Electroanalytic evaluation of physiological interactions between Lactobacilli and *Staphylococcus aureus*

Malika Toleubekova, BSc Biotechnology

Submitted in fulfilment of the requirements for the degree of Master of Science in Biomedical Engineering

School of Engineering and Digital Sciences
Department of Chemical & Materials Engineering
Nazarbayev University

53 Kabanbay Batyr Avenue,
Astana, Kazakhstan, 010000

Lead Supervisor: Professor Tri Pham
Co-supervisor: Professor Dana Akilbekova
Co-supervisor: Professor Almagul Kushugulova
External Supervisor: Professor Enrico Marsili

April 2023

Declaration

I hereby, declare that this manuscript, entitled "Electroanalytic evaluation of physiological interactions between Lactobacilli and *Staphylococcus aureus*", is the result of my own work except for quotations and citations which have been duly acknowledged.

I also declare that, to the best of my knowledge and belief, it has not been previously or concurrently submitted, in whole or in part, for any other degree or diploma at Nazarbayev University or any other national or international institution.

Malika Toleubekova
April 2023

Abstract

Studying individual bacterial species in co-culture is crucial for understanding the specific properties and behaviours that emerge from the interactions between microorganisms in the natural environment. The present study aimed to evaluate the physiological and electrochemical properties of a co-culture of Lactobacilli and *Staphylococcus aureus*.

To facilitate equal growth of the two antagonistic species, Compound Media was developed. The results showed that Lactobacilli species inhibited the planktonic growth and biofilm formation of *S. aureus* in co-culture under both aerobic and anaerobic conditions in CM media. Among the three tested Lactobacilli species, *Lactobacillus plantarum* was found to exhibit significant antimicrobial activity against *S. aureus*, indicating that the metabolites secreted by Lactobacilli could be used as a natural antimicrobial agent.

To enhance the electroactivity of bacterial cells, a redox mediator known as 2-hydroxy-1,4-naphthoquinone (HNQ) was introduced into the culture media. The inclusion of HNQ at a concentration of 20 μM increased the current and total charge of both individual and co-cultured cells. The current and total charge of *S. aureus* were higher than those of *L. plantarum,* indicating a greater proliferation rate of *S. aureus*. The data suggested that HNQ at 20 μM concentration had a minimal impact on the growth and biofilm formation of both individual and co-cultured cells.

These findings provide insights into the physiological interactions between Lactobacilli and S. aureus, which can aid in the development of new tools and approaches for combating bacterial infections. The electrochemical approach used in this study can also be applied to investigate interactions between different microorganisms and understand their physiological and electrochemical properties.

Acknowledgments

I would like to express my sincerest gratitude to Dr. Abitaeva Gulyaim for generously providing the strains used in this research. Without her help, this project would not have been possible.

I am also immensely grateful to Professor Tri Pham and Professor Enrico for their invaluable guidance, mentorship, and support throughout this project. Their expertise and insights have been instrumental in shaping this research and guiding me towards new avenues of exploration.I would like to thank Dr. Almagul Kushugulova and Dr. Obinna Ajunwa for their unwavering support and encouragement throughout this project. Their expertise and guidance have been invaluable in helping me navigate through the various challenges of this research.

Additionally, I would like to acknowledge the hard work and contributions of MSc students Alua Shagirova, Altynay Utebaeva, and BSc student Ainur Kenessova. Their dedication and commitment to this project have been essential in achieving our research goals. Finally, I would like to express my sincere appreciation to all the individuals who have supported me in this project. Their assistance, encouragement, and motivation have been instrumental in making this research a success.

Table of content

Chapter 1 - Introduction

Chapter 2 - Literature Review

Chapter 3 - Material and Methods

Chapter 4 - Results

List of Abbreviations

CA	Chronoamperometry
CFS	Cell-free supernatant
CFU	Colony forming unit
CM	Compounded medium
CV	Crystal violet
DPV	Differential pulse voltammetry
EET	Extracellular electron transfer
HNQ	2-hydroxy-1,4-naphthoquinone, redox mediator
LAB	Lactic acid bacteria
LB	Luria/Lennox/Luria-Bertani broth
MRS	de man, Rogosa and Sharpe broth/agar
NB	Nutrient broth
OD	Optical density
SPE	Screen-printed electrode
TSB	Tryptic Soy Extract Broth
ZOI	Zone of inhibition
SD	Standard Deviation

List of Figures and Tables

Chapter 1 - Introduction

1.1 Introduction

In the natural environment, bacteria do not exist in an individual manner but instead interact with other microorganisms in their surroundings, leading to distinct properties and behaviours compared to individual pure cultures. Therefore, it is necessary to study microorganisms under conditions of co-culturing to better understand their properties and behaviours in the environments. *Staphylococcus aureus* is a Gram-positive bacterium that poses a significant risk to human health. By adapting to various environmental conditions, *S. aureus* may cause a wide range of infections in various organs and have abilities to survive and adapt to a variety of environmental conditions. The ability to form a decisive and intense biofilm structure increases their resistance to medicaments, such as antibiotics[1].

Numerous strategies are currently under development to inhibit the cellular proliferation and biofilm formation of S. aureus under infectious conditions [5-6]. One such strategy includes the application of bacterial species interactions, namely antagonism against *S. aureus* by bacteria that are favourable to humans. Lactobacilli are a well-known type of bacteria that have positive effects on humans [2]. Lactobacilli are Lactic Acid Bacteria (LAB) members with antagonistic potential against a variety of human diseases due to their ability to create organic acids, bacteriocins, and other antimicrobial metabolites [3].

In some cases, the interactions between lactobacilli and *S. aureus* have been physiologically characterised [4,9,7], but it has been discovered that a critical mode by which lactobacilli negatively affect pathogens such as *S. aureus* is by producing metabolites that can cause oxidative shock to oppose cells while leaving the producing cells unaffected [5]. This mechanism is related to the electrochemical potentials of the cells.

When cells multiply on electron-conducting surfaces, their electrochemical potentials may be investigated in great detail [6]. Despite the fact that cellular electrochemical potentials may be critical indicators of specific mechanisms by which lactobacilli antagonistic activities against *S. aureus* can be measured, only a few studies have thoroughly evaluated the electrochemical functions associated with heterogeneous interactions within *S. aureus* and Lactobacilli species biofilms[19,21,45].

According to the above reasoning is obvious that electrochemical techniques could be applied to assess the biofilm activity of co-cultured bacterial cells in a non-destructive and continuous manner, by establishing the groundwork for the application of electrochemical methods in clinical antimicrobial testing.

1.2 Aim and Objectives

The aim of this research is to investigate the interactions between *S. aureus* and Lactobacilli using an electrochemical approach to assess physiological changes in individuals and co-cultures. Additionally, to describe the physiological and structural changes in S. aureus biofilms that occur while co-culturing with different Lactobacilli species under electrofementation.

The main objectives of this thesis are

1. Determination of optimum electrochemical and physiological conditions for single independent cultures of Lactobacilli and *S. aureus*.
2. Establishment of optimum physiological conditions for co-cultures of Lactobacilli and *S. aureus*
3. Determination of biofilm formation by co-cultures of Lactobacilli and *S. aureus*
4. Electrochemical analysis of the biofilms formed by co-cultures of Lactobacilli and *S. aureus* and determining the possible presence of antimicrobial metabolites (bacteriocins, organic acids) within the co-cultures.

1.3 Thesis statement

Electrochemical analysis can bring new understandings about cellular metabolic activities within the heterogeneous biofilm environment of *S. aureus* and Lactobacilli species. Electroanalytical evaluation of *S. aureus* mixed biofilms can develop new strategies in antimicrobial testing. It can be hypothesized the bacterial cells of Lactobacilli species embedded in a mixed biofilm could induce antimicrobial stress in *S. aureus*.

Chapter 2 - Literature Review

2.1 The Role of Lactobacilli in the Human Microbiome

Lactic acid bacteria (LAB) are microorganisms that are widely considered to be safe and have been used in food processing and medical health. LAB has natural antibacterial properties and can produce organic acids, bacteriocins, H2O2 (hydrogen peroxide), and other metabolites that have antibacterial effects [3,7]. Research has shown that LAB can inhibit the growth of several bacteria, including Methicillin-resistant *S. aureus* [19-30], and *Pseudomonas aeruginosa* [42] by producing antimicrobial substances.

Lactobacilli are the predominant bacterial species in the human microbiome, especially in the gut intestinal and vaginal microbiota [6-7]. It has been shown that Lactobacilli species show electron transfer activity by increasing NAD^+ and NADH metabolites and it allows the creation of more ATP to accumulate biomass relatively rapidly [8]. LAB is well-known for its capacity to produce diverse types of probiotics. According to the latest research works, probiotics are a potential new strategy for combating biofilms [7-10]. Probiotics can be described as "live bacterial strains that bestow a health benefit on the host when supplied in suitable doses" [7]. Lactic acid bacteria (LAB), are the most prevalent bacterial strain with confirmed probiotic action, including *Lactobacillus fermentum*, *Lactobacillus brevis*, and *Lactobacillus plantarum* [9].

Lactic Acid Bacteria could proliferate in various conditions by utilising various carbon sources for glucose formation. Also, LAB can produce a high amount of lactic acid along with other important metabolites, such as exopolysaccharides, organic acids, bacteriocins, and related enzymes. Thus, most of them are characterized as homofermentative bacterial strains. These critical metabolites can create a physiologically restrictive environment for the pathogenic strains. Their metabolites can generate low-level pH, changes in redox potential, and so on [10-12].

2.2. *Staphylococcus aureus:* A Pathogenic Bacterium Responsible For Infectious Diseases.

S. aureus is a pathogenic bacterium that can cause severe infections and has developed resistance to many antibiotics. This makes it important to explore the use of non-pathogenic LAB to manage *S. aureus* biofilms. *S. aureus* is a Gram-positive bacterium that can adapt to different environments and cause infections in various organs. Its ability to form a strong biofilm structure makes it more resistant to antibiotics and other treatments[5].

S. aureus clones vary genetically, and the processes causing biofilm development are unclear. Such investigations serve as the foundation for developing targeted therapeutic approaches. The analysis of temporal variations in the biofilm transcriptome of three MRSA (methicillin-resistant *Staphylococcus aureus*) clones: USA300, MRSA-15, and ST239 reveal substantial variation in gene expression during biofilm development [15]. Based on the results of 24 h of biofilm development, the clfA gene was the only one that was highly elevated across all three strains, indicating that it is an essential target for disrupting early biofilms.

Furthermore, gene expression studies in *S. aureus* have shown that biofilms can grow from microaerobic to anaerobic in comparison to planktonic cells [36]. This study has significant medical implications since *S. aureus* forms a biofilm on biomedical implants and equipment, resulting in repeated infections and further medical complications. The observation that anaerobiosis activates the intercellular adhesion (ica) operon of *S. aureus*, resulting in polysaccharide intercellular adhesin-based biofilms, begged the issue of whether additional metabolic alterations occurred in these biofilms [15,36].

2.3 Microbial Interactions And Biofilm Formation: Implications For Human Health And The Role Of Probiotics In Antibiotic-Free Therapy

Microbial interactions and communication are enthralling but still little-understood complex biological systems. The investigation of microbial consortia and their inner interactions is vital for understanding, assessing, and controlling natural and synthetic microbial consortia. All bacterial cells in biofilms can interact and regulate themselves by forming a complex consortium with features distinct from the original platonic cells. Furthermore, bacteria with a strong biofilm formation capacity can adhere securely to a living or non-living surface and produce extracellular matrix compounds [7]. This feature makes biofilm highly recalcitrant to any treatment. On the one hand, this allows probiotics and other non-pathogenic bacteria to create healthy microbiological niches and help host the immune system. On the other hand, it gives the ability to pathogenic cells to increase resistance to antimicrobial medications such as antibiotics. Thus, in terms of human health, the related studies of alternative strategies to suppress and control biofilm formation are vitally important. Antibiotic-free therapies have grown in reputation in past years, owing to the discovery and spreading of novel antibiotic-resistant strains. Among these treatment options, the usage of probiotics appears to be a viable option for the prevention of healthcare-associated infection.

Recent research indicates that probiotics can be used not only to prevent infections but also as alternative treatments for the treatment of diseases caused by multi-drug-resistant organisms [20-30]. Lactobacilli, as previously said, is considered one of the most efficient probiotics for the control and prevention of urogenital, oral and intestinal infections. [20-22, 23-27]. Furthermore, the potential of probiotic strains to form metabolic active compounds with antimicrobial properties, secretions of polypeptides that degrade carbohydrate receptors, and production of biosurfactants to cope against pathogenic microbes for nutrient and adhesion sites results in immunomodulation, inhibition of pathogen adhesion, invasion, and growth [22-26, 30]. Table 2.3 is a list of recent studies where LAB was used as an alternative therapeutic agent to prevent and treatment of infectious diseases. Most of the studies described observed biofilm formation and cross-interaction between the species by physiological and biochemical methods. Hence, the bioelectrochemical properties of bacteria in single and mixed cultures are understudied.

Table 2.3: The list of latest studies where probiotics were described as an alternative therapeutic agent to prevent and treatment of infectious diseases.

	Applied probiotic strain(s)	**Pathogenic bacteria strain(s)**	**Results**	**Ref.**
1	*L. plantarum and L. rhamnosus*	*Escherichia coli* and *S. aureus*	The study demonstrated that probiotics significantly inhibited *E. coli* and *S. aureus* biofilm formation ability on urinary tract devices.	[20]
2	*L. plantarum*	*Escherichia coli*	Application of *L.plantarum* biofilm on the surface of urinary tract device significantly reduces adhesion and biofilm formation of pathogenic *E.coli*	[21]

3	*L. gasseri* and *L. rhamnosus*	non-*albicans Candida* species	This study suggests the application of probiotic Lactobacilli species as a potential antimicrobial agent against *Candida* biofilms.	[20, 35]
5	*L. plantarum 108*	*S. mutans* and *C. albicans*	The probiotic strain cell-free supernatant demonstrated the capacity to inhibit mixed-culture biofilm formation by *S. mutants* and *C. albicans*.	[22]
6	*L. fermentum* and *L. plantarum*	*S. aureus*	This study conducted that *L. fermentum* active metabolites can regulate the expression of operons that are responsible for virulence and pathogen factors of *S. aureus.*	[24]
7	*L. rhamnosus* GG and *Streptococcus salivarius* K12	*S. aureus* and *S. epidermidis*	The cell-free supernatant derived from *S. salivarius K12* demonstrate the capacity to terminate planktonic growth and prevent early biofilm formation by *S. epidermidis.*	[26]
9	*L. plantarum* Y3	*E. coli* U12	In this study*, L. plantarum* formed biofilm was suggested as a potential method of antimicrobial coatings. According to the results, *L. plantarum* pre-coating was able to inhibit biofilm formation in microtitre plates.	[29]
10	*L.plantarum* and ethanol extracts of *Lawsonia inermis*	*S. aureus*	This study revealed that a mixture of *L. plantarum* and *L. inermis* was able to inhibit the culturability of S. aureus and decrease the anti-inflammatory effects of the wound healing process.	[27]
12	*Lactobacillus species*	*S. aureus*	The study conducted that all probiotic strains have the capacity to inhibit the growth of *S. aureus* individually. However, all probiotics did not suppress *S. aureus* in co-culture with human osteoblast cells.	[28]
13	*L. rhamnosus* in Galleria mellonella	*S. aureus or E.coli.*	In this study, *L. rhamnosus* was shown to induce better protection in Galleria mellonella against infection with *S. aureus* and *E. coli.*	[32]
14	*L. rhamnosus, L. casei, and L.*	*Candida albicans*	These results suggested that applied Lactobacilli significantly suppressed	[31]

	acidophilus		microbial growth of pathogens at the planktonic stage. However, they cannot affect mature biofilm, but the ability to reduce formation at an early stage was shown.	

2.4 Electrochemical Signalling And Membrane Potential In Bacterial Biofilm Formation

One of the serious and persistent public health concerns is bacterial biofilms, which show both inherited genetic resistance traits and a preexisting inherent tolerance to standard antibiotic treatment. As a result, there is a rising need for novel drug delivery methods to increase the potency of antimicrobial medications.

Bacterial extracellular membranes serve a crucial part in their overall physiology. The bacterial outer cell surface supports exchange processes as well as adhesive properties. These processes involve a number of electrochemical and immunological interactions that occur on the cell membrane. In these cases, it is worth mentioning that bacteria require a proton motive force to grow and survive. The proton motive force is formed in the bacterial cell membrane during energy generation. This proton motive force in the inner membrane produces potential charges in the membrane, resulting in bacterial electrochemical signalling [38]. Bacteria in their free-living planktonic condition can form biofilms, and sessile cells cling to the substrate and promote the biofilm [39].

Bacterial biofilms are organised colonies comprising billions of densely compacted cells. Bacteria in a biofilm can interact with one another via several types of communication mechanisms, collectively known as "quorum sensing." [40]. For bacteria, quorum sensing is a method of cell-to-cell signalling [38]. It has recently been proven that bacterial cell-to-cell communication is based on ion channel-mediated electrical signalling [38]. Cell-to-cell contact within a biofilm community has been allowed by this electrical signalling [38-41].

2.5 Electroanalytic Method Used In Studying Microbial Interactions

Electromicrobiology is a contemporary field of research that explores the interaction between microorganisms and electronic devices, as well as investigates the electrochemical characteristics of microorganisms. By conducting an electrochemical analysis of bacterial cells, it is possible to evaluate the electron transfer proteins' catalytic and electrochemical properties, analyze cellular metabolic processes, and predict antibiotic resistance in bacterial cells [40].

Electrogenic microorganisms are a group of bacteria that have the ability to transfer electrons to or from their surroundings, often through specialized appendages called pili or extracellular electron transport proteins. Extracellular electron transfer (EET) is a process by which microorganisms transfer electrons outside of their cells to extracellular electron acceptors, such as metal oxides, humic substances, or electrodes [41,47]. The rate of EET can be influenced by several factors, including the redox state of the electron acceptor, the electrode potential, and the availability of redox mediators.

Electrogenic microorganisms are found in a variety of environments, including soil, sediment, wastewater, and the human gut. Some well-known electrogenic microorganisms include *Geobacter sulfurreducens*, *Shewanella oneidensis*, and *P.aeruginosa.* These bacteria have been extensively studied for their ability to produce electricity and their potential use in various biotechnological applications [43-45].

Electrochemical sensors are a promising method for investigating antibiotic susceptibility because they can detect without the need for labels, are inexpensive to produce, and can be integrated with microfluidics and simple electronics for real-time data collection[49]. One effective technique is electrochemical impedance spectroscopy (EIS), which measures the impedance of the electrode-electrolyte interface across different frequencies to provide information about electron transfer properties and diffusional behaviour. Changes in impedance can indicate bacterial growth over time, making it a valuable method for identifying microorganisms like *S. aureus* and *P. aeruginosa* [45-48]

Another useful technique is differential pulse voltammetry (DPV). DPV is a technique that can measure the redox activity of bacterial cells, enzymes, or electron transport proteins by applying voltage pulses to the electrode and measuring the resulting current. It can also detect the presence of specific molecules or ions in bacterial cultures by analyzing their electrochemical response [48]. Moreover, many important compounds in culture media, such as glucose, urea, and peptone, are not electroactive, so the right combination of these compounds allows the creation of a suitable environment for detecting electroactive microorganisms. In summary, an electroanalysis is a valuable tool for studying the electrochemical behaviour of bacterial cells and detecting their proliferation and evaluation in various environments.

Microbial sensors are capable of detecting bacterial cells through contact with an electrode, which converts their biochemical signals into electric potentials. The resulting signal can be measured using amperometric sensors, which determine the current generated by an electroactive species at a fixed voltage, indicating its concentration in the solution. This technique is commonly employed to identify microorganisms that are resistant to antibiotics. It involves subjecting a test substance to an oxidation-reduction reaction at a constant potential while monitoring the change in current over a period of time[48-50]. Furthermore, voltammetry methods like cyclic voltammetry (CV) are frequently utilized to detect antibiotic-resistant bacteria. CV can determine both the redox peak potential and current simultaneously, enabling the investigation of reversible redox processes.

Furthermore, the utilization of potentiostat-controlled methodologies is progressively gaining recognition in the realm of studying microbial interactions. The potentiostat-controlled electrochemical system uses potentiostat to control the electrical potential of the bacterial cells. In this type of system, a potentiostat is used to apply a potential to the bacterial cells, and the current that flows through the cells is measured [49-50]. Figure 2.1 describes the general working setup of the potentiostat for detecting bacterial interaction. Potentiostats can also be used to study the effects of different environmental factors on microbial interactions, such as changes in pH, temperature, and substrate availability. They can be used to monitor the growth of microbial populations and to determine the optimal conditions for microbial activity.

2.6 Potentiostat and Screen-Printed Electrodes: Tools For Studying Microbial Electrochemical Interactions.

In potentiostat, microorganisms are cultivated as biofilms on the working electrode. The electrode potential can be accurately regulated, thereby enabling researchers to investigate the impacts of different electrode potentials on electron exchange transfer (EET) rates [16-18,48-49]. This method is demonstrated as an effective technique for microbial bioelectrochemical systems, which provides valuable insights into the electron transfer mechanisms inside of microorganisms.

As mentioned before, potentiostat is a regulatory system of the electrical potential between the reference and working electrodes. Potentiostat has the ability of evaluation the electric current flow which runs between the working and counter electrodes. Recently, economically beneficial additive printing methods, i.e. screen-printing or 3D printing methods are considered effective techniques for producing electrode systems and sensors for lower prices[48-49]. Among the mentioned methods, screen-printed electrodes (SPEs) have a lot of efficient benefits over classical electrodes, which include straightforward fabrication and cleaning, dependability, economically favourable, consistent and fast-acting [51-55].

In electrochemical measurements, a reference electrode is a fundamental component for accurate potential measurements. In the case of SPEs, a reference electrode is integrated into the design, typically as a three-electrode system consisting of a working electrode, a reference electrode, and a counter electrode [49-50]. One common type of reference electrode used with SPEs is the Ag/AgCl electrode, which is commonly used in aqueous environments. This electrode is stable, easily prepared, and has a well-defined potential.

The working electrode is the site of the electrochemical reaction in an SPE. It is typically composed of a conductive material, including but not limited to carbon, gold, or platinum, and is coated with a layer of a specific material to facilitate the desired electrochemical reaction [50-49]. Carbon in the form of graphite is the most commonly used material for the working electrode in SPEs due to its good electrical conductivity, chemical stability, and low cost. The sensitivity of the working electrode is a critical factor, as it refers to the ability of the electrode to detect small changes in the electrochemical signal, which can be influenced by the electrode material, modification layer, and electrochemical conditions.

In SPEs, the counter electrode plays a crucial role in closing the electrical circuit in the electrochemical reaction, and it is optimized to have a large surface area to minimize electrical resistance. Typically, the counter electrode is made of a conductive material such as silver, platinum, or carbon [51-54]. The performance of the counter electrode is fundamental to ensuring the accuracy and sensitivity of the electrochemical measurement. Hence, an ideal counter electrode is designed to have low resistance, high stability, and minimal interference with the electrochemical signal, which are critical characteristics to consider in optimizing SPEs.

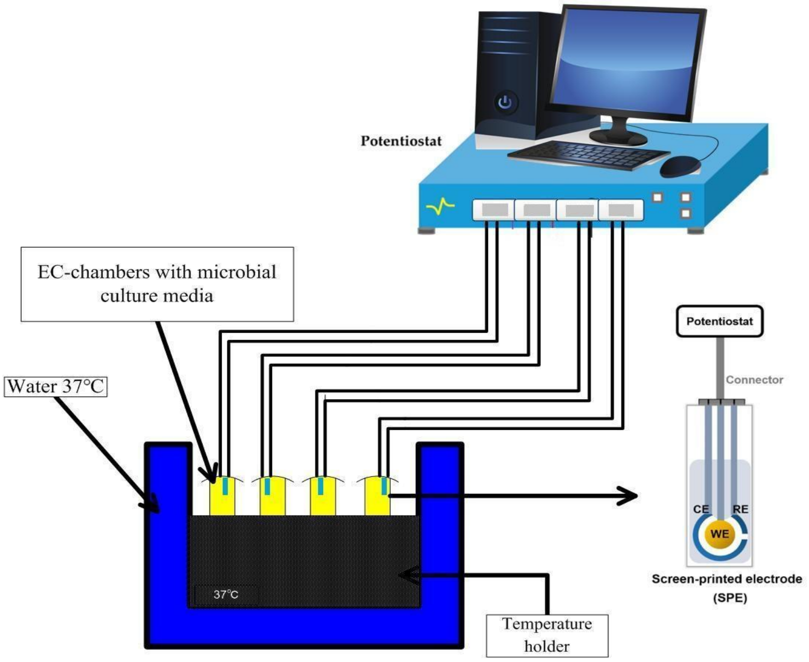


Figure 2.1: The schematic representation of a potentiostat setup for electrochemical measurements of bacterial cells.

Various microorganisms can transfer electrons to electrodes with the aid of mediators, such as methyl viologen, neutral red, or thionine [53,56]. These mediators can accept electrons from the redox-active components within cells and relay them to the electrodes. However, despite the potential usefulness of this electrical interaction, its practical benefits have yet to be established. This is partly because the mediators themselves can be unstable and toxic, making them unsuitable for large-scale industrial processes. Additionally, the use of these mediators in open environments is not possible due to their limited effectiveness.

Overall, electroanalytical techniques such as cyclic voltammetry, impedance spectroscopy, and potentiometry can be used to study the interaction between *S. aureus* and LAB at the electrochemical level. These techniques can provide valuable information on the metabolic activity, cell membrane properties, and membrane potential of the microorganisms, and can help in understanding the mechanisms of microbial interactions.

Chapter 3 - Materials and Methods

3.1 Characterization of microbial strains

The four strains used in this study were obtained through the generous provision of Dr Abitayeva Gulyaim from the Republican Collection of Microorganisms (RCM) in Astana, Kazakhstan. *Lactobacillus plantarum* B-RKM0003 was isolated from the healthy vaginal microbiota, while *Lactobacillus fermentum* B-RKM0103 was obtained from the commercial medication "Lactobacterin". *Lactobacillus brevis* B-RKM0414 was extracted from a dairy product. The RCM collection test strain *Staphylococcus aureus* ATCC 6538-P B-RKM0039 was also utilized in this research. All strains were stored in MRS broth with 20% glycerol and 10% sucrose at -20°C for preservation.

3.2 Culture medium and growth conditions

In this study, five different microbial culture media were tested for their ability to support optimal growth of Lactobacilli and *S.aureus*. The media tested included de Man, Rogosa and Sharpe Broth/Agar (MRS, Hi-Media, India), Trypticasein Soy Broth (TSB, Condalab, Spain), Luria/Lennox/Luria-Bertani Broth (LB, Millipore, Switzerland), Nutrient Broth (NB, Millipore, Switzerland), and a Compounded Medium consisting of pancreatic digest of casein (12 g/mL), yeast extract (3 g/mL), dextrose (6 g/mL), dipotassium hydrogen phosphate (2 g/mL), and sodium chloride (2 mg/mL).

All experiments were conducted under controlled environmental conditions, including both aerobic and anaerobic conditions at a constant temperature of 37 °C for 24 and 48 h. The pH of all culture media was adjusted to a range of 6.8-7.0. The optical density (OD) at a wavelength of 600 nm was used to measure the growth of both Lactobacilli and S. aureus. The co-culturing experiment involved inoculating *S. aureus* and lactic acid bacteria (LAB) in a 1:1 proportion into 1 mL wells, with initial OD of 0.601. The bacterial strains were incubated in 48-well plates. The growth kinetics were measured by the microplate reader “Gen5TM”

3.3 Antimicrobial Activity of Lactobacilli species

The agar well diffusion method was chosen to detect the antimicrobial activities of supernatants isolated from Lactobacillus strains. First, Muller-Hilton agar(MH Condalab, Spain) plates were swabbed on the surface with *S. aureus* bacterial cultures OD = 0.1. Agar wells with a diameter of 6 mm were prepared, and 100 µL of cell-free supernatants from isolated lactobacilli were added to each well. The wells were incubated for 24 hours at 37 °C, after which the areas where bacterial growth was inhibited were measured and recorded. Cell-free culture supernatants (CFSs) were obtained from aerobic and anaerobic cultured Lactic acid bacteria. CFSs were obtained by centrifuging at 13000 rpm for 15 min at 20 °C. Lactobacillus CFSs were adjusted to pH 7.0 with 1M NaOH. The following 4 conditions were observed: 1) Centrifuged, crude cell-free culture supernatant (CFSs) with pH adjustment to 7.0.; 2) Filtered CFS. Centrifuged CFSs were passed through a sterile 0.22 µm filter (Millipore, Billerica, MA, United States); 3) Centrifuged, CFS was heated at 80°C for 15 minutes; 4) Centrifuged CFS was filtered and heated at 80 °C for 15 minutes; 5) MRS broth without cells, adjusted to pH 7.0 was used as the control.

3.4 Biofilm Formation

In this study, biofilm formation was performed using a static approach, both under aerobic and anaerobic conditions. The compounded media (CM) liquid was subjected to autoclaving at a temperature of 121 °C for a duration of 15 minutes. Subsequently, *S. aureus* and lactic acid bacteria (LAB) were inoculated into the CM medium with an initial optical density (OD) of 0.1 and incubated at 37 °C for a period of 48 hours in a 48-well plate. The co-culturing experiment involved inoculating *S. aureus* and lactic acid bacteria (LAB) in a 1:1 proportion into 1 mL wells.

3.5 The assessment of biofilm formation

The classical microbiological crystal violet (CV) test was used to measure biofilm production on cultured biofilms in microtiter plates [62]. To assess the production of biofilm, the medium was aspirated from the 48 wells and rinsed twice with distilled water. The 48-well plate was then flipped over onto a piece of filter paper and allowed to air dry for 30 minutes. Next, a 0.1% CV solution was added and incubated for 30 minutes. Following this, the CV was washed off with distilled water, and the bacterial biofilm was incubated with 0% of acetic acid for 15 min. Finally, the absorbance was measured at a wavelength of 600 nm.

3.6 Electroanalysis

The short-term bioelectrochemical response of different strains was assessed using differential pulse voltammetry (DPV) and cyclic amperometry (CA) techniques. Electrochemical measurements were performed with a computer-controlled VSC multi-channel potentiostat (Bio-Logic, France) to measure electrogenicity. The EC Lab® software (Biologic, France) was used to calculate the electrical charge output in (mC) for each experiment. Screen-Printed Carbon Electrodes (SPE Ref. C110, Metrohm DropSens, Spain) were utilized in CA experiments. The SPEs were connected to a computer-controlled multichannel potentiostat and placed in an electrochemical (EC) chamber with a capacity of 10 mL (8 mL working volume), which contained 8 mL of CM and a cell suspension with an (OD) of 0.5 at 600 nm. The system was maintained for 48 h at 37 ℃ temperature and the working electrode was poised at 400 mV vs. Ag pseudo-reference electrode. DPV analysis was carried out immediately after the inoculation and at the 48h mark, with the following parameters set for DPV analysis: Ei = -0.4 V and Ef = 0.4 V.

3.7 The assessment of biofilm formation on SPEs after electrofermentation

The biofilms formed on the carbon SPEs after electroanalysis were quantified using the crystal violet assay and fluorescent inverted microscope “ZEISS Axio Zoom. V16. All images were taken using an objective magnification of 63x and an exposure time of 400 ms. The crystal violet fluorescence was measured using 592 nm excitation and 612 nm emission.

3.8 Image Processing

The pictures were saved in CZI (Carl Zeiss Image) format using and then analyzed using ImageJ and MATLAB R2015 software. For one-dimensional intensity signal analysis, the images were transformed into grayscale by averaging the red (crystal violet) and bright-field colour channels. A new code was created using MATLAB to quantify the bacterial surface coverage and total fluorescent intensity of the biofilms.

3.9 Statistical Analysis

The experiments were conducted with three biological replicates and six technical replicates, and the data were analyzed using GraphPad Prism 5 and OriginPro8.5 software. The mean and standard deviation were used to present the data. The differences between the untreated and treated biofilms were evaluated using GraphPad Prism through a One-way ANOVA analysis ranked by Tukey's multiple comparison tests. The statistical significance was indicated as follows: *$P<0.05$, **$P<0.01$, ***$P<0.001$, or ****$P<0.0001$, while "ns" denoted no significant difference.

Chapter 4 - Results

4.1 Determination of optimum physicochemical and physiological conditions for single species cultures of Lactobacilli and *S. aureus*.

This research work contains four significant parts. In the beginning, microbiological optimisation works will be done. Growth rates of *L. fermentum* B-RKM0103*, S. aureus* ATCC 6538-P B-RKM0039*, L. plantarum* B-RKM0003 *and L. brevis* B-RKM0414 on six different culture mediums will be studied.

- de Man, Rogosa and Sharpe Broth/Agar (MRS, Hi-Media, India**)**
- Trypticasein Soy Broth (TSB, Condalab, Spain)
- Luria/Lennox/Luria-Bertani Broth (LB, Millipore, Switzerland)
- Nutrient Broth - (NB, Millipore, Switzerland)
- Compounded medium (Pancreatic digest of casein 12 g/mL*(Hi-Media, India)*,Yeast extract 3 g/mL *(Sigma Aldrich, Kazakhstan)*, Dextrose-6 g/mL *(Sigma Aldrich, Kazakhstan)*, Dipotassium hydrogen phosphate 2 g/mL *(Sigma Aldrich, Kazakhstan)*, Sodium Chloride 2 mg/mL *(Sigma Aldrich, Kazakhstan).*
- Mannitol Salt Agar - (MSA, MerckMillipore , Switzerland)

All of the experiments were performed under controlled environmental conditions such as aerobic and anaerobic. The constant temperature was 37 °C for 24 and 48 h. The pH of all culture mediums is adjusted to 6.8-7.0. The growth of LAB and *S. aureus* were studied by measuring optical density (OD) at λ = 600 nm. The objective of this part is the determination of optimum physiological growth conditions for single independent cultures of Lactobacilli and *S. aureus.*

Based on the results, five growth conditions were selected for determining optimal conditions for the proliferation of mixed cultures, including MRS pH 7 at 37 °C, LB pH 7 at 37 °C, NB pH 7 at 37 °C, CM pH 7 at 37 °C, and TSB pH 7 at 37 °C. The planktonic growth (24 h) of four bacterial cells on the five different culture media under anaerobic and aerobic growth conditions is summarized in (Figure 4.1).

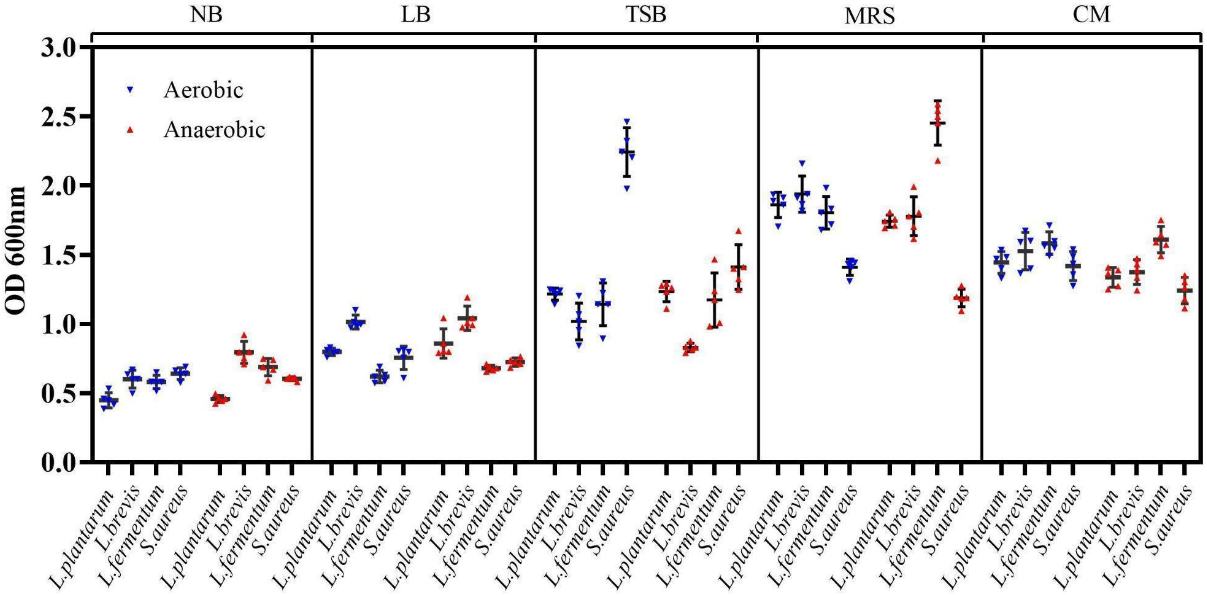


Figure 4.1: The OD of planktonic growth for four bacterial cells on five different culture media under both anaerobic and aerobic growth conditions after 48 h.

MRS is a complex culture medium that contains a wide range of complex carbon and nitrogen sources. The results revealed that MRS with an initial pH=7 resulted in much higher growth for all Lactobacilli species than the other media testes, under both aerobic and anaerobic conditions. This result was consistent with the report by the latest research [66-67] and the highest growth kinetics and bacteriocin inhibitory effect was acquired in MRS broth culture media. However, *S. aureus* grew in MRS at lower OD than the LAB tested. In the other media, all strains grow to a comparable OD, except for TSB under aerobic conditions, which resulted in higher growth for *S. aureus* than all the LAB tested.

The compound medium (CM) was chosen as one of the growth conditions for determining optimal conditions for the proliferation of mixed cultures. Because it provided a balanced nutrient composition for the growth of both Lactobacilli and *Staphylococcus aureus*. When conducting co-cultivation studies, it is important to choose a culture medium that can support the growth of both bacterial species equally, without favouring the growth of one species over the other. The optimal medium components obtained for achieving an equal growth of two different bacteria were as follows: pancreatic digest of casein, yeast extract, glucose, sodium chloride (NaCl), and dipotassium phosphate (K2HPO4). Carbon and nitrogen sources provide important nutrients for bacterial growth. Based on the observations from the latest article [13,67], glucose, NaCl, and K2HPO4 were chosen as the carbon, nitrogen, and phosphorus sources. It was previously reported that peptones in microbial media provide the main sources of nitrogen and carbon [34]. It is a valuable source of amino acids and peptides for culture media in microbiology. They also emphasized that adding yeast extract to the culture medium could cause to increase in the specific growth rate and lactose consumption of Lactobacilli.

4.2 Determine optimum physiological conditions for co-cultures of Lactocailli and *Staphylococcus aureus*

The next part of the research was held to determine optimal physiochemical conditions for the co-culture of *S. aureus* and Lactobacilli strains. Firstly, cells were incubated for 24 and 48 h within the compound medium in 48 well plates under static conditions. The growth of single species and co-culture was measured through OD600. Based on results from objective 1, it was decided to carry out the subsequent experiments under aerobic conditions, which resulted in higher growth. According to the last experiments, CM (Compounded medium) showed the optimal growth rate in aerobic conditions for every strain that was examined.

For the further establishment of quantitative estimation, LAB and SA cultures were selected for co-culturing in CM in aerobic conditions, because *S. aureus* is less active under anaerobic conditions than Lactobacillus species. Also, we are not able to create anaerobic conditions in the plate reader. Optical densities at OD 600 nm were measured during incubation at 37 °C every 20 min and growth curves were created. Figure 4.2, Figure 4.3, and Figure 4.5 demonstrate growth curves of single and co-cultured bacterial cells in a Compound Medium (pH=7.0) in 24 and 48 h at 37 °C.

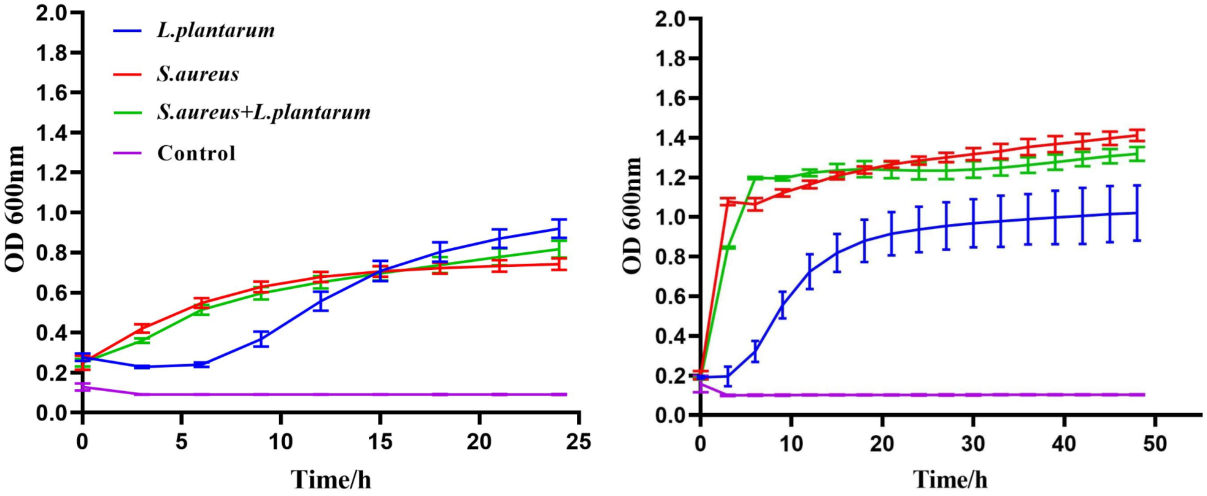


Figure 4.2: Planktonic growth of L. plantarum, S. aureus and co-culture of them at 24 h and 48 h.

As a result of 24 h growth, we can observe that the duration of the lag phase for LAB was different. Importantly, the duration of the lag phases was different for LAB in CM. As we can see, *L. fermentum* demonstrated a reduction in lag phase duration, whereas *L. plantarum* had an extended lag phase for five hours. *L. fermentum* may have been better adapted to the culture medium, allowing for a more rapid transition to exponential growth. On the other hand, *L. plantarum* may have required more time to adjust to the culture conditions before beginning exponential growth. Adapting certain strains beforehand to a new growth medium may lead to a decrease in the duration of the lag phase. This is because these strains may need to undergo genetic alterations in order to adjust their metabolism and growth to the new medium[11].

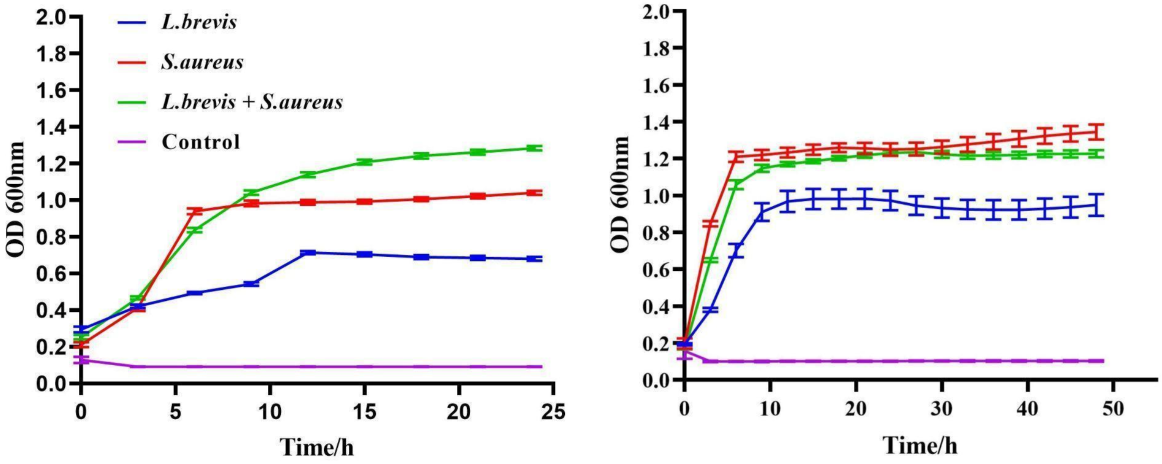


Figure 4.3: Real-time measurement of aerobic static growth of L. brevis, S. aureus and co-culture of them at 24 h and 48 h.

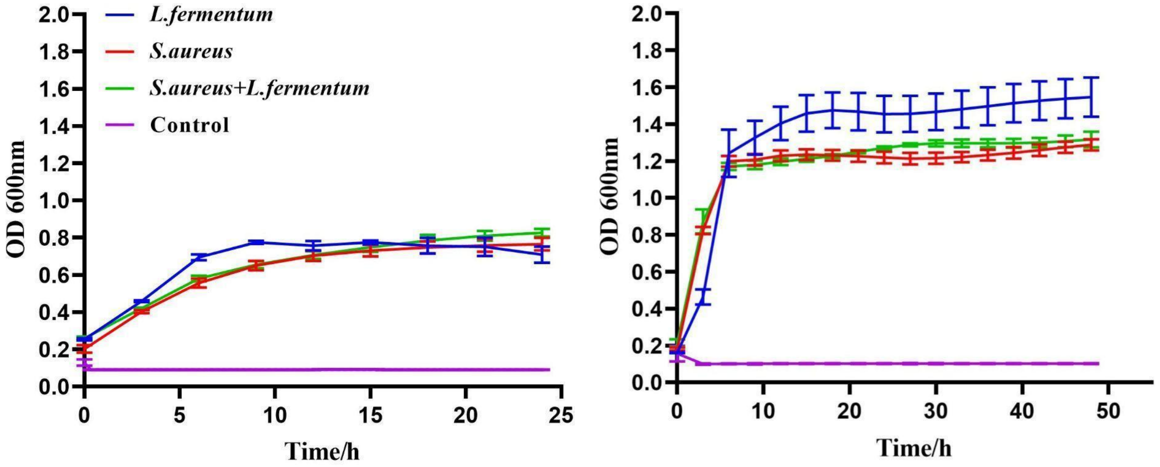


Figure 4.4: Real-time measurement of aerobic static growth of L. fermentum, S. aureus and co-culture of them at 24 h and 48 h.

Reported data confirms that the maximum biomass and metabolic concentrations of Lactobacillus spp. were obtained after 52 hours [13]. Thus, it was decided to study the growth kinetics of LAB and S. aureus individually in 48 h and in parallel to observe the co-cultivation of *S. aureus* with three different LAB.

Co-cultured strains were used further for colony-forming unit assay counting experiments. Strains were co-cultivated in CM medium with initial OD600mn 0.1-0.2. Continuously, after 48 h hours of incubation, cultures were diluted by ten-fold serial dilutions using phosphate-buffered saline (PBS, pH 7.4). Then, 0.1 mL of prepared dilutions of cultures were plated on MSA and MRS agar on Petri plates. Dilutions were poured and plated by using disposable spreaders. Conditions for incubation of plates were 37 °C for 48 h in aerobic and anaerobic conditions. Results are provided in Appendix 1.

4.3 Biofilm formation by *S. aureus* and LAB under aerobic and anaerobic conditions.

Under aerobic conditions, the biofilm formation of LAB is relatively high due to their ability to utilize oxygen for growth and metabolism [56-60]. However, *S. aureus* shows that in the presence of oxygen, it forms more robust biofilms than LAB (Figure 4.5). It can be explained that *S. aureus* has several virulence factors that promote biofilm formation, such as the polysaccharide intercellular adhesin (PIA) and the biofilm-associated protein (Bap). These factors enable *S. aureus* to attach to surfaces and to form a biofilm matrix that protects it from the immune system and antibiotics [61,63].

Under anaerobic conditions, the biofilm formation of both LAB and *S. aureus* is lower than under aerobic conditions (Figure 4.5). However, both species formed visible biofilm under anaerobic conditions. Because these bacteria can utilize alternate electron acceptors such as nitrate or fumarate for respiration [63]. Additionally, in the absence of oxygen, some bacterial strains may switch to fermentation mode, leading to the accumulation of extracellular polymeric substances (EPS), which are key components of biofilms [58].

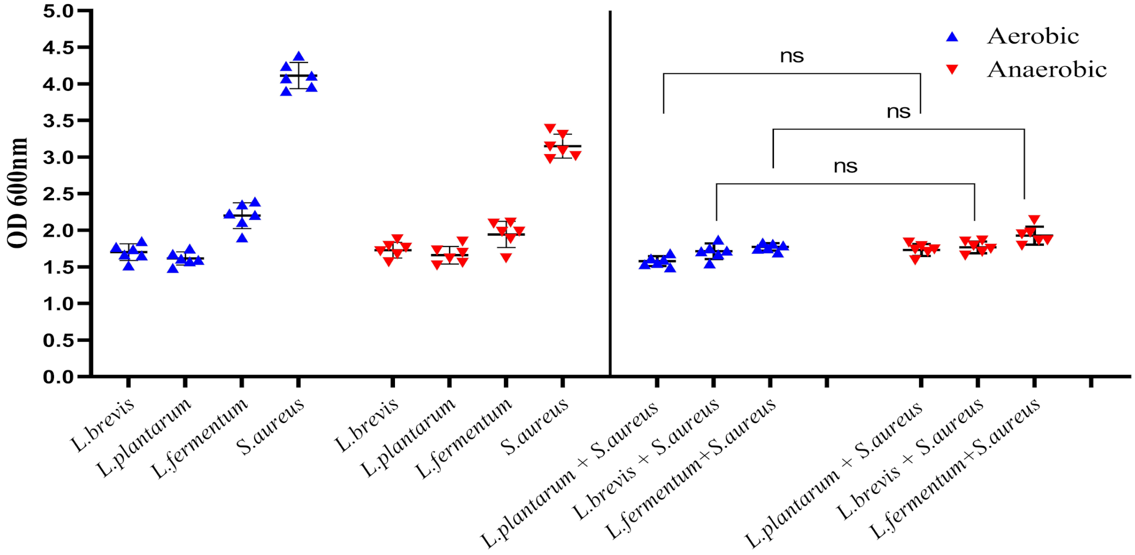


Figure 4.5: Biofilm formation by S. aureus and LAB and co-culture under aerobic and anaerobic conditions in CM media for 48 h.

There were no significant differences in the biofilm formation of bacterial cells in co-culture under both conditions (Figure 4.5). The competition for oxygen and nutrients between the two bacterial species may have influenced the biofilm formation of the mixed culture. Therefore, our findings suggest that the biofilm of *S. aureus* is inhibited by Lactobacilli species. Under both conditions, the production of organic acids by LAB may actually create an unfavourable microenvironment for the growth and biofilm formation of *S. aureus* under anaerobic conditions [3,21]. This is because the organic acids can lower the pH of the environment, which can inhibit the growth of *S. aureus*.

4.4. Antimicrobial activity of Cell- Free Supernatants of Lactobacilli against *S.aureus.*

All LAB isolates were tested for antimicrobial activity against *S. aureus*. Cell-free supernatants (CFSs) were obtained from aerobic and anaerobic cultured Lactobacilli. Overall, CFSs were tested under four different conditions. However, only supernatant from *L. plantarum* showed antimicrobial activity under all tested conditions (Figure 4.6-4.7). The findings from this results are consistent with those reported in recent articles, which have demonstrated that lactic acid bacteria (LAB) isolates exhibit a zone of inhibition (ZOI) in the range of 15.7 ± 1.2 to 6.3 ± 1.5 mm when tested against S. aureus without any pH adjustment[57,69].

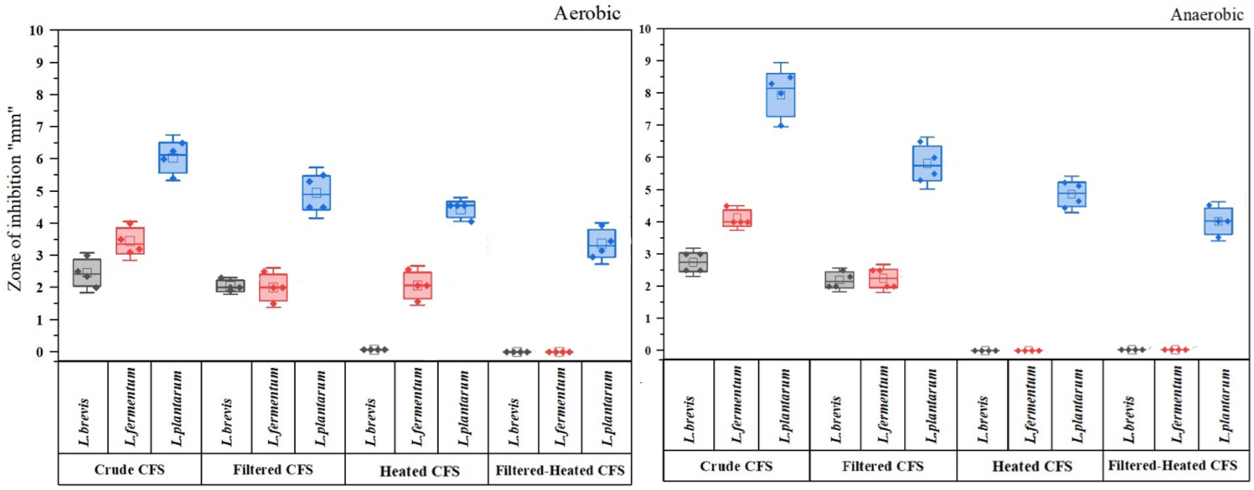


Figure 4.6: Zone of inhibition by CFSs from Lactobacillus isolates against S. aureus by well diffusion assays. CFSs were obtained from Lactobacilli cultured under both aerobic and anaerobic conditions, and pH was adjusted to 7.

The highest ZOI values were observed for supernatant from *L. plantarum* with the range of 7.9 ± 1.4 to 6.5 ± 1.2 with pH 7.0. However, CFS from *L. plantarum* without pH adjustment shows larger ZOI in the range of 11.5 ± 1.5 to 8.5 ± 1.3. Further, results demonstrated that oxygen availability during lactobacilli cultivation affects the antibacterial activity of the CFSs. Anaerobic conditions may provide a more favourable environment for the production of antibacterial substances, leading to a stronger antibacterial effect. The antibacterial activity of filtered CFSs obtained from *L. plantarum* was significantly higher against *S. aureus* compared to *L. brevis* and *L. fermentum*. The mean ZOI for *L. plantarum* CFSs produced under aerobic and anaerobic conditions were 4.95 ± 0.6 mm and 5.8 ± 1.2 mm, respectively, whereas those for *L. brevis* and *L. fermentum* were only slightly above 2 mm.

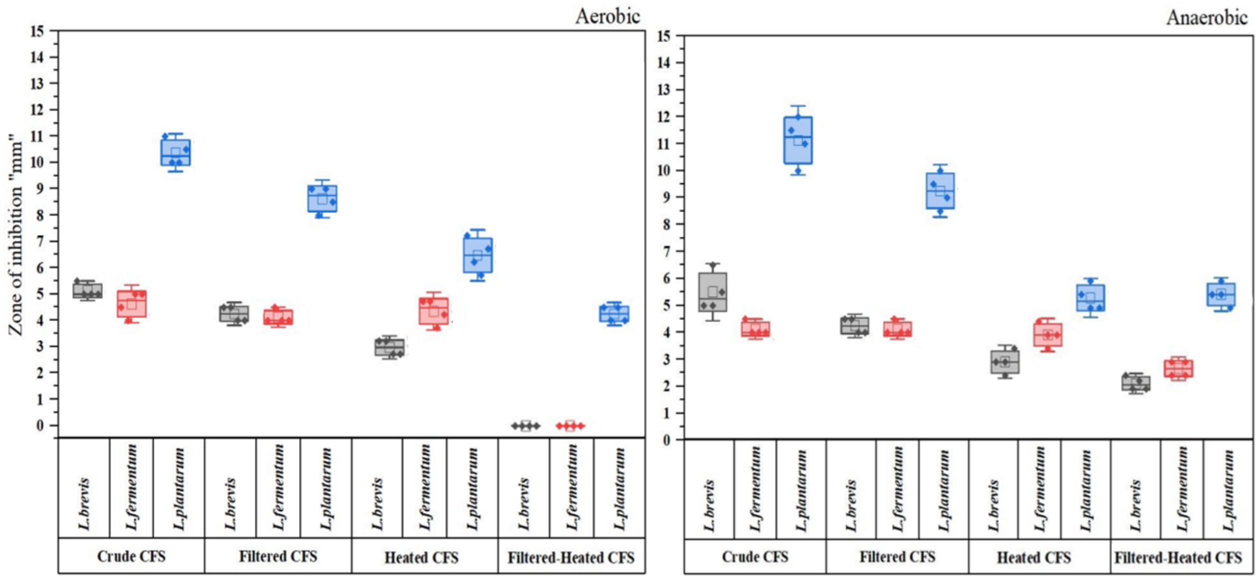


Figure 4.7: Zone of inhibition by CFSs from Lactobacillus isolates against S. aureus by agar well diffusion assays. CFSs were obtained from Lactobacilli cultured under both aerobic and anaerobic conditions.CFSs with initial pH<4.5.

It was found that crude CFSs from *L. plantarum* with an initial pH < 4.5 showed a greater ZOI compared to the CFSs with an adjusted pH of 7. This suggests that the antibacterial activity of the CFSs is influenced by the pH level, with a more acidic environment being more favourable for inhibiting the growth of *S. aureus*. It has been reported that Lactobacilli strains inhibit the growth of Gram-negative pathogenic bacteria [20]. This growth-inhibiting activity has generally been attributed to the fact that Lactobacillus spp. lower the pH and/or produce lactic acid.

4.5 Performance of the electrochemical system with 2-hydroxy-1,4-naphthoquinone (HNQ) redox mediator.

The growth dynamics of LAB and *S. aureus* were observed in separate cultures as well as in co-culture, with and without the addition of HNQ. The bacteria *S. aureus, L. plantarum*, and a co-culture of both were inoculated in compounded media (CM) and incubated at 37 °C for 24 h. The bacterial cells were resuspended in CM broth and added into a 48-well plate. The compound HNQ was mixed with the bacterial suspension at concentrations of 20, 50, 100, and 200 µM. The growth of each bacterial culture was observed with and without HNQ. The real-time microbial growth analysis system was used to record the dynamic growth of *S. aureus, L. plantarum*, and the co-culture of both. All experiments were performed in three independent biological replicates (n=3) and the error bars visualize the standard deviation. Our results show (Figure 4.8) that only 20 μM HNQ concentration is not significantly impacted on growth dynamic of *L. plantarum*, whereas concentrations above inhibit bacterial growth (Figure 4.8).

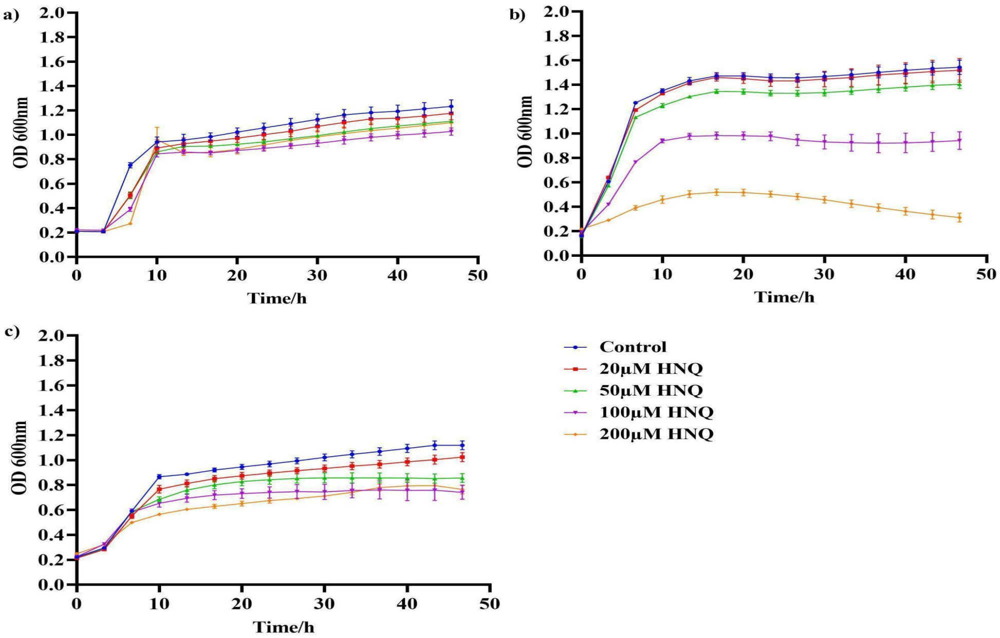


Figure 4.8: Planktonic growth of bacterial cells with different concentrations of HNQ for 48h in CM media. (a) S. aureus, (b) L. plantarum, and (c) co-culture. Control is bacterial cells without HNQ.

As we can see, in these cases HNQ in 20 μM concentration has no significant impact on bacterial growth (Figure 4.8). According to the results, the 20 μM concentration of HNQ is found more suitable for the electro-fermentation of *L. plantarum* and *S. aureus.* However, concentrations above 20 μM inhibited biofilm formation in both species.

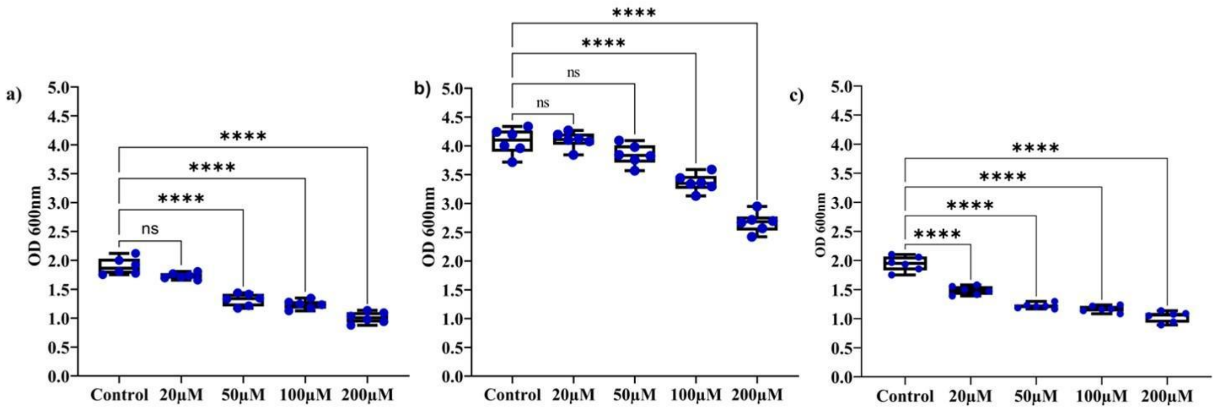


Figure 4.9: Biofilm formation of S. aureus, L. plantarum, and co-culture with different concentrations of HNQ in 48-well plates for 48 hours in CM media. (a) L. plantarum, (b) S.aureus, and (c) co-culture. Control samples are bacterial cells grown without HNQ.

Our results have shown that HNQ can inhibit biofilm formation by *S. aureus* and LAB in a dose-dependent manner (Figure 4.9). At concentrations of 20 µM, HNQ did not significantly reduce biofilm formation by *S. aureus*, and at higher concentrations (100-200 µM), it inhibited biofilm formation. HNQ shows an inhibitory effect on biofilm formation by *L. plantarum* and co-culture of *S. aureus* and *L. plantarum*, even at low concentrations (Figure 4.9).

4.6 Electrochemical analysis

The research involved bioelectrochemical tests that employed LAB and *S. aureus* cells, which were cultivated at 37 °C as biofilms on electrodes maintained at oxidative potential (400 mV vs. Ag). The outcomes of the experiments revealed that the maximum current increased with the introduction of HNQ, (Figure 4.10). Previous investigations have demonstrated that bacterial growth is an essential requirement for electroactivity, and the selection of an appropriate potential of 0.4 V was based on prior insights into the electrophysiology of electrogenic bacteria[68,45].

We performed CA and DPV measurements for individual bacterial cells and co-culture. The objective was to investigate the electrochemical behaviour of single species and co-culture (Appendix 3).

To ensure that the CM did not contain any electroactive substances, the CA and DPV of the medium without cells were measured. Single species and co-culture were then measured using the same experimental setup. Our results showed that the CA and DPV measurements significantly differed between single bacterial cells and mixed cultures.

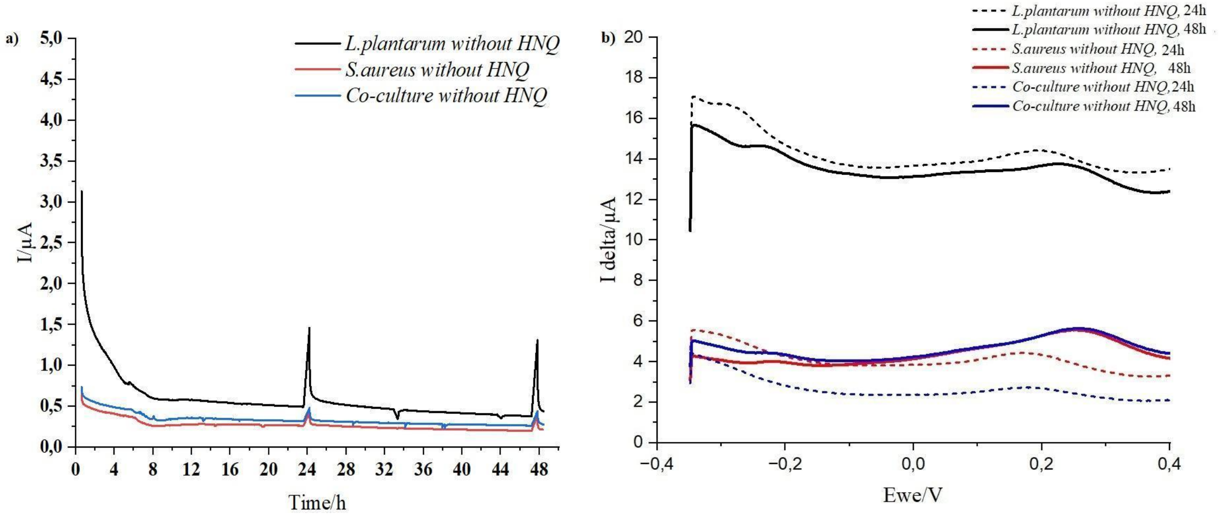


Figure 4.10: a) Chronoamperometry results for L. plantarum, S. aureus and co-culture (L. plantarum + S. aureus) without 20 HNQ, 400mV. b) DPV of L. plantarum, S. aureus and co-culture (L. plantarum + S. aureus) with and without 20 HNQ, 400mV.

DPV was conducted to analyze the redox active species present in the electrochemical system after every 24 h of growth for a total of 48 h.During electro-fermentation with HNQ, two primary peaks were observed, at -0.1 and 0.3 V, respectively (Figure 4.11).

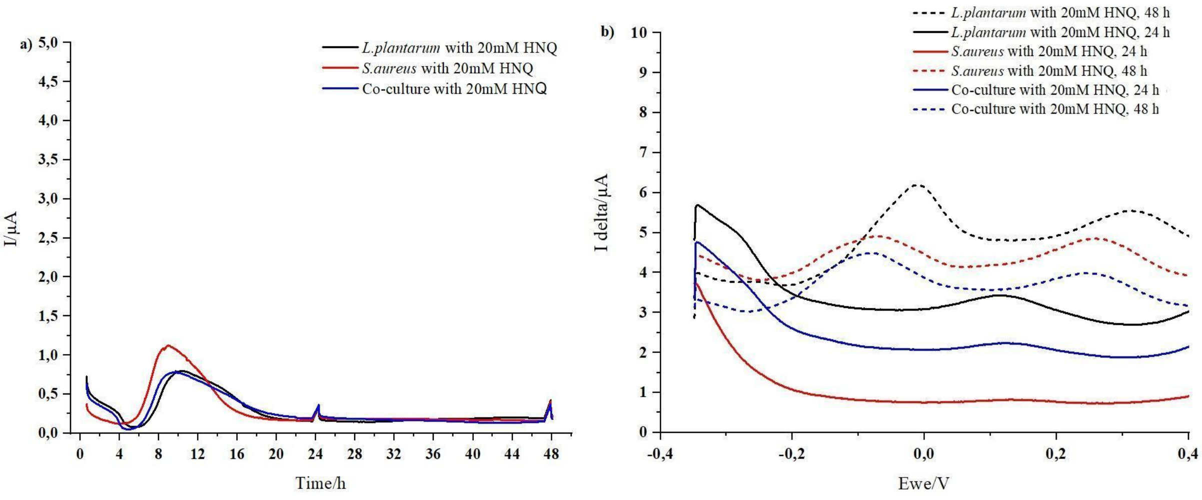


Figure 4.11. a) CA results for L. plantarum, S. aureus and co-culture (L. plantarum + S. aureus) with 20 µM HNQ, 400mV. b) DPV of L. plantarum, S. aureus and co-culture (L. plantarum + S. aureus) with 20 µM HNQ, 400mV.

According to the findings, *S. aureus* had the highest charge output (Figure 4.12) in presence of 20 µM HNQ, followed by *L. plantarum* and co-culture with 20 µM HNQ. After the insertion of HNQ, the current for *S. aureus*, *L. plantarum*, and the co-culture exhibited a single peak after 9 hours of cultivation (Figure 4.11). This suggests that it may be due to the exponential growth phase of bacterial cells. That being said, it is possible that the DPV results for lactobacilli could be higher than for *S. aureus*, depending on the specific experimental conditions. Lactobacilli are known to produce a variety of electroactive compounds, such as quinones and flavins, which could contribute to an increase in the DPV signal [53]. *S. aureus* is also capable of producing electroactive compounds, the levels and types of these compounds may be different from those produced by lactobacilli.

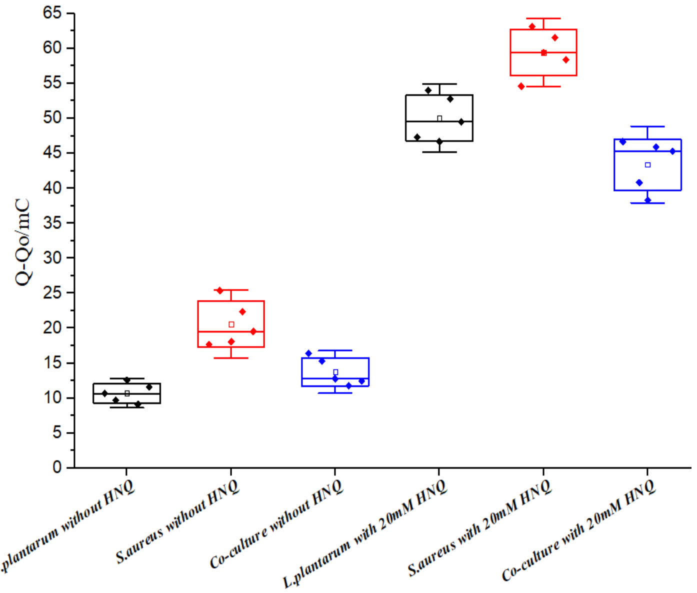


Figure 4.12: Charge outputs for L. plantarum and S. aureus at E = 0.4 V after 48 h growth in CM with and without HNQ.

4.7 Quantification of biofilm on the SPEs

Our results from fluorescence microscopy showed that *S. aureus, L. plantarum* and co-culture were able to form biofilms on screen-printed electrodes (SPEs). The biofilms were analyzed using a fluorescence intensity range of 300 to 1000. Biofilm formed by *S. aureus* covered the entire area of the SPE, while the biofilm formed by *L. plantarum* did not exhibit high coverage percentage. Co-culture of *S. aureus* and *L. plantarum* resulted in a biofilm formation on the SPE with lower coverage compared to *S. aureus* alone.

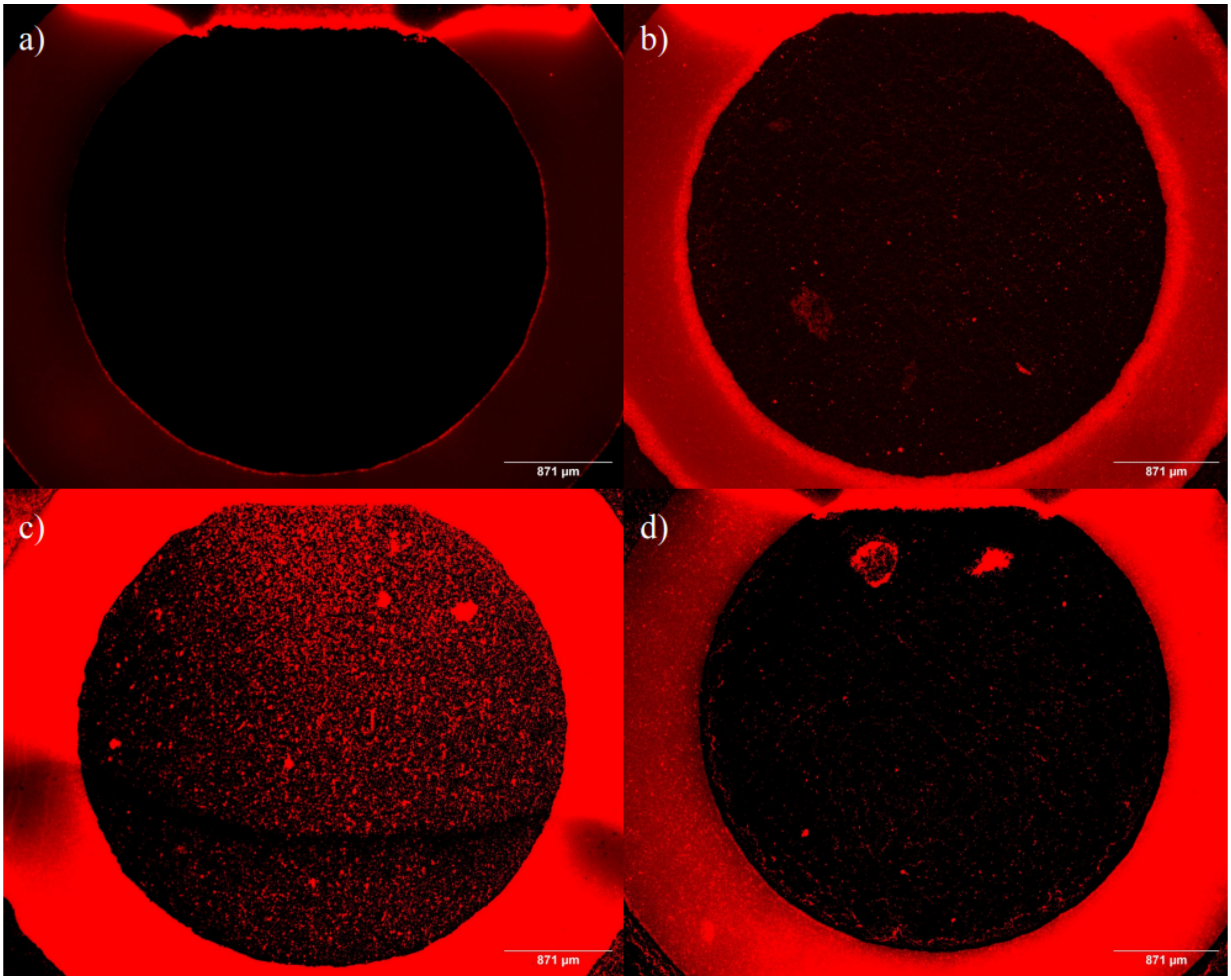


Figure 4.13: Fluorescence microscopy images of SPEs stained with 0.1% w/v crystal violet. (a) Empty SPE without cells. (b) SPE with L. plantarum biofilm formed in CM media at 400mV without HNQ. Coverage percentage ± 16.4%. (c) SPE with S. aureus biofilm formed in CM

media at 400mV without HNQ. Coverage percentage ± 99.6%. (d) SPE with co-culture biofilm formed in CM media at 400mV without HNQ. Coverage percentage ± 53.4%.

Our results showed that HNQ significantly reduced the biofilm formation of both *S. aureus* and *L. plantarum* on SPEs. The inhibition of biofilm formation was found to be dose-dependent, with higher concentrations of HNQ resulting in greater inhibition (Figure 4.13). In addition, we observed that the co-culture of *S. aureus* and *L. plantarum* resulted in biofilm formation on the SPEs, which was also inhibited by HNQ in co-culture. The minimum inhibitory concentration of HNQ was not significantly affected on biofilm formation of individual species (Figure 4.14)

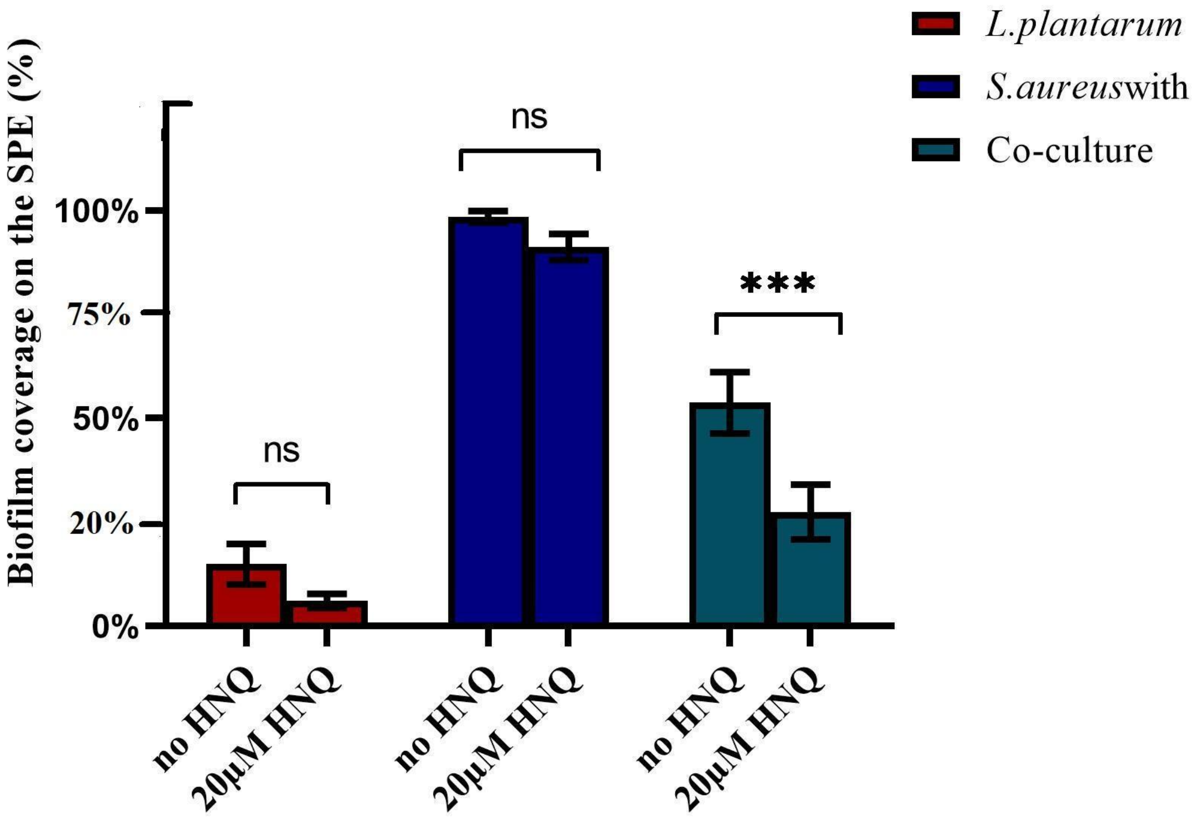


Figure 4.14: Biofilm formation on SPEs of S. aureus, L. plantarum and co-culture with and without 20 µM HNQ.

Chapter 5 - Discussion

MRS is a selective medium that is specifically designed to support the growth of LAB, including Lactobacillus species. Similarly, TSB can support the proliferation of a wide range of bacterial species, including Staphylococcus species. However, TSB may not be suitable for all Lactobacilli species, as some strains may have specific nutritional requirements that are not met by TSB.

To avoid bias in co-cultivation studies, it may be necessary to use a neutral or non-selective medium that can support the growth of both bacterial species equally. Another option is to use a mixed media that contains nutrients that are suitable for both bacterial species, such as the CM medium.

Regardless of the culture medium used, it is important to carefully design co-cultivation experiments to control for factors that can affect bacterial growth and interaction, such as pH, temperature, oxygen availability, and initial bacterial inoculum ratios. This ensures that the results of the study accurately reflect the interaction between the two bacterial species.

NB and LB are general-purpose nutritional media used to develop a broad variety of microorganisms including bacteria, fungi and yeasts. As we can see, *S. aureus* and LAB grew in the NB and LB, however, the final biomass concentration was lower than in the remaining four conditions. Thus, it may cause poor biofilm formation and metabolite extraction.

One of the results showed that LAB growth is affected by three important key factors: temperature, media composition and pH [58,67]. The optimal pH for Lactobacilli to proliferate was pH 7.0-8.5. Furthemore, at $pH<5.0$, the cell biomass and antimicrobal activity of Lactobacilli were low, whereas the optimum pH for those LAB to grow was $pH \geq 6.5$ [13,10].

The presence or lack of oxygen can have a substantial impact on bacteria's capacity to make energy, which can eventually alter biofilm development. When oxygen is not accessible for reduction, bacterial biofilms' metabolic activity can decrease[57,66]. As a result, oxygen availability is a key environmental component that can influence biofilm composition and growth. Decreased oxygen levels can cause active dispersal, which is critical for biofilm survival. Bacterial cells in the bottom of a biofilm receive less oxygen than those on the surface, which can promote detachment from the biofilm's deeper layers [58-60].

Another research paper that appeared in Microbial Ecology described the effect of oxygen availability on *L. brevis* biofilm formation in wine [61]. The researchers discovered that *L. brevis* developed more biofilm in the presence of oxygen than in the absence of oxygen. They suggested that this was due to oxygen stimulating the formation of polysaccharides, which are key components of the extracellular matrix.

Moreover, the importance of an air-liquid interface has been identified as a significant component that promotes bacterial adhesion to surfaces, and studies have demonstrated that the availability of oxygen and nutrients influences biofilm formation [65]. In a co-culture, the growth of pathogenic microorganisms can be inhibited through competition for nutrients and oxygen or by production of antagonistic compounds by other bacteria, for example LAB [41,37]. Overall, The presence of oxygen and nutrients, along with the synthesis of inhibitory substances, can have significant impacts on the formation and avoidance of biofilms.

The process of biofilm formation is a complex mechanism that can be studied from various perspectives. Bacterial biofilms have the ability to form in environments with rich nutrients, as well as in nutrient-deprived conditions. However, biofilms formed under starvation conditions tend to have a much more prominent EPS matrix [46,53,58]. Indeed, investigation of the ability of bacterial strains to form biofilms under different development phases is important. The reason is that their properties may vary depending on whether they are in the biofilm or planktonic phase. The differences in metabolites produced by the cells in each phase can account for these variations. The presence or absence of oxygen, for instance, can impact the ability of bacteria to attach to submerged surfaces and grow as biofilms [58].

Our findings imply that using biocompatible mediators like HNQ can influence both the electrical fermentation process and bacterial growth. Prior research has shown that HNQ works as a redox mediator to facilitate electron transport in microbial cultures. Also, it has been shown that at low doses, HNQ has little effect on bacterial cell metabolism [60].

According to our results, suprnatants from *L. plantarum* with an initial pH < 4.5 showed a greater ZOI compared to the CFSs with an adjusted pH of 7. This suggests that the antibacterial activity of the CFSs is influenced by the pH level, with a more acidic environment being more favourable for inhibiting the growth of *S. aureus*. Lactobacilli strains have been shown to inhibit the development of Gram-negative (E.coli, P.aeruginosa) and Gram-positive (S.aureus, S.epidermis) pathogenic bacteria [20-27]. Its growth-inhibitory function has been attributed to Lactobacilli's ability to reduce the pH of the media and/or create acids and bacteriocins.

Lactic acid has the ability to make the outer membrane of Gram-negative pathogens more porous, which makes it easier for antimicrobial molecules to enter the bacteria. As a result, the bacteria become more vulnerable to these antimicrobial substances**.**[63]. However, this mechanism may not be effective against Gram-positive pathogens like *S. aureus,* which have a different cell wall structure compared to Gram-negative bacteria. This could be a possible explanation for why *S. aureus* was not affected by the CFSs of *L. brevis* and *L. fermentum*.

The findings of the study indicate that *S. aureus* exhibited the highest total charge and current in individual cultivation, compared to *L. plantarum* and co-culture. However, the co-culture electrochemical properties were significantly higher than those observed for *L. plantarum.* According to the findings, *S. aureus* had the highest charge output in presence of 20 μM HNQ, followed by *L. plantarum* and co-culture with 20 μM HNQ. After the insertion of HNQ, the current fo*r S. aureus*, *L. plantarum*, and the co-culture exhibited a single peak after 9 hours of cultivation. This can be explained by the active proliferation of bacterial cells in exponential phase [43-44]. That being said, it is possible that the DPV results for lactobacilli could be higher than for *S. aureus*, depending on the specific experimental conditions. Lactobacilli are known to produce a variety of electroactive compounds as secondary metabolites, such as lacit acid and flavins, which could contribute to an increase in the DPV signal [53,47]. *S. aureus* is also capable of producing electroactive compounds, the levels and types of these compounds may be different from those produced by lactobacilli.

This research highlights the need to acknowledge the limitations of electroanalysis. Firstly, electroactive microorganisms typically generate low levels of current, and more precise detection methods are required to obtain accurate measurements. Secondly, minor changes in environmental conditions, such as temperature, pH, or initial inoculum size, can have a significant impact on the efficiency and stability of the electrofermentation process.

Additionally, there are limitations to the biofilm quantification assay. The assessment of biofilm formation in a 48-well plate using crystal violet staining may not be entirely precise due to the significant loss of biofilm during the washing steps. This loss can result in inaccurate measurements and may not fully reflect the true biological activity of the biofilm. Furthermore, the quantification of biofilms formed on SPEs needs improvement, as this method only provides surface coverage measurements and does not account for depth or viability of the biofilm.

Chapter 6 - Conclusion

In conclusion, our study has determined that the optimal physiochemical conditions for co-culturing S. aureus and Lactobacilli are achieved in Compounded Medium (CM) under aerobic conditions. These conditions were found to show optimal growth of Lactobacilli spp, with an average optical density (OD) of 1.5 ± 0.5, and S. aureus, with an average (OD) of 1.4 ± 0.2, after 48 hours of incubation. These findings provide valuable insights into optimizing microbiological growth conditions for the co-culture of two or more antagonistic strains. Under aerobic conditions, LAB and *S. aureus* form biofilms, but *S. aureus* forms a more substantial biofilm biomass due to its virulence factors. Under anaerobic conditions, both species form weaker biofilms but LAB can inhibit *S. aureus* biofilm formation uder both conditions, by producing organic acids that lower the pH.

Interestingly, among the three LAB tested, *L. plantarum* was the only strain that exhibited antimicrobial activity against *S. aureus* under all four tested conditions. The growth of LAB and *S. aureus* were observed in separate cultures as well as in co-culture, with and without the addition of HNQ. HNQ inhibited bacterial growth at concentrations above 20 µM. At concentrations of 20 µM, HNQ did not significantly reduce biofilm formation by *S. aureus*, but at higher concentrations (100-200 µM), it did.

The study involved bioelectrochemical tests using LAB and *S. aureus* cells, which were grown in oxidative potential. The inclusion of HNQ increased the maximum current during bioelectrochemical experiments. Bacterial growth was found to be crucial for electroactivity. DPV analysis showed two primary peaks in the bioelectrochemical system, with *S. aureus* showing the highest charge output when treated with 20 µM HNQ. It was observed that *S. aureus* produced a biofilm that spread across the complete surface of the SPE, whereas *L. plantarum* had a lower percentage of coverage. Co-culture of *S. aureu*s and *L. plantarum* resulted in a biofilm formation on the SPE with lower coverage compared to *S. aureus* alone. Moreover, our results showed that HNQ significantly reduced the biofilm formation of both *S. aureus* and *L. plantarum* on SPEs in a dose-dependent manner.

Overall, the findings of this study have important implications for the development of strategies for controlling and managing bacterial biofilm formation. The optimal growth conditions for LAB and *S. aureus* co-culture identified in this study can serve as a starting point for developing more complex co-culture models to investigate the mechanisms of interaction between various bacterial species.

Future studies could focus on investigating the specific changes in the electrochemical properties of *S. aureus* by adding different concentrations of *L. plantarum* CFSs to the media. This could help to identify any potential synergistic effects between the two microorganisms and provide a better understanding of their interactions in electro-fermentation processes. Moreover, further research could explore how electro-fermentation may affect the biofilm adhesion properties of bacterial cells. This could provide insights into the mechanisms underlying biofilm formation and potentially lead to the development of novel strategies to prevent or control biofilm growth.

Bibliography

[1] Parolin, C., Croatti, V., Laghi, L., Giordani, B., Tondi, M. R., De Gregorio, P. R., Foschi, C., & Vitali, B. (2021, October 29). Lactobacillus Biofilms Influence Anti-Candida Activity. *Frontiers in Microbiology*, *12*. https://doi.org/10.3389/fmicb.2021.750368

[2] Sieuwerts, S., Molenaar, D., van Hijum, S. A. F. T., Beerthuyzen, M., Stevens, M. J. A., Janssen, P. W. M., Ingham, C. J., de Bok, F. A. M., de Vos, W. M., & van Hylckama Vlieg, J. E. T. (2010, December). Mixed-Culture Transcriptome Analysis Reveals the Molecular Basis of Mixed-Culture Growth in *Streptococcus thermophilus* and *Lactobacillus bulgaricus*. *Applied and Environmental Microbiology*, *76*(23), 7775–7784. https://doi.org/10.1128/aem.01122-10

[3] Aoudia, N., Rieu, A., Briandet, R., Deschamps, J., Chluba, J., Jego, G., Garrido, C., & Guzzo, J. (2016, February). Biofilms of Lactobacillus plantarum and Lactobacillus fermentum: Effect on stress responses, antagonistic effects on pathogen growth and immunomodulatory properties. *Food Microbiology*, *53*, 51–59. https://doi.org/10.1016/j.fm.2015.04.009

[4] De MAN, J. C., ROGOSA, M., & SHARPE, M. E. (1960, April). A MEDIUM FOR THE CULTIVATION OF LACTOBACILLI. *Journal of Applied Bacteriology*, *23*(1), 130–135. https://doi.org/10.1111/j.1365-2672.1960.tb00188.x

[5] Sapri, H. F., Sani, N. A. M., Neoh, H. M., & Hussin, S. (2013, January 22). Epidemiological Study on Staphylococcus aureus Isolates Reveals Inverse Relationship between Antibiotic Resistance and Virulence Repertoire. *Indian Journal of Microbiology*, *53*(3), 321–322. https://doi.org/10.1007/s12088-013-0357-4

[6] Silva, G. O., Pennafirme, S., da Costa Pereira, D., Waite, C. C., Lopes, R. T., Lima, I. C., & Crapez, M. A. (2020, December). Monitoring of bacterial community structure and growth: An alternative tool for biofilm microanalysis. *Biofilm*, *2*, 100034. https://doi.org/10.1016/j.bioflm.2020.100034

[7] Human Microbiome Project Consortium. Structure, function and diversity of the healthy human microbiome. *Nature*. 2012;486(7402):207-214. Published 2012 Jun 13. doi:10.1038/nature11234

[8] Tejedor-Sanz, S., Stevens, E. T., Li, S., Finnegan, P., Nelson, J., Knoesen, A., Light, S. H., Ajo-Franklin, C. M., & Marco, M. L. (2022, February 11). Extracellular electron transfer increases fermentation in lactic acid bacteria via a hybrid metabolism. *ELife*, *11*. https://doi.org/10.7554/elife.70684

[9] Lazarenko, L., Babenko, L., Sichel, L. S., Pidgorskyi, V., Mokrozub, V., Voronkova, O., & Spivak, M. (2012, February 25). Antagonistic Action of Lactobacilli and Bifidobacteria in Relation to Staphylococcus aureus and Their Influence on the Immune Response in Cases of Intravaginal Staphylococcosis in Mice. *Probiotics and Antimicrobial Proteins*, *4*(2), 78–89. https://doi.org/10.1007/s12602-012-9093-z

[10] Halloran, K., & Underwood, M. A. (2019, August). Probiotic mechanisms of action. *Early Human Development*, *135*, 58–65. https://doi.org/10.1016/j.earlhumdev.2019.05.010

[11] Ng, S. C., Hart, A. L., Kamm, M. A., Stagg, A. J., & Knight, S. C. (2009, February). Mechanisms of action of probiotics: Recent advances. *Inflammatory Bowel Diseases*, *15*(2), 300–310. https://doi.org/10.1002/ibd.20602

[12] The Role of Prebiotics and Probiotics in Human Health: A Systematic Review with a Focus on Gut and Immune Health. (2022, May 31). *Food &Amp; Nutrition Journal*, *7*(1). https://doi.org/10.29011/2575-7091.100245

[13] De Mesquita, A. P., Costa, C. G. F., Frutuoso, J., Pinheiro, I. O., Mota, A. C. S., Franchitti, A., & Ximenes, E. A. (2017). Activity of metabolites produced by new strains of Lactobacillus in modified de Man, Rogosa and Sharpe (MRS) medium against multidrug-resistant bacteria. African Journal of Microbiology Research, 11(8), 345–355. https://doi.org/10.5897/ajmr2017.8437

[14] Legras A et al. Nosocomial infections: prospective survey of incidence in five French intensive care units.Intensive Care Medicine, 1998, 24:1040–1046

[15] Tomlinson, B. R., Malof, M. E., & Shaw, L. N. (2021). A global transcriptomic analysis of *Staphylococcus aureus* biofilm formation across diverse clonal lineages. *Microbial genomics*, *7*(7), 000598. https://doi.org/10.1099/mgen.0.000598

[16] Qin Y, He Y, She Q, Larese‑Casanova P, Li P, Chai Y. Heterogeneity in respiratory electron transfer and adaptive iron utilization in a bacterial biofilm. Nat Commun. 2019;10:3702.

[17] Stratford JP, Edwards CLA, Ghanshyam MJ, Malyshev D, Delise MA, Hayashi Y, et al. Electrically induced bacterial membrane‑potential dynamics correspond to cellular proliferation capacity. Proc Natl Acad Sci U S A. 2019;116:9552‑7.

[18] Gries CM, Bose JL, Nuxoll AS, Fey PD, Bayles KW. The Ktr potassium transport system in Staphylococcus aureus and its role in cell physiology, antimicrobial resistance and pathogenesis. Mol Microbiol. 2013;89:760‑73.

[19]Piewngam, P., & Otto, M. (2020). Probiotics to prevent Staphylococcus aureus disease? *Gut Microbes*, *11*(1), 94-101. https://doi.org/10.1080/19490976.2019.1591137

[20] Carvalho, F. M., Mergulhão, F. J. M., & Gomes, L. C. (2021). Using Lactobacilli to Fight *Escherichia coli* and *Staphylococcus aureus* Biofilms on Urinary Tract Devices. *Antibiotics (Basel, Switzerland)*, *10*(12), 1525. https://doi.org/10.3390/antibiotics10121525

[21] Carvalho, F. M., Teixeira-Santos, R., Mergulhão, F. J. M., & Gomes, L. C. (2021). Effect of *Lactobacillus plantarum* Biofilms on the Adhesion of *Escherichia coli* to Urinary Tract Devices. *Antibiotics (Basel, Switzerland)*, *10*(8), 966. https://doi.org/10.3390/antibiotics10080966

[22] Tan Y, Leonhard M, Moser D, Ma S, Schneider-Stickler B. Inhibitory effect of probiotic lactobacilli supernatants on single and mixed non-albicans Candida species biofilm. *Arch Oral Biol*. 2018;85:40-45. doi:10.1016/j.archoralbio.2017.10.002

[23] Ghane, M., Babaeekhou, L., & Ketabi, S. S. (2020). Antibiofilm Activity of Kefir Probiotic Lactobacilli Against Uropathogenic *Escherichia coli* (UPEC). *Avicenna journal of medical biotechnology*, *12*(4), 221–229.

[24] Srivastava N, Ellepola K, Venkiteswaran N, Chai LYA, Ohshima T,Seneviratne CJ. Lactobacillus Plantarum 108 Inhibits Streptococcus mutans and Candida albicans Mixed-Species Biofilm Formation. *Antibiotics (Basel)*. 2020;9(8):478. Published 2020 Aug 4. doi:10.3390/antibiotics9080478

[25] Melo, T. A., Dos Santos, T. F., de Almeida, M. E., Junior, L. A., Andrade, E. F., Rezende, R. P., Marques, L. M., & Romano, C. C. (2016). Inhibition of Staphylococcus aureus

biofilm by Lactobacillus isolated from fine cocoa. *BMC microbiology*, *16*(1), 250. https://doi.org/10.1186/s12866-016-0871-8

[26] Gélinas, M., Museau, L., Milot, A., & Beauregard, P. B. (2021). The *de novo* Purine Biosynthesis Pathway Is the Only Commonly Regulated Cellular Pathway during Biofilm Formation in TSB-Based Medium in Staphylococcus aureus and Enterococcus faecalis. *Microbiology spectrum*, *9*(3), e0080421. https://doi.org/10.1128/Spectrum.00804-21

[27]Frickmann, H., Klenk, C., Warnke, P., Redanz, S., & Podbielski, A. (2018). Influence of Probiotic Culture Supernatants on In Vitro Biofilm Formation of Staphylococci. *European journal of microbiology & immunology*, *8*(4), 119–127. https://doi.org/10.1556/1886.2018.00022

[28] Patel M, Siddiqui AJ, Hamadou WS, et al. Inhibition of Bacterial Adhesion and Antibiofilm Activities of a Glycolipid Biosurfactant from *Lactobacillus rhamnosus* with Its Physicochemical and Functional Properties.*Antibiotics (Basel)*. 2021;10(12):1546. Published 2021 Dec 17. doi:10.3390/antibiotics10121546

[29] Mekky AF, Hassanein WA, Reda FM, Elsayed HM. Anti-biofilm potential of *Lactobacillus plantarum* Y3 culture and its cell-free supernatant against multidrug-resistant uropathogen *Escherichia coli* U12. *Saudi J Biol Sci*. 2022;29(4):2989-2997. doi:10.1016/j.sjbs.2022.01.014

[30] Elebeedy, D., Ghanem, A., El-Sayed, M., Fayad, E., Abu Ali, O. A., Alyamani, A., & Sayed Abdelgeliel, A. (2022). Synergistic Antimicrobial Effect of *Lactiplantibacillus plantarum* and *Lawsonia inermis* Against *Staphylococcus aureus*. *Infection and drug resistance*, *15*, 545–554. https://doi.org/10.2147/IDR.S342976

[31] Chandran, C., Tham, H. Y., Abdul Rahim, R., Lim, S. H. E., Yusoff, K., & Song, A. A. (2022). *Lactococcus lactis* secreting phage lysins as a potential antimicrobial against multi-drug resistant *Staphylococcus aureus*. *PeerJ*, *10*, e12648. https://doi.org/10.7717/peerj.12648

[32] Kalaycı Yüksek, F., Gümüş, D., Gündoğan, G.İ. *et al.* Cell-Free *Lactobacillus* sp Supernatants Modulate *Staphylococcus aureus* Growth, Adhesion and Invasion to Human Osteoblast (HOB) Cells. *Curr Microbiol* 78, 125–132 (2021). https://doi.org/10.1007/s00284-020-02247-1

[33] Jorjão, A.L., de Oliveira, F.E., Leão, M.V.P. *et al.* Effect of Lactobacillus rhamnosus on the response of *Galleria mellonella* against *Staphylococcus aureus* and *Escherichia coli* infections. *Arch Microbiol* 200, 383–389 (2018). https://doi.org/10.1007/s00203-017-1441-7

[34] Fallah, M., Bahram, S., & Javadian, S. R. (2015). Fish peptone development using enzymatic hydrolysis of silver carp by-products as a nitrogen source in Staphylococcus aureus media. *Food science & nutrition*, *3*(2), 153–157. https://doi.org/10.1002/fsn3.198

[35] Matsubara, V.H., Wang, Y., Bandara, H.M.H.N. *et al.* Probiotic lactobacilli inhibit early stages of *Candida albicans* biofilm development by reducing their growth, cell adhesion, and filamentation. *Appl Microbiol Biotechnol* 100, 6415–6426 (2016). https://doi.org/10.1007/s00253-016-7527-3

[36] Vlaeminck, J., Lin, Q., Xavier, B. B., De Backer, S., Berkell, M., De Greve, H., Hernalsteens, J., & Goossens, H. (2022). The dynamic transcriptome during maturation of biofilms formed by methicillin-resistant Staphylococcus aureus. *Frontiers in Microbiology*, *13*. https://doi.org/10.3389/fmicb.2022.882346

[37] Cramton, S. E., Ulrich, M., Götz, F., & Döring, G. (2001). Anaerobic conditions induce expression of polysaccharide intercellular adhesin in Staphylococcus aureus and Staphylococcus epidermidis. *Infection and immunity*, *69*(6), 4079–4085. https://doi.org/10.1128/IAI.69.6.4079-4085.2001

[38] Bradbeer C. (1993). The proton motive force drives the outer membrane transport of cobalamin in Escherichia coli. *Journal of bacteriology*, *175*(10), 3146–3150. https://doi.org/10.1128/jb.175.10.3146-3150.1993

[39] Rollet, C., Gal, L., & Guzzo, J. (2009). Biofilm-detached cells, a transition from a sessile to a planktonic phenotype: a comparative study of adhesion and physiological characteristics in Pseudomonas aeruginosa. *FEMS microbiology letters*, *290*(2), 135–142. https://doi.org/10.1111/j.1574-6968.2008.01415.x

[40] Liu, J., Prindle, A., Humphries, J., Gabalda-Sagarra, M., Asally, M., Lee, D. Y., Ly, S., Garcia-Ojalvo, J., & Süel, G. M. (2015). Metabolic co-dependence gives rise to collective oscillations within biofilms. *Nature*, *523*(7562), 550–554. https://doi.org/10.1038/nature14660

[41] Prindle, A., Liu, J., Asally, M., Ly, S., Garcia-Ojalvo, J., & Süel, G. M. (2015). Ion channels enable electrical communication in bacterial communities. *Nature*, *527*(7576), 59–63. https://doi.org/10.1038/nature15709

[42] Sikorska, H., & Smoragiewicz, W. (2013). Role of probiotics in the prevention and treatment of meticillin-resistant Staphylococcus aureus infections. In *International Journal of Antimicrobial Agents* (Vol. 42, Issue 6, pp. 475–481). Elsevier B.V. https://doi.org/10.1016/j.ijantimicag.2013.08.003

[43] Lovley, D. R. (2012). Electromicrobiology. *Annual Review of Microbiology*, *66*, 391–409. https://doi.org/10.1146/annurev-micro-092611-150104

[44] Busalmen, J. P., Esteve-Nuñez, A., & Feliu, J. M. (2008). Whole cell electrochemistry of electricity-producing microorganisms evidence an adaptation for optimal exocellular electron transport. *Environmental Science and Technology*, *42*(7), 2445–2450. https://doi.org/10.1021/es702569y

[45]. Ajunwa, O. M., Odeniyi, O. A., Garuba, E. O., Nair, M., Marsili, E., & Onilude, A. A. (2022). Evaluation of extracellular electron transfer in Pseudomonas aeruginosa by co-expression of intermediate genes in NAD synthetase production pathway. *World Journal of Microbiology and Biotechnology*, *38*(5). https://doi.org/10.1007/s11274-022-03274-9

[46] Hu, Y., Wang, Y., Han, X., Shan, Y., Li, F., & Shi, L. (2021). Biofilm Biology and Engineering of Geobacter and Shewanella spp. for Energy Applications. In *Frontiers in Bioengineering and Biotechnology* (Vol. 9). Frontiers Media S.A. https://doi.org/10.3389/fbioe.2021.786416

[47] Wang, W. S., Huang, H. Y., Chen, S. C., Ho, K. C., Lin, C. Y., Chou, T. C., Hu, C. H., Wang, W. F., Wu, C. F., & Luo, C. H. (2011). Real-time telemetry system for amperometric and potentiometric electrochemical sensors. *Sensors*, *11*(9), 8593–8610. https://doi.org/10.3390/s110908593

[48] Ward, A. C., Hannah, A. J., Kendrick, S. L., Tucker, N. P., MacGregor, G., & Connolly, P. (2018). Identification and characterisation of Staphylococcus aureus on low cost screen printed

carbon electrodes using impedance spectroscopy. *Biosensors and Bioelectronics*, *110*, 65–70. https://doi.org/10.1016/j.bios.2018.03.048

[49] Brosel-Oliu, S., Mergel, O., Uria, N., Abramova, N., van Rijn, P., & Bratov, A. (2019). 3D impedimetric sensors as a tool for monitoring bacterial response to antibiotics. *Lab on a Chip*, *19*(8), 1436–1447. https://doi.org/10.1039/c8lc01220b

[50] Batchelor-Mcauley, C., Kätelhçn, E., Barnes, E. O., Compton, R. G., Aborda, E., & Molina, A. (2015). *Recent Advances in Voltammetry*. www.chemistryopen.org

[51] Brett, C. M. A., & Brett, A. M. Oliveira. (1993). *Electrochemistry : principles, methods, and applications*. Oxford University Press.

[52] Madhu, S., Ramasamy, S., & Choi, J. (2022). Recent Developments in Electrochemical Sensors for the Detection of Antibiotic-Resistant Bacteria. In *Pharmaceuticals* (Vol. 15, Issue 12). MDPI. https://doi.org/10.3390/ph15121488

[53] Bard, A. J., & Faulkner, L. R. (n.d.). *Electrochemical methods : fundamentals and applications*.

[54] Du, C. X., Han, L., Dong, S. L., Li, L. H., & Wei, Y. (2016). A novel procedure for fabricating flexible screen-printed electrodes with improved electrochemical performance. *IOP Conference Series: Materials Science and Engineering*, *137*(1). https://doi.org/10.1088/1757-899X/137/1/012060

[55] Tan, C., Nasir, M. Z. M., Ambrosi, A., & Pumera, M. (2017). 3D Printed Electrodes for Detection of Nitroaromatic Explosives and Nerve Agents. *Analytical Chemistry*, *89*(17), 8995–9001. https://doi.org/10.1021/acs.analchem.7b01614

[56]. Lovley, D. R. (2006). Bug juice: Harvesting electricity with microorganisms. In *Nature Reviews Microbiology* (Vol. 4, Issue 7, pp. 497–508). Nature Publishing Group. https://doi.org/10.1038/nrmicro1442

[57] Wang, G., & Zeng, H. (2022). Antibacterial Effect of Cell-Free Supernatant from Lactobacillus pentosus L-36 against Staphylococcus aureus from Bovine Mastitis. *Molecules*, *27*(21). https://doi.org/10.3390/molecules2721762

[58] Chang, Y. W., Fragkopoulos, A. A., Marquez, S. M., Kim, H. D., Angelini, T. E., & Fernández-Nieves, A. (2015). Biofilm formation in geometries with different surface curvature and oxygen availability. *New Journal of Physics*, *17*. https://doi.org/10.1088/1367-2630/17/3/033017

[59] Ahimou, F., Semmens, M. J., Haugstad, G., & Novak, P. J. (2007). Effect of protein, polysaccharide, and oxygen concentration profiles on biofilm cohesiveness. *Applied and Environmental Microbiology*, *73*(9), 2905–2910. https://doi.org/10.1128/AEM.02420-06

[60] Toyofuku, M., Inaba, T., Kiyokawa, T., Obana, N., Yawata, Y., & Nomura, N. (2016). Environmental factors that shape biofilm formation. In *Bioscience, Biotechnology and Biochemistry* (Vol. 80, Issue 1, pp. 7–12). Japan Society for Bioscience Biotechnology and Agrochemistry. https://doi.org/10.1080/09168451.2015.1058701

[61] Russo, P., Fernández De Palencia, P., Romano, A., Fernández, M., Lucas, P., Spano, G., & Lápez, P. (2012). Biogenic amine production by the wine Lactobacillus brevis IOEB 9809 in systems that partially mimic the gastrointestinal tract stress. *BMC Microbiology*, *12*. https://doi.org/10.1186/1471-2180-12-247

[62] Ebert, C., Tuchscherr, L., Unger, N., Pöllath, C., Gladigau, F., Popp, J., Löffler, B., & Neugebauer, U. (2021). Correlation of crystal violet biofilm test results of Staphylococcus aureus clinical isolates with Raman spectroscopic read-out. *Journal of Raman Spectroscopy*, *52*(12), 2660–2670. https://doi.org/10.1002/jrs.6237

[63] Ponomareva, A. L., Buzoleva, L. S., & Bogatyrenko, E. A. (2018). Abiotic Environmental Factors Affecting the Formation of Microbial Biofilms. *Biology Bulletin*, *45*(5), 490–496. https://doi.org/10.1134/S106235901805014

[64] Peter A. Vandenbergh, Lactic acid bacteria, their metabolic products and interference with microbial growth, *FEMS Microbiology Reviews*, Volume 12, Issue 1-3, September 1993, Pages 221–237, https://doi.org/10.1111/j.1574-6976.1993.tb00020.x

[65] Alakomi, H. L., E. Skytta, M. Saarela, T. Mattila-Sandholm, K. Latva-Kala, and I. M. Helander. 2000. Lactic acid permeabilizes gram-negative bacteria by disrupting the outer membrane. Appl. Environ. Microbiol. 66:2001–2005

[66] Smetanková, J., Hladíková, Z., Valach, F., Zimanová, M., Kohajdová, Z., Greif, G., & Greifová, M. (2018). Influence of aerobic and anaerobic conditions on the growth and metabolism of selected strains of Lactobacillus plantarum. *Acta Chimica Slovaca*, *5*(2), 204–210. https://doi.org/10.2478/v10188-012-0031-1

[67] Wijtzes, T., de Wit, J. C., Huis in 't Veld, J. H. J., van 't Riet, K., & Zwietering, M. H. (1995). Modelling bacterial growth of Lactobacillus curvatus as a function of acidity and temperature. *Applied and Environmental Microbiology*, *61*(7), 2533–2539. https://doi.org/10.1128/aem.61.7.2533-2539.1995

[68] Amanda, C. de M., Cintia, R. R. C., Jessica, F., Irapuan, O. P., Amanda, M., Adelisa, A. F., & Eulalia, A. X. (2017). Activity of metabolites produced by new strains of Lactobacillus in modified de Man, Rogosa and Sharpe (MRS) medium against multidrug-resistant bacteria. *African Journal of Microbiology Research*, *11*(8), 345–355. https://doi.org/10.5897/ajmr2017.8437

[68] Eghtesadi, N., Olaifa, K., Perna, F. M., Capriati, V., Trotta, M., Ajunwa, O., & Marsili, E. (2022). Electroactivity of weak electricigen Bacillus subtilis biofilms in solution containing deep eutectic solvent components. *Bioelectrochemistry*, *147*. https://doi.org/10.1016/j.bioelechem.2022.108207

69 Bekele, E. (2014). *Isolation and screening of antibacterial producing lactic acid bacteria from traditionally fermented drinks (" Ergo " and " Tej ") in Gondar town, Northwest Ethiopia The Genomic, Epigenomic, and Metagenomic Non-communicable Disorder Initiative for Africa (GEMINI Africa) View project Antibiotic searching project View project*. https://www.researchgate.net/publication/282706115

Appendix 1

Compounded medium (CM) showed the optimal growth rate in aerobic conditions for every strain that was examined. For the further establishment of quantitative estimation, LAB and *S. aureus* cultures were selected for co-culturing in CM in aerobic conditions.

Co-cultured strains were used further for colony-forming unit assay counting experiments. Also, Mannitol Salt Agar and de Mann Rogosa and Sharpe agar media were used. Counting experiments, strains were co-cultivated in CM medium with initial OD600nm 0.1-0.2. Continuously, after 24 h of incubation, cultures were diluted by ten-fold serial dilutions using phosphate-buffered saline (PBS, pH 7.4). Then, 0.1mL of prepared dilutions of cultures were plated on MSA and MRS agar on Petri plates. Dilutions were poured and plated by using disposable spreaders. Conditions for incubation of plates were 37 °C for 48 h in aerobic and anaerobic conditions. Results are provided in the given tables (Table 1-4). Counting was held by the formula given below:

cfu/ml = (no. of colonies x dilution factor) / volume of culture plate

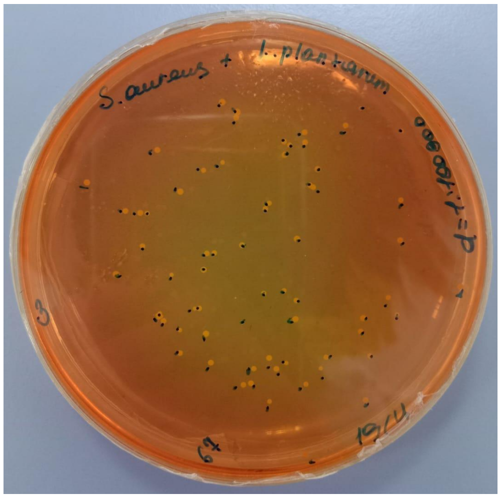
Table A.1 Colony-forming unit assay counts of LAB and S. aureus co-cultured under aerobic conditions.

№	Samples on MSA (Mannitol Salt Agar). Condition aerobic	no. of colonies	dilution factor	CFU/ml
1	*L. plantarum + S. aureus*	67	10^5	$6.7 * 10^8$
2	*L. brevis + S. aureus*	128	10^5	$1.28 * 10^8$
3	*L. fermentum + S. aureus*	97	10^5	$9.7 * 10^8$

Table A.2 Colony-forming unit assay counts of LAB and S. aureus co-cultured under aerobic conditions.

№	Samples on MSA (Mannitol Salt Agar). Condition anaerobic	no. of colonies	dilution factor	CFU/ml
1	*L. plantarum + S. aureus*	45	10^5	$4.5*10^7$
2	*L. brevis + S. aureus*	83	10^5	$8.3*10^7$
3	*L. fermentum + S. aureus*	104	10^5	$1.04*10^8$

Obtained results showed the growth rate and CFU number of *S. aureus* in aerobic conditions was more elevated, which means that there was no or little antimicrobial activity of LAB against *S. aureus*. Hence, in anaerobic conditions growth rate of *S. aureus* was increased due to the antimicrobial activity of LAB.



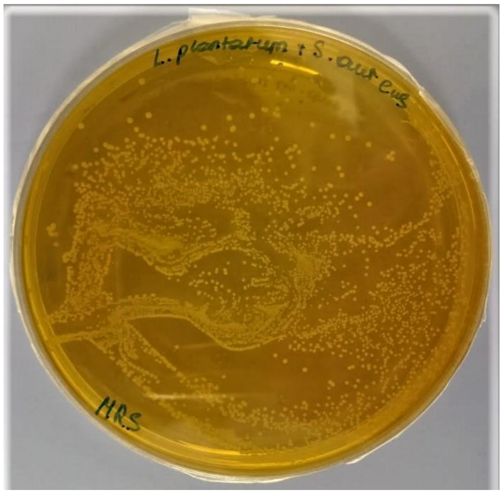


Figure A.1: The petri dish contains a 10^5 dilution of L. plantarum + S. aureus at a 1:10000 ratio. The petri dish yielded a count of 67 colonies of S. aureus on MSA.

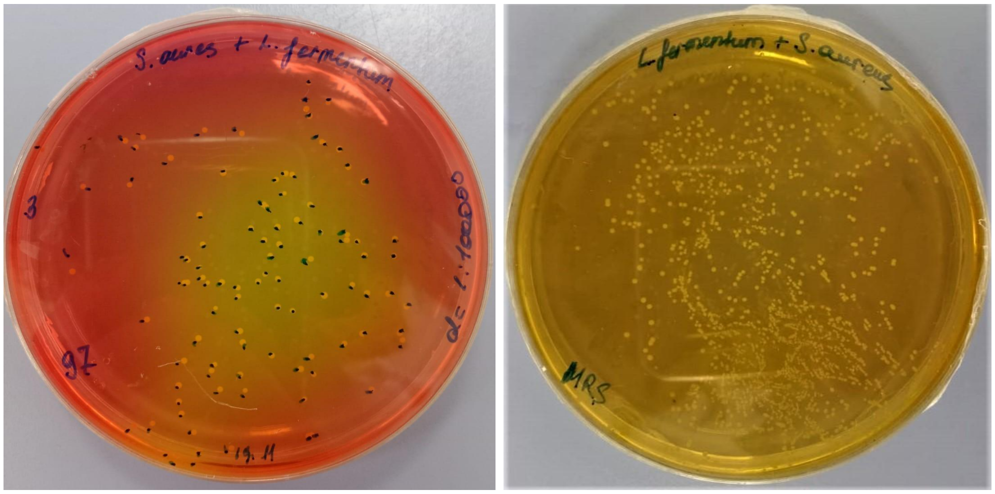


Figure A.2 The petri dish contains a 10^5 dilution of L. fermentum + S. aureus at a 1:10000 ratio. The petri dish yielded a count of 97 colonies of S. aureus on MSA.

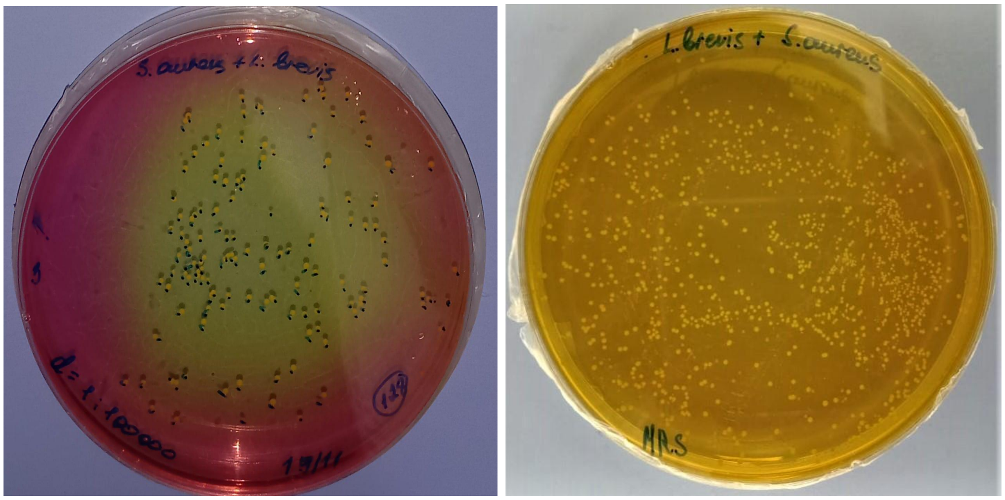


Figure A.3 The petri dish contains a 10^5 dilution of L. brevis + S. aureus at a 1:10000 ratio. The petri dish yielded a count of 128 colonies of S. aureus on MSA.

Appendix 2

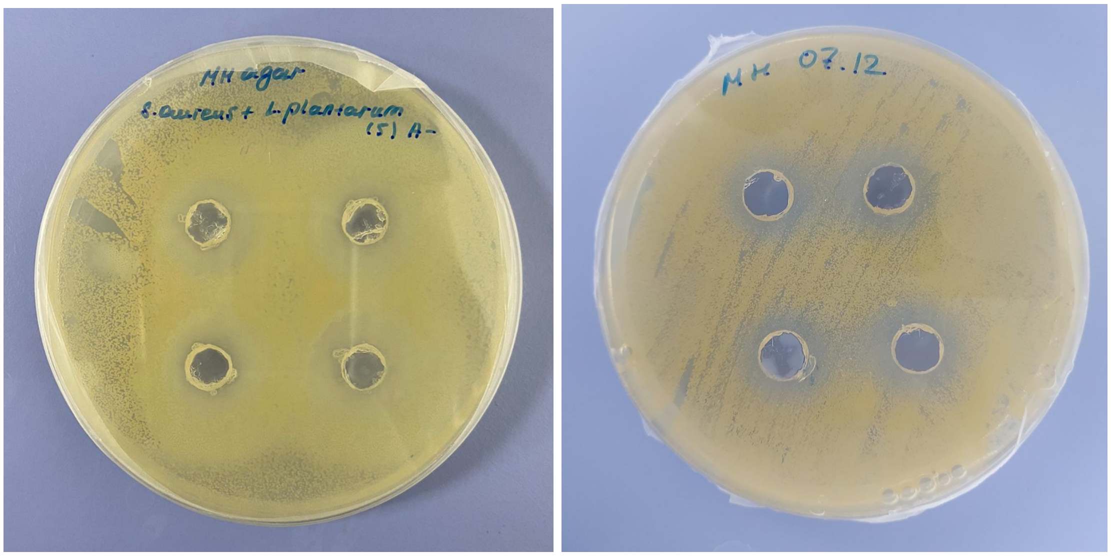


Figure A.4:. Antibacterial efficacy of *L. plantarum* supernatant from the anaerobic condition. Zone of inhibition in mm (Mean ± SD). 6.15 ± 0.20. CFSs with pH equal to 7.

Appendix 3

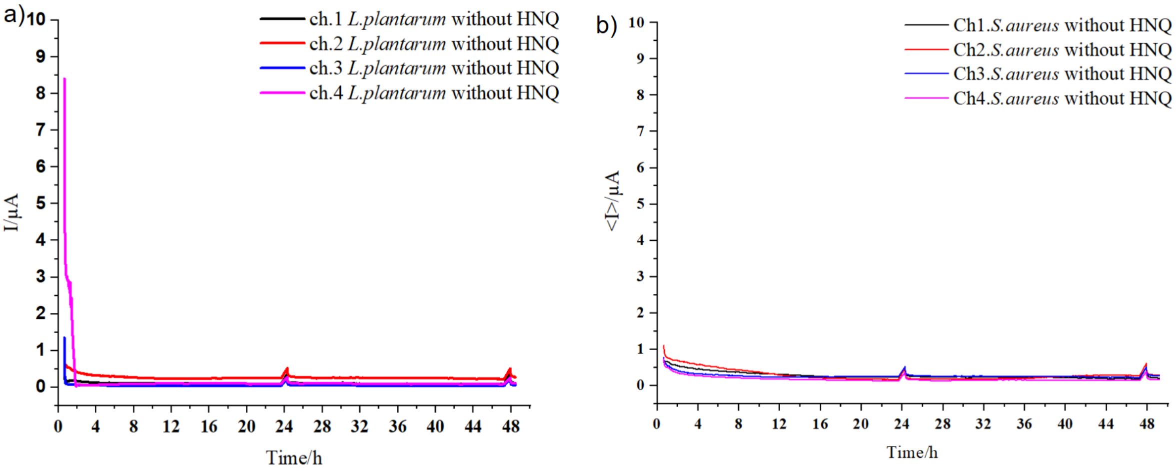


Figure A.5. CA results for individual bacterial cells. a) L. plantarum without HNQ. 400mV. b) S. aureus without HNQ. 400mV.

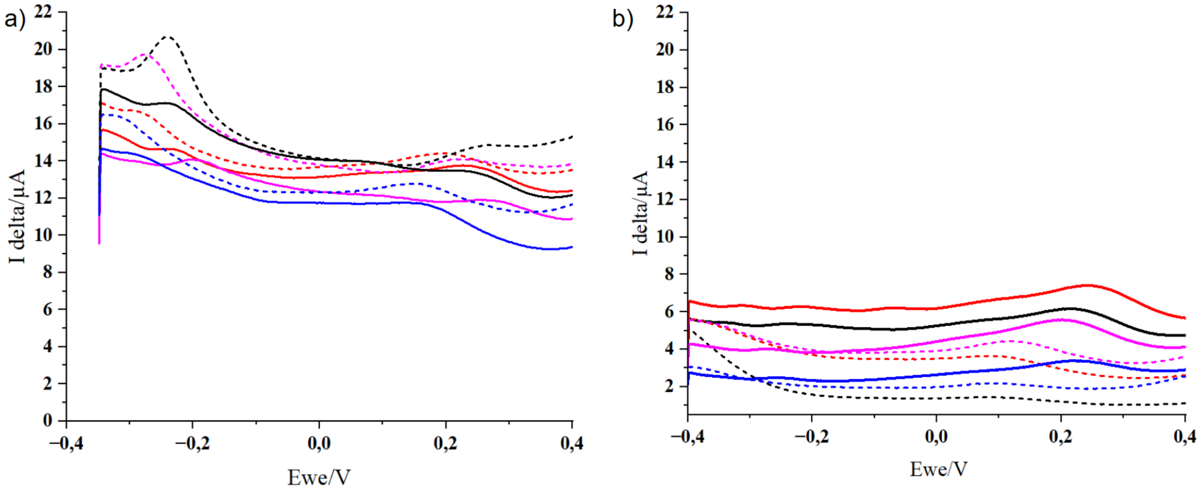
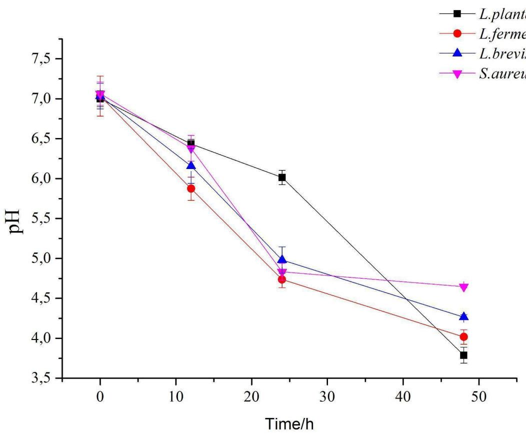


Figure A.6. DPV results for *L. plantarum*, *S. aureus* and co-culture in two different time frames. Dash line(0-24h), Solid line (24-48h). a) L.plantarum with four replicates. b) S.aureus with four replicates.

Appendix 4



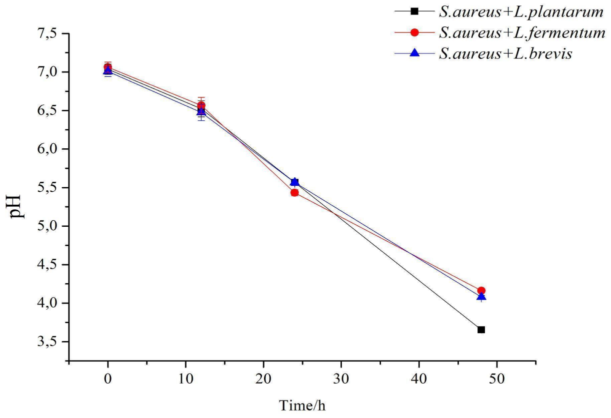


Figure A.7: Decrease of pH values of LAB and S.aureus cultivated under aerobic conditions at 37 °C for 48 hours.

Table A.3: Biofilm coverage on SPEs

Sample (without HNQ)	**Biofilm coverage, %**
L. plantarum	14.71±0.14
S. aureus	99.97±0.08
Co-culture	57.67±0.12

Appendix 5

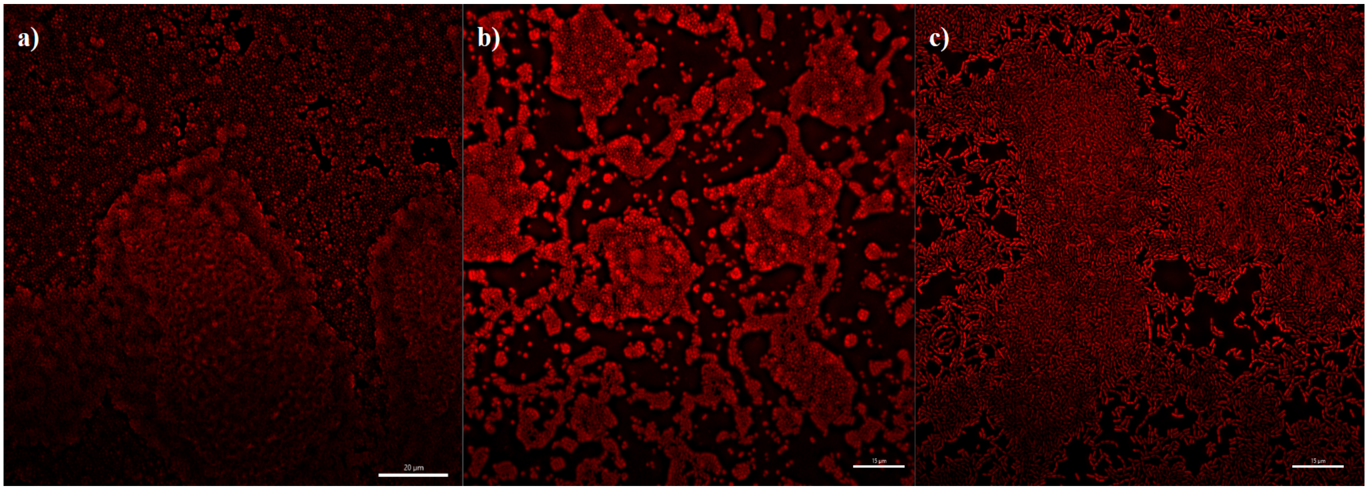


Figure A.8: ***Fluorescence microscopy images of biofilms stained with 0.1% w/v crystal violet in the 8- well ibidi plates under 63x magnification. a) Biofilm of S.aureus;b) Biofilm of co-culture; c) Biofilm of L.plantarum.***