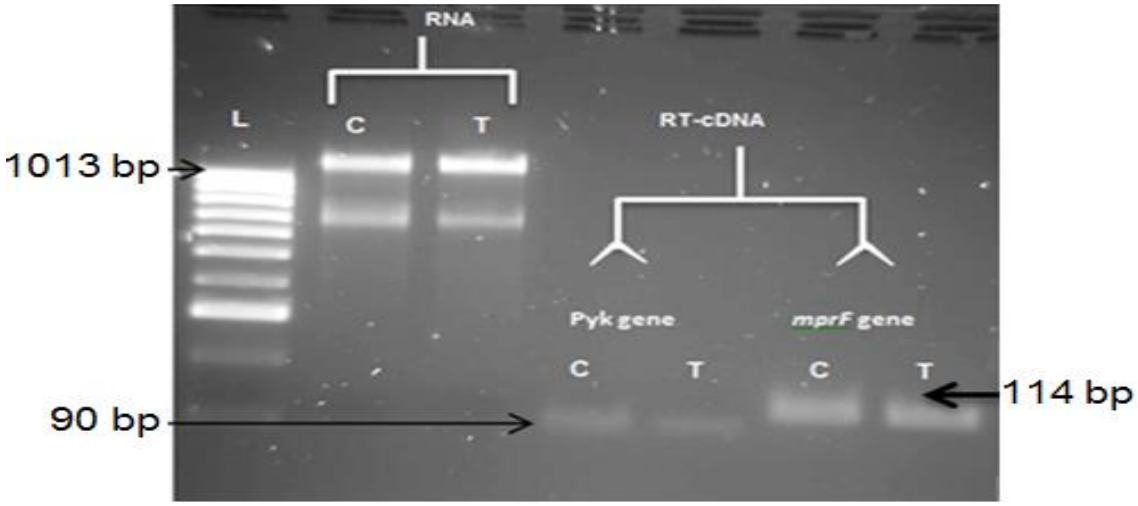


Figure 4.9 Agarose gel electrophoresis of RNA samples and amplification cDNA. L: ladder, (C): control without treatment, T: treatment (hBD-2), *Pyk*: pyruvate kinase gene (housekeeping gene), and *mprF*: modified peptide resistant factor gene (target gene).

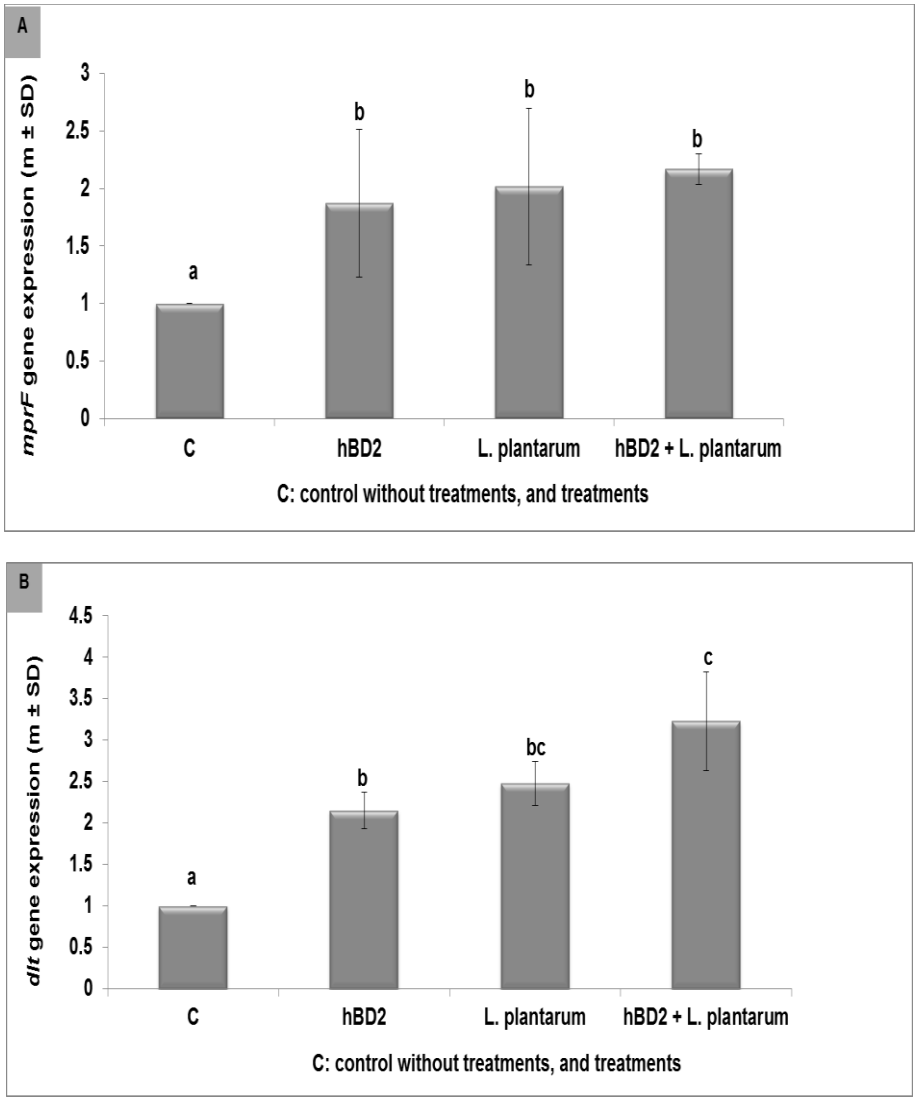


Figure 4.10 Gene expressions for MRSA with the mixture of hBD-2 and culture of *L. plantarum* with some genes, and as follows: (A): *MprF*, and (B): *dlt* gene expression of MRSA 33591 was demonstrated up-regulation with hBD-2, *L. plantarum* and mixture hBD-2 with *L. plantarum*. (C): control without treatments. The data are mean ± SD from three independent experiments, mean value with same superscript letters within each gene are not significantly different.

4.4 Discussion

Cationic AMPs are necessary compounds of the immune system. AMPs protect the host by exerting bactericidal activity, modulating the immune response, molecular signaling and facilitating the communication between innate and acquired immunity (Goytia *et al.*, 2013). Currently, researchers have observed that there are interactions between host mucosal cells and extracellular proteins / antimicrobial peptides produced by various probiotic genera, e.g. *Bifidobacterium*, *Lactobacillus* and *E. coli* strain Nissle 1917 (Sánchez *et al.*, 2010). Scheel *et al.* (2008) reported that numerous *Lactobacillus* strains and other probiotic bacteria called VSL#3 stimulate the secretion of hBD-2 into the culture media. Recombinant hBD-2, in a way similar to that of all defensins, exhibits antimicrobial activities against Gram positive and Gram negative bacteria (Veldhuizen *et al.*, 2008).

In the current study, hBD-2 activity was tested against limited number of strains of *S. aureus* and *P. aeruginosa*.

The present study observed that increasing incubation time from 2 h to 5 h with hBD-2 increased its effectiveness compared with 2 h incubation, especially with 10 µg / ml concentrations, while it was less effective at 2.5 and 5 µg / ml concentrations. Incubation time at 5 h with 2.5 and 5 µg / ml hBD-2 was found to have a similar bactericidal effect as at 2 h incubation time with 5 µg / ml and 10 µg / ml concentration, respectively, for most species.

Interestingly, MRSA showed no effect with low hBD-2 concentrations, such as 2.5 and 5 µg / ml, but was affected when the concentration was increased to 10 - 30 µg / ml. Furthermore, the activity increased when *L. plantarum* was mixed with different hBD-2 concentrations, compared with the activity of hBD-2 alone. The reason for the small effect may be due to some resistant factors in MRSA, such as altered cell surface charge, production of proteases or external trapping of AMPs and others (Nizet, 2006). Furthermore, the activity increased when *L. plantarum* was mixed with different hBD-2 concentrations, compared to the activity of hBD-2 alone.

The current study revealed that *S. aureus* was sensitive to hBD-2. Pazgier *et al.* (2006) reported that the antimicrobial activity of recombinant hBD-2 is largely dependent on the type of microorganism. For example, in this study recombinant hBD-2 was more effective against *S. aureus* and *P. aeruginosa* than against MRSA strains. Several studies conducted to evaluate the effectiveness of recombinant hBD-2 against pathogens demonstrated susceptibility of these microorganisms and were compatible with the findings of the current study. Among these studies were those conducted by Midorikawa *et al.* (2003) and Harder *et al.* (2000), who described the susceptibility of *S. aureus* and mucoid *P. aeruginosa* to hBD-2. In addition, Iwase *et al*. (2010) and Sandiford and Upton (2012) observed that there were some AMPs produced from *S. epidermidis*, epidermicin biosynthesis serine protease (Esp), which were able to kill some Gram positive bacteria. On the other hand, some studies were not compatible with the current study, such as the study conducted by Peschel *et al.*(2001), which reported that the pathogen *S. aureus* is insensitive to defensins, and that the reason may be

due to *mpr*F gene or others. *Mpr*F thus constitutes a novel virulence factor, which may be of general importance for pathogens and represents a potential new method of attacking different multidrug resistant bacteria.

Moreover, this study showed that combining hBD-2 and *L. plantarum* was more effective compared to hBD-2 alone, particularly when the incubation time increased to 5 h. The reason may be due to fact that the mechanism of action for both on target the cell membrane.

In addition, *L. plantarum* produces many substances, such as organic acids, which may have an impact on pathogens (Fu and Mathews, 1999) and may act together with hBD-2 and other substances against pathogens. Chen *et al*. (2005), demonstrated that there is synergistic effect of AMPs, such as hBDs 1, 2 and 3 and acidic pH against *S. aureus* and *E. coli*. This effect was significantly better and enhanced in acidic conditions.

AMPs strongly disturb the anionic lipid component of bacterial membranes in the presence of acidic pH (organic acids) and cause bacterial lysis. This opinion was supported by Mason *et al*. (2006). Alakomi *et al.* (2000) revealed that the mode of action for lactic acid has been observed to permeabilize the outer cell membrane of Gram negative bacteria by causing the release of important substances, e.g. LPS, leading to loss of viability. As a result of permeabilization, the action of LA may facilitate the activity of other antimicrobial factors. In addition, Sugiarto and Yu (2004) reported that the mode of action for hBD-2 represented changes of the electric potential. Peptides will pass across the cell membrane and consequently accumulate into dimers. Pore complex will be formed because of the breaking of the H^+ bonds between the amino acids in the terminal end of the strands connecting

AMPs (e.g. defensins) monomers. Formation of pore complex will cause membrane depolarization and cell membrane lysis, followed by an efflux of cellular substances and the death of bacterial cells (van Dijk *et al.*, 2008).

In the current study, *L. plantarum* demonstrated significant resistance to hBD-2 compared to pathogens. Hugo *et al.* (2006) observed that some non-pathogenic and potentially probiotic *Lactobacillus* species, such as *L. delbrueckii* subsp. *Lactis*, were also able to resist the inhibitory effect of antimicrobial peptides, for example hBD-2. Furthermore, Venkatesan *et al.* (2012) observed that probiotics had high antibiotic resistance activity, antimicrobial activity and antioxidant activity. This may be due to several causes, whether *in vivo* or *in vitro*, such as interference of the chosen probiotic microorganisms with the host's cells and the immune system response (Hugo *et al.*, 2006), the type of microorganisms, the conditions of study and other factors.

In relation to the effect of NaCl on hBD-2 activity, the current study demonstrated that NaCl decreased hBD-2 activity, especially with high NaCl concentrations (150 mM). Inactivation of hBD-2 may be due to the salt combining with protein, and causing precipitation. Kuehner *et al.* (1996) mentioned that a molecular thermodynamic model was developed for salt induced protein precipitation. Several studies are compatible with the current study, such as that conducted by Goldman *et al.* (1997), who mentioned that the antimicrobial activity of hBD-2 is reduced by using NaCl concentration, and reported that even 150 mM of NaCl was sufficient to reduce the bacterial effect of hBD-2 by at least 15 fold.

When *L. plantarum* and hBD-2 were mixed with NaCl, some antibacterial activity against pathogens remained; this may be due to *L. plantarum* not being adversely affected by such conditions. Rao *et al.* (2004) reported that *Lactobacillus* species, such as *L. plantarum* possess the ability to grow at high NaCl concentration and different initial pH values. At the same time, *Lactobacillus* strains were able to ferment glucose in up to 8% NaCl and produce antimicrobial substances, e.g. LA and bacteriocins.

L. plantarum was selected for the present study because of its ability to produce antimicrobial substances, for example LA, and to tolerate harsh conditions. *L. plantarum* performed better than other species of *Lactobacillus*, and has been shown to diminish the negative effect of pathogens (Leroy and De Vuyst, 2004; Anderson *et al.*, 2010).

The effect of hBD-2, *L. plantarum* and mixture *L. plantarum* on expression of the *mprF* and *dlt* genes were different. The expressions of *mprF* and *dlt* genes were increased compared with the negative control (i.e. no probiotic or hBD-2). The results showed that the expression of inducer *mprF* was strongly up-regulated; no significant differences were observed between expression of this gene and *dlt* gene when hBD-2 and *L. plantarum* were used synergistically.

Interestingly, *mprF* and *dlt* gene expression were increased in level but without functioning. This may be due to the mode of action of hBD-2 and antimicrobial substances (produced by *L. plantarum*) in acidic pH, and play an important role by overlapping with cell wall syntheses (obstruction). Acidic pH is better and encourages peptides in function (Chen *et al*., 2005). The results in the current study are supported with a study conducted by Chen *et*

al. (2005), which demonstrated that there is synergistic effect of AMPs, such as hBD-1, 2 and 3 in acidic pH conditions against *S. aureus* and *E. coli*. This effect was significantly enhanced in acidic conditions (pH 4.6), as well as resembling normal skin pH. Seidenari and Giusti (1995) and Ring *et al.* (2000) reported that the significance of pH in the skin is that it acts as a defence mechanism against pathogens. In addition, the alteration of skin pH is one of the pathological factors involved in skin conditions such as eczema. However, in the present study was found that the antibacterial activity of hBD-2 could be to some extent enhanced in acidic conditions (organic acids, bacteriocin and others produce by *L. plantarum*).A study conducted by Lemaire *et al*. (2007) observed that low pH prevents the expression of some genes responsible for cell wall synthesis, such as those responsible for PBP2. In this case, non-functioning PBP2 is produced in conjunction with reduced recruitment of PBP2a in facilitating resistance. This may be due to the effect of acidic pH on genes such as *mprF* and *dlt*, as well as differences in expression to *mprF* and *dlt* genes. However, further investigations with a wide range of MRSA strains are required to explain this.

As for *dlt* gene, D-alanine (cationic charge) may be no transfer to techoic acids (anionic charge) to reduce negative charge, and then increase affinity between hBD-2 and cell wall. This case is leading to increase affinity between MRSA cell wall and hBD-2 (Nizet, 2006), and other antimicrobial substances (organic acids, bacteriocins and others). On the other hand, Lehrer and Ganz, (2002) mentioned that in the case resistant bacteria, the *dlt* gene exists in other Gram positives, such as *S. aureus* and is involved in the transfer of D-alanine into TAs. Bacteria overexpressing the *dlt* gene may

possess large amounts of d-alanine esters in their TA, thus binding cationic peptides less well and as a result may be less sensitive to the effects of AMPs such as hBD-2. Moreover, Peschel and Vincent (2001) demonstrated that the *mprF* gene mutant was more sensitive to killing by defensins, and was found to lack a positively charged membrane phospholipid derivative enriched in the amino acid L-lysine. Furthermore, Peschel *et al*. (2001) observed that increase binding of AMPs with bacterial cell wall in the case of absence lysylphosphotidylglycerol.

4.5 Conclusions

The current study was observed that hBD-2 had a significant impact on different bacterial human skin pathogens, particularly with a long incubation period at 37 °C for 5 h. In addition, this study suggests that when hBD-2 is used in conjunction with *L. plantarum,* synergistic antimicrobial activities are observed, in particular against MRSA, which frequently colonizes the skin; this synergistic / additive effect is possibly influenced by acidic conditions caused by the growth of *L. plantarum*, which may have corresponded to normal skin pH although this was not confirmed with appropriate measurements during the study. Therefore, further study of the effect of pH is required. Moreover, although there was a negative impact on the effectiveness of hBD-2 in the presence of NaCl, especially at high concentrations (150 mM), there was a positive impact when hBD-2 and *L. plantarum* were used with NaCl against pathogens. Finally, there is the importance of hBD-2 in getting rid of bacterial infections through the results of

gene expression, which demonstrated up-regulation of the *mprF* and the *dlt* genes in MRSA.

Chapter 5

Restoration of the susceptibility of methicillin-resistant *Staphylococcus aureus* (MRSA) to beta-Lactam (β-lactam) antibiotics by probiotic *Lactobacillus plantarum*

The results have been presented at V International Conference on Environmental, Industrial, and Applied Microbiology, Medicine College, Madrid University, Madrid, Spain, 2nd - 4th October 2013 and the abstract published in the conference proceedings. The results have also been published as paper (Restoration of the susceptibility of methicillin-resistant *Staphylococcus aureus* (MRSA) to β-Lactam antibiotics by probiotic *Lactobacillus plantarum* (*L. plantarum*). In: A. Méndez-Vilas, An Industrial, medical and environmental applications of microorganisms: current status and trends. Wageningen Academic Publishers, ISBN: 978-90-8686-243-6, ISBN E-book: 978-90-8686-795-0, pp. 585 - 589.

Chapter 5: Restoration of the susceptibility of methicillin-resistant *Staphylococcus aureus* (MRSA) to beta-Lactam (β-lactam) antibiotics by probiotic *Lactobacillus plantarum*

5.1 Introduction

S. aureus is an important bacterium amongst other bacterial pathogens in humans and animals, and it causes a broad spectrum of diseases, ranging from skin and soft tissue infection, bone / joint infection, myositis, endocarditis, pneumonia and bacteraemia, to life threatening infections like necrotizing fasciitis, septicaemia and toxic shock syndrome-1 (TSS-1) (Lowy, 1998). Fluit *et al.* (2001) reported that some *S. aureus* infections represent a challenge to treatment, particularly those genera which contain MRSA and related beta-lactams. Currently, MRSA is spreading endemically and has been diagnosed in most hospitals around the world, accounting for 40 - 70% of global nosocomial *S. aureus* infections in intensive care units (Blomquist, 2006). In the last decade, MRSA has become an increasing danger, not only in hospitals, but also in general society (Fridkin *et al.*, 2005; Moran *et al.*, 2006). Infections caused by MRSA represent a serious threat to humans and animals and they are of major concern to health authorities. In addition, MRSA has been identified as the main nosocomial pathogen in several parts of the world (Simor *et al.*, 2001; Graffunder and Venezia, 2002). Libert *et al.* (2008) reported that in addition to its influence on farm animals, MRSA infections in humans have been associated with increased mortality and morbidity and increased length of hospitalization. Roghmann and McGrail (2006) found that in the period 2000 - 2006 the prolonged therapeutic use of

antibiotics in humans and the administration of antibiotics as promoters of growth in the diet of farm animals have been associated with the development of antimicrobial-resistant bacteria. The majority of antimicrobials (antibiotics) are given to patients randomly and without previous consultation by physicians, and this has led to an emergence of methicillin resistance. One of the main strategies which has developed recently to solve this problem is the use of probiotic microorganisms (Tagg and Dierksen, 2003; Roghmann and McGrail, 2006). Alvarez-Olmos and Oberhelman (2001) observed that antibiotic resistance is almost always attributable to *S. aureus* infections where there has been chronic exposure to antibiotics. This has led to a renewed emphasis on environmental methods to prevent diseases, which makes probiotic microorganisms a very interesting area for more research. Probiotic bacteria, for example LAB, are promising because they generate antimicrobial substances, e.g. bacteriocins and some enzymes, that are able to prevent biofilm formation and the growth of pathogens. For example, Ammor *et al.* (2006) reported that some probiotic LAB are highly antagonistic to biofilm forming MRSA. Maragkoudakis *et al.* (2006) reported that useful effects were conferred by a probiotic of *Lactobacillus* species through the inhibition of Gram positive and Gram negative pathogenic bacteria. Charlier *et al.* (2008) demonstrated that even low acidifying strains of the probiotic *Lactococcus lactis* were able to exert inhibitory effects during the early stages of culture.

Probiotic LAB were reported to exert a highly inhibitory effect on *S. aureus* growth. Many reasons have been proposed for the inhibition of *S. aureus* by

probiotic LAB substances, such as the production of organic acids (Gilliland and Speck, 1977), bacteriocins, H_2O_2 and others (Velraeds *et al.*, 1996).

There are two types *S. aureus*, methicillin susceptible *S. aureus* (MSSA) and methicillin resistant *S. aureus* (MRSA). MSSA possesses the *mecA* gene, which encodes four types of PBP (1 - 4) (Ubukata *et al.*, 1989). In MSSA strains, all PBPs are inactivated in the presence of β-lactam antibiotics, leading to cell wall death that is caused by the inhibition of the transpeptidase step reaction, one of the final steps in cell wall biosynthesis. In contrast, MRSA survives in the presence of β-lactams because of an extra PBP (PBP2a) that is specific to MRSA and has a low binding affinity to all β-lactams. It retains its activity in the presence of β-lactams, allowing MRSA cell wall biosynthesis to continue. The *MecR1-MecI* system was identified as a regulatory factor for PBP2a expression (García-Castellanos *et al.*, 2004). Furthermore, a genetic mobile element containing the *mecA* gene, called staphylococcal chromosome *mec* (*SCCmec*), is believed to be transferred between staphylococci (Hanssen and Ericson Sollid, 2006). Deurenberg and Stobberingh (2008) demonstrated that *SCCmec* is classified into seven groups, and *SCCmec* is suitable for epidemiological studies of MRSA.

The aims of the current study were to evaluate the antimicrobial activity of some probiotic *Lactobacillus* species against MRSA and, in addition, to explore the role of probiotics in the modulation of methicillin resistance in MRSA, using culture supernatant of *L. plantarum*.

5.2 Material and methods

Methicillin sodium sulphate ($C_{17}H_{20}N_2O_6S$) powder (1 mg / ml) was purchased from (Sigma-Aldrich Ltd., UK). Antibiotic discs were obtained from Oxoid, UK. Antibiotics include cefotaxime 30 µg (CTX), ampicillin 10 µg (AMN), tetracycline 30 µg (TET), ciprofloxacin 10 µg (CIP), azithromycin 15 µg (AZM), lincomycin 2 µg (MY), streptomycin 10 µg (S), chloramphenicol 20 µg (CHP), gentamicin 10 µg (GN) and sulpha trimethoprim 25 µg (SXT). In addition, MHB, MRSB and NB were used. Several acids were used in the current study: lactic acid (88%; LA), acetic acid (100%; AA), citric acid (99.5%; CA), and propionic acid (99%; PA), which were supplied by Sigma (Sigma-Aldrich Ltd., UK). Sodium lactate 71% was also used, and supplied by Merck - BDH, UK. Microtitre plates were obtained from Sterilin Ltd. (Stone, UK). PBS tablets and lysostaphin provided by Sigma, UK. All tests were replicated three times under the same identical experimental conditions.

5.2.1 *Lactobacillus* species and methicillin resistant *S. aureus* used in this study

The following *Lactobacillus* species were used in the current study: *L. plantarum*, *L. casei* and *L. acidophilus* (10^7 CFU / ml). These species were selected because they showed the greatest antimicrobial activity, aggregation, biofilm formation against bacterial human skin pathogens, as reported in Chapter 4. Three strains of MRSA (10^6 CFU / ml) were also used, as follows: MRSA 12493, MRSA 33591 and MRSA 25691, as outlined in Table 2.1.

5.2.2 Agar well diffusion and agar spot methods

To assess the antimicrobial activity of selected probiotic *Lactobacillus* species, several *Lactobacillus* species and MRSA strains were used in this experiment. *L. casei*, *L. acidophilus* and *L. plantarum* were tested as the probiotics, as listed in Table 2.1. In addition, MRSA 33591 and MRSA 12493 were used as the pathogens. *Lactobacillus* species were grown on MRSB and MRS agar, MRSA strains were grown on NB, as described in sections 2.2.4 - 2.2.5.

5.2.3 Effect of different organic acids on MRSA

LA was used in different concentrations (*v / v*) (0.0312%, 0.0156% and 0.0078%). Other acids (1.5%) (*v / v*) were also used, namely: AA (pH 4.15), CA (pH 6) and PA (pH 5.1). Organic acids were used with methicillin discs in different concentrations (10, 15 and 20 µg / ml) on MRSA, by incorporation into the growth medium. Acids in different concentrations were mixed with 10 ml NA, which was then left to solidify. After that 100 µl of MRSA was spread onto the solid medium. Then different methicillin discs were fixed on the agar, and plates incubated at 37 °C for 24 h, as shown in Figure 5.1. After incubation, the diameter of the inhibition zone was measured (in mm) with Vernier callipers (Figure 2.1). A clear zone of 2 mm or more around a spot was considered as positive (Tahara *et al.*, 1996). Aliquots of fresh NA without organic acids were used as controls.

5.2.4 Effect of *L. plantarum* supernatant on methicillin resistance and other antibiotics

In order to assess the effect of probiotics on MRSA antibiotic resistance, the supernatant of *L. plantarum* was harvested. Different volumes of supernatant (200 and 400 µl) were mixed with NA (10 ml). The medium was then left to solidify, and afterwards 100 µl MRSA (10^6 CFU / ml) was spread on the agar surface. Next, antibiotic discs of methicillin and other antibiotics (CTX, CIP, AMN, TET, AZM, MY, S, C, GN and SXT) were fixed onto the agar, and were incubated at 37 °C for 24 h, as described in Figure 5.1. Inhibition zones were measured using Vernier callipers, as mentioned in section 5.2.3. Aliquot of fresh NA without supernatant of *L. plantarum* was used as a control.

5.2.5 Effect of neutralised *L. plantarum* supernatant, MRSB and sodium lactate on methicillin resistance

L. plantarum supernatant was neutralised (pH 7.0) by adding 1 M NaOH and applied against MRSA 33591 by incorporation into the medium. 200 and 400 µl of neutralised supernatant *L. plantarum*, 200 µl 71% sodium lactate, 200 and 400 µl MRSB were applied against MRSA 33591. The procedure was the same as that outlined in section 5.2.4. Then antibiotic discs were fixed on the agar, and plates incubated at 37 °C for 24 h, as shown in Figure 5.1. Inhibition zones were measured using Vernier callipers, as outlined in section 5.2.3. Aliquot of fresh NA without neutralised supernatant of *L. plantarum*, sodium lactate or MRSB was used as a control.

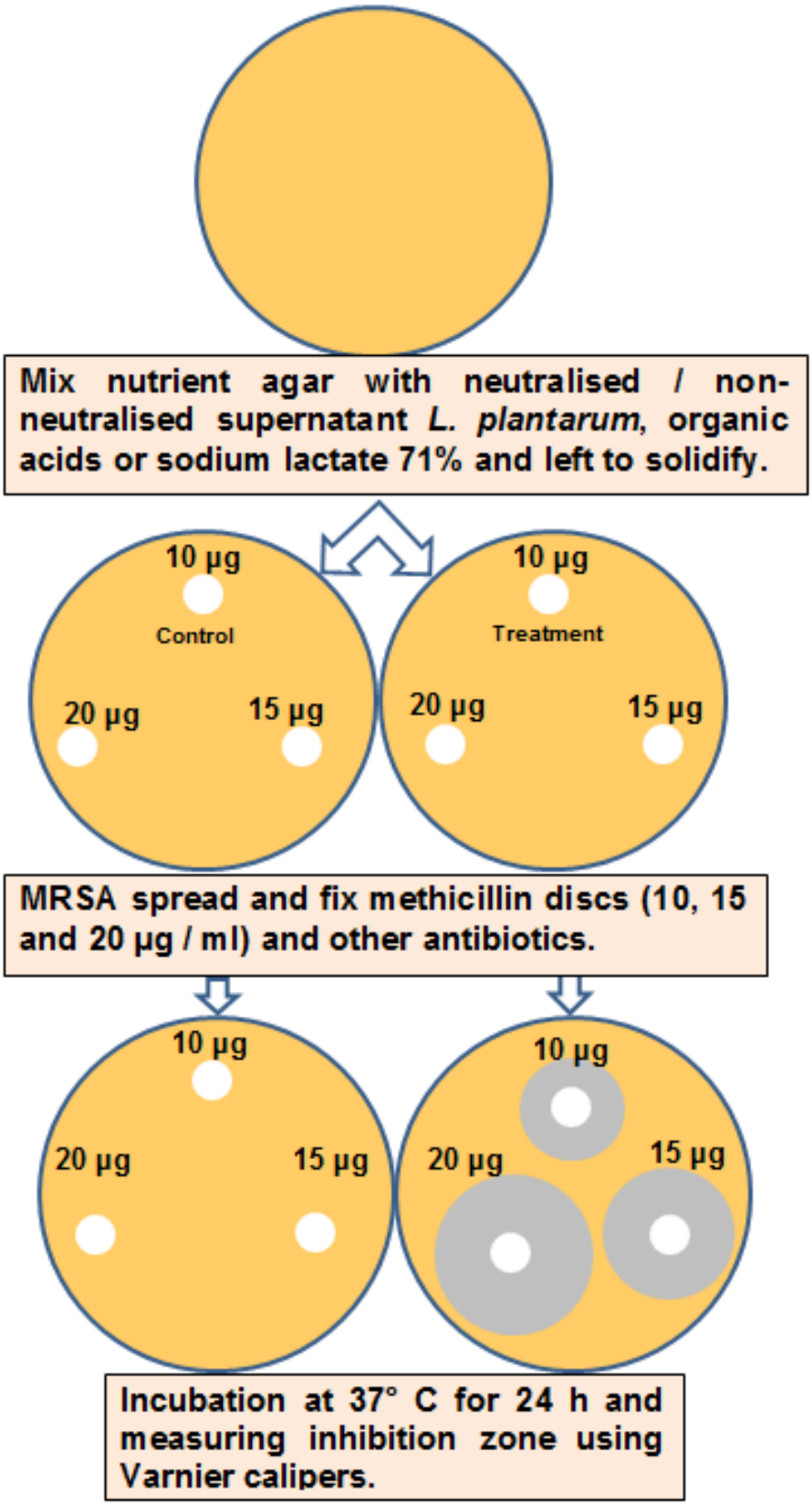


Figure 5.1 Procedure to determine the effect neutralised / non-neutralised supernatant *L. plantarum*, organic acids and sodium lactate 71% with different concentrations methicillin, which are observed the antimicrobial activity of different treatments against MRSA.

5.2.6 Determination of the minimum inhibitory concentration of methicillin

The minimum inhibitory concentration (MIC) of methicillin was determined using a microtitre method (American National Standards Institute 1991). The methicillin stock solution was prepared using methicillin sodium sulphate (1 mg / ml) in sterile distilled water. Muller-Hinton broth (950 µl) and 50 µl of MRSA 33591 (10^7 CFU / ml) were mixed, and distributed into each well at optical density 0.5. Methicillin solution in different concentrations: 0.001, 0.002, 0.004, 0.006,0.008, 0.01, 0.012, 0.014, 0.016, 0.02, 0.024, 0.028, 0.032, 0.036, 0.04, 0.08, 0.150, 0.200, 0.25, 0.3, 0.35 and 0.4 µg / ml were put into microtitre plate wells, then incubated at 37 °C for 24 h. The growth density was determined using a Magellan Plate Reader (OD at 595 nm; Molecular devices, UK Ltd., UK). Aliquots of fresh MHB and MRSA without methicillin were used as control.

5.2.7 Determination of MIC for methicillin against MRSA with supernatant of *L. plantarum*

The MIC of methicillin was determined to be 0.042 µg / ml using a microtitre method, as described in section 5.2.6. Ten µl of bacterial suspension (10^7 CFU / ml) was inoculated in each well at OD (0.5), along with 8.5 µl methicillin antibiotic solution (0.042 µg / ml). Different volumes (1, 2, 4, 6, 8, 10 and 12 µl) of filtered supernatant of *L. plantarum* (pH 3.73) were added to MHB up to a total volume of 200 µl. In addition, aliquots of fresh MHB, with / without methicillin were used as controls. Plates were incubated at 37 °C for 24 h. The growth density was determined, as described in section 5.2.6.

5.2.8 Modulation of methicillin resistance genes by probiotics

5.2.8.1 RNA extraction from bacterial cells

MRSA 33591 cells (10^7 CFU / ml) were cultured at 37 °C for 18 - 20 h in 20 ml final volume, containing: 17.15 ml MHB, 850 µl methicillin (0.042 µg / ml), 1 ml broth MRSA culture bacteria and 1 ml *L. plantarum* supernatant (pH 3.73). MRSA cells were harvested by centrifuging at 2772 × *g* (Harrier 18 / 80, MSE, UK.) for 10 minutes, and washed twice using PBS. Pellets were re-suspended in 1 ml PBS and the OD of the suspension was adjusted to 1.0 at 595 nm by adding an appropriate volume of PBS. Total RNA was extracted using the Rneasy Protect Bacteria Mini Kit (Qiagen, UK) according to the manufacturer's instructions. A control set was prepared and incubated at 37 °C for 18 - 20 h. The cells were centrifuged at 13000 x *g* (Heraeus Fresco 21 Microcentrifuge, USA) for 3 minutes, and washed twice using PBS, as described in section 4.2.4.1. Finally, RNA concentration and quality were evaluated using a NanoDrop spectrophotometer, as outlined in Appendix 2 (Figure 2.2).

5.2.8.2 Agarose gel electrophoresis

In order to determine the quality of RNA, agarose gel electrophoresis was used, as described in section 4.2.4.2.

5.2.8.3 Reverse transcription and cDNA synthesis

Reverse transcription (RTs) was performed to create cDNA. This was carried out using a High Capacity RNA-to-cDNA 50 x Kit (Fisher Scientific, UK Ltd.), according to the manufacturer's instructions, as described in section 4.2.4.3.

5.2.8.4 Quantitative RT - PCR (qRT - PCR)

qPCR was carried out to semi-quantitatively to analyse gene expression levels in the experiment relative to the reference gene (the control). PCR amplification was performed with primers, with pyruvate kinase (*Pyk*) as a housekeeping gene, and *mecR1*, *mecA* and PBP2 as target genes (Table 5.1). The target primer's genes was designed using the mRNA sequences, which were obtained from a primer blast in the NCBI (National Centre for Biotechnology Information), as described in section 4.2.4.4.

Table 5.1. Primers sequences (housekeeping and target genes) used for qRT-PCR reactions.

Primer	Primer sequence (5´…3´)	Product size (bp)	Primer concentration (pmol)	Reference
mecR1, F	5´-GTC GTT CAT TAA GAT ATG ACG 3´	310	10	Suzuki *et al*. (1993)
mecR1, R	5´- GTC TCC ACG TTA ATT CCA TT 3´	310	"	"
mecA, F	5´ GGC AAT ATT ACC GCA CCT CA 3´	214	"	Mulvey *et al*. (2005)
mecA, R	5´ GTC TGC CAC TTT CTC CTT GT 3´	214	"	"
PBP2, F	5´- TGT GAA GAG AAC GAT TAT TAA G 3´	824	"	Boyl Varva *et al*. (2003)
PBP2, R	5´- ATG AAT TAT ACT CAG AAT CTT GAT 3´	824	"	"
Pyk, F	5´- GCA TCT GTA CTC TTA CGT CC 3´	90	"	Theis *et al*. (2007)
Pyk, R	5´ GGT GAC TCC AAG TGA AGA 3´	90	"	"

5.2.8.5 Normalisation of qRT - PCR data

In most cases, the data were normalised using the instrument software with auto-normalisation. However, the data were also normalized manually, producing the relative quantification (RQ) values. The data was first normalized against the housekeeping gene (*Pyk*). This was achieved by subtracting the cycle threshold (C_T) value of the housekeeping gene from the C_T value target genes (*mecR1, mecA* and PBP2) to produce the ΔC_T, as outlined in section 4.2.4.5.

5.2.9 Statistical analysis

Databases were subjected to balanced analysis of variance (ANOVA) and *post hoc* LSD) tests using Minitab v.16 and SPSS, as described in section 4.2.5.

5.3 Results

5.3.1 Agar well diffusion method

The antimicrobial activity of *Lactobacillus* species; *L. casei*, *L. acidophilus* and *L. plantarum* was tested against MRSA strains by measuring the inhibition zones generated around the MRSA growth. Overall, the BCB *L. plantarum* produced the greatest inhibition zones with MRSA 12493 (9.80 ± 0.28 mm) and MRSA 33591 (9.70 ± 0.57 mm) compared to *L. casei* and *L. acidophilus*. Moreover, the CFS *Lactobacillus* species demonstrated lower inhibition compared to BCB, and *L. plantarum* was more effective (4.70 ± 0.28 mm) against MRSA 12493 compared to other species, as outlined in Table 5.2.

The statistical analysis showed high significant differences ($P < 0.05$) with pathogens, probiotics, and treatments (BCB and CFS).

Table 5.2 Zones of inhibition (mm; mean ± SD) for MRSA strains with a broth culture (BCB) and cell free supernatant (CFS) of probiotic *Lactobacillus* species.

	MRSA 33591		MRSA 12493	
Probiotics	BCB	CFS	BCB	CFS
L. plantarum	9.70 ± 0.57$^{a, 1}$	4.20 ± 0.28$^{a, 1}$	9.80 ± 0.28$^{a, 1}$	4.70 ± 0.28$^{a, 1}$
L. acidophilus	8.00 ± 1.00$^{a, 1}$	3.50 ± 0.50$^{a, b, 1}$	8.20 ± 0.28$^{b, 1}$	4.00 ± 0.76$^{a, 1}$
L. casei	7.00 ± 0.57$^{b, 1}$	3.70 ± 0.28$^{b, 1}$	9.00 ± 0.57$^{b, 2}$	4.20 ± 0.28$^{a, 1}$

Values with the same superscript letters in each column are not significantly different, while mean values with same superscript numbers within each row are not significantly different.

5.3.2 Agar spot method

The inhibitory effect of BCB was also measured using the agar spot method. The Inhibition zones of MRSA strains using *Lactobacillus* broth growing in MRS 2% glucose were significant ($P < 0.05$) with *L. plantarum* (10.70 ± 0.57 mm) against MRSA 12493 compared to other *Lactobacillus* species. However, the effect of *Lactobacillus* in MRS 0.2% glucose produced a minor effect on MRSA strains. *L. plantarum* was more effective (2.30 ± 0.28 mm), compared to other species, as outlined in Table 5.3.

The statistical analyses observed high significant differences ($P < 0.05$) with pathogens, probiotics, and treatments (modified and unmodified MRS).

Table 5.3 Zones of inhibition (mm; mean ± SD) on modified and unmodified MRS for probiotic *Lactobacillus* species against MRSA strains.

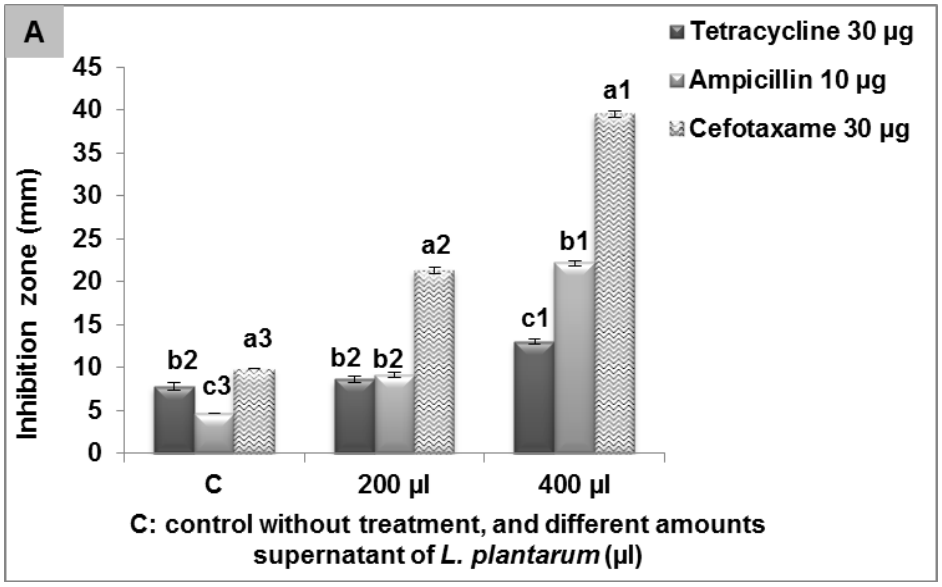
	MRSA 33591		MRSA 12493	
Probiotics	Unmodified	Modified	Unmodified	Modified
L. plantarum	$9.50 \pm 0.50^{a, 2}$	$2.30 \pm 0.28^{a, 1}$	$10.70 \pm 0.57^{a, 1}$	$2.30 \pm 0.28^{a, 1}$
L. acidophilus	$8.70 \pm 0.28^{b, 2}$	$1.70 \pm 0.57^{a, b, 1}$	$10.00 \pm 0.50^{a, 1}$	$2.00 \pm 0.50^{a, 1}$
L. casei	$7.80 \pm 0.76^{b, 1}$	$0.80 \pm 0.28^{b, 1}$	$8.00 \pm 0.50^{b, 1}$	$0.90 \pm 0.52^{b, 1}$

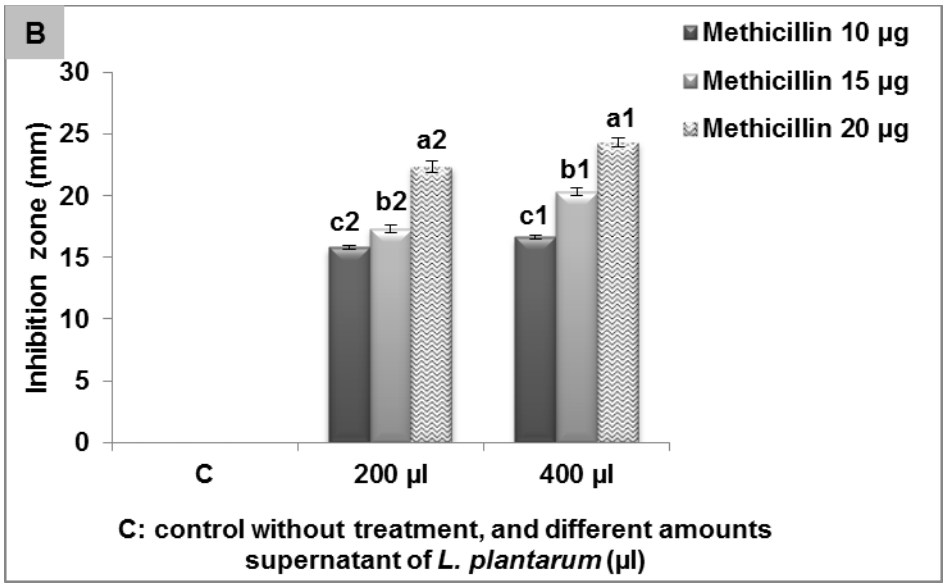
Values with the same superscript letters in each column are not significantly different, while mean values with same superscript number within each row are not significantly different.

5.3.3 Effect of *L. plantarum* supernatant with methicillin and different antibiotics on MRSA 33591

The results varied depending on the type and dose of antibiotics and the amounts of supernatant of *L. plantarum*. The results were significant ($P < 0.05$) with 400 µl supernatant of *L. plantarum*, as presented in Figure 5.2 (A - C). The results were as follows: cefotaxime (39.5 ± 0.5 mm), methicillin 20 µg

(24.33 ± 0.76 mm), ampicillin (22.16 ± 0.28 mm), methicillin 15 µg (20.33 ± 0.57 mm) and tetracycline (13 ± 0.5 mm), as outlined in Figure 5.2 (A - C), and Appendix 3 (Figures 3.3 - 3.4).





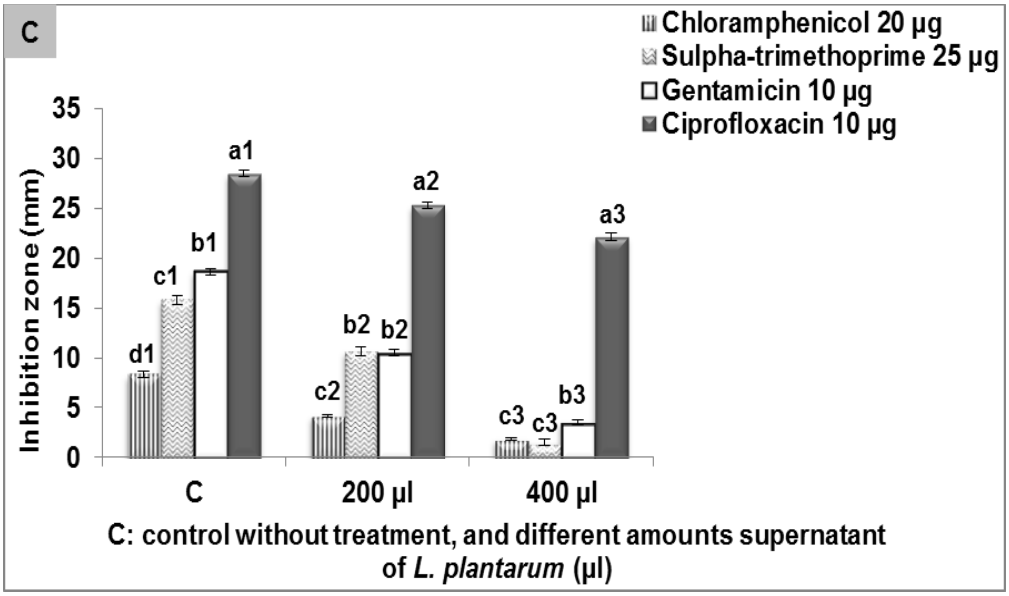


Figure 5.2 Zone of inhibition (mm; mean ± SD) for different antibiotics with different amounts supernatant of *L. plantarum* (200 and 400 µl) against MRSA 33591 (A) cefotaxime, ampicillin and tetracycline (B) different methicillin concentrations (10, 15 and 20 µg / ml), and (C) ciprofloxacin, gentamicin, sulpha-trimethoprime and chloramphenicol. The statistical analyses showed significant differences with antibiotics, supernatant and overlap between antibiotic with supernatant of *L. plantarum*. The data are mean ± SD from three independent identical experiments. Values with the same subscript letters with different antibiotics are not significantly different, while values with the same subscript numbers in different amounts supernatant of *L. plantarum* are not significantly different.

The results with some antibiotics demonstrated that they were less effective when the amount of supernatant of *L. plantarum* was increased to 400 µl, as follows: ciprofloxacin (22.16 - 28.5 mm), gentamicin (2.3 - 18.6 mm), chloramphenicol (1.83 - 8.33 mm) and sulphatrimethoprime (1.5 - 15.83 mm), as outlined in Figure 5.2 C. In addition, some results were not significant (resistance and no inhibition zone) with the following antibiotics: azithromycin, lincocin and streptomycin.

5.3.4 Effect of lactic acid and other organic acids on MRSA

LA was used to assess its activity on MRSA growth; three different LA concentrations in combination with three concentrations of methicillin were applied. The greatest inhibition zones were observed with 20 µg / ml methicillin, when the medium contained 0.0312% LA (31 ± 1 mm), as presented in Figure 5.3 A and Appendix 3 (Figure 3.1).

Moreover, organic acids such as AA, PA and CA were used against MRSA in order to evaluate the role of pH in diminishing methicillin resistance in MRSA. The MRSA was exposed to 1.5%, as a final concentration of each acid. The results demonstrated the strongest inhibition zones with methicillin discs (20 µg / ml) against MRSA, especially with AA (38.5 ± 0.5 mm), whereas, methicillin discs used with CA (3.83 ± 0.28 mm) and displayed weak or no effect on MRSA growth. Furthermore, PA produced significant inhibition (28.5 ± 0.5 mm) of MRSA, as outlined in Figure 5.3 B and Appendix 3 (Figure 3.2).

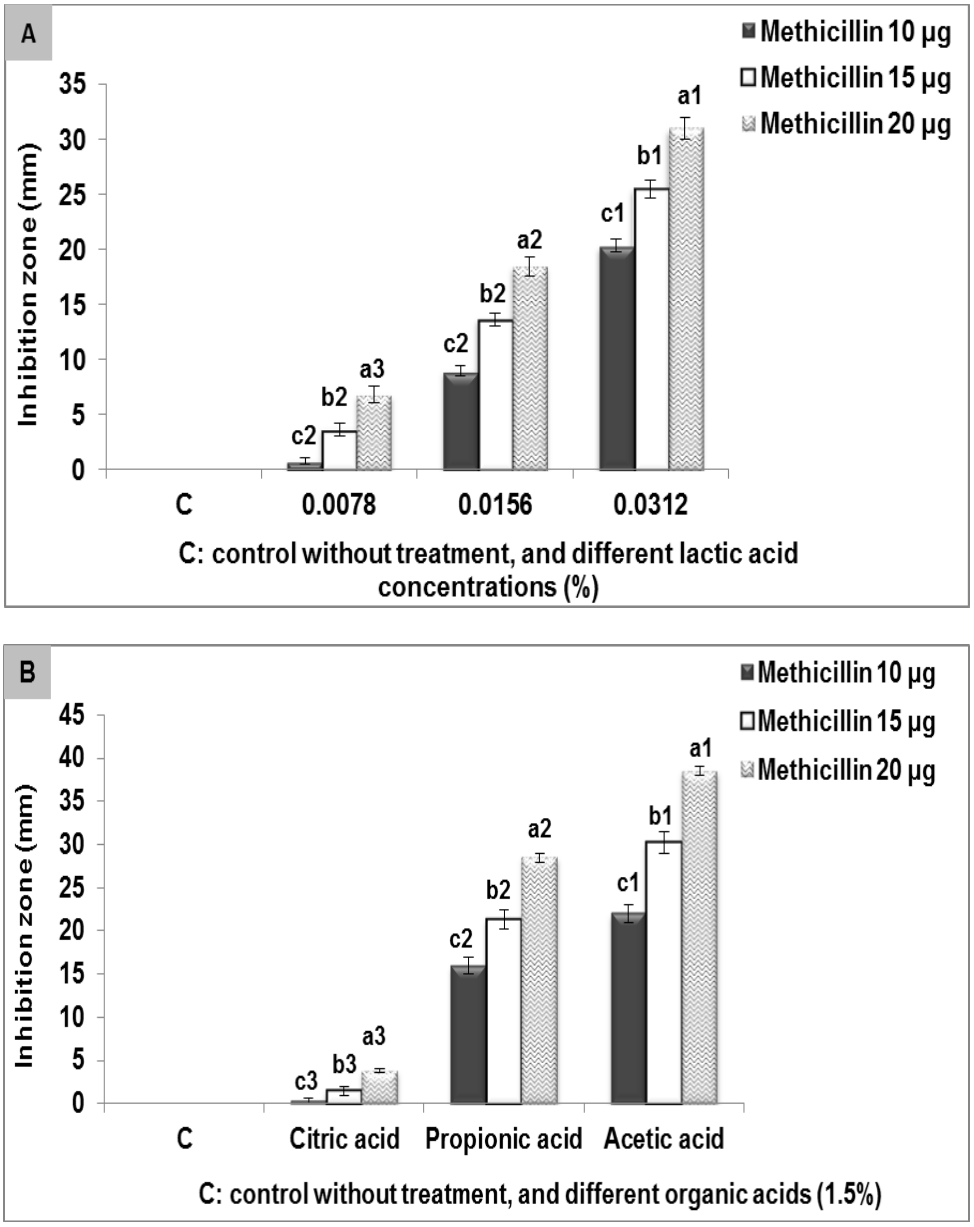


Figure 5.3 Zone of inhibition (mm; mean ± SD) for different concentrations of methicillin on NA (A) different concentrations LA, and (B) different organic acids 1.5% (AA, PA and CA) against MRSA. The statistical analyses found significant differences with methicillin, organic acids, and overlap between them. The data are mean ± SD from three independent identical experiments. Values with the same superscript letters with different amounts of organic acids are not significantly different, while value with the same superscript numbers in antibiotics are not significantly different.

5.3.5 Effect of neutralised supernatant of *L. plantarum*, sodium lactate, and MRSB, in conjunction with different concentrations of methicillin, against MRSA 33591

A neutralised supernatant of *L. plantarum*, sodium lactate 71% and MRSB were applied to MRSA in order to appraise the role of antimicrobial substances in reducing methicillin resistance in MRSA. The results demonstrated that the strongest inhibition of MRSA growth was with sodium lactate, and 20 µg / ml of methicillin more than the neutralised supernatant of *L. plantarum*, at different volumes (200 and 400 µl), as outlined in Figure 5.4 and Appendix 3 (Figure 3.5). No effect was detected using the MRSB with any concentration of methicillin, as outlined in Appendix 3 (Figure 3.6).

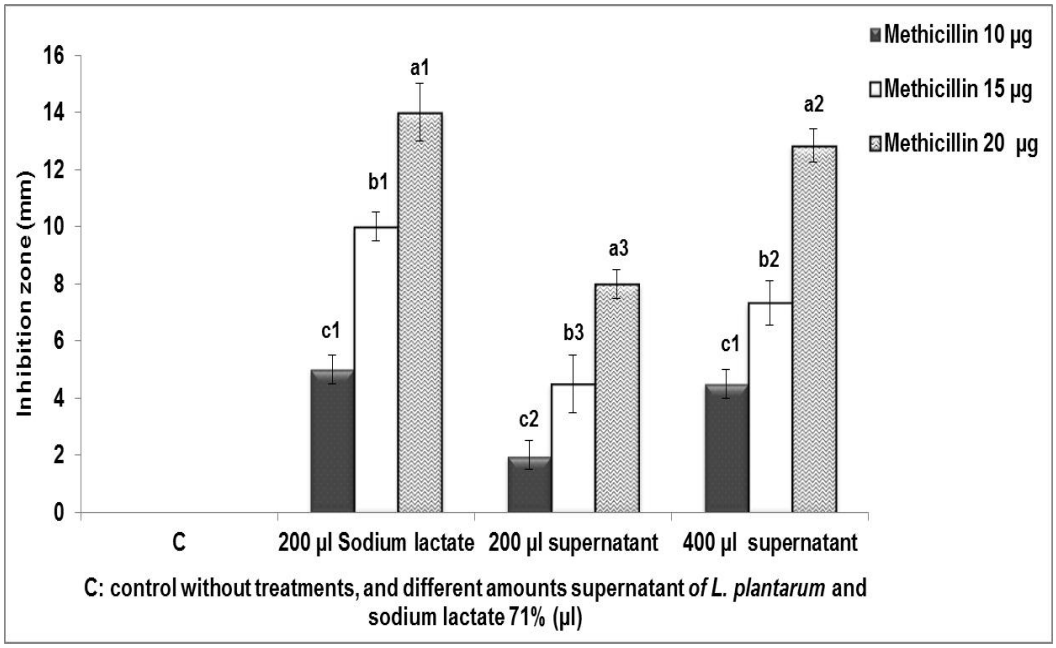


Figure 5.4 Zone of inhibition (mm; mean ± SD) for different methicillin concentrations against MRSA. Different volumes of neutralised supernatant of *L. plantarum* (200 and 400 µl) and sodium lactate 71% (200 µl) were mixed with media. Then methicillin discs fixed, and incubated at 37 ºC for 24 h. The statistical analyses observed significant differences between neutralised supernatant of *L. plantarum*, sodium lactate, and overlap between them. The data are mean ± SD from three independent identical experiments. Values with the same superscript letters in different amounts of neutralised supernatant of *L .plantarum* and sodium lactate are not significantly different, while mean values with the same superscript numbers in antibiotics are not significantly different.

5.3.6 MICs for methicillin and supernatant of *L. plantarum*

The MIC of methicillin MRSA was calculated in order to determine the sub-lethal concentration, which can modulate genes mediating methicillin resistance, followed by assessment of the role of CFS on the expression of those genes. The observed results indicated that 0.32 µg / ml of methicillin was the lethal concentration. However, to determine the effect of the supernatant of *L. plantarum*, a concentration of methicillin below MIC was selected. This concentration was 0.04 µg / ml. This level was chosen because microorganisms grow at this concentration. The MIC was obtained by the combination of different methicillin concentrations, and different volumes the supernatant of *L. plantarum* to the final pH 3.73. The observed results showed that 12 µl supernatant of *L. plantarum* and 0.04 µg / ml methicillin was the lethal concentration. A concentration of supernatant of *L. plantarum* and methicillin below MIC was selected. This concentration was 0.04 µg / ml and 10 µl the supernatant of *L. plantarum*.

5.3.7 *MecR1*, *mec*A, and PBP2a gene expression using qRT-PCR

In order to assess whether the CFS affected the expression of genes (*mecR1*, *mecA* and PBP2) mediating methicillin resistance in MRSA, the RNA of MRSA was successfully extracted, as outlined in Figure 5.5 and Appendix 2 (Figure 2.2) and cDNA was synthesized, using conventional PCR assay and qRT - PCR. The results displayed MRSA growth in presence of supernatant of *L. plantarum* and methicillin and a significant inhibition of *mecR1* gene expression compared to control samples, whereas, *mecA* and PBP2 were

strongly up-regulated after MRSA was treated with supernatant of *L. plantarum* and methicillin, as outlined in Figure 5.6 (A - C).

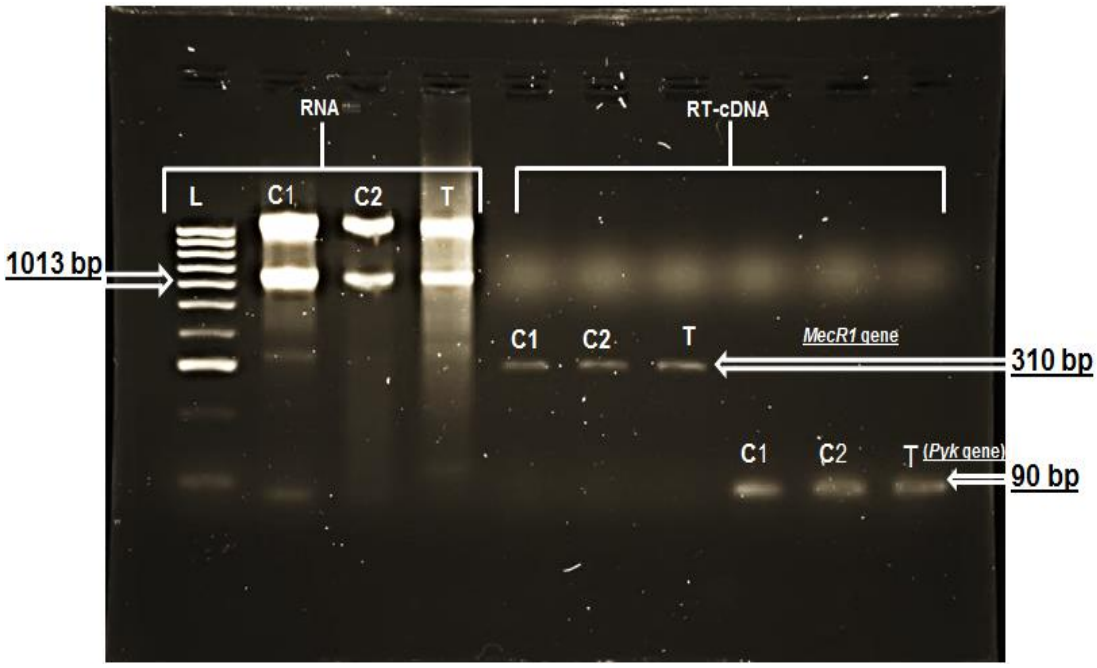


Figure 5.5 Agarose gel electrophoresis of RNA samples and amplification of cDNA. L: ladder (1013 bp), C 1: control 1 (without treatment), C 2: control 2 (with methicillin), T: treatment (with methicillin and supernatant of *L. plantarum*), *Pyk*: pyruvate kinase gene (housekeeping gene) and *mecA*: *mecA* genes (target genes).

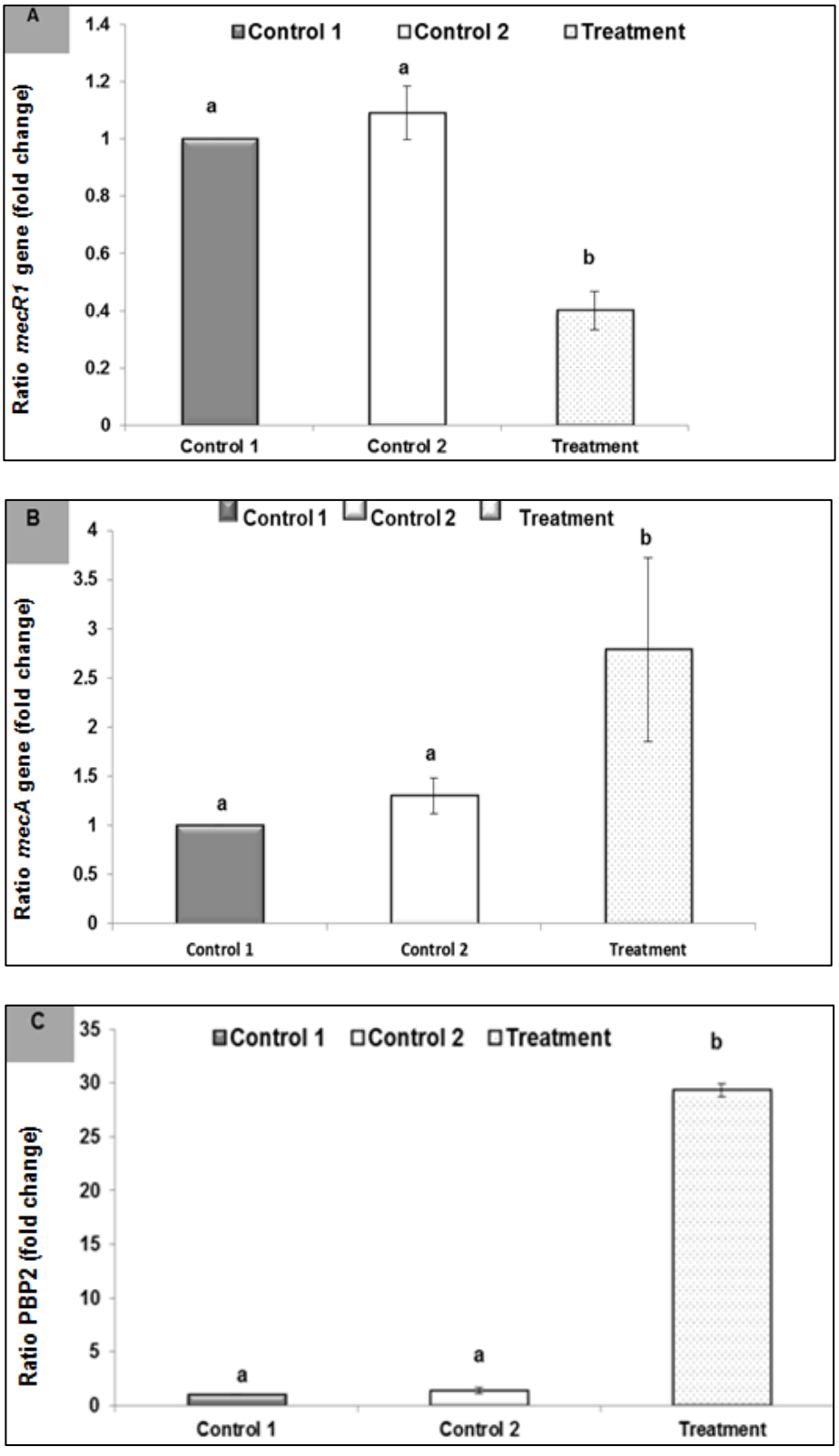


Figure 5.6 Gene expressions for MRSA with the mixture of methicillin and culture supernatant of *L. plantarum*, and as follows: (A) *mecR1* (down-regulation), (B) *mecA* (up-regulation) and (C) PBP2 (up-regulation), control 1 (without treatment), control 2 (with methicillin) and treatment (with methicillin and supernatant of *L. plantarum*). The statistical analyses observed significant differences. The data are mean ± SD from three independent experiments. Values with the same superscript letters are not significantly different.

5.4 Discussion

Probiotic lactobacilli are currently used to treat a range of microbiological diseases, to inhibit of growth of pathogenic microbes and for the modulation of the GIT immune system against harmful microorganisms (Fooks and Gibson, 2002). In the current study, three *Lactobacillus* species were used to study their effect on MRSA resistance to methicillin. The present study examined the antimicrobial activity of probiotic *Lactobacillus* species using both BCB and CFS.

The results demonstrated an inhibitory effect of *Lactobacillus* species against MRSA strains. The majority of MRSA strains appeared to be more sensitive to BCB compared to CFS, but to varying degrees, and *L. plantarum* displayed the strongest inhibition zone compared to other species. The antimicrobial activity of probiotics for different *Lactobacillus* species is represented by the production of numerous anti-bacterial substances, such as organic acids, bacteriocins, CO_2, H_2O_2 or other substances as antibiotics (Vallor *et al.*, 2001), as described in section 2.4.

As for different organic acids and supernatant *L. plantarum* results, the effect of LA was very effective and significant. This antagonism is believed to be due to the action of LA on the bacterial cytoplasmic membrane, which interferes with the maintenance of membrane potential and inhibits active transport (Sheu *et al*., 1972; Eklund, 1989; De Vuyst and Vandamme, 1994). Moreover, Cherrington *et al.* (1991) reported that the cause may be mediated both by dissociated and un-dissociated acid. As well as the pH effect, un-dissociated LA collapses the electrochemical proton gradient, causing bacteriostasis and finally the death of the target bacteria (Eklund, 1989).

Furthermore, LA has been shown to permeabilize the external membrane of Gram negative bacteria by the release of LPS, leading to loss of viability (Arqués *et al*., 2004).

Several studies have observed the effect of organic acids on pathogens, e.g. the study conducted by Domenech *et al.* (1992), which showed that four *S. aureus* strains incubated at 37 °C for 24 h in broth progressively acidified with different organic acids (lactic, citric, propionic, pyruvic, and ascorbic acids), and survival in different rates. Another study, carried out by Nihei *et al.* (1993), reported that petroleum jelly containing 3% AA was sufficient to remove MRSA on cutaneous lesions. Several factors may explain the susceptibility *S. aureus* to antibiotics after treatment with acids. One of these explanations was revealed in a study conducted by Sabath *et al.* (1972), which reported that the growth of bacterial cells in medium at pH 5.2 before antibiotic susceptibility testing at pH 7.4 failed to suppress antibiotic resistance, and indicates (a) that the gene determining methicillin resistance was not eliminated during growth at acid pH and (b) that suppression of resistance is easily reversed. There is another opinion that the acid medium itself prevents methicillin resistant bacterial cells from growing, whether the antibiotic is present or absent. Recently, Lemaire *et al.* (2008) observed that the susceptibility of MRSA strains to β-lactams, especially methicillin can be restored by acidic pH (the relationship between pH and the function of PBP-2a was examined). In addition, acidic pH has the following effects: (a) β-lactam antibiotics interact with PBP-2a with high affinity, and (b) PBP-2a undergoes a conformational alteration in the presence of β-lactam antibiotics consistent with the opening of the active site from the closed conformation.

Remarkably, the results for non-neutralised *L. plantarum* supernatant (pH 4.85) were the same as with different organic acids, and were highly significant. Several products in metabolism of *Lactobacillus* species, which contain mainly LA and AA (Thanh *et al.*, 2009). In addition, many products in addition to organic acids are produced by *L. plantarum* strains, for example bacteriocin, which contributes to the microbial activity, and a broad spectrum of antimicrobial activity against pathogens has previously been observed (Van Thu *et al.*, 2011; Sankar *et al.*, 2012). Furthermore, Lee *et al.* (2013) revealed that bacteriocins KU24 produced by some LAB, such as *Lactococcus lactis* KU24, exhibited inhibitory effects against MRSA. Interestingly, Sabath *et al.* (1972) reported that the susceptibility of MRSA to traditional β-lactams can be almost fully restored if MRSA is grown at pH 5.5 or lower. This fact was first described in the early 1970s in relation to healthcare-associated MRSA (HA-MRSA) growing in broth and exposed to β-lactams, e.g. penicillins or first generation cephalosporins. This has now proved to be the case with other β-lactams, including carbapenems, and also applies to CA-MRSA (Lemaire *et al.*, 2007). Hartman and Tomasz (1984) and Lemaire *et al.* (2007) demonstrated that this influence of low pH is due to (1) reduced copy numbers of PBP-2a (reduced or even explicit absence of the expression of PBP-2a) and (2) a lack of transpeptidase activity of PBP-2a at acidic pH. Lemaire *et al.* (2007) mentioned that the first reason be can rejected, since the expression of *mecA* and the immunodetection of the encoded PBP-2a are both unaffected by low pH. Therefore, the second reason cannot be ruled out and is probably the most acceptable. In fact, the active site of this protein is protected under normal conditions, which is the

main reason for the lack of effective inhibition of the enzyme by traditional β-lactams.

In the current study, the results for ciprofloxacin, chloramphenicol, gentamicin and trimethoprim were different and MRSA became resistant rather than sensitive. This may be because there is overlap between supernatant and antibiotics, and then supernatant destroyed and disrupted role of antibiotics. In addition, some antibiotics, such as azithromycin, lincocin and streptomycin were still resistant without any changes with regardless of the amount of supernatant used. Many reasons may be behind this and further investigations are required to explain this.

The results with neutralised (pH 7) *L. plantarum* supernatant and sodium lactate 71% were significant. The activity of inhibitors in the supernatant of *Lactobacillus* species is due to substances produced by these species, such as organic acids, and also to other inhibitory substances, such as CO_2, H_2O_2, bacteriocins and others (Ogunbanwo, 2005). Van Thu *et al.* (2011) and Sankar *et al.* (2012) reported that several products in addition to organic acids produced by various strains of *L. plantarum*, e.g. bacteriocin, participate in inhibitory activity, and have a broad spectrum of antimicrobial activity against pathogens. In the current study, no activity against MRSA was observed in MRS broth. The reasons may be due to the absence of probiotic *L. plantarum* (probiotic *Lactobacillus* species ferment sugar to organic acids and produce other substances), as mentioned previously.

In the current study, the effects of non-neutralised supernatant of *L. plantarum* on the expression of genes contributing to methicillin resistance were studied. The results revealed that the expression of inducer gene

mecR1 was strongly downregulated after exposure to CFS and methicillin compared to controls, which potentially means weak expression of *mecA* was induced by *mecR1*. Moreover, increasing *mecA* gene expression in the control sample may have been due to unexplained factors, as has been previously observed (Chen *et al.*, 2011). However, the gene expression data recorded a strong increase in both PBP2 and *mecA* genes. This is probably attributable to different regulatory pathways of *mecA* gene expression. Moreover, several studies report that there are several regulators for the *mecA* gene. One such study, carried out by Arede *et al.* (2012), observed that no correlation was found between the existence of functional *mecR1-mecI* genes and the level of β-lactams resistance in a representative collection of epidemic MRSA, and display that the *mecA* gene regulatory locus consists. The *mecR2* function is necessary for the full induction of *mecA* expression, which compensates for the incompetent induction of the *mecA* by the *mecR1*, and allows the best expression of β-lactams resistance in MRSA with functional *mecR1-mecI* regulatory genes (Figure 5.7).

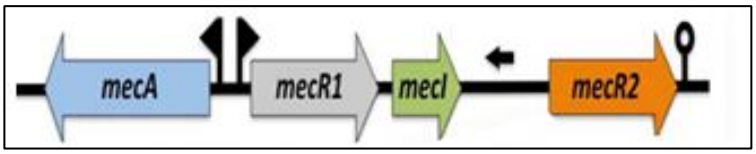


Figure 5.7 *MecA* regulation by *mecR2* instead of *mecR1-mecI* and *blaR1-blaI* pathways, adapted from Arede *et al*. (2008).

In addition, there are other regulators of *mecA,* as mentioned in other studies. Two studies carried out by Ryffel *et al.* (1992) and Hiramatsu (1995), reported that the *blaR1-blaI* complex (concerned with β-lactase production) is

able to regulate the expression of the *mecA* gene. MRSA that have a dysfunctional regulatory region can either express the *mecA* constitutively or they can use the β-lactamase regulatory genes to optimally express the *mecA*, due to *blaR1* being a strong inducer of the *mecA*, and the *blaI* a weak repressor. Hiramatsu (1995) revealed that the *mecR1* and *mecI* genes have a high degree of homology to the *blaR1-blaI* genes (Figure 5.8).

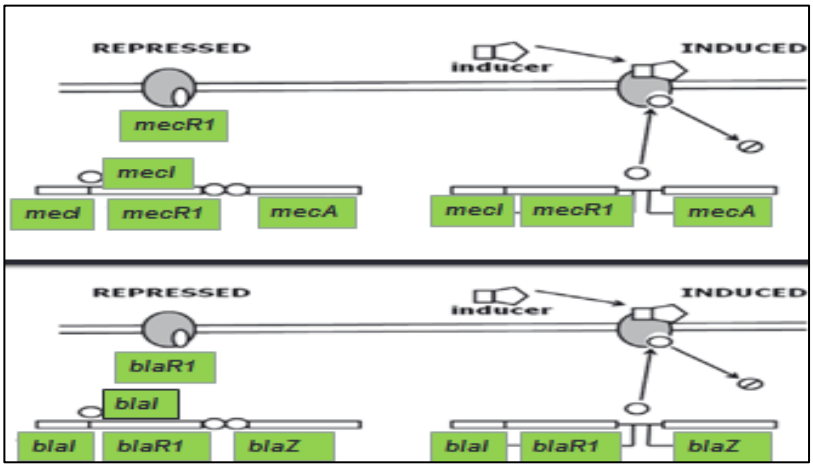


Figure 5.8 Regulation of *mecA* by *mecI-mecR1* and *blaI-blaR1*. *MecI* represses transcription of the *mecA* and *mecRI-mecI* operon. Upon binding of the β-lactam to the sensor domain of *mecRI*, the intracellular peptidase domain cleaves the *mecI* repressor, which triggers *mecA* and *mecI* transcription. An analogous system controls *blaZ* and *blaRI-blaI* expression. Despite *blaI* and *mecI* repressors being interchangeable and both recognizing *mec* and *bla* regulatory sequences, cleavage of the expression regulators by *blaRI* and *mecRI* is specific, adapted from Berger-Bächi and Rohrer (2002).

The current study is compatible with one carried out by Lemaire *et al.* (2007), which observed that low pH prevents PBP2 from functioning (PBP2 is produced, but does not function) in conjunction with the PBP2a, and / or difficulties its recruitment. At the same time, Lemaire *et al.* (2007) explained that further studies must be conducted and with the expansion of knowledge it may be found that there are reasons other than acid for this phenomenon

(synthetic organic acids). This means that substances secreted by *L. plantarum,* such as bacteriocins, may have an effect on methicillin resistance. The *mecR1* gene was strongly down-regulated after exposure to CFS with methicillin compared to controls, but the *mecA* and PBP2 genes were observed to be up-regulated after exposure to CFS and methicillin.

Generally, the signal transducing integral membrane protein, *mecR1* helps initiate the expression of the antibiotic-resistant *mecA* gene, which encodes the PBP2a. Blocking the *mecR1* regulatory pathway may be a novel strategy for combating MRSA.

5.5 Conclusions

The current study demonstrated that the probiotic *L. plantarum* has a significant antimicrobial effect against MRSA, as has been reported in previous chapters. Furthermore, the present study reveals that some organic acids have a significant effect on MRSA due to acidic pH. Interestingly, the restoration of MRSA antibiotic sensitivity after treatment with supernatant of *L. plantarum* remained until the removal of the impact of the acid pH, using neutralised supernatant of *L. plantarum* and sodium lactate. This means the effects were due not only to acidic pH, but also to other substances found within the supernatant of *L. plantarum*. The results indicated that the *mec*R1-*mec*I-*mecA*-PBP2 signalling pathway was obstructed, using supernatant of *L. plantarum* and methicillin, and that the susceptibility of MRSA to some antibiotics, especially β-lactam antibiotics, can be restored.

Chapter 6
General discussion and recommended future work

Chapter 6: General discussion and recommended future work

6.1 General discussion

The skin is the principal barrier against different microbial infections, it interacts with the external environment, and is colonized with a numerous population of microorganisms. The vast majority of colonizing normal flora are represented by bacteria, whether Gram positive or Gram negative (Harder *et al.*, 1997). Skin and soft tissue infections are often a problem confronted in clinical practice, which range from mild to serious life threatening infections (Hersh *et al.*, 2008; Swartz, 2004). In the last six years, resistance to antibiotics has increased due to several reasons such as prolonged therapeutic use of antibiotics, drugs administered to patients randomly and without previous consultation by physicians (Roghmann and McGrail, 2006). As is well known, the importance of skin diseases has increased recently, most notably the resistance to conventional therapies and most antibiotics of skin pathogens such as MRSA. The lack of response to traditional treatments and increasing concerns over antibiotic resistance has led to the search for possible alternative solutions.

One potential area of investigation to alleviate this problem is the use of probiotic microorganisms (Roghmann and McGrail, 2006; Tagg and Dierksen, 2003) such as LAB, whose inhibitory activity is due to several mechanisms, including production of several substances, such as organic acids, H_2O_2, bacteriocins and others (Malago and Koninkx, 2011).

Probiotics are defined as live microorganism food supplements or microbial components, which have been observed to have beneficial effects on health

(Salminen *et al.*, 1998). Probiotic microorganisms have been widely used for the treatment/ or prevention of gut disorders, but a growing number of clinical studies suggest that probiotic approaches provoke a systemic effect which extends beyond the gut and may even affect selected functions of the skin (Ouwehand *et al.*, 2002b). Other applications of probiotics are few, particularly regarding the UGT and the skin regions (Reid, 1999). Therefore, the aim of this study was to investigate the application of a wide range of probiotics on the skin pathogens, which is considered a novel approach to look at the role of probiotics.

In this thesis, there were four lines of investigation to look for and identify a number of possible direct interactions between probiotic *Lactobacillus* species and bacterial human skin pathogens: Firstly, the evaluation of antimicrobial activity of eight probiotic *Lactobacillus* species against five bacterial human skin pathogens using agar well diffusion and agar spot methods. Secondly, the assessment of the ability of probiotics *Lactobacillus* species and pathogens to aggregate, form biofilms and adhere to keratin. Thirdly, the evaluation of probiotic *L. plantarum* and investigation of the synergism between *L. plantarum* and hBD-2 against human bacterial skin pathogens. Finally, antimicrobial activity of probiotic *Lactobacillus* species against MRSA, and the role of probiotics in the modulation of methicillin resistance in MRSA was investigated.

It was originally envisaged that at least one of these four lines of investigation would be successful and would subsequently constitute the chief line of research for the thesis. After initial studies, a decision was made to persist with each of the four lines of investigation. Moreover, many reports indicated

that the probiotic *Lactobacillus* species are antagonistic towards pathogens through a combination of mechanisms such as the production of antimicrobial substances and inhibition of adherence.

In the first line of investigation, the results showed that the majority of strains of human skin pathogens used appeared sensitive to the probiotic *Lactobacillus* species used (*L. casei*, *L. salivarius*, *acidophilus* and *L. plantarum*), but to varying degrees. *Lactobacillus* species were able to inhibit the growth of pathogens. The agar spot method gave better results than the well diffusion method, in terms of the inhibitions zones observed and the subsequent clarity of results. The increased antimicrobial activity of the *L. plantarum* on unmodified MRS (2% glucose) suggested that the activity was due mainly to LA production. Probiotic *Lactobacillus* species may potentially provide solutions to antibiotic resistance in some human skin pathogens. The current study suggests that following further research, it may be possible to exploit the inhibitory mechanisms of the probiotic microorganisms studied, either alone or in combination with each other and/ or other antimicrobial compounds.

The second line of investigation demonstrated that compounds secreted by probiotics may interfere with a pathogen's ability to adhere to a surface and impede growth. Aggregation of bacteria to each other has been correlated with adhesion. Auto-aggregation and co-aggregation are considered a prerequisite for colonization and infection of the cutaneous region by numerous pathogens. In the current study, probiotic *Lactobacillus* species and pathogens revealed no significant co-aggregation or adhesion to keratins, but significantly interfered with biofilm formation. *L. casei* demonstrated the

highest aggregation, whether in auto-aggregation or co-aggregation. The majority of auto-aggregation may be attributed to adherence properties (Boris *et al*., 1997). Some reports observed that there are structures on the surface of some *Lactobacillus* species and pathogens for example S-layer proteins in *Lactobacillus*, which play an important role in aggregation, and these contribute to 15 - 20% of the total cellular protein content (Pouwels *et al*., 1998; Sa´ra and Sleytr 2000).

Overall, in the current study, there were interactions between probiotic *Lactobacillus* species and skin pathogens when mixed together. Probiotic *Lactobacillus* species (*L. casei, L. salivarius, L. acidophilus* and *L. plantarum*) exerted a higher effect than individual strains alone. The majority of *Lactobacillus* species displayed co-aggregation with human skin pathogens (e.g. *S. aureus* strains or *P. aeruginosa*) because may be have different structures on the surface of these bacteria. The reduction of pathogens can either be by competition for adhesion sites or other growth factors (Comelli *et al*., 2002). Furthermore, *Lactobacillus* species have revealed low adherence with keratin when compared to pathogens.

Metabolites produced by some LAB include bio-surfactants (Heinemann *et al.*, 2000). Bio-surfactants produced by various strains of lactobacilli are complex biological mixtures that have been shown to inhibit the adhesion of pathogens to both biomaterial and epithelial cell surfaces (Velraeds *et al.*, 1998). The mucoid exopolysaccharide matrix, pili, flagella and LPS on the surface of *P. aeruginosa* (adhesion factors) (Gupta *et al.*, 1994) and glycocalyx polymer in *Pr. acnes* act as a protective exo-skeleton natural barrier (Burkhart and Burkhart, 2003). Moreover, these structures are

believed to play an important role in the adhesion, especially in the initial steps (Greene and Klaenhammer, 1994). It is likely that the lactobacilli used in this study interfered with these adhesion processes. Furthermore, Tahmourespour and Kermanshahi (2011) demonstrated that *L. acidophilus* was able to interfere in the adhesion and biofilm formation of the *Streptococcus mutans*.

The third line of study looked at interactions between pathogenic bacteria, antimicrobial peptides (hBD-2) and probiotic *Lactobacillus* species (*L. plantarum*). Probiotic bacteria play an important role in activating antimicrobial peptides and inducing their expression e.g. *E. coli* Nissle 1917 and several LAB of different species activated expression of the hBD-2 (Wehkamp *et al*., 2004). In the current study, hBD-2 showed high antimicrobial activity (as a bactericide) and this was significantly increased in the presence of *L. plantarum* demonstrating a degree of synergism between them. Goytia *et al.* (2013) mentioned that antimicrobial peptides protect the host by exerting bactericidal activity, modulating the immune system response, molecular signaling, and facilitating the communication between native and acquired immunity. Interestingly, hBD-2 and *L. plantarum* were more effective compared to hBD-2 alone, especially with longer incubation periods of 5 h. The reason may be due to synergism between *L. plantarum* (acidic pH) and hBD-2, and all of them the mechanism of action on cell membrane. In addition, the acidic pH caused the production of organic acids may be creating a suitable environment for the work of peptides, whether hBD-2 or other substances produced by *L. plantarum* (Chen *et al*., 2005). The mode of action for organic acids (LA and AA) is observed to

permeabilize the outer bacterial cell membrane of Gram negative bacteria by releasing important substances, such as LPS, leading to loss of viability, and the action of LA may facilitate the activity of other antimicrobial factors (Alakomi *et al*., 2000).

There is an important role for NaCl on hBD-2 activity, in that it has a negative effect on antimicrobial activity. The reason may be that NaCl ultimately inactivates or disrupts the action of antimicrobial peptides, such as hBD-2. The reason is probably due to the ability of NaCl to precipitate the peptide. Inactivation of hBD-2 may due to the salt combining with protein, and precipitating hBD-2.

When *L. plantarum* and hBD-2 were mixed with NaCl there was some effectiveness against pathogens as *L. plantarum* was not affected by the presence of NaCl; indeed *Lactobacillus* strains were able to ferment glucose in up to 8% NaCl and produce antimicrobial activity. This means that whilst NaCl works to inactivate bacteriocins, hBD-2 and other peptides, other non-protein antimicrobial substances are not affected. Therefore, the antimicrobial activity in this case was considered to be due to *L. plantarum* alone.

Gene expression of *mprF* and *dlt* was highly upregulated when hBD-2 and *L. plantarum* were used together compared with controls or hBD-2 alone. This effect was enhanced in an acidic environment (pH 4.6), which resembles the normal pH of skin. The significance of pH in skin has been considered in terms of its ability to defend against pathogens, and the alteration of skin pH is one of the pathological factors involved in skin infections (Eberlein-König *et al.*, 2000). In addition, pH may affect the structure of hBD-2, causing the alteration of their antimicrobial efficacy (Johansson *et al.*, 1998).

The fourth and final line of investigation searched for restoration of sensitivity of methicillin resistant *Staphylococcus aureus* to β-lactams by the supernatant of *L. plantarum*, which was selected because it had the highest anti-microbial activity in earlier investigations.

Interestingly, sensitivity of MRSA to β-lactams was restored using non-pH neutralised supernatant of *L. plantarum*. This may be due to the final products of metabolism of *Lactobacillus* species, which contain mainly organic acids or bacteriocins (Thanh *et al.*, 2009; Lee *et al.*, 2013). These substances participate in inhibitory activity and they have a broad spectrum of antimicrobial activity against pathogens. The influence of acidic pH could be attributed to one of the following reasons; reduced copy numbers of the PBP2a or lack of transpeptidase activity of PBP2a, the gene is expressed but not translated into protein, or the low pH could interfere with translocation of the protein into the cell wall. The first reason can be discounted because of the expression of *mecA* (Lemaire *et al.*, 2007). Although the current study cannot rule out any of the other possible mechanisms it is thought that interference with transpeptidase activity is the most likely.

Numerous organic acids were applied to find out whether susceptibility of MRSA to methicillin could be restored. Cherrington *et al.* (1991) reported that the activity might be mediated both by dissociated and un-dissociated acid. As well as the pH effect, Eklund (1989) showed that un-dissociated LA collapses the electrochemical proton gradient, causing bacteriostasis and finally the death of the target bacteria.

To avoid the effect of pH, MRSA was exposed to pH-neutralised CFS of *L. plantarum* and sodium lactate. Significant inhibition of methicillin resistance

was observed suggesting that the supernatant of *L. plantarum* consists of several compounds that can actively inhibit the methicillin resistance pathway. The effect of sodium lactate also strongly supported this hypothesis. Maas *et al.* (1989) demonstrated that there are two possible mechanisms for the activity of sodium lactate. Firstly, the presence of high levels of lactate ions may shift the pyruvate reduction-to-lactate reaction closer to its thermodynamic equilibrium, thus inhibiting a major anaerobic energy metabolism pathway that is essential for growth. Secondly, in pathogens, lactate efflux from the bacterial cell may be coupled to ATP generation from protein transfer across bacterial cell membranes.

In the current study, *mecR1* was strongly downregulated after exposure to the supernatant of *L. plantarum* and methicillin. Furthermore, Chen *et al.* (2011) observed that increase expression of control due to the *mecA* gene could be constitutive or inducible. In addition, expression of PBP2 was similar to *mecA* (upregulated). Furthermore, the PBP2 is encoded by *mecA*, and the pathway of expression of both genes is the same. MRSA cells became very sensitive to methicillin after exposure to CFS of *L. plantarum*, which can be attributed to PBP2 dysfunction because of the drastic change in pH caused by organic acids that are present in CFS. There is another opinion which states that organic acids may not be the only factor as some restoration of sensitivity to methicillin was observed when MRSA was exposed to pH-neutralised CFS and sodium lactate. The results showed that there is significant inhibition in methicillin resistance, and suggest that the supernatant consists of compounds that can actively inhibit the methicillin resistance pathway, in addition to other compounds, such as bacteriocins

and/ or others; this requires future research. Generally, low pH prevents PBP2 from functioning in conjunction with PBP2a and/ or difficulties its recruitment (Lemaire *et al.*, 2007).

In conclusion, probiotics, especially *L. plantarum* may be considered a possible avenue for investigation into new treatments of pathogen diseases for skin and soft tissue. Probiotics can inhibit the growth of pathogens in several ways. Furthermore, probiotics can produce several antimicrobial substances, which inhibit or impede the growth of pathogens. In addition, its activation of tissue to stimulate and encourage the production of antimicrobial peptides such as hBD-2, which obstruct growth or kill pathogens enhance its antimicrobial properties.

In certain cases there may be some reluctance to applying live bacteria to infected skin, even though they may be regarded as safe. Therefore, it is useful to note that many of the beneficial properties of the lactobacilli studies here were achieved using cell free supernatants. The use of metabolites from lactobacilli rather than the organisms themselves may be more acceptable in clinical situations, and this aspect needs more investigation.

Remarkably, some lactobacilli species (*L. plantarum*) restore sensitivity of pathogens, such as methicillin resistant *S. aureus* to β-lactam antibiotics, such as methicillin and others, using culture supernatant of *L. plantarum*. Probiotic *Lactobacillus* species can apply as successful solutions to bacterial antibiotic resistance.

Finally, probiotics can apply to treat resistant bacteria for antibiotics, such as MRSA without any side effects, safety, available in everywhere and low cost, in compared to antibiotics.

6.2 Recommendations and ideas for future work

The main areas to be highlighted are:

1. There is some knowledge accumulated on the application of probiotic in the skin diseases but this is still limited and the research should continue. For example, little is known about the immunological response of the human skin to probiotics. Therefore, it would be of benefit to undertake immunological studies of the skin, including the effect of probiotics on the modulation of immunity via their impact on the innate and adaptive immune response.

2. Test more than one genus of probiotic microorganisms against bacterial human skin pathogens to determine which is better in comparison to probiotic *Lactobacillus* species, possessing greater beneficial properties against pathogens.

3. Test different types of keratin *in vivo* and *in vitro* (on cell lines and tissue models) to monitor the real action of bacterial adhesion. The majority of researchers have demonstrated that the results are better *in vivo* compared to results *in vitro*.

4. Further types of the antimicrobial peptides (AMPs) should be developed and tested, which may have greater effectiveness compared to hBD-2 e.g. cathelicidins or others.

5. Appraise the role secreted protein and cell lysates for probiotics on pathogens exclusion.

6. Evaluate the role of antimicrobial substances that are produced by some *Lactobacillus* species on pathogens using the transcriptomic techniques such as a Microarray technique and/ or real time PCR.

7. Test a wide range of antibiotics against large numbers MRSA. In addition, use of a large number of genes in gene expression and observe the changes, either upregulation or downregulation. In addition, must be study the role of cell wall proteins, which played an important role in all changes that event in MRSA cell wall.

References

Abudu, L., Blair, I., Fraise, A., and Cheng, K. (2001). Methicillin-resistant *Staphylococcus aureus* (MRSA): a community-based prevalence survey. *Epidemiology and Infection* **126**, 351-356.

Adams, B. (2002). Dermatologic disorders of the athlete. *Sports Medicine* **32**, 309-321.

Agerberth, B., Lee, J. Y., Bergman, T., Carlquist, M., Boman, H. G., Mutt, V., and Jornvall, H. (1991). Amino acid sequence of PR 39. *European Journal of Biochemistry* **202**, 849-854.

Alakomi, H. L., Skyttä, E., Saarela, M., Mattila-Sandholm, T., Latva-Kala, K., and Helander, I. (2000). Lactic acid permeabilizes gram-negative bacteria by disrupting the outer membrane. *Applied of Environmental Microbiology* **66**, 2001-2005.

Alander, M., Korpela, R., Saxelin, M., Vilpponen-Salmela, T., Mattila-Sandholm, T., and Von Wright, A. (1997). Recovery of *Lactobacillus rhamnosus* GG from human colonic biopsies. *Letters in Applied Microbiology* **24**, 361-364.

Altenhoefer, A., Oswald, S., Sonnenborn, U., Enders, C., Schulze, J., Hacker, J., and Oelschlaeger, T. A. (2004). The probiotic *Escherichia coli* strain Nissle 1917 interferes with invasion of human intestinal epithelial cells by different enteroinvasive bacterial pathogens. *FEMS Immunology and Medical Microbiology* **40**, 223-229.

Alvarez-Olmos, M. I., and Oberhelman, R. A. (2001). Probiotic agents and infectious diseases: a modern perspective on a traditional therapy. *Clinical Infectious Diseases* **32**, 1567-1576.

Ammor, S., Tauveron, G., Dufour, E., and Chevallier, I. (2006). Antibacterial activity of lactic acid bacteria against spoilage and pathogenic bacteria isolated from the same meat small-scale facility: 1-Screening and characterization of the antibacterial compounds. *Food Control* **17**, 454-461.

Anas, M., Eddine, H. J., and Mebrouk, K. (2008). Antimicrobial Activity of *Lactobacillus* Species Isolated from Algerian Raw Goat's Milk Against *Staphylococcus aureus. World Journal of Dairy and Food Sciences* **3**, 39-49.

Anderson, R. C., Cookson, A. L., McNabb, W. C., Kelly, W. J., and Roy, N. C. (2010). *Lactobacillus plantarum* DSM 2648 is a potential probiotic that enhances intestinal barrier function. *FEMS Microbiology Letters* **309**, 184-192.

Arêde, P., Milheiriço, C., de Lencastre, H., and Oliveira, D. C. (2012). The Anti-Repressor *mecR2* promotes the proteolysis of the *mecA* repressor and enables optimal expression of β-lactam resistance in MRSA. *PLoS Pathogens* **8**, 1-17.

Arqués, J. L., Fernández, J., Gaya, P., Nuñez, M., Rodríguez, E., and Medina, M. (2004). Antimicrobial activity of reuterin in combination with nisin against food-borne pathogens. *International Journal of Food Microbiology* **95**, 225-229.

Balcázar, J. L., Vendrell, D., De Blas, I., Ruiz-Zarzuela, I., Gironés, O., and Múzquiz, J. L. (2007). *In vitro* competitive adhesion and production of antagonistic compounds by lactic acid bacteria against fish pathogens. *Veterinary Microbiology* **122**, 373-380.

Bals, R., Wang, X., Wu, Z., Freeman, T., Bafna, V., Zasloff, M., and Wilson, J. M. (1998). Human beta-defensin 2 is a salt-sensitive peptide antibiotic expressed in human lung. *Journal of Clinical Investigation* **102**, 874-880.

Bardan, A., Nizet, V., and Gallo, R. L. (2004). Antimicrobial peptides and the skin. *Expert Opinion on Biological Therapy* **4**, 543-549.

Barrett, D., Leimkuhler, C., Chen, L., Walker, D., Kahne, D., and Walker, S. (2005). Kinetic characterization of the glycosyltransferase module of *Staphylococcus aureus PBP2*. *Journal of Bacteriology* **187**, 2215-2217.

Barrett, F. F., McGehee Jr, R. F., and Finland, M. (1968). Methicillin-resistant *Staphylococcus aureus* at Boston city hospital. *New England Journal of Medicine* **279**, 441-448.

Bek-Thomsen, M., Lomholt, H. B., and Kilian, M. (2008). Acne is not associated with yet-uncultured bacteria. *Journal of Clinical Microbiology* **46**, 3355-3360.

Berger-Bächi, B., and Rohrer, S. (2002). Factors influencing methicillin resistance in staphylococci. *Archives of Microbiology* **178**, 165-171.

Berger-Bächi, B., and Tschierske, M. (1998). Role of Fem factors in methicillin resistance. *Drug Resistance Updates* **1**, 325-335.

Bernet, M., Brassart, D., Neeser, J., and Servin, A. (1994). *Lactobacillus acidophilus* LA 1 binds to cultured human intestinal cell lines and inhibits cell attachment and cell invasion by enterovirulent bacteria. *Gut* **35**, 483-489.

Besselink, M., van Santvoort, H., Buskens, E., Boermeester, M., van Goor, H., Timmerman, H., Nieuwenhuijs, V., Bollen, T., van Ramshorst, B., and Witteman, B. (2008). Probiotic prophylaxis in predicted severe acute pancreatitis: a randomised, double-blind, placebo-controlled trial. *The Lancet* **371**, 651-659.

Bezkorovainy, A. (2001). Probiotics: determinants of survival and growth in the gut. *The American Journal of Clinical Nutrition* **73**, 399S-405S.

Blomquist, P. H. (2006). Methicillin-resistant *Staphylococcus aureus* infections of the eye and orbit (an American Ophthalmological Society thesis). *Transactions of the American Ophthalmological Society* **104**, 322-345.

Bodera, P., and Chcialowski, A. (2009). Immunomodulatory effect of probiotic bacteria. *Recent Patents on Inflammation and Allergy Drug Discovery* **3**, 58-64.

Bodey, G. P., Bolivar, R., Fainstein, V., and Jadeja, L. (1983). Infections caused by *Pseudomonas aeruginosa. Review of Infectious Diseases* **5**, 279-313.

Boirivant, M., and Strober, W. (2007). The mechanism of action of probiotics. *Current Opinion in Gastroenterology* **23**, 679-692.

Bojar, R., and Holland, K. (2002). Review: the human cutaneous microflora and factors controlling colonisation. *World Journal of Microbiology and Biotechnology* **18**, 889-903.

Boman, H. G., Agerberth, B., and Boman, A. (1993). Mechanisms of action on *Escherichia coli* of cecropin P1 and PR-39, two antibacterial peptides from pig intestine. *Infection and Immunity* **61**, 2978-2984.

Boris, S., Suarez, J., and Barbes, C. (1997). Characterization of the aggregation promoting factor from *Lactobacillus gasseri*, avaginal isolate. *Journal of Applied Microbiology* **83**, 413-420.

Bouhss, A., Trunkfield, A. E., Bugg, T. D., and Mengin Lecreulx, D. (2007). The biosynthesis of peptidoglycan lipid-linked intermediates. *FEMS Microbiology Reviews* **32**, 208-233.

Boyle-Vavra, S., Yin, S., Challapalli, M., and Daum, R. S. (2003). Transcriptional induction of the penicillin-binding protein 2 gene in *Staphylococcus aureus* by cell wall-active antibiotics oxacillin and vancomycin. *Antimicrobial Agents Chemotherapy* **47**, 1028-1036.

Braat, H., van den Brande, J., van Tol, E., Hommes, D., Peppelenbosch, M., and van Deventer, S. (2004). *Lactobacillus rhamnosus* induces peripheral hyporesponsiveness in stimulated CD4+ T cells via modulation of dendritic cell function. *American Journal of Clinical Nutrition* **80**, 1618-1625.

Brogden, K. A. (2005). Antimicrobial peptides: pore formers or metabolic inhibitors in bacteria? *Nature Reviews Microbiology* **3**, 238-250.

Brown, J., Shriner, D., Schwartz, R., and Janniger, C. (2003). Impetigo: an update. *International Journal of Dermatology* **42**, 251-255.

Buivydas, A., Pasanen, T., Senčilo, A., Daugelavičius, R., Vaara, M., and Bamford, D. H. (2013). Clinical isolates of *Pseudomonas aeruginosa* from superficial skin infections have different physiological patterns. *FEMS Microbiology Letters* **343**, 183-189.

Burkhart, C. G., and Burkhart, C. N. (2007). Expanding the microcomedone theory and acne therapeutics: *Propionibacterium acnes* biofilm produces biological glue that holds corneocytes together to form plug. *Journal of the American Academy of Dermatology* **57**, 722-724.

Burkhart, C. N., and Burkhart, C. G. (2003). Microbiology's principle of biofilms as a major factor in the pathogenesis of acne vulgaris. *International Journal of Dermatology* **42**, 925-927.

Butmarc, J., Yufit, T., Carson, P., and Falanga, V. (2004). Human β-defensin-2 expression is increased in chronic wounds. *Wound Repair and Regeneration* **12**, 439-443.

Cadieux, P., Burton, J., Gardiner, G., Braunstein, I., Bruce, A. W., Kang, C. Y., and Reid, G. (2002). *Lactobacillus* strains and vaginal ecology. *JAMA: The Journal of the American Medical Association* **287**, 1940-1941.

Cai, H., Rodríguez, B. T., Zhang, W., Broadbent, J. R., and Steele, J. L. (2007). Genotypic and phenotypic characterization of *Lactobacillus casei* strains isolated from different ecological niches suggests frequent recombination and niche specificity. *Microbiology* **153**, 2655-2665.

Cálix-Lara, T. F., Rajendran, M., Talcott, S. T., Smith, S. B., Miller, R. K., Castillo, A., Sturino, J. M., and Taylor, T. M. (2014). Inhibition of *Escherichia coli* O157: H7 and *Salmonella enterica* on spinach and identification of antimicrobial substances produced by a commercial Lactic Acid Bacteria food safety intervention. *Food Microbiology* **38**, 192-200.

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

Cebra, J. J. (1999). Influences of microbiota on intestinal immune system development. *The American Journal of Clinical Nutrition* **69**, 1046S-1051S.

Chambers, H. F. (2003). Solving staphylococcal resistance to β-lactams. *Trends in Microbiology* **11**, 145-148.

Chan, Y. R., and Gallo, R. L. (1998). PR-39, a syndecan-inducing antimicrobial peptide, binds and affects p130Cas. *Journal of Biological Chemistry* **273**, 28978-28985.

Charlier, C., Even, S., Gautier, M., and Le Loir, Y. (2008). Acidification is not involved in the early inhibition of *Staphylococcus aureus* growth by *Lactococcus lactis* in milk. *International Dairy Journal* **18**, 197-203.

Chen, H., and Hoover, D. (2003). Bacteriocins and their food applications. *Comprehensive Reviews in Food Science and Food Safety* **2**, 82-100.

Chen, L. F., Chopra, T., and Kaye, K. S. (2011). Pathogens resistant to antibacterial agents. *Medical Clinics of North America* **95**, 647-676.

Chen, X., Niyonsaba, F., Ushio, H., Okuda, D., Nagaoka, I., Ikeda, S., Okumura, K., and Ogawa, H. (2005). Synergistic effect of antibacterial agents human β-defensins, cathelicidin LL-37 and lysozyme against *Staphylococcus aureus* and *Escherichia coli*. *Journal of Dermatological Science* **40**, 123-132.

Cherrington, C., Hinton, M., Mead, G., and Chopra, I. (1991). Organic acids: chemistry, antibacterial activity and practical applications. *Advance in Microbial Physiology* **32**, 87-108.

Chiller, K., Selkin, B. A., and Murakawa, G. J. (2001). Skin microflora and bacterial infections of the skin. J*ournal of Investigative Dermatology Symposium Proceedings*, 6, 170-174.

Christensen, G. D., Simpson, W., Younger, J., Baddour, L., Barrett, F., Melton, D., and Beachey, E. (1985). Adherence of coagulase-negative staphylococci to plastic tissue culture plates: a quantitative model for the adherence of staphylococci to medical devices. *Journal of Clinical Microbiology* **22**, 996-1006.

Christensen, L. D., van Gennip, M., Rybtke, M. T., Wu, H., Chiang, W.-C., Alhede, M., Høiby, N., Nielsen, T. E., Givskov, M., and Tolker-Nielsen, T. (2013). Clearance of *Pseudomonas aeruginosa* foreign-body biofilm infections through reduction of the c-di-GMP level in the bacteria. *Infection and Immunity* **81**, 7205-7213.

Christiansen, P., Petersen, M., Kask, S., Moller, P., Petersen, M., Nielsen, E., Vogensen, F., and Ardo, Y. (2005). Anticlostridial activity of *Lactobacillus* isolated from semi-hard cheeses. *International Dairy Journal* **15**, 901-909.

Church, D., Elsayed, S., Reid, O., Winston, B., and Lindsay, R. (2006). Burn wound infections. *Clinical Microbiology Reviews* **19**, 403-434.

Coconnier, M., Lievin, V., Hemery, E., and Servin, A. (1998). Antagonistic activity against *Helicobacter* infection in vitro and in vivo by the human *Lactobacillus acidophilus* strain LB. *Applied and Environmental Microbiology* **64**, 4573-4580.

Cogen, A., Nizet, V., and Gallo, R. (2008). Skin microbiota: a source of disease or defence? *British Journal of Dermatology* **158**, 442-455.

Cole, A. M., and Ganz, T. (2000). Human antimicrobial peptides: analysis and application. *Biotechniques* **29**, 822-843.

Collado, M. C., Gueimonde, M., Hernandez, M., Sanz, Y., and Salminen, S. (2005). Adhesion of selected *Bifidobacterium* strains to human intestinal mucus and the role of adhesion in enteropathogen exclusion. *Journal of Food Protection* **68**, 2672-2678.

Collado, M. C., Meriluoto, J., and Salminen, S. (2008). Adhesion and aggregation properties of probiotic and pathogen strains. *European Food Research and Technology* **226**, 1065-1073.

Collado, M. C., Surono, I., Meriluoto, J., and Salminen, S. (2007). Indigenous Dadih Lactic Acid Bacteria: Cell Surface Properties and Interactions with Pathogens. *Journal of Food Science* **72**, M89-M93.

Collins, J. W., La Ragione, R. M., Woodward, M. J., and Searle, L. E. (2009). 30. Application of Prebiotics and Probiotics in Livestock. *Prebiotics and Probiotics Science and Technology* **9**, 1123-1192.

Comelli, E. M., Guggenheim, B., Stingele, F., and Neeser, J. R. (2002). Selection of dairy bacterial strains as probiotics for oral health. *European Journal of Oral Sciences* **110**, 218-224.

Crawford, J. (1979). Probiotics in animal nutrition. In *Proceedings of 1979 Arkansas Nutrition Conference*, pp. 45-55.

Cremonini, F., Di Caro, S., Nista, E., Bartolozzi, F., Capelli, G., Gasbarrini, G., and Gasbarrini, A. (2002). Meta analysis: the effect of probiotic administration on antibiotic associated diarrhoea. *Alimentary Pharmacology &Therapeutics* **16**, 1461-1467.

Cribier, B., Piemont, Y., and Grosshans, E. (1994). Staphylococcal scalded skin syndrome in adults: A clinical review illustrated with a new case. *Journal of the American Academy of Dermatology* **30**, 319-324.

Curbelo, Y. G., García, Y., López, A., and Boucourt, R. (2005). Probióticos: una alternativa para mejorar el comportamiento animal. *Revista Cubana de Ciencia Agrícola* **39**, 129-140.

Czerucka, D., Piche, T., and Rampal, P. (2007). Review article: yeast as probiotics *Saccharomyces boulardii*. *Alimentary Pharmacology & Therapeutics* **26**, 767-778.

Daeschel, M., Andersson, R., and Fleming, H. (1987). Microbial ecology of fermenting plant materials 1. *FEMS Microbiology Letters* **46**, 357-367.

Daeschel, M., and Klaenhammer, T. R. (1985). Association of a 13.6-megadalton plasmid in *Pediococcus pentosaceus* with bacteriocin activity. *Applied and Environmental Microbiology* **50**, 1538-1541.

Dagan, R., and Bar-David, Y. (1992). Double-blind study comparing erythromycin and mupirocin for treatment of impetigo in children: implications of a high prevalence of erythromycin-resistant *Staphylococcus aureus* strains. *Antimicrobial Agents and Chemotherapy* **36**, 287-292.

De Keersmaecker, S., Verhoeven, T., Desair, J., Marchal, K., Vanderleyden, J., and Nagy, I. (2006). Strong antimicrobial activity of *Lactobacillus rhamnosus GG* against *Salmonella typhimurium* is due to accumulation of lactic acid. *FEMS Microbiology Letters* **259**, 89-96.

De Vuyst, L., and Leroy, F. (2007). Bacteriocins from lactic acid bacteria: production, purification, and food applications. *Journal of Molecular Microbiology and Biotechnology* **13**, 194-199.

De Vuyst, L., and Vandamme, E. (1994). Antimicrobial potential of lactic acid bacteria. *Bacteriocins of Lactic Acid Bacteria*, 91-142.

DeBoer, S., and O'Connor, A. (2004). Prehospital and emergency department burn care. *Critical Care Nursing Clinics of North America* **16**, 61-73.

Del Re, B., Sgorbati, B., Miglioli, M., and Palenzona, D. (2000). Adhesion, autoaggregation and hydrophobicity of 13 strains of *Bifidobacterium longum*. *Letters in Applied Microbiology* **31**, 438-442.

Deurenberg, R. H., and Stobberingh, E. E. (2008). The evolution of *Staphylococcus aureus*. *Infection, Genetics and Evolution* **8**, 747-763.

Devriese, L., Vancanneyt, M., Baele, M., Vaneechoutte, M., De Graef, E., Snauwaert, C., Cleenwerck, I., Dawyndt, P., Swings, J., and Decostere, A. (2005). *Staphylococcus pseudintermedius sp*. nov., a coagulase-positive species from animals. *International Journal of Systematic and Evolutionary Microbiology* **55**, 1569-1573.

Dieleman, L., Goerres, M., Arends, A., Sprengers, D., Torrice, C., Hoentjen, F., Grenther, W., and Sartor, R. (2003). *Lactobacillus* GG prevents recurrence of colitis in HLA-B27 transgenic rats after antibiotic treatment. *Gut* **52**, 370-376.

Domenech, A., Hernandez, F. J., Orden, J. A., Goyache, J., Lopez, B., Suarez, G., and Gomez-Lucia, E. (1992). Effect of six organic acids on staphylococcal

growth and enterotoxin production. *Zeitschrift für Lebensmitteluntersuchung und-Forschung A* **194**, 124-128.

Douglas, L. J. (2003). *Candida* biofilms and their role in infection. *Trends in Microbiology* **11**, 30-36.

Doyle, A., Beigi, B., Early, A., Blake, A., Eustace, P., and Hone, R. (1995). Adherence of bacteria to intraocular lenses: a prospective study. *British Journal of Ophthalmology* **79**, 347.

Drake, D., and Montie, T. C. (1988). Flagella, motility and invasive virulence of *Pseudomonas aeruginosa*. *Journal of General Microbiology* **134**, 43-52.

Dunne, C., Murphy, L., Flynn, S., O'Mahony, L., O'Halloran, S., Feeney, M., Morrissey, D., Thornton, G., Fitzgerald, G., and Daly, C. (1999). Probiotics: from myth to reality. Demonstration of functionality in animal models of disease and in human clinical trials. *Antonie Van Leeuwenhoek* **76**, 279-292.

Eady, E. A., and Ingham, E. (1994). *Propionibacterium acnes*-friend or foe? *Reviews in Medical Microbiology* **5**, 163-170.

Eberlein-König, B., Schäfer, T., Huss-Marp, J., Darsow, U., Möhrenschlager, M., Herbert, O., Abeck, D., Krämer, U., Behrendt, H., and Ring, J. (2000). Skin Surface pH, Stratum Corneum Hydration, Trans-epidermal Water Loss and Skin Roughness Related to Atopic Eczema and Skin Dryness in a Population of Primary School Children: Clinical Report. *Acta Dermato-Venereologica* **80,** 188-191.

Eklund, T. (1989). Organic acids and esters. *Mechanisms of Action of Food Preservation Procedures*, 161-200.

Elkin, S., and Geddes, D. (2003). Pseudomonal infection in cystic fibrosis: the battle continues. *Expert Review of Anti-Infective Therapy* **1**, 609-618.

Ennahar, S., Sashihara, T., Sonomoto, K., and Ishizaki, A. (2000). Class IIa bacteriocins: biosynthesis, structure and activity. *FEMS Microbiology Reviews* **24**, 85-106.

Ernst, R. K., Adams, K. N., Moskowitz, S. M., Kraig, G. M., Kawasaki, K., Stead, C. M., Trent, M. S., and Miller, S. I. (2006). The *Pseudomonas aeruginosa* lipid A deacylase: selection for expression and loss within the cystic fibrosis airway. *Journal of Bacteriology* **188**, 191-201.

File, T. M. (2008). Methicillin-resistant *Staphylococcus aureus* (MRSA): focus on community-associated MRSA. *Southern African Journal of Epidemiology and Infection* **23**, 13-15.

Fitzpatrick, F., Humphreys, H., and O'Gara, J. P. (2005). Evidence for icaADBC-independent biofilm development mechanism in methicillin-resistant *Staphylococcus aureus* clinical isolates. *Journal of Clinical Microbiology* **43**, 1973-1976.

Fluit, A., Wielders, C., Verhoef, J., and Schmitz, F. J. (2001). Epidemiology and susceptibility of 3,051 *Staphylococcus aureus* isolates from 25 university hospitals participating in the European SENTRY study. *Journal of Clinical Microbiology* **39**, 3727-3732.

Flynn, S., van Sinderen, D., Thornton, G. M., Holo, H., Nes, I. F., and Collins, J. K. (2002). Characterization of the genetic locus responsible for the production of ABP-118, a novel bacteriocin produced by the probiotic bacterium *Lactobacillus salivarius* subsp. *salivarius* UCC118. *Microbiology* **148**, 973-984.

Fooks, L., and Gibson, G. (2002). Probiotics as modulators of the gut flora. *British Journal of Nutrition* **88**, 39-50.

Foolad, N., Brezinski, E. A., Chase, E. P., and Armstrong, A. W. (2013). Effect of Nutrient Supplementation on Atopic Dermatitis in ChildrenA Systematic Review of Probiotics, Prebiotics, Formula, and Fatty AcidsNutrient Supplementation and Atopic Dermatitis. *JAMA Dermatology* **149**, 350-355.

Foster, T. B., S. (1996). Medical Microbiology. *University of Texas Medical Branch, Galveston*.

Foster, T. J., and Höök, M. (1998). Surface protein adhesins of *Staphylococcus aureus*. *Trends in Microbiology* **6**, 484-488.

Freney, J., Kloos, W., Hajek, V., Webster, J., Bes, M., Brun, Y., and Vernozy-Rozand, C. (1999). Recommended minimal standards for description of new staphylococcal species. *International Journal of Systematic Bacteriology* **49**, 489-502.

Fridkin, S. K., Hageman, J. C., Morrison, M., Sanza, L. T., Como-Sabetti, K., Jernigan, J. A., Harriman, K., Harrison, L. H., Lynfield, R., and Farley, M. M. (2005). Methicillin-resistant *Staphylococcus aureus* disease in three communities. *New England Journal Medicine* **352**, 1436-1444.

Frohm, M., Agerberth, B., Ahangari, G., Ståhle-Bäckdahl, M., Lidén, S., Wigzell, H., and Gudmundsson, G. H. (1997). The expression of the gene coding for the antibacterial peptide LL-37 is induced in human keratinocytes during inflammatory disorders. *Journal of Biological Chemistry* **272**, 15258-15263.

Fu, W., and Mathews, A. (1999). Lactic acid production from lactose by *Lactobacillus plantarum*: kinetic model and effects of pH, substrate, and oxygen. *Biochemical Engineering Journal* **3**, 163-170.

Fuller, R. (1989). Probiotics in man and animals. *The Journal of Applied Bacteriology* **66**, 365-378.

Fuller, R., and Gibson, G. R. (1997). Modification of the intestinal microflora using probiotics and prebiotics. *Scandinavian Journal of Gastroenterology. Supplement* **222**, 28-31.

Gaisford, W. C., and Reynolds, P. E. (1989). Methicillin resistance in *Staphylococcus epidermidis*. *European Journal of Biochemistry* **185**, 211-218.

Galdeano, C. M., and Perdigon, G. (2006). The probiotic bacterium *Lactobacillus casei* induces activation of the gut mucosal immune system through innate immunity. *Clinical and Vaccine Immunology* **13**, 219-226.

Gallo, R. L., and Nakatsuji, T. (2011). Microbial symbiosis with the innate immune defense system of the skin. *Journal of Investigative Dermatology* **131**, 1974-1980.

Gallo, R. L., Ono, M., Povsic, T., Page, C., Eriksson, E., Klagsbrun, M., and Bernfield, M. (1994). Syndecans, cell surface heparan sulfate proteoglycans, are induced by a proline-rich antimicrobial peptide from wounds. *Proceedings of the National Academy of Sciences* **91**, 11035-11039.

Ganz, T. (2003). Defensins: antimicrobial peptides of innate immunity. *Nature Reviews Immunology* **3**, 710-720.

García-Castellanos, R., Mallorquí-Fernández, G., Marrero, A., Potempa, J., Coll, M., and Gomis-Rüth, F. X. (2004). On the transcriptional regulation of methicillin resistance *MecI* repressor in complex with its operator. *Journal of Biological Chemistry* **279**, 17888-17896.

Gatesoupe, F. (1999). The use of probiotics in aquaculture. *Aquaculture* **180**, 147-165.

Ghadban, G. (2002). Probiotics in broiler production-A review. *Archiv fur Geflugelkunde* **66**, 49-58.

Gilliland, S., and Speck, M. (1977). Antagonistic action of *Lactobacillus acidophilus* toward intestinal and foodborne pathogens in associative cultures. *Journal of Food Protection* **40**, 820-823.

Goffin, C., and Ghuysen, J.-M. (1998). Multimodular penicillin-binding proteins: an enigmatic family of orthologs and paralogs. *Microbiology and Molecular Biology Reviews* **62**, 1079-1093.

Goldman, M. J., Anderson, G. M., Stolzenberg, E. D., Kari, U. P., Zasloff, M., and Wilson, J. M. (1997). Human β-defensin-1 is a salt-sensitive antibiotic in lung that is inactivated in cystic fibrosis. *Cell* **88**, 553-560.

Gołek, P., Bednarski, W., and Lewandowska, M. (2007). Characteristics of adhesive properties of *Lactobacillus* strains synthesising biosurfactants. *Polish Journal of Naturition Sciences* **22**, 333-342.

Gotz, F. (2004). Staphylococci in colonization and disease: prospective targets for drugs and vaccines. *Current Opinion in Microbiology* **7**, 477-487.

Goytia, M., Kandler, J. L., and Shafer, W. M. (2013). Mechanisms and Significance of Bacterial Resistance to Human Cationic Antimicrobial Peptides. In *Antimicrobial Peptides and Innate Immunity* 23, 219-254. Springer.

Graffunder, E. M., and Venezia, R. A. (2002). Risk factors associated with nosocomial methicillin-resistant *Staphylococcus aureus* (MRSA) infection including previous use of antimicrobials. *Journal of Antimicrobial Chemotherapy* **49**, 999-1005.

Granato, D., Perotti, F., Masserey, I., Rouvet, M., Golliard, M., Servin, A., and Brassart, D. (1999). Cell surface-associated lipoteichoic acid acts as an adhesion factor for attachment of *Lactobacillus johnsonii* La1 to human enterocyte-like Caco-2 cells. *Applied and Environmental Microbiology* **65**, 1071-1077.

Green, S. K., Schroth, M. N., Cho, J. J., Kominos, S. D., and Vitanza-Jack, V. B. (1974). Agricultural plants and soil as a reservoir for *Pseudomonas aeruginosa*. *Applied Microbiology* **28**, 987-991.

Greene, J. D., and Klaenhammer, T. R. (1994). Factors involved in adherence of lactobacilli to human Caco-2 cells. *Applied and Environmental Microbiology* **60**, 4487-4494.

Grice, Kong, H. H., Renaud, G., Young, A. C., Bouffard, G. G., Blakesley, R. W., Wolfsberg, T. G., Turner, M. L., and Segre, J. A. (2008). A diversity profile of the human skin microbiota. *Genome Research* **18**, 1043-1050.

Grice, E. A., Kong, H. H., Conlan, S., Deming, C. B., Davis, J., Young, A. C., Bouffard, G. G., Blakesley, R. W., Murray, P. R., and Green, E. D. (2009). Topographical and temporal diversity of the human skin microbiome. *science* **324**, 1190-1192.

Guarner, F., and Schaafsma, G. (1998). Probiotics. *International Journal of Food Microbiology* **39**, 237-238.

Guéniche, Benyacoub, J., Buetler, T., Smola, H., and Blum, S. (2006a). Supplementation with oral probiotic bacteria maintains cutaneous immune homeostasis after UV exposure. *European Journal of Dermatology* **16**, 511-517.

Guéniche, Hennino, A., Goujon, C., Dahel, K., Bastien, P., Martin, R., Jourdain, R., and Breton, L. (2006b). Improvement of atopic dermatitis skin symptoms by Vitreoscilla filiformis bacterial extract. *European Journal of Dermatology* **16**, 380-384.

Gueniche, A., Benyacoub, J., Philippe, D., Bastien, P., Kusy, N., Breton, L., Blum, S., and Castiel-Higounenc, I. (2010). *Lactobacillus paracasei* CNCM I-2116 (ST11) inhibits substance P-induced skin inflammation and accelerates skin barrier function recovery in vitro. *European Journal of Dermatology* **20**, 731-737.

Gueniche, A., Cathelineau, A. C., Bastien, P., Esdaile, J., Martin, R., Queille Roussel, C., and Breton, L. (2008). Vitreoscilla filiformis biomass improves seborrheic dermatitis. *Journal of the European Academy of Dermatology and Venereology* **22**, 1014-1015.

Gupta, S., Berk, R., Masinick, S., and Hazlett, L. (1994). Pili and lipopolysaccharide of *Pseudomonas aeruginosa* bind to the glycolipid asialo GM1. *Infection and Immunity* **62**, 4572-4579.

Gupta, V., and Garg, R. (2009). Probiotics. *Indian Journal of Medical Microbiology* **27**, 202-209.

Gusils, C., Cuozzo, S., Sesma, F., and Gonzalez, S. (2002). Examination of adhesive determinants in three species of *Lactobacillus* isolated from chicken. *Canadian Journal of Microbiology* **48**, 34-42.

Gutsmann, T., Hagge, S. O., Larrick, J. W., Seydel, U., and Wiese, A. (2001). Interaction of CAP18-derived peptides with membranes made from endotoxins or phospholipids. *Biophysical Journal* **80**, 2935-2945.

Haller, D., Blum, S., Bode, C., Hammes, W., and Schiffrin, E. (2000). Activation of human peripheral blood mononuclear cells by nonpathogenic bacteria in vitro: evidence of NK cells as primary targets. *Infection and Immunity* **68**, 752-759.

Hanssen, A. M., and Ericson Sollid, J. U. (2006). SCCmec in staphylococci: genes on the move. *FEMS Immunology and Medical Microbiology* **46**, 8-20.

Harder, J., Bartels, J., Christophers, E., and Schroder, J. (1997). A peptide antibiotic from human skin. *Nature* **387**, 861-861.

Harder, J., Meyer-Hoffert, U., Teran, L. M., Schwichtenberg, L., Bartels, J., Maune, S., and Schroder, J.-M. (2000). Mucoid *Pseudomonas aeruginosa*, TNF-α, and IL-1 β, but Not IL-6, Induce Human β-Defensin-2 in Respiratory Epithelia. *American Journal of Respiratory Cell and Molecular Biology* **22**, 714-721.

Hartman, B. J., and Tomasz, A. (1984). Low-affinity penicillin-binding protein associated with beta-lactam resistance in *Staphylococcus aureus*. *Journal of Bacteriology* **158**, 513-516.

Hassan, M., Kjos, M., Nes, I. F., Diep, D. B., and Lotfipour, F. (2012). Natural antimicrobial peptides from bacteria: characteristics and potential applications to fight against antibiotic resistance. *Journal of Applied Microbiology* **113**, 723-736.

Heinemann, C., Hylckama Vlieg, J. E., Janssen, D. B., Busscher, H. J., Mei, H. C., and Reid, G. (2000). Purification and characterization of a surface binding protein from *Lactobacillus fermentum* RC 14 that inhibits adhesion of *Enterococcus faecalis* 1131. *FEMS Microbiology Letters* **190**, 177-180.

Hersh, A. L., Chambers, H. F., Maselli, J. H., and Gonzales, R. (2008). National trends in ambulatory visits and antibiotic prescribing for skin and soft-tissue infections. *Archives of Internal Medicine* **168**, 1585-1591.

Hessle, C., Hanson, L., and Wold, A. (1999). Lactobacilli from human gastrointestinal mucosa are strong stimulators of IL 12 production. *Clinical and Experimental Immunology* **116**, 276-282.

Hiramatsu, K. (1995). Molecular evolution of MRSA. *Microbiology and Immunology* **39**, 531-543.

Holzapfel, W. H., Haberer, P., Geisen, R., Björkroth, J., and Schillinger, U. (2001). Taxonomy and important features of probiotic microorganisms in food and nutrition. *The American Journal of Clinical Nutrition* **73**, 365s-373s.

Hooper, L., and Gordon, J. (2001). Commensal host-bacterial relationships in the gut. *Science* **292**, 1115-1118.

Houtsmuller, U., and Deenen, L. v. (1965). On the amino acid esters of phosphatidyl glycerol from bacteria. *Biochimica et Biophysica Acta (BBA)-Lipids and Lipid Metabolism* **106**, 564-576.

Huang, L. C., Redfern, R. L., Narayanan, S., Reins, R. Y., and McDermott, A. M. (2007). In vitro activity of human β-defensin 2 against *Pseudomonas aeruginosa* in the presence of tear fluid. *Antimicrobial Agents and Chemotherapy* **51**, 3853-3860.

Hugo, A., De Antoni, G., and Pérez, P. (2006). *Lactobacillus delbrueckii* subsp *lactis* strain CIDCA 133 inhibits nitrate reductase activity of *Escherichia coli*. *International Journal of Food Microbiology* **111**, 191-196.

Ibler, K. S., and Kromann, C. B. (2014). Recurrent furunculosis-challenges and management: a review. *Clinical, Cosmetic and Investigational Dermatology* **7**, 59-64.

Iwase, T., Uehara, Y., Shinji, H., Tajima, A., Seo, H., Takada, K., Agata, T., and Mizunoe, Y. (2010). *Staphylococcus epidermidis* Esp inhibits *Staphylococcus aureus* biofilm formation and nasal colonization. *Nature* **465**, 346-349.

Iwatsuki, K., Yamasaki, O., Morizane, S., and Oono, T. (2006). Staphylococcal cutaneous infections: invasion, evasion and aggression. *Journal of Dermatological Science* **42**, 203-214.

Izadpanah, A., and Gallo, R. L. (2005). Antimicrobial peptides. *Journal of the American Academy of Dermatology* **52**, 381-390.

Johanson Jr, W. G., Higuchi, J. H., Chaudhuri, T. R., and Woods, D. E. (1980). Bacterial adherence to epithelial cells in bacillary colonization of the respiratory tract. *The American Review of Respiratory Disease* **121**, 55-63.

Johansson, J., Gudmundsson, G. H., Rottenberg, M. n. E., Berndt, K. D., and Agerberth, B. (1998). Conformation-dependent antibacterial activity of the naturally occurring human peptide LL-37. *Journal of Biological Chemistry* **273**, 3718-3724.

Johnson, S., Goddard, P., Iliffe, C., Timmins, B., Rickard, A., Robson, G., and Handley, P. (2002). Comparative susceptibility of resident and transient hand bacteria to para-chloro-meta-xylenol and triclosan. *Journal of Applied Microbiology* **93**, 336-344.

Jones, D. E., and Bevins, C. (1992). Paneth cells of the human small intestine express an antimicrobial peptide gene. *Journal of Biological Chemistry* **267**, 23216-23225.

Jones, D. E., and Bevins, C. L. (1993). Defensin-6 mRNA in human Paneth cells: implications for antimicrobia peptides in host defense of the human bowel. *FEBS Letters* **315**, 187-192.

Jyoti, B., Suresh, A., and Venkatesh, K. (2003). Diacetyl production and growth of *Lactobacillus rhamnosus* on multiple substrates. *World Journal of Microbiology and Biotechnology* **19**, 509-514.

Kagan, B. L., Ganz, T., and Lehrer, R. I. (1994). Defensins: a family of antimicrobial and cytotoxic peptides. *Toxicology* **87**, 131-149.

Kaila, M., Isolauri, E., Soppil, E., Virtanen, E., Laine, S., and Arvilommi, H. (1992). Enhancement of the circulating antibody secreting cell response in human diarrhea by a human *Lactobacillus* strain. *Pediatric Research* **32**, 141-144.

Kalalou, I., Faid, M., and Touhami Ahami, A. (2004). Extending shelf life of fresh minced camel meat at ambient temperature by *Lactobacillus dlbrueckii* subsp. *delbrueckii. Electronic Journal of Biotechnology* **7**, 5-6.

Kalliomäki, M., Salminen, S., Arvilommi, H., Kero, P., Koskinen, P., and Isolauri, E. (2001). Probiotics in primary prevention of atopic disease: a randomised placebo-controlled trial. *The Lancet* **357**, 1076-1079.

Kellum, R. E., Strangfeld, K., and Ray, L. F. (1970). Acne vulgaris: studies in pathogenesis: triglyceride hydrolysis by Corynebacterium acnes in vitro. *Archives of Dermatology* **101**, 41-47.

Kenward, M., Brown, M., and Fryer, J. (1979). The Influence of Calcium or Manganese on the Resistance to EDTA, Polymyxin B or Cold Shock, and the Composition of *Pseudomonas aeruginosa* Grown in Glucose or Magnesium depleted Batch Cultures. *Journal of Applied Microbiology* **47**, 489-503.

Kirkup, B. (2006). Bacteriocins as oral and gastrointestinal antibiotics: theoretical considerations, applied research, and practical applications. *Current Medicinal Chemistry* **13**, 3335-3350.

Klaenhammer, T. R. (1988). Bacteriocins of lactic acid bacteria. *Biochimie* **70**, 337-349.

Klein, G., Pack, A., Bonaparte, C., and Reuter, G. (1998). Taxonomy and physiology of probiotic lactic acid bacteria. *International Journal of Food Microbiology* **41**, 103-125.

Koch, A. (1994). Growth Measurement In *Methods for General Molecular Bacteriology* (G. Germaine, R. Murray, W. Wood, and N. Krieg, Eds.) **132,** 248-277. American Soc. Microbiol. (ASM), Washington.

Kollath, W. (1954). Diagnosis of intestinal dysbacteria]. *Zentralblatt für Bakteriologie, Parasitenkunde, Infektionskrankheiten und Hygiene. 1. Abt. Medizinisch-hygienische Bakteriologie, Virusforschung und Parasitologie. Originale* **161**, 501-503.

Kong, H. H., and Segre, J. A. (2012). Skin microbiome: looking back to move forward. *Journal of Investigative Dermatology* **132**, 933-939.

Kopp-Hoolihan, L. (2001). Prophylactic and therapeutic uses of probiotics: a review. *Journal of the American Dietetic Association* **101**, 229-241.

Kos, B., Šušković, J., Vuković, S., Šimpraga, M., Frece, J., and Matošić, S. (2003). Adhesion and aggregation ability of probiotic strain *Lactobacillus acidophilus* M92. *Journal of Applied Microbiology* **94**, 981-987.

Kotzin, B., Leung, D., Kappler, J., and Marrack, P. (1993). Superantigens and their potential role in human disease. *Advances in Immunology* **54**, 99-166.

Krediet, T., Jones, M., Janssen, K., Gerards, L., and Fleer, A. (2001). Prevalence of molecular types and mecA gene carriage of coagulase-negative staphylococci in a neonatal intensive care unit: relation to nosocomial septicemia. *Journal of Clinical Microbiology* **39**, 3376-3378.

Kreig, N., Holt, J. G., Murray, R., Breener, D., Bryant, M., Moulder, J., Pfennig, N., Sneath, P., and Staley, J. (1984). *Bergey's Manual of Systematic Bacteriology. Volume 1*. Williams and Wilkins.

Krishnakumari, V., Singh, S., and Nagaraj, R. (2006). Antibacterial activities of synthetic peptides corresponding to the carboxy-terminal region of human β-defensins 1–3. *Peptides* **27**, 2607-2613.

Kristian, S. A., Dürr, M., Van Strijp, J. A., Neumeister, B., and Peschel, A. (2003). MprF-mediated lysinylation of phospholipids in *Staphylococcus aureus* leads to protection against oxygen-independent neutrophil killing. *Infection and Immunity* **71**, 546-549.

Kuehner, D. E., Blanch, H. W., and Prausnitz, J. M. (1996). Salt-induced protein precipitation: Phase equilibria from an equation of state. *Fluid Phase Equilibria* **116**, 140-147.

Labischinski, H. (1992). Consequences of the interaction of β-lactam antibiotics with penicillin binding proteins from sensitive and resistant *Staphylococcus aureus* strains. *Medical Microbiology and Immunology* **181**, 241-265.

Lasagno, M., Beoletto, V., Sesma, F., Raya, R., and Font, D. E. V. (2002). Selection of bacteriocin producer strains of lactic acid bacteria from a dairy environment. *The New Microbiologica* **25**, 37-44.

Lee, N. K., Jin Han, E., Jun Han, K., and Paik, H. D. (2013). Antimicrobial Effect of Bacteriocin KU24 Produced by *Lactococcus lactis* KU24 against Methicillin Resistant *Staphylococcus aureus*. *Journal of Food Science* 78, M465-M469.

Lee, W., Shalita, A. R., and Poh-Fitzpatrick, M. B. (1978). Comparative studies of porphyrin production in *Propionibacterium acnes* and *Propionibacterium granulosum*. *Journal of Bacteriology* **133**, 811-815.

Lee, Y. K. (2009). Selection and maintenance of probiotic microorganisms. *Handbook of Probiotics and Prebiotics*, 177-187.

Lehrer, R. I., Daher, K., Ganz, T., and Selsted, M. (1985). Direct inactivation of viruses by MCP-1 and MCP-2, natural peptide antibiotics from rabbit leukocytes. *Journal of Virology* **54**, 467-472.

Lehrer, R. I., and Ganz, T. (2002). Defensins of vertebrate animals. *Current Opinion in Immunology* **14**, 96-102.

Lehrer, R. I., Lichtenstein, A. K., and Ganz, T. (1993). Defensins: antimicrobial and cytotoxic peptides of mammalian cells. *Annual Review of Immunology* **11**, 105-128.

Lemaire, S., Fuda, C., Van Bambeke, F., Tulkens, P. M., and Mobashery, S. (2008). Restoration of Susceptibility of Methicillin-resistant *Staphylococcus aureus* to β-Lactam Antibiotics by Acidic pH. *Journal of Biological Chemistry* **283**, 12769-12776.

Lemaire, S., Van Bambeke, F., Mingeot-Leclercq, M. P., Glupczynski, Y., and Tulkens, P. M. (2007). Role of acidic pH in the susceptibility of intraphagocytic methicillin-resistant *Staphylococcus aureus* strains to meropenem and cloxacillin. *Antimicrobial Agents and Chemotherapy* **51**, 1627-1632.

Lengkey, H., and Adriani, L. (2009). Effects of milk fermented with *Lactobacillus acidophilus* and *Bifidobacterium* spp., on lactic acid and acetic acid content and on *Staphylococcus aureus* and *Pseudomonas aeruginosa*. *Biotechnology in Animal Husbandry* **25**, 719-724.

Leroy, F., and De Vuyst, L. (2004). Lactic acid bacteria as functional starter cultures for the food fermentation industry. *Trends in Food Science and Technology* **15**, 67-78.

Levy, Bens, M. S., Craun, G. F., Calderon, R. L., and Herwaldt, B. L. (1998). Surveillance for waterborne-disease outbreaks-United States, 1995-1996. *Morbidity and Mortality Weekly Report* **47**, 1-33.

Levy, P. Y., Fenollar, F., Stein, A., Borrione, F., Cohen, E., Lebail, B., and Raoult, D. (2008). *Propionibacterium acnes* postoperative shoulder arthritis: an emerging clinical entity. *Clinical Infectious Diseases* **46**, 1884-1886.

Leyden, J., McGinley, K., Nordstrom, K., and Webster, G. (1987). Skin microflora. *The Journal of Investigative Dermatology* **88**, 65s-72s.

Leyden, J., McGinley, K., and Vowels, B. (1998). *Propionibacterium acnes* colonization in acne and nonacne. *Dermatology* **196**, 55-58.

Li, M. Y., Hua, Y., Wei, G. H., and Qiu, L. (2014). Staphylococcal Scalded Skin Syndrome in Neonates: An 8 Year Retrospective Study in a Single Institution. *Pediatric Dermatology* **31**, 43-47.

Libert, M., Elkholti, M., Massaut, J., Karmali, R., Mascart, G., and Cherifi, S. (2008). Risk factors for meticillin resistance and outcome of *Staphylococcus aureus* bloodstream infection in a Belgian university hospital. *Journal of Hospital Infection* **68**, 17-24.

Lilly, D. M., and Stillwell, R. H. (1965). Probiotics: growth-promoting factors produced by microorganisms. *Science* **147**, 747-748.

Lim, D., and Strynadka, N. C. (2002). Structural basis for the β lactam resistance of PBP2a from methicillin-resistant *Staphylococcus aureus*. *Nature Structural and Molecular Biology* **9**, 870-876.

Lindgren, S. E., and Dobrogosz, W. J. (1990). Antagonistic activities of lactic acid bacteria in food and feed fermentations. *FEMS Microbiology Letters* **87**, 149-163.

Link-Amster, H., Rochat, F., Saudan, K., Mignot, O., and Aeschlimann, J. (1994). Modulation of a specific humoral immune response and changes in intestinal flora mediated through fermented milk intake. *FEMS Immunology and Medical Microbiology* **10**, 55-63.

Liu, A. Y., Destoumieux, D., Wong, A. V., Park, C. H., Valore, E. V., Liu, L., and Ganz, T. (2002). Human &bgr;-Defensin-2 Production in Keratinocytes is Regulated by Interleukin-1, Bacteria, and the State of Differentiation. *Journal of Investigative Dermatology* **118**, 275-281.

Liu, C., and Huang, H. (2012). In vitro anti-propionibacterium activity by curcumin containing vesicle system. *Chemical and Phamaceutical Bulletin* **61**, 419-425.

Lowy (1998). *Staphylococcus aureus* infections. *New England Journal of Medicine* **339**, 520-532.

Lyczak, J. B., Cannon, C. L., and Pier, G. B. (2000). Establishment of *Pseudomonas aeruginosa* infection: lessons from a versatile opportunist. *Microbes and Infection* **2**, 1051-1060.

Maas, M., Glass, K., and Doyle, M. (1989). Sodium lactate delays toxin production by *Clostridium botulinum* in cook-in-bag turkey products. *Applied and Environmental Microbiology* **55**, 2226-2229.

Maassen, C., van Holten-Neelen, C., Balk, F., Heijne den Bak-Glashouwer, M. J., Leer, R. J., Laman, J. D., Boersma, W. J. A., and Claassen, E. (2000). Strain-dependent induction of cytokine profiles in the gut by orally administered *Lactobacillus* strains. *Vaccine* **18**, 2613-2623.

Mack, D. R., Michail, S., Wei, S., McDougall, L., and Hollingsworth, M. A. (1999). Probiotics inhibit enteropathogenic *E. coli* adherence in vitro by inducing intestinal mucin gene expression. *American Journal of Physiology-Gastrointestinal and Liver Physiology* **276**, G941-G950.

Mah, T.-F. C., and O'Toole, G. A. (2001). Mechanisms of biofilm resistance to antimicrobial agents. *Trends in Microbiology* **9**, 34-39.

Malago, J. J., and Koninkx, J. F. J. G. (2011). Probiotic-Pathogen Interactions and Enteric Cytoprotection. *Probiotic Bacteria and Enteric Infections* **6**, 289-311.

Maragkoudakis, P. A., Zoumpopoulou, G., Miaris, C., Kalantzopoulos, G., Pot, B., and Tsakalidou, E. (2006). Probiotic potential of *Lactobacillus* strains isolated from dairy products. *International Dairy Journal* **16**, 189-199.

Marples, M. J. (1969). Life on the human skin. *Scientific American* **220**, 108-115.

Mason, A. J., Gasnier, C., Kichler, A., Prévost, G., Aunis, D., Metz-Boutigue, M.-H., and Bechinger, B. (2006). Enhanced membrane disruption and antibiotic action against pathogenic bacteria by designed histidine-rich peptides at acidic pH. *Antimicrobial Agents and Chemotherapy* **50**, 3305-3311.

Mayor, S. (1999). England sets standards to reduce hospital acquired infection. *BMJ: British Medical Journal* **319**, 1392-1401.

McCaig, L., McDonald, L., Mandal, S., and Jernigan, D. (2006). *Staphylococcus aureus*-associated skin and soft tissue infections in ambulatory care. *Emerging Infectious Diseases* **12**, 1715-1723.

McGowan, J. E. (2006). Resistance in nonfermenting gram-negative bacteria: multidrug resistance to the maximum. *The American Journal of Medicine* **119**, S29-S36.

McGrath, J., Eady, R., and Pope, F. (2010). Anatomy and organization of human skin. *Rook's Textbook of Dermatology* **1**, 3.1-3.15.

McManus, A., Mason, A., McManus, W., and Pruitt, B. (1985). Twenty-five year review of *Pseudomonas aeruginosa* bacteremia in a burn center. *European Journal of Clinical Microbiology and Infectious Diseases* **4**, 219-223.

Melish, M. (1982). Staphylococci, streptococci and the skin: review of impetigo and staphylococcal scalded skin syndrome. *Semin Dermatology* **1**, 101-109.

Melish, M., and Glasgow, L. (1970). The staphylococcal scalded skin syndrome: development of an experimental model. *New England Journal of Medicine* **282**, 1114-1119.

Mercenier, A., Pavan, S., and Pot, B. (2003). Probiotics as biotherapeutic agents: present knowledge and future prospects. *Current Pharmaceutical Design* **9**, 175-191.

Meurman, J., Antila, H., Korhonen, A., and Salminen, S. (1995). Effect of *Lactobacillus rhamnosus* strain GG (ATCC 53103) on the growth of *Streptococcus sobrinus* in vitro. *European Journal of Oral Sciences* **103**, 253-258.

Midorikawa, K., Ouhara, K., Komatsuzawa, H., Kawai, T., Yamada, S., Fujiwara, T., Yamazaki, K., Sayama, K., Taubman, M. A., and Kurihara, H. (2003). *Staphylococcus aureus* susceptibility to innate antimicrobial peptides, β-defensins and CAP18, expressed by human keratinocytes. *Infection and Immunity* **71**, 3730-3739.

Miettinen, M., Vuopio-Varkila, J., and Varkila, K. (1996). Production of human tumor necrosis factor alpha, interleukin-6, and interleukin-10 is induced by lactic acid bacteria. *Infection and Immunity* **64**, 5403-5405.

Millette, M., Luquet, F. M., Ruiz, M. T., and Lacroix, M. (2008). Characterization of probiotic properties of *Lactobacillus* strains. *Dairy Science and Technology* **88**, 695-705.

Mishra, V., and Prasad, D. (2005). Application of in vitro methods for selection of *Lactobacillus casei* strains as potential probiotics. *International Journal of Food Microbiology* **103**, 109-115.

Moran, G. J., Krishnadasan, A., Gorwitz, R. J., Fosheim, G. E., McDougal, L. K., Carey, R. B., and Talan, D. A. (2006). Methicillin-resistant *S. aureus* infections

among patients in the emergency department. *New England Journal of Medicine* **355**, 666-674.

Morelli, L. (2000). In vitro selection of probiotic lactobacilli: a critical appraisal. *Current Issues in Intestinal Microbiology* **1**, 59-67.

Morris, S., and Nicholls, J. (1978). An evaluation of optical density to estimate fungal spore concentrations in water suspensions. *Strain* **1**, 1240-1242.

Mulvey, M. R., MacDougall, L., Cholin, B., Horsman, G., Fidyk, M., Woods, S., and Group, S. C.-M. S. (2005). Community-associated methicillin-resistant *Staphylococcus aureus*, *Canada*. *Emerging Infectious Diseases* **11**, 844-850.

Murakami, M., Ohtake, T., Dorschner, R. A., Schittek, B., Garbe, C., and Gallo, R. L. (2002). Cathelicidin anti-microbial peptide expression in sweat, an innate defense system for the skin. *Journal of Investigative Dermatology* **119**, 1090-1095.

Naimi, T. S., LeDell, K. H., Como-Sabetti, K., Borchardt, S. M., Boxrud, D. J., Etienne, J., Johnson, S. K., Vandenesch, F., Fridkin, S., and O'Boyle, C. (2003). Comparison of community-and health care-associated methicillin-resistant *Staphylococcus aureus* infection. *JAMA: The Journal of the American Medical Association* **290**, 2976-2984.

Neurath, M. F. (2007). IL-23: a master regulator in Crohn disease. *Nature Medicine* **13**, 26-28.

Ng, S., Hart, A., Kamm, M., Stagg, A., and Knight, S. (2009). Mechanisms of action of probiotics: recent advances. *Inflammatory Bowel Diseases* **15**, 300-310.

Nihei, Y., Hasegawa, T., and Sato, M. (1993). The efficacy of 3% acetic acid vaseline for troublesome methicillin-resistant *Staphylococcus aureus* (MRSA) skin infection in a case of vesicular pemphigoid. *The Japanies Journal of Dermatology* **103**, 27-32.

Niku Paavola, M. L., Laitila, A., Mattila Sandholm, T., and Haikara, A. (1999). New types of antimicrobial compounds produced by *Lactobacillus plantarum*. *Journal of Applied Microbiology* **86**, 29-35.

Noordiana, N., Fatimah, A., and Mun, A. (2013). Antibacterial agents produced by lactic acid bacteria isolated from Threadfin Salmon and Grass Shrimp. *International Food Research Journal* **20**, 117-124.

Nowroozi, J., Mirzaii, M., and Norouzi, M. (2004). Study of *Lactobacillus* as probiotic bacteria. *Iranian Journal of Public Health* **33**, 1-7.

O'Brien, L. M., Walsh, E. J., Massey, R. C., Peacock, S. J., and Foster, T. J. (2002). *Staphylococcus aureus* clumping factor B (ClfB) promotes adherence to human type I cytokeratin 10: implications for nasal colonization. *Cellular Microbiology* **4**, 759-770.

O'Hara, A., Shanahan, F., and Barrett, K. (2007). Mechanisms of action of probiotics in intestinal diseases. *The Scientific World Journal* **7**, 31-46.

Ogston, A., and Witte, W. (1984). On abscesses. *Reviews of Infectious Diseases* **6**, 122-128.

Ogunbanwo, S. (2005). Functional properties of lactic acid bacteria isolated from ogi and fufu, two Nigerian fermented foods. *Advance in Food Sciences* **27**, 14-21.

Oku, Y., Kurokawa, K., Ichihashi, N., and Sekimizu, K. (2004). Characterization of the *Staphylococcus aureus mprF* gene, involved in lysinylation of phosphatidylglycerol. *Microbiology* **150**, 45-51.

Orrhage, K., Brismar, B., and Nord, C. (1994). Effect of supplements with *Bifidobacterium longum* and *Lactobacillus acidophilus* on the intestinal

microbiota during administration of clindamycin. *Microbial Ecology in Health and Disease* **7**, 17-25.

Otto, M. (2009). Staphylococcus epidermidis-the'accidental'pathogen. *Nature Reviews Microbiology* **7**, 555-567.

Ouwehand, Isolauri, E., and Salminen, S. (2002a). The role of the intestinal microflora for the development of the immune system in early childhood. *European Journal of Nutrition* **41**, 132-137.

Ouwehand, Kirjavainen, P. V., Shortt, C., and Salminen, S. (1999). Probiotics: mechanisms and established effects. *International Dairy Journal* **9**, 43-52.

Ouwehand, Salminen, S., and Isolauri, E. (2002b). Probiotics: an overview of beneficial effects. *Antonie Van Leeuwenhoek* **82**, 279-289.

Ouwehand, A., Båtsman, A., and Salminen, S. (2003). Probiotics for the skin: a new area of potential application? *Letters in Applied Microbiology* **36**, 327-331.

Pan, C.-Y., Chen, J.-Y., Lin, T.-L., and Lin, C.-H. (2009). In vitro activities of three synthetic peptides derived from epinecidin-1 and an anti-lipopolysaccharide factor against *Propionibacterium acnes Candida albicans*, and *Trichomonas vaginalis*. *Peptides* **30**, 1058-1068.

Parish, M., and Davidson, P. (1993). Methods for evaluation. *Food Science and Technology-New York-Marcl Dekker.*, 597-597.

Parker, R. (1974). Probiotics, the other half of the antibiotic story. *Animal Nutrition and Health* **29**, 4-8.

Parvez, S., Malik, K., Ah Kang, S., and Kim, H. Y. (2006). Probiotics and their fermented food products are beneficial for health. *Journal of Applied Microbiology* **100**, 1171-1185.

Pascual, L. M., Daniele, M. B., Ruiz, F., Giordano, W., Pájaro, C., and Barberis, L. (2008). *Lactobacillus rhamnosus* L60, a potential probiotic isolated from the human vagina. *The Journal of General and Applied Microbiology* **54**, 141-148.

Pasparakis, M., Haase, I., and Nestle, F. O. (2014). Mechanisms regulating skin immunity and inflammation. *Nature Reviews Immunology* **14**, 289-301.

Pazgier, M., Hoover, D., Yang, D., Lu, W., and Lubkowski, J. (2006). Human β-defensins. *Cellular and Molecular Life Sciences* **63**, 1294-1313.

Peschel, A., Jack, R. W., Otto, M., Collins, L. V., Staubitz, P., Nicholson, G., Kalbacher, H., Nieuwenhuizen, W. F., Jung, G., and Tarkowski, A. (2001). *Staphylococcus aureus* resistance to human defensins and evasion of neutrophil killing via the novel virulence factor *MprF* is based on modification of membrane lipids with l-lysine. *The Journal of Experimental Medicine* **193**, 1067-1076.

Peschel, A., Otto, M., Jack, R. W., Kalbacher, H., Jung, G., and Götz, F. (1999). Inactivation of the dlt Operon in *Staphylococcus aureus* Confers Sensitivity to Defensins, Protegrins, and Other Antimicrobial Peptides. *Journal of Biological Chemistry* **274**, 8405-8410.

Piddock, L. J. V. (1990). Techniques used for the determination of antimicrobial resistance and sensitivity in bacteria. *Journal of Applied Microbiology* **68**, 307-318.

Pinho, M. G., De Lencastre, H., and Tomasz, A. (2001). An acquired and a native penicillin-binding protein cooperate in building the cell wall of drug-resistant staphylococci. *Proceedings of the National Academy of Sciences* **98**, 10886-10891.

Pivarcsi, A., and Homey, B. (2005). Chemokine networks in atopic dermatitis: traffic signals of disease. *Current Allergy and Asthma Reports* **5**, 284-290.

Plata, K., Rosato, A. E., and Wegrzyn, G. (2009). *Staphylococcus aureus* as an infectious agent: overview of biochemistry and molecular genetics of its pathogenicity. *Acta Biochimica Polonica* **56**, 597-612.

Platz, J., Beisswenger, C., Dalpke, A., Koczulla, R., Pinkenburg, O., Vogelmeier, C., and Bals, R. (2004). Microbial DNA induces a host defense reaction of human respiratory epithelial cells. *The Journal of Immunology* **173**, 1219-1223.

Postollec, F., Norde, W., De Vries, J., Busscher, H., and Van der Mei, H. (2006). Interactive forces between co-aggregating and non-co-aggregating oral bacterial pairs. *Journal of Dental Research* **85**, 231-234.

Pothoulakis, C., Kelly, C. P., Joshi, M. A., Gao, N., O'Keane, C. J., Castagliuolo, I., and Lamont, J. T. (1993). *Saccharomyces boulardii* inhibits *Clostridium difficile* toxin A binding and enterotoxicity in rat ileum. *Gastroenterology-Baltimore then Philadelphia* **104**, 1108-1108.

Pouwels, P. H., Leer, R. J., Shaw, M., Heijne den Bak-Glashouwer, M. J., Tielen, F. D., Smit, E., Martinez, B., Jore, J., and Conway, P. L. (1998). Lactic acid bacteria as antigen delivery vehicles for oral immunization purposes. *International Journal of Food Microbiology* **41**, 155-167.

Projan, S., and Novick, R. (1997). The molecular basis of pathogenicity. *The Staphylococci in Human Disease*, 55-81.

Raj, P. A., and Dentino, A. R. (2002). Current status of defensins and their role in innate and adaptive immunity. *FEMS Microbiology Letters* **206**, 9-18.

Rangavajhyala, N., Shahani, K., Sridevi, G., and Srikumaran, S. (1997). Nonlipopolysaccharide components) of *Lactobacillus addophilus* stimulate (s) the production of interleukin-1 and tumor necrosis factor- by murine macrophages. *Nutrition and Cancer* **28**, 130-134.

Rao, M., Pintado, J., Stevens, W., and Guyot, J.-P. (2004). Kinetic growth parameters of different amylolytic and non-amylolytic *Lactobacillus* strains under various salt and pH conditions. *Bioresource Technology* **94**, 331-337.

Ray, L. F., and Kellum, R. E. (1970). *Corynebacterium acnes* From Human Skin: Identification by Morphologic, Cultural, Biochemical, Serological, and Chromatographic Methods. *Archives of Dermatology* **101**, 36-40.

Reid, G. (1999). Potential preventive strategies and therapies in urinary tract infection. *World Journal of Urology* **17**, 359-363.

Reid, G. (2001). Probiotic agents to protect the urogenital tract against infection. *American Journal of Clinical Nutrition* **73**, 437S-443S.

Reid, G. (2008). Probiotic lactobacilli for urogenital health in women. *Journal of Clinical Gastroenterology* **42**, S234-S236.

Reid, G., Jass, J., Sebulsky, M., and McCormick, J. (2003). Potential uses of probiotics in clinical practice. *Clinical Microbiology Reviews* **16**, 658-672.

Reyed, R. M. (2007). Probiotics: A new strategies for prevention and therapy of diarrhea disease. *Journal of Application Sciences Research* **3**, 291-299.

Richardson, M. (2002). Understanding the structure and function of the skin. *Nursing times* **99**, 46-48.

Ring, J., Eberlein-König, B., Schäfer, T., Huss-Marp, J., Darsow, U., Möhrenschlager, M., Herbert, O., Abeck, D., Krämer, U., and Behrendt, H. (2000). Skin surface pH, stratum corneum hydration, trans-epidermal water loss and skin roughness related to atopic eczema and skin dryness in a population of primary school children: clinical report. *Acta Dermatology-Venerology* **80**, 188-191.

Roghmann, M. C., and McGrail, L. (2006). Novel ways of preventing antibiotic-resistant infections: What might the future hold? *American Journal of Infection Control* **34**, 469-475.

Rohde, H., Burandt, E. C., Siemssen, N., Frommelt, L., Burdelski, C., Wurster, S., Scherpe, S., Davies, A. P., Harris, L. G., and Horstkotte, M. A. (2007). Polysaccharide intercellular adhesin or protein factors in biofilm accumulation of *Staphylococcus epidermidis* and *Staphylococcus aureus* isolated from prosthetic hip and knee joint infections. *Biomaterials* **28**, 1711-1720.

Rolinson, G. (1961). "Celbenin"-resistant staphylococci. *British Medical Journal* **1**, 125-126.

Rook, G., and Brunet, L. (2005). Microbes, immunoregulation, and the gut. *Gut* **54**, 317-320.

Rosenfeldt, V., Benfeldt, E., Nielsen, S., Michaelsen, K., Jeppesen, D., Valerius, N., and Paerregaard, A. (2003). Effect of probiotic *Lactobacillus* strains in children with atopic dermatitis* 1. *Journal of Allergy and Clinical Immunology* **111**, 390-395.

Ruzin, A., Severin, A., Moghazeh, S. L., Etienne, J., Bradford, P. A., Projan, S. J., and Shlaes, D. M. (2003). Inactivation of mprF affects vancomycin susceptibility in *Staphylococcus aureus*. *Biochimica et Biophysica Acta (BBA)-General Subjects* **1621**, 117-121.

Ryan, M. P., Jack, R. W., Josten, M., Sahl, H. G., Jung, G., Ross, R. P., and Hill, C. (1999). Extensive post-translational modification, including serine to D-alanine conversion, in the two-component lantibiotic, lacticin 3147. *Journal of Biological Chemistry* **274**, 37544-37550.

Ryffel, C., Kayser, F. H., and Berger-Bächi, B. (1992). Correlation between regulation of mecA transcription and expression of methicillin resistance in staphylococci. *Antimicrobial Agents of Chemotherapy* **36**, 25-31.

Saarela, Mogensen, G., Fondén, R., Mättö, J., and Mattila-Sandholm, T. (2000). Probiotic bacteria: safety, functional and technological properties. *Journal of Biotechnology* **84**, 197-215.

Saavedra, J. M., Bauman, N., Perman, J., Yolken, R., and Oung, I. (1994). Feeding of *Bifidobacterium bifidum* and *Streptococcus thermophilus* to infants in hospital for prevention of diarrhoea and shedding of rotavirus. *The Lancet* **344**, 1046-1049.

Sabath, L., Wallace, S. J., and Gerstein, D. A. (1972). Suppression of intrinsic resistance to methicillin and other penicillins in Staphylococcus aureus. *Staphylococcus aureus. Antimicrobial Agents and Chemotherapy* **2**, 350-355.

Salminen, S., Bouley, C., Boutron, M., Cummings, J., Franck, A., Gibson, G., Isolauri, E., Moreau, M., Roberfroid, M., and Rowland, I. (1998). Functional food science and gastrointestinal physiology and function. *British Journal of Nutrition* **80**, 147-171.

Sánchez, B., Urdaci, M. C., and Margolles, A. (2010). Extracellular proteins secreted by probiotic bacteria as mediators of effects that promote mucosa–bacteria interactions. *Microbiology* **156**, 3232-3242.

Sanders, M. (2000). Considerations for use of probiotic bacteria to modulate human health. *The Journal of Nutrition* **130**, 384S-390S.

Sandiford, S., and Upton, M. (2012). Identification, characterization, and recombinant expression of epidermicin NI01, a novel unmodified bacteriocin produced by *Staphylococcus epidermidis* that displays potent activity against staphylococci. *Antimicrobial Agents and Chemotherapy* **56**, 1539-1547.

Sanford, M., Widmer, A., Bale, M., Jones, R., and Wenzel, R. (1994). Efficient detection and long-term persistence of the carriage of methicillin-resistant *Staphylococcus aureus*. *Clinical Infectious Diseases* **19**, 1123-1128.

Sankar, N. R., Priyanka, V. D., Reddy, P. S., Rajanikanth, P., Kumar, V. K., and Indira, M. (2012). Purification and Characterization of Bacteriocin Produced by *Lactobacillus plantarum* Isolated from Cow Milk. *International Journal of Microbiology Research* **3**, 133-137.

Sara, M., and Sleytr, U. B. (2000). S-layer proteins. *Journal of Bacteriology* **182**, 859-868.

Satorius, A. E., Szafranski, J., Pyne, D., Ghanesan, M., Solomon, M. J., Newton, D. W., Bortz, D. M., Younger, J. G., and Younger, J. G. (2013). Complement C5a Generation by Staphylococcal Biofilms. *Shock* **39**, 336-342..

Scheffers, D. J., and Pinho, M. G. (2005). Bacterial cell wall synthesis: new insights from localization studies. *Microbiology and Molecular Biology Reviews* **69**, 585-607.

Schillinger, U., and Lucke, F. K. (1989). Antibacterial activity of *Lactobacillus sake* isolated from meat. *Applied and Environmental Microbiology* **55**, 1901-1906.

Schlee, M., Harder, J., Köten, B., Stange, E., Wehkamp, J., and Fellermann, K. (2008). Probiotic lactobacilli and VSL# 3 induce enterocyte β-defensin 2. *Clinical and Experimental Immunology* **151**, 528-535.

Schrezenmeir, J., and de Vrese, M. (2001). Probiotics, prebiotics, and synbiotics-approaching a definition. *The American Journal of Clinical Nutrition* **73**, 361S-364S.

Schröder, J. M., and Harder, J. (1999). Human beta-defensin-2. *The International Journal of Biochemistry and Cell Biology* **31**, 645-651.

Seidenari, S., and Giusti, G. (1995). Objective assessment of the skin of children affected by atopic dermatitis: a study of pH, capacitance and TEWL in eczematous and clinically uninvolved skin. *Acta Dermato-Venereologica* **75**, 429-433.

Shanson, D. (1990). Septicaemia in patients with AIDS. *Transactions of the Royal Society of Tropical Medicine and Hygiene* **84**, 14-16.

Sheil, B., McCarthy, J., O'Mahony, L., Bennett, M., Ryan, P., Fitzgibbon, J., Kiely, B., Collins, J., and Shanahan, F. (2004). Is the mucosal route of administration essential for probiotic function? Subcutaneous administration is associated with attenuation of murine colitis and arthritis. *Gut* **53**, 694-700.

Shetty, P. H., and Jespersen, L. (2006). *Saccharomyces cerevisiae* and lactic acid bacteria as potential mycotoxin decontaminating agents. *Trends in Food Science and Technology* **17**, 48-55.

Sheu, C. W., Konings, W. N., and Freese, E. (1972). Effects of acetate and other short-chain fatty acids on sugar and amino acid uptake of *Bacillus subtilis*. *Journal of Bacteriology* **111**, 525-530.

Silva, M., Jacobus, N., Deneke, C., and Gorbach, S. (1987). Antimicrobial substance from a human *Lactobacillus* strains. *Antimicrobial Agents and Chemotherapy* **31**, 1231-1233.

Simmering, R., and Blaut, M. (2001). Pro-and prebiotics-the tasty guardian angels? *Applied Microbiology and Biotechnology* **55**, 19-28.

Simor, A. E., Ofner-Agostini, M., Bryce, E., Green, K., McGeer, A., Mulvey, M., and Paton, S. (2001). The evolution of methicillin-resistant *Staphylococcus aureus* in Canadian hospitals: 5 years of national surveillance. *Canadian Medical Association Journal* **165**, 21-26.

Singh, P. K., Jia, H. P., Wiles, K., Hesselberth, J., Liu, L., Conway, B.-A. D., Greenberg, E. P., Valore, E. V., Welsh, M. J., and Ganz, T. (1998). Production of β-defensins by human airway epithelia. *Proceedings of the National Academy of Sciences* **95**, 14961-14966.

Soleimani, Kermanshahi, R. K., Yakhchali, B., and Sattari, T. N. (2010). Antagonistic activity of probiotic lactobacilli against *Staphylococcus aureus* isolated from bovine mastitis. *African Journal of Microbiology Research* **4**, 2169-2173.

Somerville, D. A., and Noble, W. (1974). Resident and transient bacteria of the skin. *Journal of Cutaneous Pathology* **1**, 260-264.

Soomro, A., Masud, T., and Anwaar, K. (2002). Role of lactic acid bacteria (LAB) in food preservation and human health–a review. *Pakistan Journal of Nutrition* **1**, 20-24.

Spano, G., and Massa, S. (2006). Environmental stress response in wine lactic acid bacteria: beyond *Bacillus subtilis*. *Critical Reviews in Microbiology* **32**, 77-86.

Speller, D., Johnson, A., James, D., Marples, R., Charlett, A., and George, R. (1997). Resistance to methicillin and other antibiotics in isolates of *Staphylococcus aureus* from blood and cerebrospinal fluid, England and Wales, 1989-95. *The Lancet* **350**, 323-325.

Staubitz, P., Neumann, H., Schneider, T., Wiedemann, I., and Peschel, A. (2004). MprF mediated biosynthesis of lysylphosphatidylglycerol, an important determinant in staphylococcal defensin resistance. *FEMS Microbiology Letters* **231**, 67-71.

Stefani, S., Chung, D. R., Lindsay, J. A., Friedrich, A. W., Kearns, A. M., Westh, H., and MacKenzie, F. M. (2012). Meticillin-resistant *Staphylococcus aureus* (MRSA): global epidemiology and harmonisation of typing methods. *International Journal of Antimicrobial Agents* **39**, 273-282.

Steidler, L., Hans, W., Schotte, L., Neirynck, S., Obermeier, F., Falk, W., Fiers, W., and Remaut, E. (2000). Treatment of murine colitis by *Lactococcus lactis* secreting interleukin-10. *Science* **289**, 1352-1355.

Steiner, H., Hultmark, D., Engström, Å., Bennich, H., and Boman, H. (1981). Sequence and specificity of two antibacterial proteins involved in insect immunity. *Nature* **292**, 246 - 248.

Steinhoff, M., Brzoska, T., and Luger, T. A. (2001). Keratinocytes in epidermal immune responses. *Current Opinion in Allergy and Clinical Immunology* **1**, 469-476.

Stepanovil , S., Vukovi, D., Hola, V., Bonaventura, G. D. I., Djuki, S., Irkovi, I., and Ruzicka, F. (2007). Quantification of biofilm in microtiter plates: overview of testing conditions and practical recommendations for assessment of biofilm production by staphylococci. *Apmis* **115**, 891-899.

Stewart, P. S., and William Costerton, J. (2001). Antibiotic resistance of bacteria in biofilms. *The Lancet* **358**, 135-138.

Stoodley, P., Sauer, K., Davies, D., and Costerton, J. (2002). Biofilms as complex differentiated communities. *Annual Reviews in Microbiology* **56**, 187-209.

Storici, P., and Zanetti, M. (1993). A cDNA derived from pig bone marrow cells predicts a sequence identical to the intestinal antibacterial peptide PR-39. *Biochemical and Biophysical Research Communications* **196**, 1058-1065.

Strid, J., Hourihane, J., Kimber, I., Callard, R., and Strobel, S. (2004). Disruption of the stratum corneum allows potent epicutaneous immunization with protein antigens resulting in a dominant systemic Th2 response. *European Journal of Immunology* **34**, 2100-2109.

Strid, J., and Strobel, S. (2005). Skin barrier dysfunction and systemic sensitization to allergens through the skin. *Current Drug Targets-Inflammation; Allergy* **4**, 531-541.

Strom, K., Sjogren, J., Broberg, A., and Schnurer, J. (2002). *Lactobacillus plantarum* MiLAB 393 produces the antifungal cyclic dipeptides cyclo (L-Phe-L-Pro) and cyclo (L-Phe-trans-4-OH-L-Pro) and 3-phenyllactic acid. *Applied and Environmental Microbiology* **68**, 4322-4327.

Stulberg, D., Penrod, M., and Blatny, R. (2002). Common bacterial skin infections. *American Family Physician* **66**, 119-128.

Sugiarto, H., and Yu, P. L. (2004). Avian antimicrobial peptides: the defense role of β-defensins. *Biochemical and Biophysical Research Communications* **323**, 721-727.

Sung, J. M.-L., Lloyd, D. H., and Lindsay, J. A. (2008). *Staphylococcus aureus* host specificity: comparative genomics of human versus animal isolates by multi-strain microarray. *Microbiology* **154**, 1949-1959.

Šušković, J., Kos, B., Beganović, J., Leboš Pavunc, A., Habjanič, K., and Matošić, S. (2010). Antimicrobial activity-the most important property of probiotic and starter lactic acid bacteria. *Food Technology and Biotechnology* **48**, 296-307.

Sutton, S. (2011). Measurement of microbial cells by optical density. *Journal of Validation Technology* **17**, 46-49.

Suzuki, E., Kuwahara-Arai, K., Richardson, J., and Hiramatsu, K. (1993). Distribution of mec regulator genes in methicillin-resistant *Staphylococcus* clinical strains. *Antimicrobial Agents Chemotherapy* **37**, 1219-1226.

Swartz, M. N. (2004). Cellulitis. *New England Journal of Medicine* **350**, 904-912.

Tagg, J. R., and Dierksen, K. P. (2003). Bacterial replacement therapy: adapting 'germ warfare'to infection prevention. *TRENDS Biotechnology* **21**, 217-223.

Tahara, T., Oshimura, M., Umezawa, C., and Kanatani, K. (1996). Isolation, partial characterization, and mode of action of Acidocin J1132, a two-component bacteriocin produced by *Lactobacillus acidophilus* JCM 1132. *Applied and Environmental Microbiology* **62**, 892-897.

Tahmourespour, A., and Kermanshahi, R. K. (2011). The effect of a probiotic strain (*Lactobacillus acidophilus)* on the plaque formation of oral Streptococci. *Bosnian Journal of Basic Medical Science* **11**, 37-40.

Tahmourespour, A., Salehi, R., and Kermanshahi, R. K. (2011). *Lactobacillus acidophilus*-derived biosurfactant effect on *gtfB* and *gtfC* expression level in *Streptococcus mutans* biofilm cells. *Brazilian Journal of Microbiology* **42**, 330-339.

Takahashi, A., Wada, A., Ogushi, K., Maeda, K., Kawahara, T., Mawatari, K., Kurazono, H., Moss, J., Hirayama, T., and Nakaya, Y. (2001). Production of [beta]-defensin-2 by human colonic epithelial cells induced by *Salmonella enteritidis* flagella filament structural protein. *FEBS Letters* **508**, 484-488.

Takahashi, T., Satoh, I., and Kikuchi, N. (1999). Phylogenetic relationships of 38 taxa of the genus *Staphylococcus* based on 16S rRNA gene sequence analysis. *International Journal of Systematic and Evolutionary Microbiology* **49**, 725-728.

Tareb, R., Bernardeau, M., Gueguen, M., and Vernoux, J.-P. (2013). In vitro characterization of aggregation and adhesion properties of viable and heat-killed forms of two probiotic *Lactobacillus* strains and interaction with foodborne zoonotic bacteria, especially *Campylobacter jejuni*. *Journal of Medical Microbiology* **62**, 637-649.

Tejero-Sariñena, S., Barlow, J., Costabile, A., Gibson, G. R., and Rowland, I. (2012). In vitro evaluation of the antimicrobial activity of a range of probiotics against pathogens: Evidence for the effects of organic acids. *Anaerobe* **18,** 530-538. .

Thanh, N., Loh, D. T. C., Foo, H., Hair-Bejo, M., and Azhar, B. (2009). Effects of feeding metabolite combinations produced by *Lactobacillus plantarum* on growth performance, faecal microbial population, small intestine villus height and faecal volatile fatty acids in broilers. *British Poultry Science* **50**, 298-306.

Theis, T., Skurray, R. A., and Brown, M. H. (2007). Identification of suitable internal controls to study expression of a *Staphylococcus aureus* multidrug resistance system by quantitative real-time PCR. *Journal of Microbiological Methods* **70**, 355-362.

Tissier, H. (1907). *Traitement des infections intestinales par la méthode de transformation de la flore bactérienne de l'intestin*. *Comptes Rendus des Seances de la Societe de Biologie* **60,** 359-361.

Todd, J., Fishaut, M., Kapral, F., and Welch, T. (1978). Toxic-shock syndrome associated with phage-group-I staphylococci. *The Lancet* **312**, 1116-1118.

Toledo-Arana, A., Merino, N., Vergara-Irigaray, M., Debarbouille, M., Penades, J. R., and Lasa, I. (2005). *Staphylococcus aureus* develops an alternative, ica-independent biofilm in the absence of the arlRS two-component system. *Journal of Bacteriology* **187**, 5318-5329.

Tuohy, K. M., Probert, H. M., Smejkal, C. W., and Gibson, G. R. (2003). Using probiotics and prebiotics to improve gut health. *Drug Discovery Today* **8**, 692-700.

Ubukata, K., Nonoguchi, R., Matsuhashi, M., and Konno, M. (1989). Expression and inducibility in *Staphylococcus aureus* of the *mecA* gene, which encodes a methicillin-resistant *S. aureus*-specific penicillin-binding protein. *Journal of Bacteriology* **171**, 2882-2885.

Uchida, Y., Morita, H., Adachi, S., Asano, T., Taga, T., and Kondo, N. (2011). Bacterial meningitis and septicemia of neonate due to *Lactococcus lactis*. *Pediatrics International* **53**, 119-120.

Valerio, F., Lavermicocca, P., Pascale, M., and Visconti, A. (2004). Production of phenyllactic acid by lactic acid bacteria: an approach to the selection of strains contributing to food quality and preservation. *FEMS Microbiology Letters* **233**, 289-295.

Vallor, A. C., Antonio, M. A. D., Hawes, S. E., and Hillier, S. L. (2001). Factors associated with acquisition of, or persistent colonization by, vaginal lactobacilli: role of hydrogen peroxide production. *Journal of Infectious Diseases* **184**, 1431-1436.

Valore, E. V., Park, C. H., Quayle, A. J., Wiles, K. R., McCray Jr, P. B., and Ganz, T. (1998). Human beta-defensin-1: an antimicrobial peptide of urogenital tissues. *Journal of Clinical Investigation* **101**, 1633-1642.

van Dijk, A., Veldhuizen, E. J. A., and Haagsman, H. P. (2008). Avian defensins. *Veterinary Immunology and Immunopathology* **124**, 1-18.

Van Hoogmoed, C. G., Van der Mei, H. C., and Busscher, H. J. (2004). The influence of biosurfactants released by S. mitis BMS on the adhesion of pioneer strains and cariogenic bacteria. *Biofouling* **20**, 261-267.

Van Thu, T., Foo, H. L., Loh, T. C., and Bejo, M. H. (2011). Inhibitory activity and organic acid concentrations of metabolite combinations produced by various strains of *Lactobacillus plantarum*. *African Journal of Biotechnology* **10**, 1359-1363.

Vanbelle, M., Teller, E., and Focant, M. (1990). Probiotics in animal nutrition: a review. *Archives of Animal Nutrition* **40**, 543-567.

Vandenbergh, P. A. (1993). Lactic acid bacteria, their metabolic products and interference with microbial growth. *FEMS Microbiology Reviews* **12**, 221-237.

Vandevoorde, L., Christiaens, H., and Verstraete, W. (1992). Prevalence of coaggregation reactions among chicken lactobacilli. *Journal of Applied Microbiology* **72**, 214-219.

Veldhuizen, E. J. A., Rijnders, M., Claassen, E. A., Van Dijk, A., and Haagsman, H. P. (2008). Porcine [beta]-defensin 2 displays broad antimicrobial activity against pathogenic intestinal bacteria. *Molecular Immunology* **45**, 386-394.

Velraeds, M., Van der Mei, H., Reid, G., and Busscher, H. J. (1996). Inhibition of initial adhesion of uropathogenic *Enterococcus faecalis* by biosurfactants from *Lactobacillus* isolates. *Applied and Environmental Microbiology* **62**, 1958-1963.

Velraeds, M. M., Van De Belt-Gritter, B., van, Van der Mei, H., Reid, G., and Busscher, H. (1998). Interference in initial adhesion of uropathogenic bacteria and yeasts to silicone rubber by a *Lactobacillus acidophilus* biosurfactant. *Journal of Medical Microbiology* **47**, 1081-1085.

Venkatesan, S., Kirithika, M., Roselin, I., Ganesan, R., and Muthuchelian, K. (2012). Comparative invitro and invivo study of three probiotic organisms, *Bifidobacterium* spp., *Lactobacillus* spp., *S. cerevisiae* and analyzing its improvement with the supplementation of prebiotics. *International Journal of Plant, Animal and Environmental Sciences* **2**, 94-106.

Vergin, F. (1954). Antibiotics and probiotics]. *Hippokrates* **25**, 116-119.

Verschuere, L., Rombaut, G., Sorgeloos, P., and Verstraete, W. (2000). Probiotic bacteria as biological control agents in aquaculture. *Microbiology and Molecular Biology Reviews* **64**, 655-671.

Vesterlund, S., Paltta, J., Karp, M., and Ouwehand, A. C. (2005). Measurement of bacterial adhesion-*in vitro* evaluation of different methods. *Journal of Microbiological Methods* **60**, 225-233.

Viljanen, M., Pohjavuori, E., Haahtela, T., Korpela, R., Kuitunen, M., Sarnesto, A., Vaarala, O., and Savilahti, E. (2005). Induction of inflammation as a possible mechanism of probiotic effect in atopic eczema-dermatitis syndrome. *Journal of Allergy and Clinical Immunology* **115**, 1254-1259.

Vincze, S., Stamm, I., Monecke, S., Kopp, P., Semmler, T., Wieler, L., Lübke-Becker, A., and Walther, B. (2013). Molecular Analysis of Human and Canine *Staphylococcus aureus* Strains Reveals Distinct Extended-Host-Spectrum Genotypes Independent of Their Methicillin Resistance. *Applied and Environmental Microbiology* **79**, 655-662.

Vlková, E., Rada, V., Šmehilová, M., and Killer, J. (2008). Auto-aggregation and co-aggregation ability in bifidobacteria and clostridia. *Folia Microbiologica* **53**, 263-269.

Vora, P., Youdim, A., Thomas, L. S., Fukata, M., Tesfay, S. Y., Lukasek, K., Michelsen, K. S., Wada, A., Hirayama, T., and Arditi, M. (2004). β-defensin-2 expression is regulated by TLR signaling in intestinal epithelial cells. *The Journal of Immunology* **173**, 5398-5405.

Voss, A., Milatovic, D., Wallrauch-Schwarz, C., Rosdahl, V., and Braveny, I. (1994). Methicillin-resistant *Staphylococcus aureus* in Europe. *European Journal of Clinical Microbiology and Infectious Diseases* **13**, 50-55.

Wagner, F. (2000). Differential expression of human α- and β-defensins mRNA in gastrointestinal epithelia. *European Journal of Clinical Investigation* **30**, 695-701.

Walencka, E., Ró alska, S., Sadowska, B., and Ró alska, B. (2008). The influence of *Lactobacillus acidophilus*-derived surfactants on staphylococcal adhesion and biofilm formation. *Folia Microbiologica* **53**, 61-66.

Walker, M., Hobot, J., Newman, G., and Bowler, P. (2003). Scanning electron microscopic examination of bacterial immobilisation in a carboxymethyl cellulose and alginate dressings. *Biomaterials* **24**, 883-890.

Wang, J., Zhang, R., Zhou, L., Su, X., Hu, C., Zhu, B., and Feng, T. (2012). *Lactobacillus* inhibit adhesion of *Staphylococcus aureus* to HeLa cells. *Sheng Wu Gong Cheng Xue Bao Chinese Journal of Biotechnology* **28**, 715-725.

Weese, J. S., Archambault, M., Willey, B., Hearn, P., Kreiswirth, B., Said-Salim, B., McGeer, A., Likhoshvay, Y., Prescott, J., and Low, D. (2005). Methicillin-resistant *Staphylococcus aureus* in horses and horse personnel, 2000-2002. *Emerging Infectious Diseases* **11**, 430-435.

Wehkamp, J., Harder, J., Wehkamp, K., Wehkamp-von Meissner, B., Schlee, M., Enders, C., Sonnenborn, U., Nuding, S., Bengmark, S., and Fellermann, K. (2004). NF-κB-and AP-1-mediated induction of human beta defensin-2 in intestinal epithelial cells by *Escherichia coli* Nissle 1917: a novel effect of a probiotic bacterium. *Infection and Immunity* **72**, 5750-5758.

Weidenmaier, C., Peschel, A., Kempf, V. A., Lucindo, N., Yeaman, M. R., and Bayer, A. S. (2005). DltABCD-and MprF-mediated cell envelope modifications of *Staphylococcus aureus* confer resistance to platelet microbicidal proteins and contribute to virulence in a rabbit endocarditis model. *Infection and Immunity* **73**, 8033-8038.

Weston, S., Halbert, A., Richmond, P., and Prescott, S. (2005). Effects of probiotics on atopic dermatitis: a randomised controlled trial. *Archives of Disease in Childhood* **90**, 892-897.

White, D. C., and Frerman, F. E. (1967). Extraction, characterization, and cellular localization of the lipids of *Staphylococcus aureus*. *Journal of Bacteriology* **94**, 1854-1867.

White, S. H., Wimley, W. C., and Selsted, M. E. (1995). Structure, function, and membrane integration of defensins. *Current Opinion in Structural Biology* **5**, 521-527.

Whorwell, P., Altringer, L., Morel, J., Bond, Y., Charbonneau, D., O'Mahony, L., Kiely, B., Shanahan, F., and Quigley, E. (2006). Efficacy of an encapsulated probiotic *Bifidobacterium infantis* 35624 in women with irritable bowel syndrome. *The American Journal of Gastroenterology* **101**, 1581-1590.

Wickboldt, L., and Fenske, N. (1986). Streptococcal and staphylococcal infections of the skin. *Hospital Practice* **21**, 41-47.

Wilson, K. H., and Perini, F. (1988). Role of competition for nutrients in suppression of *Clostridium difficile* by the colonic microflora. *Infection and Immunity* **56**, 2610-2614.

Wolcott, R., Cutting, K. F., Dowd, S., and Percival, S. (2008). Surgical site infections: biofilms, dehiscence and delayed healing. *Touch Briefings, US Dermatology* **3**, 56-59.

Wollenberg, A., Wagner, M., Günther, S., Towarowski, A., Tuma, E., Moderer, M., Rothenfusser, S., Wetzel, S., Endres, S., and Hartmann, G. (2002). Plasmacytoid dendritic cells: a new cutaneous dendritic cell subset with

distinct role in inflammatory skin diseases. *Journal of Investigative Dermatology* **119**, 1096-1102.

Wollowski, I., Rechkemmer, G., and Pool-Zobel, B. (2001). Protective role of probiotics and prebiotics in colon cancer. *American Journal of Clinical Nutrition* **73**, 451S-455S.

Woo, J., and Ahn, J. (2013). Probiotic mediated competition, exclusion and displacement in biofilm formation by food borne pathogens. *Letters in Applied Microbiology* **56**, 307-313.

Yang, D., Chertov, O., and Oppenheim, J. (2001). The role of mammalian antimicrobial peptides and proteins in awakening of innate host defenses and adaptive immunity. *Cellular and Molecular Life Sciences* **58**, 978-989.

Zacharof, M., and Lovitt, R. (2012). Bacteriocins produced by lactic acid bacteria a Review Article. *APCBEE Procedia* **2**, 50-56.

Zaidi, Z., and Lanigan, S. W. (2010). Skin: Structure and Function. *Dermatology in Clinical Practice*, 1-15.

Zaiou, M., Nizet, V., and Gallo, R. L. (2003). Antimicrobial and protease inhibitory functions of the human cathelicidin (hCAP18/LL-37) prosequence. *Journal of Investigative Dermatology* **120**, 810-816.

Zakaria Gomaa, E. (2013). Antimicrobial and anti-adhesive properties of biosurfactant produced by lactobacilli isolates, biofilm formation and aggregation ability. *The Journal of General and Applied Microbiology* **59**, 425-436.

Zanetti, M., Litteri, L., Griffiths, G., Gennaro, R., and Romeo, D. (1991). Stimulus-induced maturation of probactenecins, precursors of neutrophil antimicrobial polypeptides. *The Journal of Immunology* **146**, 4295-4300.

Zarazaga, M., Dizy, M., Torres, C., and Ruiz-Larrea, F. (2004). High tolerance of wild *Lactobacillus plantarum* and *Oenococcus oeni* strains to lyophilisation and stress environmental conditions of acid pH and ethanol. *FEMS Microbiology Letters* **230**, 53-61.

Zhang, Z., Hinrichs, D. J., Lu, H., Chen, H., Zhong, W., and Kolls, J. K. (2007). After interleukin-12p40, are interleukin-23 and interleukin-17 the next therapeutic targets for inflammatory bowel disease? *International Immunopharmacology* **7**, 409-416.

Appendices

Appendix 1:

Data relevant for chapter (5 and 6) bacterial human skin pathogens pathway modulation by microbial compounds

***MecA* gene:**

1741 tctatagcgc attagaaaat aatggcaata ttaacgcacc tcacttatta aaagacacga
1801 aaaacaaagt ttggaagaaa aatattattt ccaaagaaaa tatcaatcta ttaaatgatg
1861 gtatgcaaca agtcgtaaat aaaacacata aagaagatat ttatagatct tatgcaaact
1921 taattggcaa atccggtact gcagaactca aaatgaaaca aggagaaagt ggcagacaaa

***Pyk* gene**:

1803121 gtttcaacag ctgttgcaac tgcattgttt aacaatgcat ctgtactctt acgtccttt
1803181 ttaactacag gttgaactcc ccaaacaatt gaacattgac gtgcagtttc ttcacttgga
1803241 gtcacc

***PBP2* gene**:

121 tcaaaaaaga atagaaa tgtgaagagaacg attattaag a ttattggctt catgattatt
181 gcatttttg ttgttctttt actaggtatc ttattgtttg cttattatgc ttggaaagca
241 cctgctttta ccgaagctaa attacaagat ccgattcctg caaagatata tgacaagaac
301 ggagaacttg ttaaaacatt agataatggc caaagacatg agcatgtaaa tttaaaagac
361 gtgccgaaat caatgaaaga cgcagtactt gcaactgaag acaatcgttt ctacgaacat
421 ggcgcacttg attataaacg tttattcggt gcaattggta agaacttgac tggtggattt
481 ggttctgaag gtgcctcaac attaacacaa caagttgtta aagatgcatt tttatcacaa
541 cataaatcta ttggacgtaa agctcaagaa gcttacttat catatcgttt agaacaagag
601 tatagtaaag atgatatctt ccaagtatat ctaaataaaa tttactattc tgatggcgta
661 acaggtatta aagctgctgc taagtattac tttaataaag atttaaaaga tttaaactta
721 gcggaagaag cttatttagc cggtttacct caggttccaa acaactataa tatttatgat
781 catccaaaag ctgctgaaga tcgtaaaaac actgttttat acttaatgca ttatcataaa
841 cgcattacag ataaacagtg ggaagatgct aagaaaatcg atttaaaagc gaacttagta
901 aatcgtactg ctgaagaacg tcaaaacatt gatacaa atc aagattctga gtataattca
961 t acgttaact ttgtaaaatc tgaattaatg aataataaag cattcaaaga tgaaaattta

***mecR1* gene**

1261 ataactaatt gttatatgct ttttgtagaa ctgcatctta ctttgacata ctttaagtcg
1321 ttcattaaga tatgacgatt ccaatgacga acttttaata acatcaattt gtcggaatgc
1381 cttaatcata taaaataagc acaacaaact accaaatacc catatcaaaa gaatcatata
1441 cgttatattt gaggtctcaa actgattaac attaattgct aagtctttcg taacagatga
1501 ttgttgacca tctaacatat gactaaccga agaagtcgtg tcagatacat ttcgattcat
1561 catatctttt gaaaatgtaa aattcgatat tttgtaaaat ggtattaatg gaattaacgt
1621 ggagacgagc actaataacc aaatcttatg tgacataata ttttgagtat attttatata

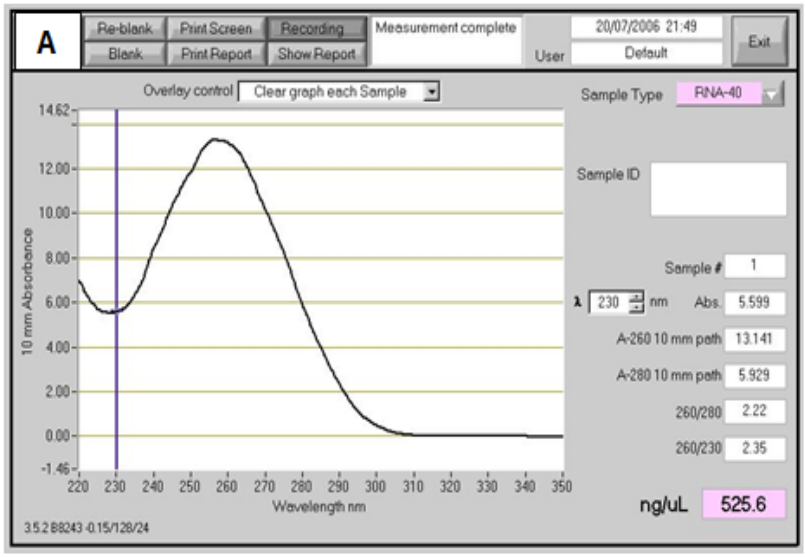
***mprF* gene**

541 atttattcaa tggttagacc acccgataaa aacaatcgtt ttgtaggatt gtactgcact
601 ttagtgtcgt gtgttgaatg gttagcagct gcagttgtat tatatttctg tggtgtaatt
661 gttgacgctc

***dltA* gene for *S. aureus*:**

781 tgtggtgaaa ttctacctca cagagcagca aaagcgttag tgagccgttt cccaagtgcg
841 acgatttaca acacatatgg tccaactgaa gctacggtag cagttacaag tattcaaatt
901 acacaagaaa tcttagatca atatccgaca ttacctgttg gcgttgaaag actaggcgca
961 agattatcta ctacagatga cggtgaactt gttattgaag gtcaaagtgt aagtttagga

Appendix 2:



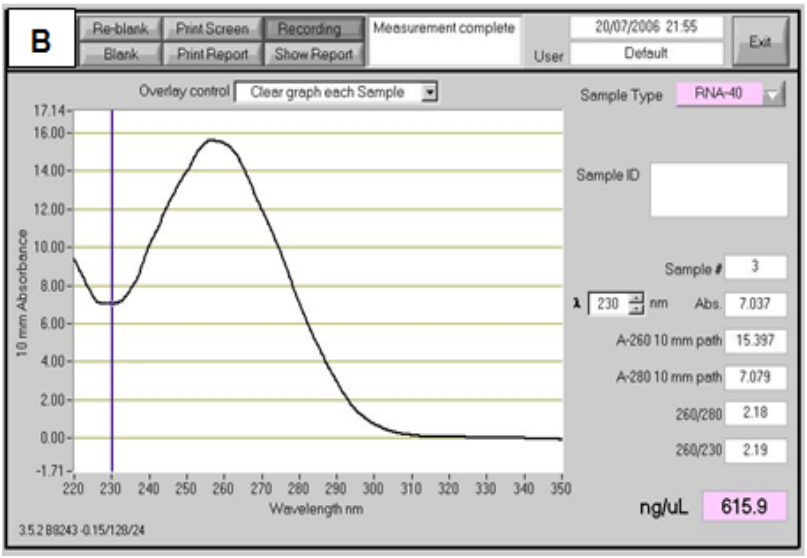


Figure 2.1 RNA purity and concentrations data, obtained following RNA extraction, for MRSA 33591 samples (A) control and (B) with hBD-2. The highlighted value number is the RNA concentration of the sample measured using a NanoDrop spectrophotometer.

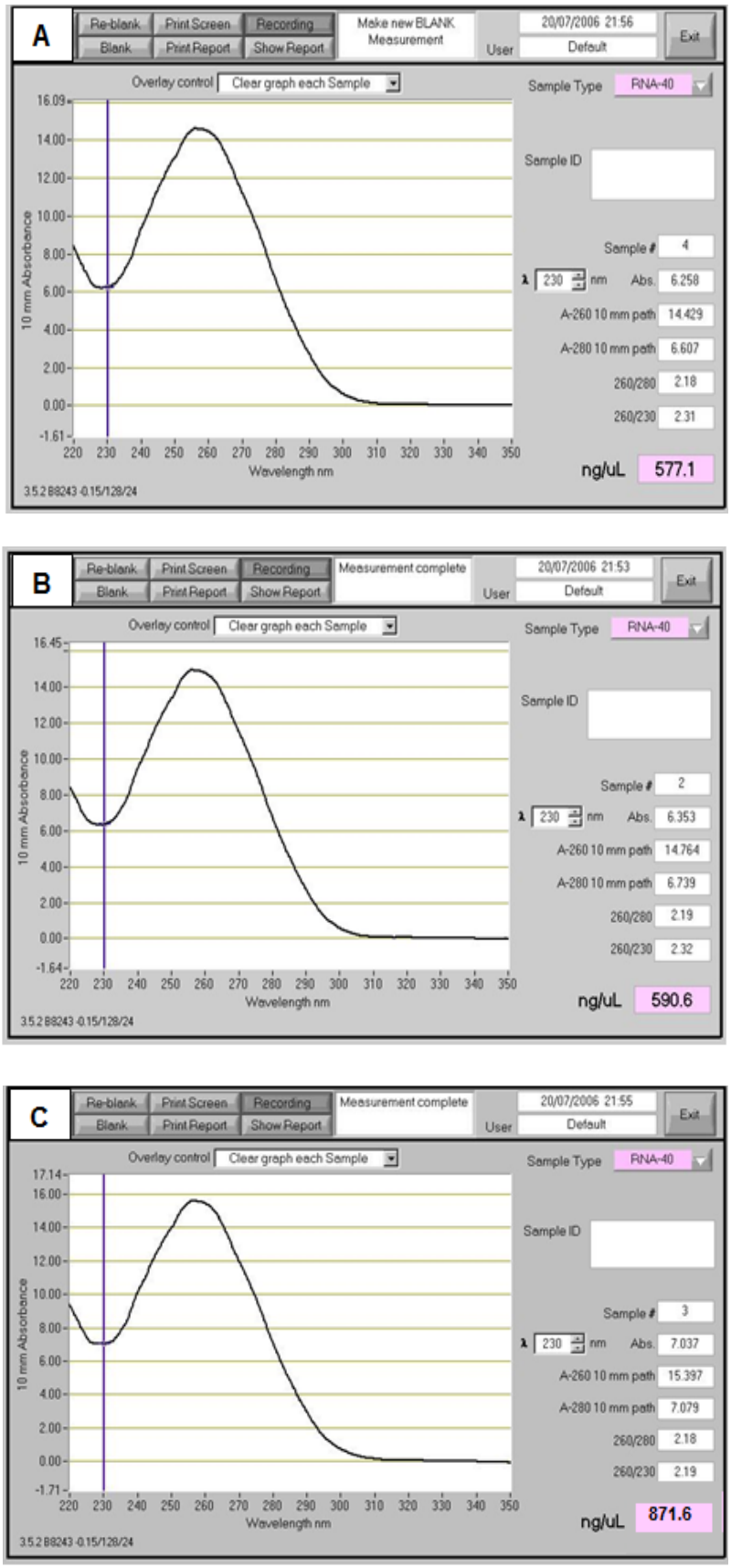


Figure 2.2 RNA purity and concentrations data, obtained following RNA extraction, from MRSA 33591 samples: (A) control 1, (B) control 2, (C) treatment. The highlighted value is the RNA concentration in the sample measured using a NanoDrop spectrophotometer.

Appendix 3:

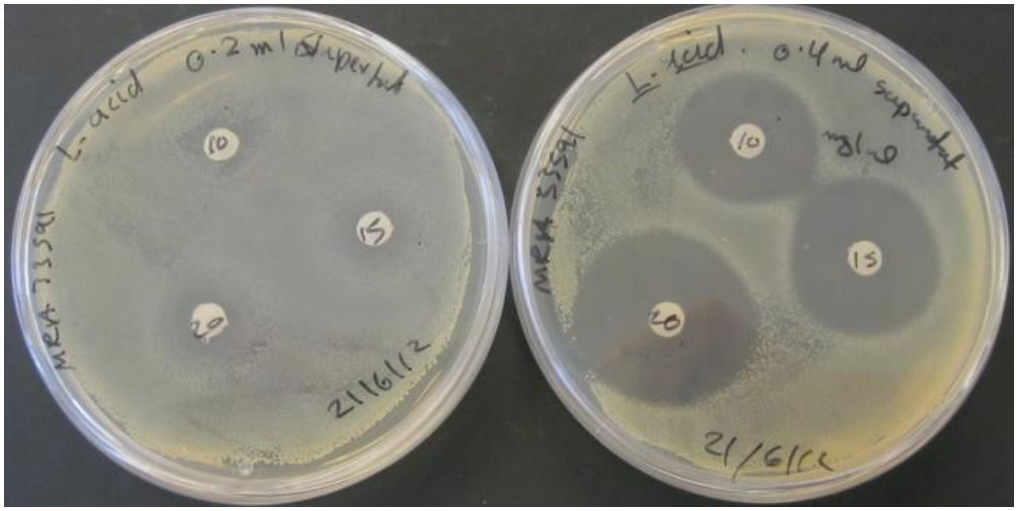


Figure 3.1 Zones of Inhibition around different concentration methicillin (10, 15 and 20µg) with 0.0312% concentration of LA against MRSA 33591

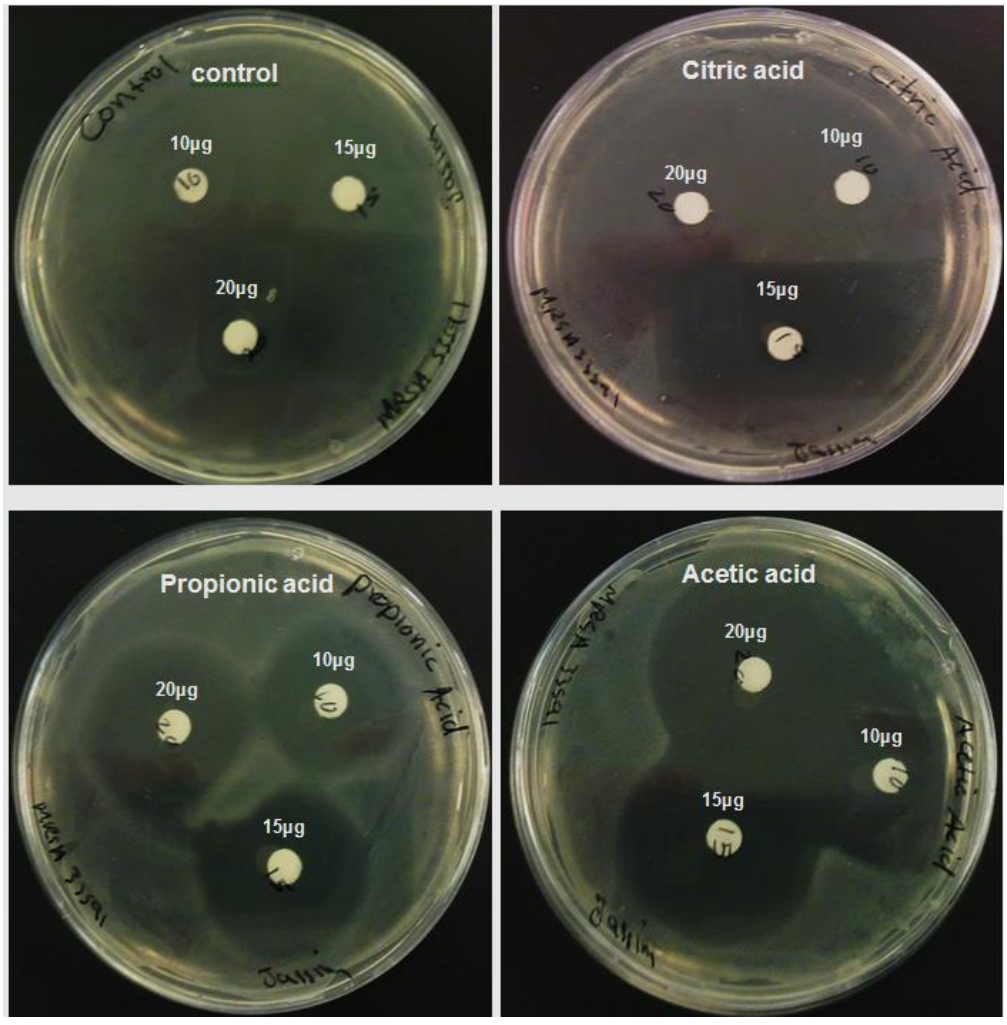


Figure 3.2 Zones of Inhibition around different concentration methicillin (10, 15 and 20µg) with 1.5% concentration of different acids against MRSA 3359.

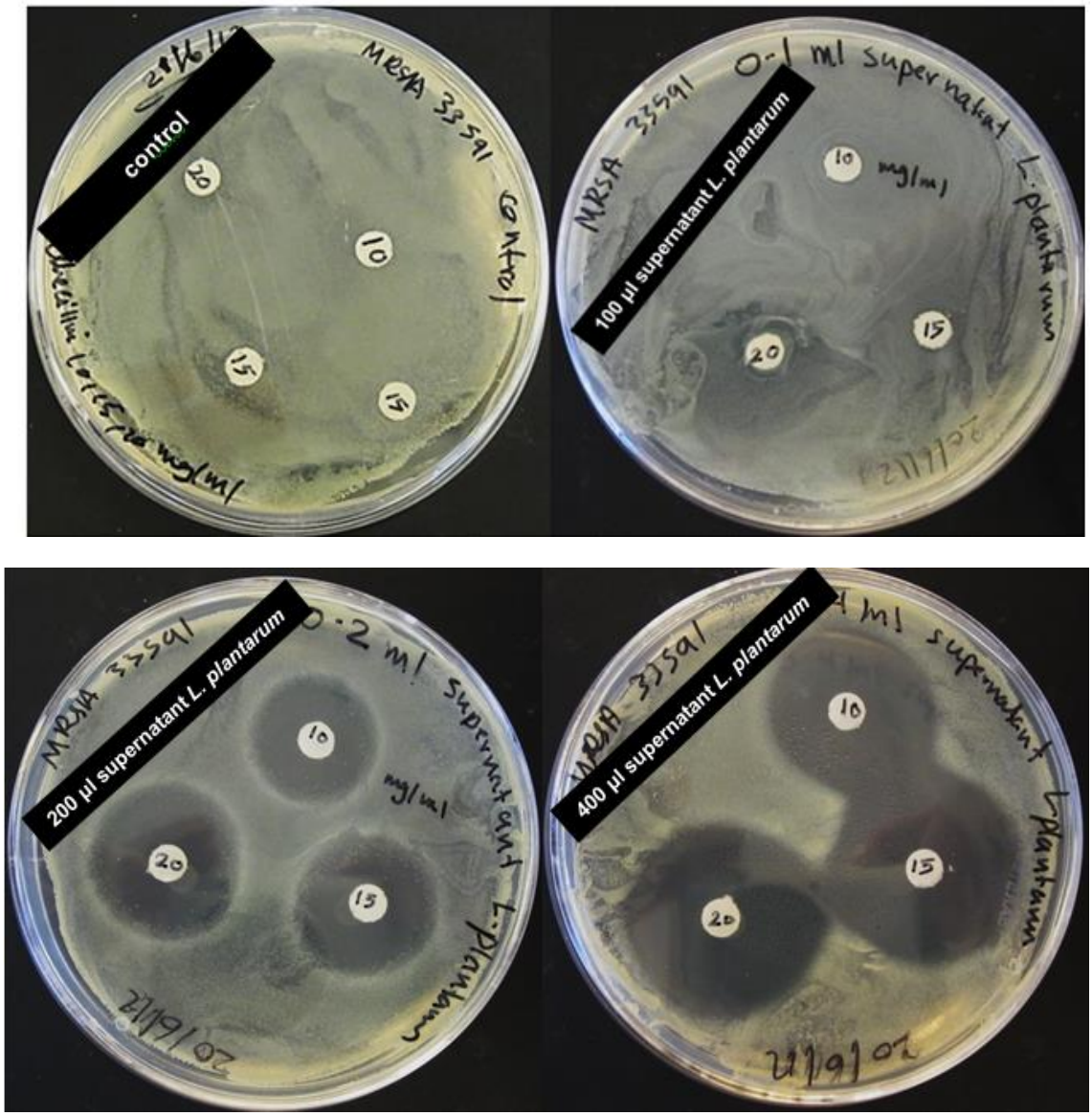


Figure 3.3 Zones of Inhibition around different concentration methicillin (10, 15 and 20µg) with different amounts supernatant of *L. plantarum* (200 and 400 µl) against MRSA 33591.

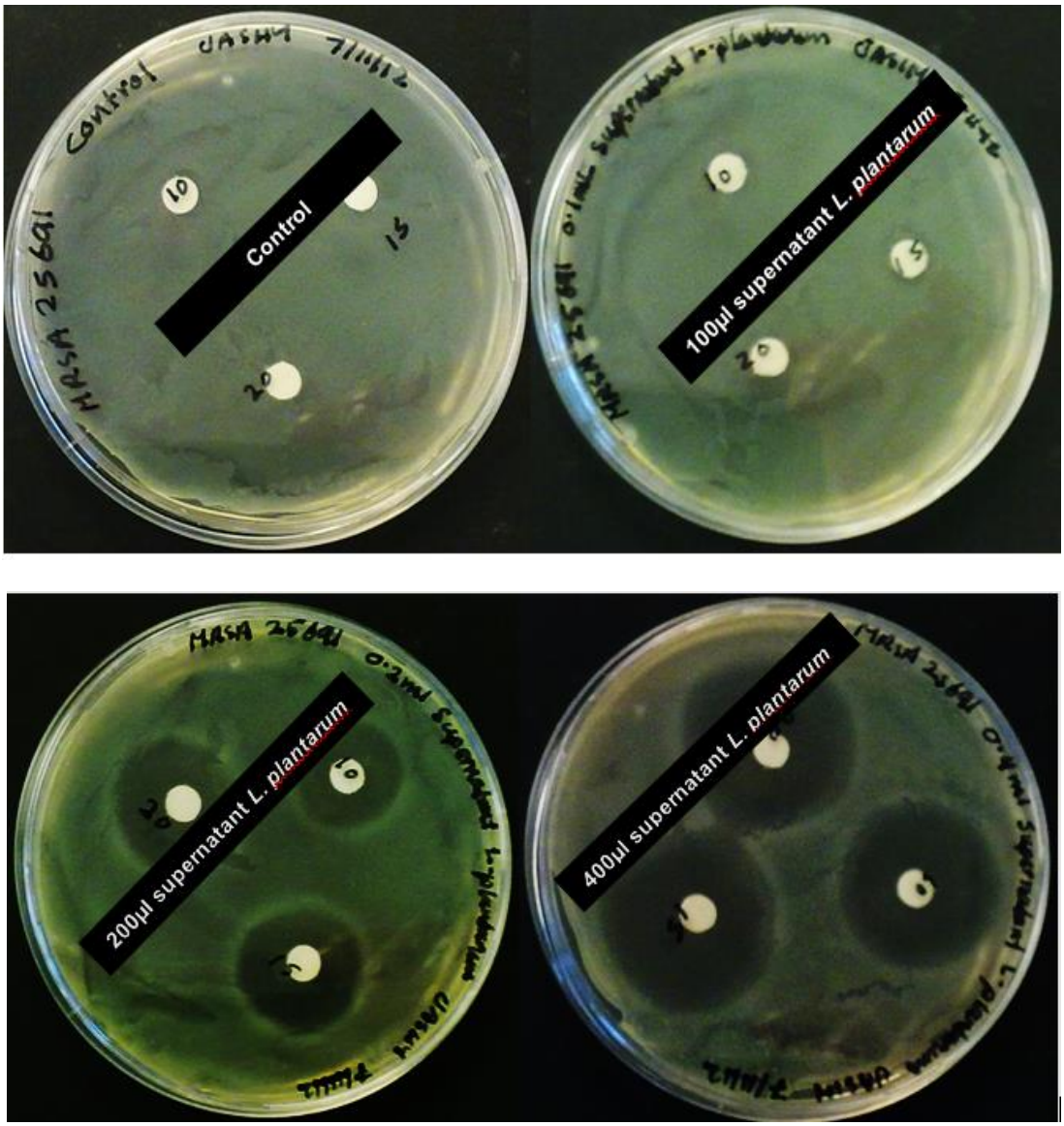


Figure 3.4 Zones of Inhibition around different concentration methicillin (10, 15 and 20 µg) with different amounts supernatant of *L. plantarum* (200 and 400 µl) against MRSA 25691 (human skin wound strain).

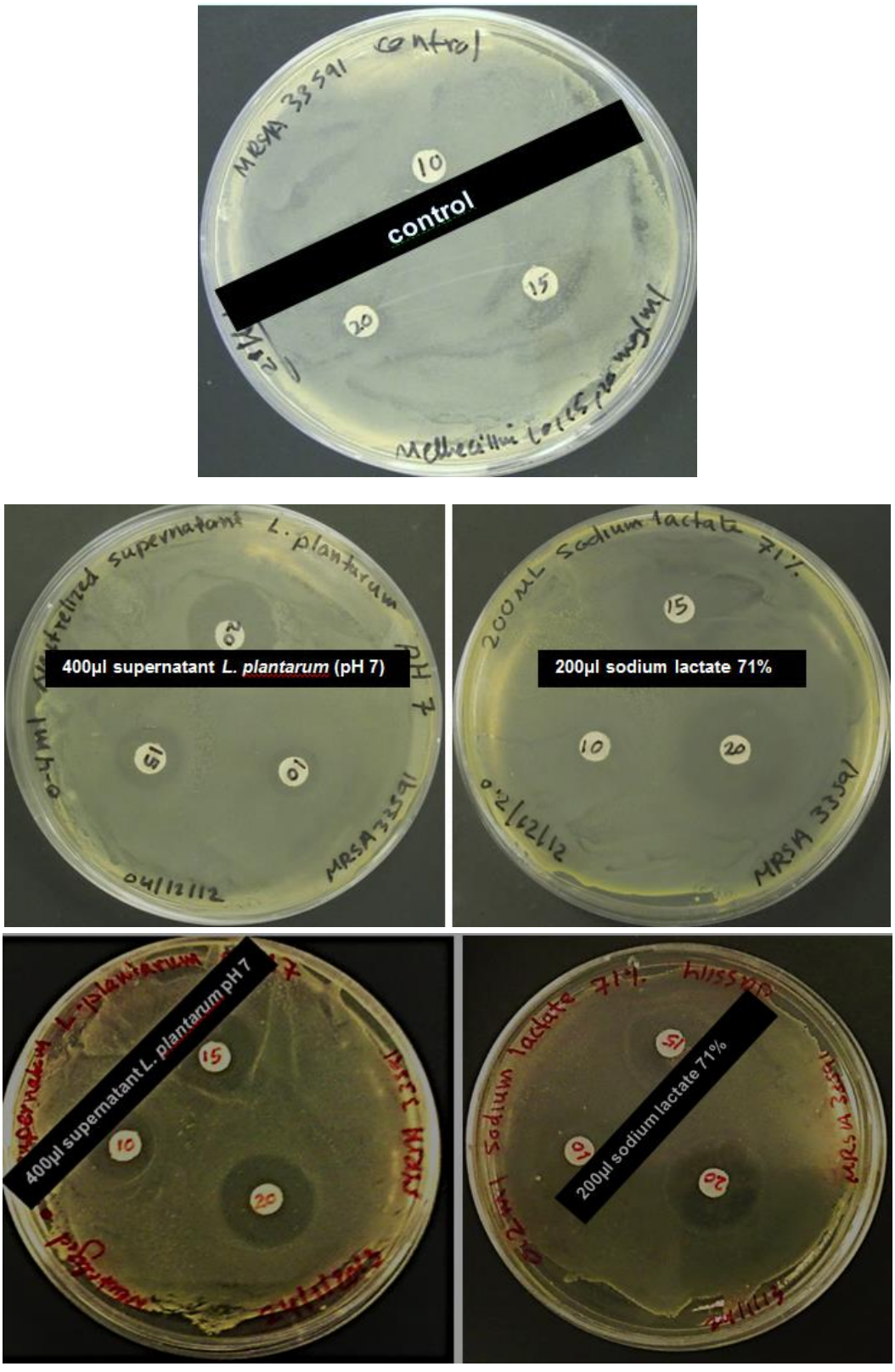


Figure 3.5 inhibition zones around different concentrations of methicillin (10, 15 and 20 µg) with 400 µl neutralised supernatant of *L. plantarum* (pH 7) and 200 µl sodium lactate 71% against MRSA 33591.

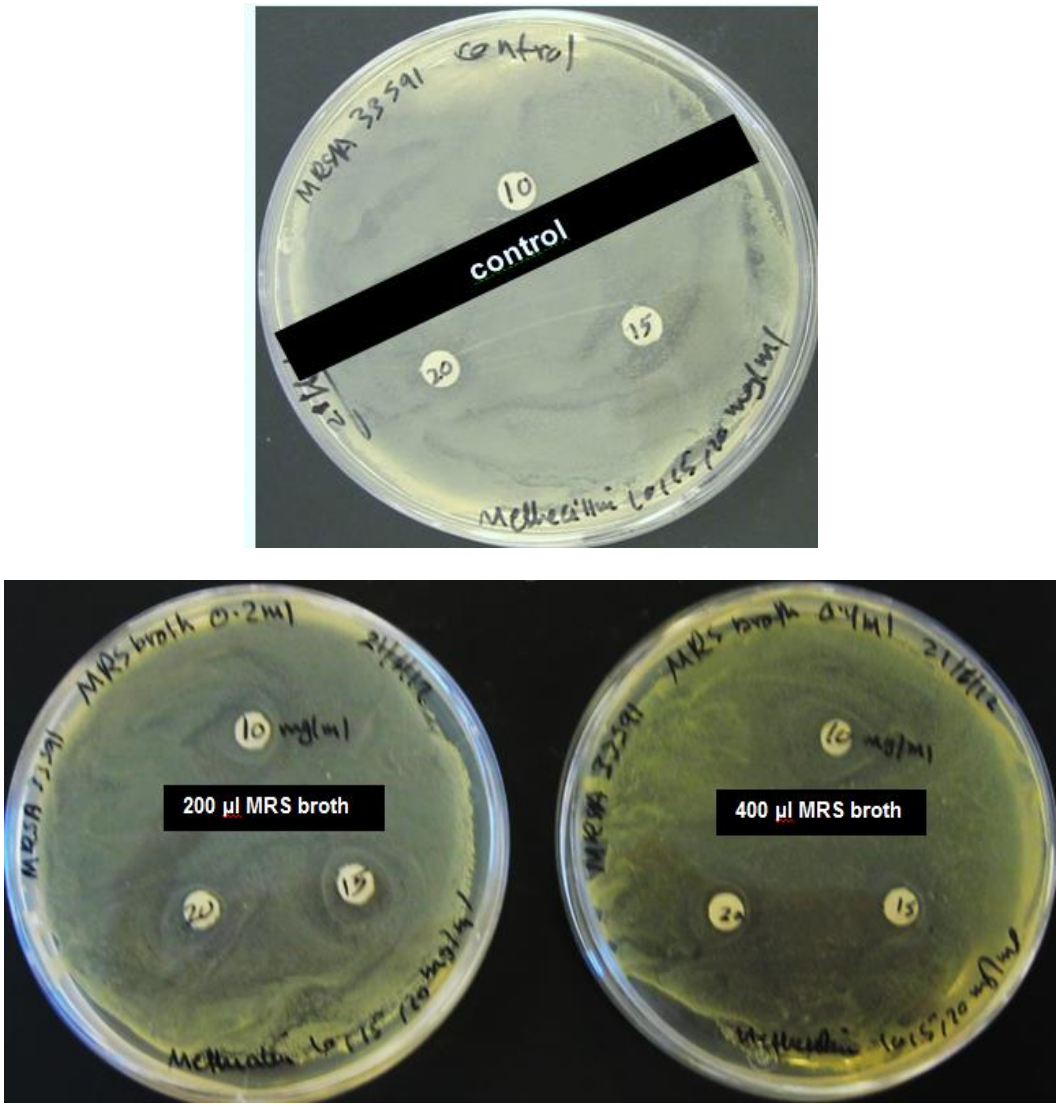
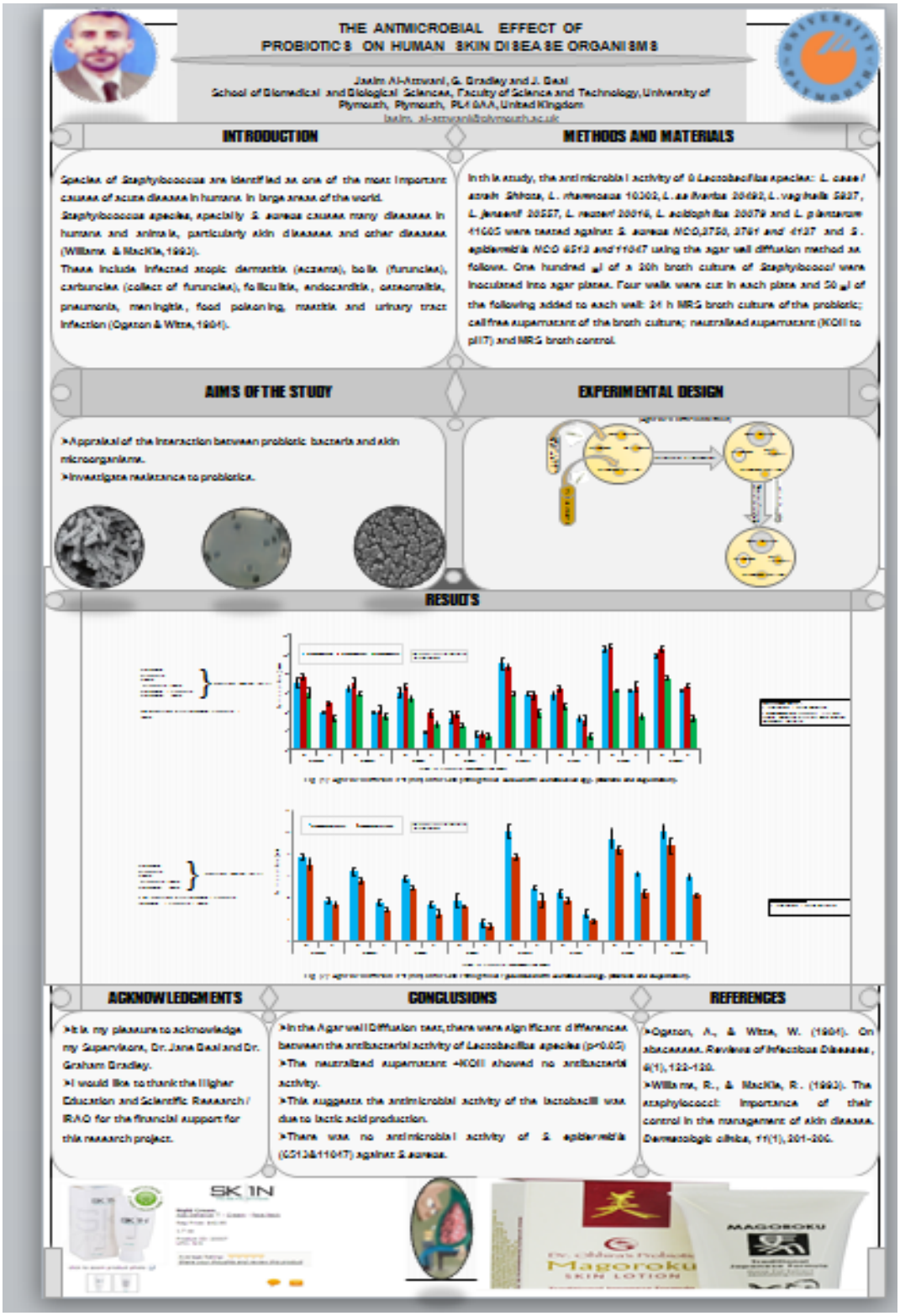


Figure 3.6 Inhibition zones were not observed around methicillin discs (10, 15 and 20 µg) within different amounts MRS broth (200 and 400 µl) against MRSA 33591.

Appendix 4:

Posters:

4.1 Chapter 2: Evaluation of antimicrobial activity of probiotic *Lactobacillus* species against bacterial human skin pathogens



4.2 Chapter 2: Evaluation of antimicrobial activity of probiotic *Lactobacillus* species against bacterial human skin pathogens.

The results have been published online (Abstract) in XVth International Congress on Animal Hygiene 2011, 3th - 7th July 2011, Vienna, Austria.

THE EFFECT OF PROBIOTICS ON HUMAN SKIN STAPHYLOCOCCAL DISEASES (Abstract)

Jasim Al-Attwani*, J. Beal, G. Bradley

*School of Biomedical and Biological Sciences, Faculty of Science and Technology, University of Plymouth, Plymouth, PL4 8AA, United Kingdom *All correspondence: jhal-attwani@plymouth.ac.uk*

INTRODUCTION

Species of *Staphylococcus* bacteria are identified as one of the most important causes of acute disease in humans. *Staphylococcus aureus* causes many diseases in humans and animals, particularly skin diseases (Williams & MacKie, 1993).

Researchers are attempting to find successful solutions to overcome microbial infections especially that are resistant to most antimicrobial drugs such as methicillin resistant *S. aureus* (MRSA) one potential area of investigation is the use of probiotics (Reid, Jass, Sebulsky, & McCormick, 2003).

MATERIAL

In this study, will be used several equipments, bacterial isolates (9 *Lactobacillus* isolates and 5 *Staphylococcus* isolates), MRS and Nutrient media.

METHODS

In the methods conducted agar well diffusion assay to observe effect; Lactobacillus species (*L. casei Shirota, L rhamnosus, L. salivarius 20492, L. vaginalis 5837, L. jensenii 20557, L. reuteri 20016, L. acidophilus 20079, L. plantarum and L. fermentum*), supernatant, KOH+ supernatant and control on pathogenic human skin *Staphylococcus aureus* and *S.epidermidis*.

RESULTS

The good results with inhibition zone (mm) assay for *Lactobacillus* species were; *L. salivarius , acidophilus, L.plantarum L. casei shirota* and *L. rhamnosus* on *S. aureus* isolate respectively, and on *S. epidermidis* were; *L.plantarum L. casei shirota, L. acidophilus, L. salivarius* and *L. rhamnosus* respectively.

CONCLUSIONS

The initial results were good and agreement with previous studies, whether on human skin diseases or in the other diseases, and can be applied on human after it experimented on animal models, then observation a positive results.

4.3 Chapter 3: Assessment of aggregation, biofilm formation, and adhesion to keratin.

The results have been published (Abstract) in NEMO Keele Conference, Keele University, Keele, UK.

4.4 Chapter 4: Antimicrobial activity of human β-defensin-2 (hBD-2) and probiotic *Lactobacillus plantarum* (*L. plantarum*) against bacterial human skin pathogens.

The results have been published abstract and article in SFAM conference 1st - 4th July, Cardiff, UK, and publish (abstract) in V International Conference on Environmental, Industrial, and Applied Microbiology, Medicine College, Madrid University, Madrid, Spain, 2nd - 4th October 2013.

Antimicrobial Activity of Human B-Defensin-2 (HBD-2) and Probiotic *Lactobacillus plantarum* (*L. plantarum*) on Bacterial Human Skin Pathogens

J. Al-Attwani , P. Waines and J. Beal
School of Biomedical and Biological Sciences, University of Plymouth, Plymouth, PL4 8AA, UK
Jasim.alattwani@plymouth.ac.uk

RESEARCH WITH PLYMOUTH UNIVERSITY

Introduction

Human β-defensin-2 (hBD-2) is a cysteine rich cationic peptide of the innate immune system that serves as an antimicrobial peptide (AMP) (Barden *et al.*, 2004). The aims of this study were to determine the antimicrobial activity of recombinant hBD-2 and synergism between hBD-2 and probiotic *L. plantarum* against human skin pathogens, *S. aureus* and *P. aeruginosa*.

Methods

Antimicrobial activity of recombinant hBD-2, alone or in the presence of *L. plantarum* or NaCl (0, 25, 50, 100 and 150 mM) was tested on the following pathogens: *P. aeruginosa* NCIB 8626, *S. aureus* 4137, MRSA ATCC 33591. Approximately 10^7 CFU/ml of each bacteria and hBD-2 (2.5, 5 and 10 µg/ml) were incubated at 37° C for 2 and 5 h and plate counts conducted on appropriate agar. The procedure was repeated for hBD-2 with *L. plantarum* and hBD-2 (7.5 µg/ml) with NaCl (0-150 mM).

Results

Human skin pathogens were very effective, especially with10 µg/ml hBD-2 for 5 h and the counts survival rates were as follows: *S. aureus* 4137 (26.5% ± 3.4%), *Pseudomonas aeruginosa* 8262 (23.3% ± 2.3%) and MRSA 33591 (30% ± 3.4%). When hBD-2 was mixed with *L. plantarum* the survival rate was significantly higher: *P. aeruginosa* 8626 (8.5% ± 2%), *S. aureus* 4137 (9.4% ± 3%) and MRSA 33591 (12% ± 3.4%). In addition, The effectiveness of hBD-2 on human skin pathogens in the presence of NaCl was studied, which demonstrated that NaCl inhibited hBD-2 activity. Recombinant hBD-2 activity was reduced in the presence of 25-150 mM NaCl.

Conclusions

These observations suggest that the recombinant hBD-2 has potential antimicrobial activity against bacterial human skin pathogens and this was greater when mixed with probiotic *L. plantarum*. In addition, there is a negative impact on the effectiveness of hBD-2 when adding sodium chloride, especially at high concentrations (150 mM).

Ongoing works

(1) Effect NaCl in different concentrations on the antimicrobial activity of hBD-2 and *L. plantarum* against human skin pathogens.

(2) Gene expression with synergism between hBD-2 and *L. plantarum* against human skin pathogens.

Acknowledgments

I would like to thank the Higher Education and Scientific Research / Iraq for the financial support for this research.

Reference

Bardan, A., V. Nizet, *et al.* (2004). "Antimicrobial peptides and the skin." Expert opinion on biological therapy 4(4): 543-549.

Appendix 5:

Training courses, conferences and taught sessions attended

5.1. Generic Skills Training completed

1. Module Postgraduate Research Skills and Methods (BIO5124), 6th - 14th December 2010.

2. GAT course, 27th January 3rd , 10th , 17th , 24th February 3rd March 2011.

3. ENV 5101 Demonstration, 29th October - 9th December 2010.

4. PowerPoint - Creating a Presentation, 22th February 2011.

5. PowerPoint - Creating a Presentation, 27th February 2011.

6. Plagiarism, 9th March 2011.

7. Word Using Master Documents, 28th March 2011.

8. Developing Professional Writing Skills, 6th April 2011.

9. Presentation for BIO5124, 27th April 2011

10. Session about Olympus Microscope, 26th May 2011.

11. The transformation of QPCR, 24th October 2011.

12. Excel 2010 Essential Features, 02nd November 2011.

13. The transformation of QPCR, Jams Hargreaves-NGOTSS team, 24th October 2011, Roland Levinsky Building, Room: 206, 13:00 - 14:00.

14. Life technologies Real time PCR training course, 23ed November 2011, Exeter trainer Chris Maddren.

15. Excel 2010 Essential Features, Jaqcui Hunter, 02nd November 2011, 10:00 - 13:00, Babbage building, Room: 322.

16. Conditional formatting and chart, 29th February 2012, 10:00-12:00

17. Excel 2010 Essential Features, Jaqcui Hunter, Babbage building, Room: 322.

18. PP. enhancing your presentation, 15th March 2012, Thursday 10:00 - 13:00, Babbage, R: 322.

19. Excel sorting filtering and pivot tables, 21st March 2012, Wednesday 13:30 - 15:30, Babbage, R: 322.

20. Writing for Research Publication, 30th March 2012, 9:00 - 12:30, Rolle Building, R: 117.

21. Word Master Documents, 3ed April 2012, 10:00-12:00, Babbage, R: 322.

22. SPSS, 29th May 2012, 13:00-17:00, Babbage Building Room: 109.

23. The CRTB Research Day, Wednesday 4th July, Portland Square, Attendance.

24. Practical techniques in molecular biology workshop, 16th - 19th July 2012, Plymouth University.

25. The pipette workshop, 3ed floor Room: 312, Davy building

26. Mass spectrometry-based proteomics and its application in malaria research, 27th November 2012, Plymouth University.

5.2. Other Skills Training completed (e.g. UKGRAD School; English / Foreign Language) and / or conferences / meetings attended

1. English Language Summer School (intensive course) / Academic writing, Cookworthy Refectory, 25th October 2010 - 25th January 2011.

2. Supporting English Language Classes (tutorial lessons), Cookworthy Refectory, 31st May 2011.

3. Postgraduate English Language Summer, Cookworthy Refectory, June 2011 July 2011.

4. English Language Summer School (intensive course) / Academic writing, Cookworthy Refectory, 25th October 2010 - 25th January 2011.

5. English Language for international Students, Cookworthy Refectory, 10th October 2011.

6. English language support for international students, Cookworthy Refectory, 3ed December 2012.

5.3: Seminar / Conference / Performance presentations

1. Marine conference, Plymouth University, 20th December 2010, Audience.

2. The Postgraduate society conference series, University of Plymouth, Roland Levinesky, 17th March - 2011, Audience.

3. Annual Research Day Conference, Plymouth University, 4th - 5th - March 2011, Audience.

4. The antimicrobial effect of probiotics on human skin diseases, Postgraduate / Society Conference Roland Levinsky / Plymouth University, 17th March 2011, Published (Abstract).

5. 2nd Marine Institute Research Conference, University of Plymouth, Sherwell Centre, 5th May 2011, Audience.

6. The Postgraduate Society Conference Series, University of Plymouth, Rolle Building Lecture Theatre, Poster, 29th June 2011, Published (Abstract).

7. The effect of probiotics on human skin staphylococcal diseases, XVth International Congress on Animal Hygiene / Vienna, Austria, Poster, 3ed July 2011, Published (Abstract).

8. Ecotoxicology Research and Innovation Centre (ERIC) conference, Portland Square Building, Plymouth University, 13th July 2012, Audience.

9. Activity of human β-defensin-2 (hBD-2) and probiotic Lactobacillus plantarum against bacterial human skin pathogens, SFAM Conference / Cardiff, UK, Poster, 1st - 4th July 2012, Published (Abstract).

10. The effect of *Lactobacillus* species on the biofilm formation by bacterial human skin pathogens, NEMO in Keele University Conference / Keele University, Oral and Poster, 9th November 2012, Published (Abstract).

11. The postgraduate society conference series, Plymouth University, Rolle Building, 12th November 2012, Audience.

12. The effect of *Lactobacillus* species on the biofilm formation by bacterial human skin pathogens, CARS Postgraduate Symposium / Plymouth University, Poster, 10th December 2012, Published (Abstract).

13. Postgraduate society 2nd conference, Plymouth University, November 2013, Audience. restoration of susceptibility of methicillin-resistant *S. aureus* (MRSA) to β-lactam antibiotics by probiotic *L. plantarum*, SoBBS Seminar Series / Babbage 006 / Plymouth University, Oral presentation, 15th May 2013.

14. The annual conference, Plymouth University, 18th June 2013, Audience.

15. Annual Conference, Plymouth University, Poster, 19th June 2013, Published (Abstract).

16. Activity of human β-defensin-2 (hBD-2) and probiotic *Lactobacillus plantarum* against bacterial human, Madrid / Spain, Poster, 2nd - 4th October 2013, Published (Abstract).

17. Restoration of susceptibility of methicillin-resistant *S. aureus* (MRSA) to β-lactam antibiotics by probiotic *L. plantarum*, Madrid / Spain, Oral presentation, 2nd - 4th October 2013, Published (Paper), ISBN: 978-90-8686-243-6, ISBN E-book: 978-90-8686-795-0, pp. 585-589.