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ANTIMICROBIAL POTENTIAL OF PROBIOTIC LACTIC ACID BACTERIA ISOLATED FROM MILK OF LACTATING COW AGAINST THE MOST CONTAMINATED BACTERIAL INFECTION OF BURNS IN HUMAN

A Thesis

Submitted to the Council of the College of Veterinary Medicine/ University of Diyala in a Partial Fulfillment of the Requirements for the Degree of Master of Science in Veterinary Medicine (Veterinary Microbiology)

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بسم الله الرحمن الرحيم

"نَرْفَعُ دَرَجَاتٍ مَنْ نَشَاءُ وَفَوْقَ كُلِّ ذِي عِلْمٍ عَلِيمٌ"

صدق الله العظيم

سورة يوسف الآية٦٧

Supervisor Certification

I certify that this thesis entitled **(Antimicrobial Potential of Probiotic Lactic Acid Bacteria Isolated from Milk of Lactating Cow Against the most Contaminated Bacterial Infection of Burns in Human)** was prepared by (**Rawan Raad Hussein)** under our supervision at the Department of Microbiology, College of Veterinary Medicine, University of Diyala, as a partial fulfillment of the requirements for the Master Degree of Science in Veterinary Medicine (Veterinary Microbiology).

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Examination Committee Certification

We, the examination committee, certify that the entitled thesis (**Antimicrobial Potential of Probiotic Lactic Acid Bacteria Isolated From Milk of Lactating Cow Against The Most Contaminated Bacterial Infection of Burns in Human)** has been examined and read through all of its contents and related topics. The committee recommends that the student passed and awarded the degree of Master of Science in Veterinary Medicine (Veterinary Microbiology).

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Dedication

I would like to dedicate this work to the one who encouraged me to persevere throughout my life, to the first and most prominent man in my life **(*my dear father*)**, to the one with whom I rise, and on whom I rest, the giving heart **(*my dear, affectionate mother*)**, to those who made an effort to help me in every step of my life **(*my dear brothers*)**.

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ABSTRACT

Burns infection is one of the most common consequences and the leading causes of death. The burn sites are particularly exposed to various infections, mostly, with bacteria that are resistant to many types of antibiotics, leading to a prolonged hospitalization, and invasive care procedures. Therefore, it is necessary to find alternative solutions to control burns infections. This study aimed to assessment antimicrobial potential of lactobacilli Cell Free Supernatants (CFS) and evaluation of their antimicrobial activity when combined with some antibiotics against the most isolated burn-contaminants bacteria. Regarding methodology, A 42 samples of cow's milk was collected from two different areas in Baghdad. Milk samples were inoculated onto MRS agar under aerobic conditions for 24 hrs at 37 ^{0} C. The bacterial isolates in this study were identify using manual and automated (VITEK2 system). The most isolated *Lactobacilli* species were *Lactobacillus plantarum* (spp.1, spp.2, spp.3) and *Lactobacillus acidophilus*. Moreover, 187 swabs were collected from patients with burns infection living in Baghdad. Each of the burn swabs were inoculated onto enriched and selective culture media. The data indicated that *Pseudomonas aeruginosa* (34.22%) and *Staphylococcus aureus* (27.27%) were the most predominant isolates, followed by *Acinetobacter baumannii* (17.11%), *Klebsiella pneumoniae* (14.44%), *Escherichia coli* (4.81%) and *Proteus mirabilis*. (1.07%). These isolates were multi-drugs resistant (MDR). However, some bacterial contaminants were sensitive to certain antibiotics. *A. baumannii*

was sensitive to Minocycline and Colistin with MIC (<= 1) and (<= 0.5), respectively, *P. aeruginosa* was sensitive only to Colistin with MIC (<= 0.5), *S. aureus* was sensitive to Linezolid, Teicoplanin, Vancomycin, Tigecycline and Nitrofurantoin with MIC (<= 0.5), (<= 0.5), (<= 0.5), (<= 0.12) and (<= 16), respectively. This study reported that when CFS was combined with the used antibiotics, the zone of inhibition increased in some antimicrobial combinations compared to using antibiotics alone. The zones of *P. aeruginosa* growth inhibition were increased when CFS of *L. plantarum* spp. 1, *L. plantarum* spp. 2, *L. plantarum* spp. 3 and *L. acidophilus* were combined with Azithromycin, the zone of inhibition increase from zero to 27.5 mm, 25.5 mm, 24.5 mm and 22.5 mm, respectively. In most cases, a high zone of bacterial growth inhibition was noticed when CFS used alone. This study data showed that the highest auto-aggregation percentages were after 24 hrs of incubation, 87.2% for *L. plantarum* spp 2. The highest co-aggregation percentages were also reported after 24 hrs incubation between *L. plantarum* spp.2 and *P. aeruginosa* (88.4%). Furthermore, the minimum inhibitory concentration (MIC90) of CFS prepared from *L. plantarum* spp. 2 against *P. aeruginosa* was 50%, inhibited 97.17%. While the MIC90 of *L. plantarum* spp.3 CFS was 50% against *A. baumanni, P. aeruginosa, K. pneumoniae,* and *S. aureus*; prevened 99.92%, 99.87%, 99.85% and 99.55%, respectively of the their growth, p-value (< 0.05), and p-value (< 0.001). No MIC90 were determined for *L. plantarum* spp. 1 and *L. acidophilus'* CFS against isolated bacteria. In addition, the Minimum Biofilm Inhibitory

Concentration (MBIC50) of lactobacilli CFS was detected in rang (25%-6.25%). The MBIC50 of CFS prepared from *L. acidophilus* against *A. baumanni P. aeruginosa*, *K. pneumonia* and *S. aureus* was ranged between 12.5% - 25%, preventing bacterial biofilm by 74.29%, 60.35%, 50.00% and 61.53%, respectively. While the MBIC50 of CFS prepared from *L. plantarum* spp. 1 were 12.5%, 6.25%, 12.5% and 25% which, prevented 67.14%, 62.92%, 63.04% and 67.91%, respectively of *A. baumanni P. aeruginosa*, *K. pneumonia* and *S. aureus* biofilm. In regards to the MBIC50 of CFS prepared from *L. plantarum* spp. 2, it was 12.5%, 6.25%, 12.5% and 25% inhibited 71.36%, 72.89%, 53.12% and 58.59%, of biofilm of *A. baumanni P. aeruginosa*, *K. pneumonia* and *S. aureus,* respectively. The MBIC50 of CFS prepared from *L. plantarum* spp. 3 against *A. baumanni P. aeruginosa*, *K. pneumonia* and *S. aureus* were; 25%, 12.5%, 6.25% and 25%, preventing 74.19%, 64.58%, 53.01% and 99.65% of the bacterial growth, respectively. The author concluded that *Lactobacilli*, alone, or in combination with some antibiotics could be used as effective-alternatives in the therapeutic applications to control burns infection-associated pathogenic bacteria.

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	biofilm	

List of Abbreviations

Letter or Symbol	Meaning
-	Negative result / not able to growth
+	Positive result / able to growth
µg	Microgram
µm	Micrometre
A	Acid
AK 30	Amikacin in 30 Mcg
ALK	Alkaline
AMR	Antimicrobial resistance
ANOVA	Analysis of variance
AX 25	Amoxcilin in 25 Mcg
AZM 15	Azithromycin (15) Mcg
BHI	Brain heart infusion
BHI	Brain Heart Infusion Broth
CFS	Cell Free Supernatant
CFU	Colony-forming unit
CPS	Capsular polysaccharide
CS 10	Coliston sulphid in 10 Mcg
D.W.	Distilled water
EPS	Extracellular polysaccharides
ESBL	extended spectrum beta-lactamase
FAO	Food and Agriculture Organization of the United Nations
H_2S	Hydrogen Sulfide
Ib	Pound
ISAPP	International Scientific Association For Probiotics And Prebiotics
Kg	Kilogram
L 10	Lincomycin in 10 Mcg
LAB	Lactic Acid Bacteria
MBIC	Minimum biofilm Inhibitory Concentration
MDR	Multidrug Resistance
ME 10	Methicillin in 10 Mcg
Mg	Milligram
MIC	Minimum Inhibitory Concentration
Mm	Millimeter
MRAB	multidrug-resistant *Acinetobacter baumannii*
MRS	De man, Rogosa and Sharpe
MRSA	methicillin-resistant Staphylococcus aureus
NA 10	Naldixic acid in 10 Mcg
Nm	Nanometer

P 10	Penicillin in 10 IU
PBS	Phosphate buffer saline
R	Resistance
Rpm	Reotation per minute
S	Sensitive
TCS	two-component systems
Tob 10	Tobramycin in 10 Mcg
TSI	Triple Sugar Iron
VA 30	Vancomycin in 30 Mcg
VRE	vancomycin-resistant *Enterococcus*
VRSA	vancomycin-resistant *S. aureus*
WELL	Use CFS alone
WHO	World Health Organization

CHAPTER ONE

INTRODUCTION

1.1 INTRODUCTION

The most large organ in human body is the skin, skin makes up around 15% of an adult human's entire body weight. It is made of Three main layers, including “from top to bottom” the epidermis with appendages, dermis and the hypodermis. These layers having their own specific functions; including (i) protection from external physical, chemical and biological factors, (ii) preventing excess water loss and (iii) thermoregulation in addition to being (iv) a sensory organ.The skin also protects the body from the entry of various microorganisms, including bacteria, viruses and fungi etc. The skin is lined with a mucous membranes. The body is easily susceptible to harm due to the exposure of the skin layers to burning or tearing (Sullivan *et al*., 2022).

One of the most common injuries to the skin is burns, it's have a significant impact on the patients in many ways; physically, psychologically, and physiologically. Burns are still considered among the top of death causes and disability in the world (Ribeiro *et al*., 2008). Microbial infections also considered as the main causes of morbidity and mortality increasing in patients burned-skin. These infections could be viral, fungal and bacterial infections, especially infections caused by multidrug-resistant strains (MDR). *Staphylococcus* spp, *beta-hemolytic Streptococcus* group A and *Enterococcus* spp. are distinguished as the most common Gram-positive bacteria involved in burn infections, While Gram-negative bacteria include *Pseudomonas*

aeruginosa, *Klebsiella spp, Stenotrophomonas spp., Enterobacter cloacae., Acinetobacter baumannii* and *Escherichia coli* (Weber *et al*., 2004; Ronat *et al*., 2014).

These pathogenic bacteria have virulence factors which allow them to colonize, reproduce, produce enzymes/toxins and causing infection, most of these infections are resistance to various traditional antimicrobial agents (Casadevall and Pirofski, 2009; Ryding, 2021).

Antimicrobial resistance (AMR) occurs when microbes develop mechanisms to protect themselves from antimicrobial effects. Each year, the infections of AMR kills millions of people. Resistant microbes infections are more difficult to be treated. A higher doses of antimicrobial drugs, in this situation potentially more toxic medications will be enough to control such infection, these methods may also be more costly. Genetic mutation play an important role in bacterial resistance development or even as a result of acquiring resistance from one species to another. Antimicrobial resistance is increasing globally as a result of a random antibiotic prescription and dispensing in developing countries (Vega and Gore, 2014).

Bacterial cells are commonly found in nature either as varied communities embedded in a complex matrix, or as simple planktonic cells. Biofilms formation is the most important virulence factor of pathogenic bacteria which colonize onto a variety of biotic (tissues) and abiotic (devices) surfaces

(Reg Bott, 2011; Jamal *et al*., 2018). Bacteria form biofilm to protect themselves from the harmful environmental circumstances such osmotic stress, metal toxicity, and antibiotic exposure (Gebreyohannes *et al*., 2019).

Pathogens that develop biofilms are linked to persistent infections in up to 80% of cases. About 90% of the biofilm mass is made up of proteins, DNA, and extracellular polysaccharides (EPS). In addition to cell stability and mediating surface adhesion, EPS also serves as a scaffold for the attachment of cells, enzymes, and antibiotics (Flemming and Wingender, 2010; Beloin, 2014).

Antibiotic-resistant diseases are growing widespread in the whole world, and this means that treatments for these diseases are becoming rare (Cerceo *et al*., 2016). It is anticipated that will be no effective antibiotic available to treat infections by 2050 if no new antibiotics are manufactured or discovered (Rolain *et al*., 2016).

The direct administration of probiotics to burn patients is a unique strategy that avoids the drawbacks of current antibiotic therapy (Argenta *et al*., 2016). According with a current definition, "Probiotics are live microorganisms that, when provided in suitable proportions, confer a health effect on the host". This definition of probiotics was created and supported by the “Food and Agriculture Organization” of the United Nations (FAO), “The World Health Organization” (WHO), and “the International Scientific Association for Probiotics and Prebiotics” (ISAPP) (Hill *et al*., 2014). Probiotic

therapy has been shown in animal models and patients to reduce infections of the middle ear, bladder, gut, and urogenital tract, in addition to its applications in the lay press and food sector (Patra *et al*., 2022).

Cow milk is a light liquid made by a cow's mammary glands. It is an infant mammal's principal source of nutrition until they have the ability to digest the other foods. It also includes a variety of other nutrients, like lactose and protein (Van *et al*., 2011). Lactic acid bacteria (LAB) grow in milk, which is one of their natural environments (Delavenne *et al.,* 2012). Lactic acid bacteria (LAB) are found in milk and milk products naturally (Chen *et al*., 2005).

The bacteria that produced lactic acid are frequently used as probiotics because of their capacity to outcompete infections. In addition, they regulating the immune response, inhibiting neutrophil and macrophage death and increasing phagocytic activity (De LeBlanc Ade *et al*., 2010; Ramos *et al*., 2010). *Lactobacillus* bacteria are the most common probiotics including; "*Lactobacillus plantarum, Lactobacillus acidophilus, Lactobacillus casei*, *Lactobacillus rhamnosus*and *Lactobacillus delbrueckii subsp. Bulgaricus*" in addition to *Bifidobacterium* genera "e.g., *Bifidobacterium longum, Bifidobacterium infantis, and Bifidobacterium animalis subsp. Lactis"*. Also, some bacterial strains from other species were reported to show a probiotic potential "e.g., *Lactococcus lactis, Propionibacterium acidilactici, Leuconostoc mesenteroides, Bacillus subtilis, Enterococcus faecium, Escherichia coli,* and

Streptococcus thermophiles" and some certain yeasts "e.g., *Saccharomyces boulardii*" (Fijan, 2014).

1.2. Objectives of the Study

1-Isolation and identification (using VITEK 2 compact system) of the followings;

a- Lactic acid bacteria (LAB) are present in milk samples collected from healthy lactating cows.

b- The most bacterial contaminants from the clinical samples collected from burns infections cases, such as *Staphylococcus* spp., *Pseudomonas aeruginosa*, *Acenitobacter baumannii* and *klebsiella pneumonia.*

2- Performing auto-aggregation of LAB and their co-aggregation with the clinical isolates of bacterial contaminants.

3- Evaluation of antimicrobial activity of Cell Free Supernatants (CFS) of LAB against burn contaminant isolates using agar well diffusion assay.

4- Evaluation of antimicrobial combinations of LAB CFS with some antibiotics against burn bacterial contaminant using a modified disk diffusion assay.

5- Determination of "Minimum Inhibitory Concentration" (MIC) and "Minimum Biofilm Inhibitory Concentration" (MBIC) CFS extracted from

isolated *Lactobacilli* spp. against biofilm formation by above mentioned bacterial contaminants.