QUANTIFICATION OF LACTOBACILLUS OBTAINED FROM AN ABIOTIC SURFACE USING SOME SOLUBILIZING AGENTS

BY

ASAMA OREZIMENA SOPHIA

PHA1405696

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DEPARTMENT OF PHARMACEUTICAL MICROBIOLOGY

FACULTY OF PHARMACY,

UNIVERSITY OF BENIN,

BENIN CITY, NIGERIA.

SUPERVISED BY

DR. E. OLOTON

JULY 2021.

DECLARATION

I, ASAMA OREZIMENA SOPHIA with the matriculation number PHA1405696 hereby declare that this project work entitled **QUANTIFICATION OF LACTOBACILLUS OBTAINED FROM ABIOTIC SURFACE USING SOME SOLUBILIZING AGENTS** is the original research work carried out by me in the department of Pharmaceutical Microbiology, Faculty of Pharmacy, University of Benin, Benin city, Nigeria.

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ASAMA OREZIMENA SOPHIA DATE

CERTIFICATION

This is to certify that this is an original research work carried out by ASAMA OREZIMENA SOPHIA in the Department of Pharmaceutical Microbiology, Faculty of Pharmacy, University of Benin, in partial fulfillment of the requirements for the award of Doctor of Pharmacy (PharmD) degree.

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DR E. OLOTON DATE

(PROJECT SUPERVISOR)

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DR (MRS) U.F BABAIWA DATE

(HEAD OF DEPARTMENT)

DEDICATION

To my brothers of blessed memory, Uwomano and Okiroro Asama. This journey would not have begun but for you. Thank you for seeing me through the toughest times.

To my mum of blessed memory, Mrs. Grace Asama. You laid the foundation for the passion that set me on this course.

Forever alive in my heart.

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I never would have made it this far but for the grace of my Father in Heaven, YAHWEH, an ever-present help in trouble, my safe place. To him be all the glory forever.

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ABSTRACT

Bacteria possess special features which help them attach to biotic and abiotic surfaces alike. Adhesion of bacteria to surfaces contribute to the spread of infections via contact with these surfaces, be it biotic or abiotic. On abiotic surfaces however, bacteria form biofilms, making them more difficult to eliminate. Solubilizing agents facilitate penetration of a substance into another in which it would be normally insoluble. They are used as cleaning agents, emulsifiers, vehicles, cosolvents, etc. Lactobacilli are a type of Gram-positive, nonsporulating bacteria. The aim of this study was to investigate the ability of certain solubilizing agents to detach bacteria attached to an abiotic (glass) surface, by quantifying the bacterial cells grown from a solution of the solubilizing agent. Minimum inhibitory concentrations of the solubilizing agents were first determined. A non-inhibitory concentration of the solubilizing agent was then used to detach lactobacilli attached to a glass surface and the resulting solution was diluted serially and plated out. Quantitative bacterial quantification was performed by determining colony forming units/ml. Spectrophotometric method was used to qualitatively quantify bacterial detachment. Results showed a higher cell count from the solubilizing agent than from the control, indicating that they are indeed well able to elicit bacterial detachment from abiotic surfaces.

TABLE OF CONTENTS

CHAPTER FIVE

CHAPTER ONE

1.0 INTRODUCTION

1.1 Lactobacillus

Lactobacillus refers to any of a group of rod-shaped, Gram-positive, non-spore forming bacteria of the genus Lactobacillus. (Makarova *et al.*, 2006). They are small, slender, non-motile and occur in chains. They are microaerophilic or anaerobic and are oxidase and catalase negative.

Like other genera in the family, Lactobacillus are characterized by their ability to produce lactic acid as a by-product of glucose metabolism. Lactobacillus is the largest genus within the group of lactic acid producing bacteria, containing over 170 species (Aguirre 1993), with about 44 species adapted to vertebrate hosts or insects. (Zheng *et al*., 2020)

They are widely distributed in animal feeds, silage, manure, milk and milk products. Various species of Lactobacillus are used commercially during the production of sour milks, cheeses, and yogurt, and they have an important role in the manufacture of fermented vegetables (pickles and sauerkraut), beverages (wine and juices), sourdough breads, and some sausages.

The amount of lactic acid produced by lactobacilli varies among species. In several species, like *L. acidophilus*, *L. casei* and *L. plantarum*, glucose metabolism is described as homofermentative, as lactic acid is the primary byproduct, making up at least 85 percent of end metabolic products. In other species however, such as *L. brevis* and *L. fermentum*, glucose metabolism is heterofermentative, with lactic acid making up about 50 percent of metabolic byproducts and ethanol, CO_2 and acetic acid making up most of the other 50 percent. Certain other heterofermentative Lactobacillus organisms are relatively inefficient in their metabolism of glucose and must derive energy from other types of organic compounds like malate, galactose and fructose (The Editors of Encyclopedia Britannica 2018).

Lactobacillus species are a part of the human mouth, gastrointestinal tract, and female genital tract floras, where they produce lactic acid and competitively inhibit pathogenic organisms (Duar *et al*., 2017). Some species of Lactobacillus are used commonly as probiotics

1.2 Beneficial Uses of Lactobacillus

1.2.1 Food Industry

Most food fermenting bacteria are Lactobacillus. They are utilized as started cultures for controlled fermentation in the production of fermented foods such as ogi, akamu, agidi, cheese, pickles, cider, yoghurt, wine, kefir, certain beers, etc. They are also used in the production of animal feeds such as silage, a type of fodder fed to ruminants, made from green foliage crops which have been preserved by acidification through fermentation (Ganzle and Zheng, 2019). The peculiar sour taste associated with foods such as yoghurt is due to the metabolization of sugar to lactic acid by lactobacillus, thus lowering the pH of the environment.

1.2.2 Inhibition of Pathogens

Lactobacillus has an inhibitory effect against pathogenic organisms like *Candida albicans* both in vivo and in vitro. This is due to the production of certain organic acids and metabolites by lactobacillus, creating an unfavorable environment for the survival of *C. albicans.* The organic acids reduce the pH of the environment, inhibiting hyphal growth of the fungus, thus reducing its virulence. It also competitively prevents the biofilm formation of the fungus (Vilela *et al*., 2015). On the other hand, antibiotic therapy may cause suppressed growth of lactobacilli, leading to an overgrowth of *Candida albicans* at sites where they cohabit e.g., digestive tract and vaginal tract.

1.2.3 Vaginal flora and Gastrointestinal Integrity

Lactobacillus dominates the human vaginal microbiota and also forms a significant portion of the gut microbiota. They form biofilms at these sites which enables them to survive harsh environmental conditions and proliferate quite well (Lin *et al*., 2018). Lactobacillus shares a mutualistic relationship with the human body, getting nutrients from the body, while inhibiting pathogens.

1.2.4 Public Health

Oral health: Some lactic acid bacteria have been associated with dental caries since lactic acid can corrode teeth. Salivary Lactobacilli count has been used for a very long time as an indicator of dental caries. Some studies show that some Lactobacilli worsen dental caries. Recent studies however show that probiotics can promote lactobacilli colonization of sites on teeth, inhibiting the ability of streptococcal pathogens to populate and cause dental decay. This study field is new though and only few studies have been published (Meurman and Stamatova, 2007). The result of studies showing evidence of the benefit of certain Lactobacilli against dental caries has led to the application of probiotic incorporation into chewing gums and lozenges (Bonifait *et al*., 2009).

Other Health Conditions: In medicine, *L. acidophilus* is sometimes used to treat/prevent conditions like bacterial vaginosis, diarrhea due to *C. difficile* infection, candida infection, urinary tract infections, lactose intolerance and irritable bowel syndrome.

Probiotics: Lactobacilli is used alone or in combination with other beneficial organisms in the production of probiotic formulations. These are used in treating a number of conditions like

irritable bowel disease, urogenital and vaginal infections, and atopic dermatitis. It is also used as an adjuvant in treatment of H. pylori infection and as an intestinal immunity booster (Ruggiero 2014).

The major mechanisms by which Lactobacillus elicits its probiotic effect include enhancement of epithelial intestinal barrier, increased adhesion to intestinal mucosa, concomitant inhibition of pathogen adhesion, competitive exclusion of pathogenic organisms, production of bacteriocins and modulation of the immune system (Collado *et al.,* 2010).

Before probiotic strains can colonize the intestinal mucosa, they must first adhere to it. Lactobacilli has various surface properties that enable it to adhere to intestinal epithelial cells and mucus. They possess proteins and surface adhesins that promote adhesion to the mucus layer (MI and MJ, 2011). The surface proteins are either attached to the bacterial cell membrane by a lipid moiety or embedded in the cell wall, while the adhesins are usually secreted.

L. reuteri produces one of the most studied mucus targeting bacterial adhesin called MUB - mucus-binding protein (Buck *et al*., 2005).

Examples of dosage forms that have Lactobacillus as a major or part of the active constituent include:

- Tablets for oral administration- these are majorly intended for action in the gastrointestinal tract. There are however studies that show that daily intake of certain Lactobacillus strains may modify the vaginal flora (Reid *et al*., 2001). Oral probiotic formulations must possess the ability to survive passage through the GIT and attach to the intestinal epithelium to colonize it.

- Pessaries and suppositories – these are solid dosage forms intended for insertion into the vagina/anus respectively. It then dissolves, soften and exert a local or systemic effect. They are used in treatments of Candida, bacterial vaginosis ad other urogenital infections.
- Powders – they may also be formulated as lyophilized powders to be mixed with milk or water and consumed orally.
- Capsules – they may be formulated as capsules for oral administration or as suppositories.

1.3 Solubilizing Agents

Solubility, which is the phenomenon of dissolution of solute in solvent to give a homogenous system, is one of the important parameters to achieve desired concentration of drug in systemic circulation for desired pharmacological response. Low aqueous solubility is the major problem encountered with formulation development of new chemical entities as well as for the generic development. More than 40% new chemical entities developed in pharmaceutical industry are practically insoluble in water. Solubility is a major challenge for formulation scientist (Sharma *et al.*, 2009; Williams *et al*., 2013).

It has long been known that the aqueous solubility of sparingly soluble or insoluble substances can be increased by adding an appropriate third component. Systematic studies using surfactants led to this phenomenon being called solubilization (Moroi, 1992).

Solubilization plays a very important role in industrial and biological processes. Solubilization may be defined as 'a particular mode of bringing into solution substances that are otherwise insoluble in a given medium, involving the previous presence of a colloidal solution whose particles take up and incorporate within or upon themselves the otherwise insoluble material' (McBain and Hutchinson, 1955). This definition is however too narrow, because the increase in

solubility is not always caused by direct introduction of colloidal particles into the system. More often, the enhanced solubility of the solubilizates as colloidal particles is due to the presence of a third component. Therefore, the term solubilization has come to have the following very broad definition: 'the preparation of a thermodynamically stable isotropic solution of a substance normally insoluble or very slightly soluble in a given solvent by the introduction of an additional amphiphilic component or components'. (Elworthy *et al*., 1968).

Before a drug can be absorbed, it must be present in the form of solution at the site of absorption. Various techniques are used for the enhancement of the solubility of poorly soluble drugs. These include pH/temperature modifications, reduction of particle size, addition of surfactants, salt formation, solid dispersion, complexation, addition of cosolvents and so forth. Selection of solubility improving technique depends on drug property, site of absorption, and required dosage form characteristics (Savjani *et al.,* 2012).

Solubilization progresses in the following step:

- Breaking interionic or intermolecular bonds of solutes.
- Separation of pharmaceutical solvents into freed up spaces to accommodate solutes.
- Interactions between pharmaceutical solvents and solutes (Pedada *et al.,* 2013).

A solubilizing agent is a substance that facilitates the dissolution of an agent in a medium in which it is otherwise insoluble. Solubilizing agents act as surfactants, increasing the solubility of one substance in another (Government of Canada *et al*., 2009).

Solubilizing agents are used in pharmaceutical formulations to improve the solubility of poorly soluble drugs, thus, improving the bioavailability of the active pharmaceutical ingredient (API). The solubility of poorly water-soluble drugs is way below the desired threshold, which lowers their absorption in the gastrointestinal tract. Also, poorly water-soluble drugs have reduced drug

efficiencies as compared to pharmaceutical formulations with appropriate solubility profiles (*Solubilizer Agents*, n.d; Savjani *et al.,* 2012).

Solubilizing agents are used in both oral and parenteral drug formulations to improve drug solubility and consequently, bioavailability. They may also act as emulsifiers in the formulation of emulsions.

1.3.1 How Solubilizing Agents Work.

Solubilizing agents are amphiphilic molecules, i.e., they contain both a hydrophilic / polar (also known as head) and a hydrophobic / non-polar region (also known as tail). The actual structure of the hydrophilic and hydrophobic parts varies, depending on the type of solubilizing agent.

The hydrophilic region may be:

- Ionic: Anionic (having a negative charge e.g., potassium laurate, sodium lauryl sulphate) or cationic (having a positive charge e.g., cetrimide, benzalkonium chloride).
- Non-ionic: having no charge, rather deriving its water solubility from highly polar groups like hydroxyl, polyoxyethylene, etc. Examples are sorbitan esters and polysorbates.
- Zwitterionic: having both a negative and a positive charge e.g., the sulfobetaines.

Despite this variety in the structure of hydrophilic and hydrophobic ends, the core principle of action is the same. When solubilizing agents are added to a liquid at low concentrations, the molecules adsorb at the interface. As the concentration increases, more molecules adsorb at the interface. When the interface is fully occupied, the excess surfactant molecules are forced into the bulk of the liquid. Further increase in surfactant concentration leads to the formation of aggregates or clusters of surfactant molecules within the liquid, known as **micelles.** The

concentration at which this happens is called the **Critical Micellar Concentration (CMC).** The hydrophobic tails of the micelles face the interior (away from the surface) to reduce their contact with water, while the hydrophilic tails face the surface to increase their contact with water (Pedada *et al*., 2013).

Micellar formation is a very important property of solubilizing agents as it facilitates the breakdown of intermolecular bonds of solutes. The CMC varies among different types of surfactants, and it affects their efficacy and required concentration for a given application. Surfactants with low CMC are more effective as they require lower concentrations to form micelles (*Dispersa,* n.d.).

1.3.2 Solubilizing Agents as Antimicrobials.

Surfactants can destroy microbial cells (including bacterial, viral and fungal cells) because they are composed of lipid membranes with embedded proteins. The phospholipids that make up cell membranes are also amphiphilic molecules. Thus, surfactants can interfere with and break up the lipid and protein components of cell membranes.

Cationic surfactants have a high affinity for the interfaces of all three microbial classes mentioned above, especially when applied at alkaline to neutral pH. Because the microbial interface is negatively charged, the positively charged cationic surfactant is pulled towards it, disrupting its integrity. They are also able to degrade the proteins and nucleic acids in the cell (Falk, 2019).

Anionic surfactants have long been utilized as antimicrobials. They are especially effective when sourced from strong acids and have an alkyl chain length which matches the cell wall lipid

structure. For example, sodium lauryl sulphate is well known to denature proteins and is used in cell lysing solutions for assays (Falk, 2019).

Zwitterionic surfactants will have a positive charge at acidic pH, increasing their affinity for microbial cells membranes, so long as the cell is below its isoelectric point. Non-ionic surfactants like surface active glycolipids are also able to disrupt cell membrane integrity, but this may vary according to the type of organism (Falk, 2019).

1.3.3 General Uses of Solubilizing Agents

Some general uses of solubilizing agents include:

Pharmaceuticals: They are used as solubilizers and emulsifiers in parenteral and oral dosage forms. Examples are, castor oil, sorbitan esters, monoglycerols, etc. They are also used with organic solvents as vehicles for drug delivery.

Disinfection: Solubilizing agents are formulated in combination with iodine to as iodophores. This causes the slow release of the iodine in solution. Iodophores penetrate the cell walls and membranes of microbes, interfering with DNA synthesis, thus killing the microbial cells. Providone-iodine is an example of a widely used iodophore for disinfection of surfaces in breweries and dairy industries.

Industry: Industrial applications of surfactants include production of paints, detergents, soaps, fabric softeners, motor oils, inks, adhesives, anti-fogs, de-inking of recycled papers, etc. They are used in the agricultural industry in the formulation of herbicides, biocides and insecticides. In the cosmetic industry, they are used in making shower gels, shampoos, hair conditioners, etc. in the petroleum industry, they are used as liquid drag reducing agents in pipelines and to mobilize oil in oil wells.

1.3.4 Types of Solubilizing Agents Used in Pharmaceuticals.

Some commonly used solubilizing agents for pharmaceutical dosage forms include:

- Water soluble organic solvents e.g., ethanol, polyethylene glycols, propylene glycol, glycerin, dimethylacetamide, dimethyl sulfoxide.
- Non-ionic surfactants e.g., cremophors, polysorbates.
- Hydrophobic lipids e.g., castor oil, olive oil, corn oil, peppermint oil.
- Organic liquids/semi solids e.g., beeswax, oleic acid.
- Cyclodextrins and phospholipids.

(*Solubilizer Agent*s, n.d.).

1.3.5 Dimethyl Sulfoxide (DMSO)

DMSO is an organosulfur compound with chemical formula $(CH_3)_2$ SO. It is a clear, colourless and odourless liquid, produced cheaply as a by-product of the paper industry. It has a wide spectrum of pharmacological activity including local Analgesia, membrane penetration, anti-inflammatory effects and as a weak bacteriostatic agent. It is used principally in pharmaceuticals as a vehicle for other drugs, enhancing their solubility and thus effects. It is also used to improve the penetration of drugs into the skin. DMSO penetrates quickly through tissues and increases the rate of absorption of compounds through biological tissues. It is used also, as a cryoprotectant. DMSO has the unusual property of producing a garlic-like taste in the mouth after contact with the skin (PubChem, n.d.).

1.3.6 Acetic Acid

Acetic acid, also called ethanoic acid, with chemical formula (CH_3COOH) is a clear, colourless organic liquid with a distinct sour taste and pungent smell. It is the main component in household vinegar. Acetic acid has a wide range of uses such as, as a raw material and solvent in the production of other chemical products used in making plastics, paints, adhesives, photographic films, etc. It is used in the food industry as a condiment and acidity regulator. It is also used as a cleaning and disinfecting agent in food processing plants. In pharmaceutical industries, acetic acid is used to produce vitamins, hormones, antibiotics and other products.Acetic acid has excellent antibacterial and anti-fungal properties (Maldonado, 2020).

1.3.7 Propylene Glycol

Propylene glycol is a viscous, colorless, almost odorless liquid with a faintly sweet taste. It has the chemical formula CH_3 $CH(OH)CH_2OH$. The glycols are generally non-irritating and have very low volatility risk. Propylene glycol is miscible with a wide range of solvents including chloroform, water and acetone (Sullivan *et al.,*2018). It is used for the production of polymers, as a food additive, in cosmetics and in pharmaceutics. It is also widely used as an antifreeze.

Propylene glycol is used as a vehicle in many oral, topical and parenteral pharmaceutical formulations. It is used as a carrier and solvent for certain drug tablets (e.g., benzodiazepines) and capsules. It is also an ingredient in certain formulations of artificial tears (Janusz and Centre, 1991; PubChem n.d.)

1.4 Justification for The Study.

For bacteria to colonize a surface, they must first attach to the surface. When bacteria attach to a surface in a moist environment, they form biofilms by secreting a slimy, glue-like substance. A biofilm is comprised of living, reproducing microorganisms (e.g., bacteria) existing as a colony or community. This biofilm structure is complex and provides protection to the member cells.

Biofilms can form on any surface if the conditions are right (moisture, nutrient and surface) such as on plastics, medical invasive devices, contact lenses, kitchen counters, rocks in streams, walls of a hot tub, living tissues, plant materials, etc. An example of biofilm formation is the plaques on our teeth surfaces. In nature, biofilms usually consist of a mixture of different kinds of bacteria as well as other organisms.

Biofilms are held together by a substance called EPS (Extra Polymeric Substances) produces extracellularly by the microbial cells. These strands of EPS are linked between cells, forming a complex 3-D structure of layers upon layers of microbial communities. This structure protects the microbial cells from attacks that would normally destroy them in a planktonic state. Thus, biofilms are more difficult to eradicate (*A Brief Introduction to Biofilms*, n.d.).

Antimicrobial treatments were developed based on studies of their effectiveness against microbes in a planktonic state, rather than in as members of a biofilm colony which they usually exist as in nature. Thus, the effectiveness of these treatments is quite limited where biofilms are involved (*A Brief Introduction to Biofilms*, n.d.).

Because biofilms are of great health and economic significance, scientists have redirected their focus to research aimed at understanding biofilms and developing effective strategies to eradicate them.

1.5 Aims and Objectives.

The aim of this study was to investigate the detachment of *Lactobacillus reuteri* from an abiotic surface using specific solubilizing agents. The specific objectives were to:

- Determine the minimum inhibitory and minimum bactericidal concentrations of certain solubilizing agents.
- Culture lactobacillus on an abiotic surface and determine its detachment from the abiotic surface with the solubilizing agents, by quantification of viable cells.

CHAPTER TWO

MATERIALS AND METHOD

2.1 MATERIALS

All materials used during this experiment were sterilized before and after use.

2.1.1 Equipment

Inoculating wire loop, autoclave (Gallenkamp, England), bacteriological incubator (Uniscope, England), digital weighing balance, Bunsen burner, refrigerator, gas cylinder, hot air oven (Gallenkamp and co Ltd), microscope, centrifuge, spectrophotometer, anaerobic jars, micropipettes (1000 microliter and 200 microliter micropipettes).

2.1.2 Glass wares

Measuring cylinders, disposable petri dishes, universal bottles, pipettes (1ml, 5ml, 10ml), Bijou bottles, disinfectant jar, glass stirrer.

Other materials include cotton wool, grease pencil, marker pens, masking tape, gloves, face mask, candle, spatula, forceps.

2.1.3 Microbiological media

MRS agar, MRS broth.

2.1.4 Reagents and Chemicals

Phosphate buffer saline (sodium hydrogen phosphate + disodium hydrogen phosphate + sodium chloride + water), dimethyl sulfoxide (DMSO), Tween 80, crystal violet, absolute ethanol, methylated spirit, disinfectant liquid.

2.1.5 Organism

Lactobacillus reuteri strain CF48-3A

Source: The organism was obtained through the NIH Biodefense and Emerging Infections Research Resources Repository, NAID.

2.2 METHOD

2.2.1 Study area

The study was conducted at the Post Graduate Pharmaceutical Microbiology laboratory of the University of Benin, Benin city.

2.2.2 Preparation of solubilizing Agents

All procedures were carried out aseptically and in a sterile zone.

Concentrations of 12.5%, 25%, 50%, 75% and 100% of each solubilizing agent (DMSO, Propylene glycol and Acetic acid) was prepared, to a total volume of 5ml for each concentration. The concentrations were labelled A (100%), B (75%), C (50%), D (25%) and E (12.5%).

2.2.3 MIC and Time-Kill Assay

The susceptibility and kill-time of the test organism to DMSO, Tween 80 and acetic acid was assessed using broth dilution assay.

A 48h MRS broth culture of the test organism was prepared by inoculating a 20ml MRS broth with 20µl of stock culture and incubating at 37ºC for 48h in an anaerobic jar. The new culture was then centrifuged at 8000rev/min for 10 minutes. After centrifugation, the supernatant was discarded. 20ml of phosphate buffer saline (PBS) was added to the pellets (lactobacilli cells) and shaken gently to disperse the cells, forming a suspension. This suspension was centrifuged again

at 8000rev/min for 10 minutes. The supernatant was again discarded and 20ml PBS added to the pellets. The mixture was shaken gently to form a colorless suspension.

A 1:10 dilution (up to 12 each, labeled 1-12 with the corresponding concentration labeled letter) was carried out for the various concentrations using MRS broth as diluent and the inoculated concentrations of solubilizing agent as test sample. The labelled bottles of diluents were arranged sequentially in rows and columns according to their corresponding concentrations, making a total of 12 rows and 5 columns.

1ml of the PBS and Lactobacillus cell suspension was first added to solubilizing agent of concentration A (100%). 30 seconds letter, 1ml of the suspension was added to solubilizing agent of concentration B (75%). The same was repeated for concentrations C (50%), D (25%) and E (12.5%) at 30 seconds interval respectively.

30 seconds after inoculation of concentration E, the broths were inoculated with 1ml of solubilizing agent corresponding to their respective concentrations as follows:

- A to A_1, B to B_1, C to C_1, D to D_1 and E to E_1, all carried out at 30 seconds interval respectively

The procedure above was repeated for the remaining rows of broths, i.e., A to A_2, B to B_2…. Up to A to A12, B to B_{12}….

All inoculations were done consecutively and at a time interval of 30 seconds respectively. This implies that the inoculation time for broths in row one was 2.5 mins, row two 5 minutes, row three 7.5 minutes, row four 10 minutes, row five 12.5 minutes, row six 15 minutes, row seven 17.5 minutes, row eight 20 minutes, row nine 22.5 minutes, row ten 25 minutes, row eleven 27.5 minutes and row twelve 30 minutes.

A 10ml sterile broth served as a negative control for the experiment.

All the inoculated broths were put into a tin container. A lit candle was placed inside the container before sealing with the lid, to create a micro-aerophilic environment. The container was then placed in the incubator at 37ºC for 48 hours.

2.2.4 Minimum Bactericidal Concentration (MBC) Assay

After 48-hour incubation, the broths were observed for growth. The broth concentrations without growth were plated out on MRS agar using drop plate method. 20µl drops was used to plate out. The inoculated agar plates were left to dry at room temperature, after which they were inverted and placed in a tin container. A lit candle was placed into the container and the container sealed to create a micro aerophilic environment. The container was then placed in the incubator at 37ºC for 48 hours.

The entire procedure was carried out for DMSO, Tween 80 and acetic acid.

2.2.5 De-adhesion Tests

2.2.6 Quantitative Determination

10ml of MRS broth was added to two universal bottles respectively. They were sterilized in the autoclave at 121ºC for 15 minutes and allowed to cool. Each was then inoculated with 20µl of 48h broth culture of the test organism. The broths were incubated in an anaerobic jar for 48h at 37ºC. After incubation, the broths were discarded, and the universal bottles were rinsed three times each with 15ml sterile water.

15ml DMSO was then poured into bottle1 and left to stand for 5 minutes. A 1:10 dilution was carried out with the DMSO solution and PBS as diluent. The DMSO solution and its dilutions were then plated out on MRS agar plates using the drop plate method. 20µl drops were used to

plate out. The inoculated plates were allowed to dry at room temperature, inverted and incubated in an anerobic container at 37^{o}C for 48hours.

The same procedure was repeated with bottle2 using PBS in place of DMSO. This served as a positive control.

2.2.7 Qualitative Determination

Two universal bottles each containing 10ml of sterile MRS broth were prepared. Bottle1 was inoculated with 20µl of test organism. It was then incubated in an anaerobic jar for 48h at 37^{o}C. After incubation, the broth was discarded. The bottle was rinsed three times with 15ml sterile water, then inverted and left to air dry.

Afterwards, the bottle was rinsed with 2% crystal violet for 60 seconds. 10ml ethanol was then poured into the bottle and left for 30 seconds while gently shaking. Spectrophotometric reading of this ethanol solution was then taken at 600nm wavelength.

The procedure was repeated for Bottle2, except that bottle2 was not inoculated with the test organism. It thus served as the control.

CHAPTER THREE

3.1 RESULTS

FOR DMSO:

The minimum inhibitory concentration (MIC) experiment showed growth in the broth medium for concentrations of 50% and below for all the experimental times. Concentrations of 75% and 100% showed no growth for all the experimental times.

The minimum bactericidal concentration (MBC) experiment showed no growth from the tested concentrations.

FOR TWEEN 80:

The MIC experiment showed growth in all the concentrations, including 100% concentration, for all the experimental times.

FOR ACETIC ACID:

The MIC experiment showed no growth in all the concentrations, including the least concentration of 12.5%, for all the experimental times.

FOR THE DE-ADHESION TESTS:

Quantitative analysis of detached *Lactobacillus reuteri* cells showed that 4.6×10^5 cfu/ml cells were obtained from the abiotic surface with DMSO while 1.3×10^4 cfu/ml cells were obtained from the abiotic surface with the control (PBS).

Qualitative analysis of detached *Lactobacillus reuteri* cells from the abiotic surface using Ethanol and a control produced an optical density of 0.665 for ethanol and 0.626 for the control.

The general results are presented in tables below:

Table 1 represents the results for the calculated volumes of water and solubilizing agent used to prepare the different concentrations of solubilizing agent.

Table 2 represents the results for minimum inhibitory concentration (MIC) of DMSO against *Lactobacillus reuteri.*

Table 3 represents the result for MIC of Tween 80 against *Lactobacillus reuteri.*

Table 4 represents result for MIC of Acetic acid against *Lactobacillus reuteri.*

Table 5 represents result for MBC of DMSO against *Lactobacillus reuteri.*

Table 6 represents result for MBC of Acetic acid against *Lactobacillus reuteri.*

Table 7 represents the result for quantitative determination of detached *L. reuteri* cells from the abiotic surface.

Table 8 represents the results for qualitative determination of detached *L. reuteri* cells from the abiotic surface.

Table 1: Dilution of solubilizing agents

	A	B	C	D	E
S.A (ml)	5	3.75	2.50	1.25	0.63
Water(ml)	0	1.25	2.50	3.75	4.37
Final volume (ml)	5	5	5	5	5
Final concentration (%)	100	75	50	25	12.5

Key:

S.A = Solubilizing agent

A = 100% concentration of S.A

B = 75% concentration of S.A

C = 50% concentration of S.A

D = 25% concentration of S.A

E =12.5% concentration of S.A

Table 2: MIC and Kill-Time Result for DMSO

Time (mins)	A	B	C	D	E
2 ½	NG	NG	G	G	G
5	NG	NG	G	G	G
7 ½	NG	NG	G	G	G
10	NG	NG	G	G	G
12 1/2	NG	NG	G	G	G
15	NG	NG	G	G	G
17 ½	NG	NG	G	G	G
20	NG	NG	G	G	G
22 ½	NG	NG	G	G	G
25	NG	NG	G	G	G
27 ½	NG	NG	G	G	G
30	NG	NG	G	G	G

Key:

NG = NO GROWTH

G = GROWTH

Table 3: MIC and Kill-Time Result for Tween 80

Time (mins)	A	B	C	D	E
2 ½	G	G	G	G	G
5	G	G	G	G	G
7 ½	G	G	G	G	G
10	G	G	G	G	G
12 1/2	G	G	G	G	G
15	G	G	G	G	G
17 ½	G	G	G	G	G
20	G	G	G	G	G
22 ½	G	G	G	G	G
25	G	G	G	G	G
27 ½	G	G	G	G	G
30	G	G	G	G	G

Key:

NG = NO GROWTH

G = GROWTH

Table 4: MIC and Kill-Time Result for Acetic acid

Time (mins)	A	B	C	D	E
2 ½	NG	NG	NG	NG	NG
5	NG	NG	NG	NG	NG
7 ½	NG	NG	NG	NG	NG
10	NG	NG	NG	NG	NG
12 1/2	NG	NG	NG	NG	NG
15	NG	NG	NG	NG	NG
17 ½	NG	NG	NG	NG	NG
20	NG	NG	NG	NG	NG
22 ½	NG	NG	NG	NG	NG
25	NG	NG	NG	NG	NG
27 ½	NG	NG	NG	NG	NG
30	NG	NG	NG	NG	NG

Key:

NG = NO GROWTH

G = GROWTH

Table 5: MBC Result for DMSO 100% and 75% concentrations

Time (mins)	A	B
2 ½	NG	NG
5	NG	NG
7 ½	NG	NG
10	NG	NG
12 1/2	NG	NG
15	NG	NG
17 ½	NG	NG
20	NG	NG
22 ½	NG	NG
25	NG	NG
27 ½	NG	NG
30	NG	NG

Key:

NG = NO GROWTH

G = GROWTH

Table 6: MBC Result for Acetic acid

Time (mins)	A	B	C	D	E
2 ½	NG	NG	NG	NG	NG
5	NG	NG	NG	NG	NG
7 ½	NG	NG	NG	NG	NG
10	NG	NG	NG	NG	NG
12 1/2	NG	NG	NG	NG	NG
15	NG	NG	NG	NG	NG
17 ½	NG	NG	NG	NG	NG
20	NG	NG	NG	NG	NG
22 ½	NG	NG	NG	NG	NG
25	NG	NG	NG	NG	NG
27 ½	NG	NG	NG	NG	NG
30	NG	NG	NG	NG	NG

Key:

NG = NO GROWTH

G = GROWTH

Table 7: Result for quantitative determination of detached *L. reuteri* cells from the abiotic surface.

	cfu/ml
PBS (control)	1.3×10^4
DMSO	4.6×10^5

Key:

cfu/ml = colony forming units per ml

PBS = Phosphate Buffer Saline

DMSO = Dimethyl Sulfoxide

Table 8: Result for qualitative determination of detached *L. reuteri* cells from the abiotic surface.

	O.D
Control	0.626
Ethanol	0.665

Key:

O.D = optical density

CHAPTER FOUR

DISCUSSION

From the results obtained, the minimum inhibitory concentration of Dimethyl Sulfoxide (DMSO) was determined to be 75% and its minimum bactericidal concentration as 75% also. This implies that at concentrations below 75%, DMSO possesses no inhibitory or bactericidal effect against *Lactobacillus reuteri.*

For Tween 80, growth was observed in all the concentrations, implying that Tween 80 has no inhibitory effect on *Lactobacillus reuteri.*

For Acetic acid, no growth was observed in any of the concentrations, implying that Acetic acid has a strong inhibitory effect against *Lactobacillus reuteri,* as even a low concentration of 12.5% inhibited bacterial growth in even as short an exposure time of 2.5 minutes. Minimum bactericidal concentration test also showed no growth for all the concentrations. Thus, acetic acid doesn't just have a strong inhibitory activity, it also has a strong bactericidal activity against *Lactobacillus reuteri.*

Determination of the minimum inhibitory concentration and minimum bactericidal concentration of the solubilizing agents was important to accurately determine a suitable concentration with which to carry out the de-adhesion test. Use of a bactericidal concentration will produce a false negative result as the organisms detached into the solubilizing agent will be killed, thus no growth will be observed. A non-bactericidal concentration on the other hand will maintain viability of the organisms after detachment, thus they will grow in a suitable media and can be quantified. Also, a solubilizing agent like acetic acid will be unsuitable for use in this kind of de-adhesion experiment performed because of its very high bactericidal property.

From the results of the de-adhesion test, the solubilizing agent (DMSO), produced a far greater cell count (35-fold) than the control (PBS) in the quantitative analysis. For the qualitative analysis, the solubilizing agent (ethanol) produced a higher optical density value than the control. This means that more organisms were present in the ethanol solution, resulting in a greater scattering of light, thus higher absorbance value and ultimately a higher optical density value.

The results of this experiment show that the solubilizing agents were able to effectively detach *Lactobacillus reuteri* cells from the abiotic surface to which they were attached.

CHAPTER FIVE

CONCLUSION

This study demonstrates that bacterial inhibitory property of solubilizing agents may vary with concentration and that solubilizing agents are able to effectively detach *Lactobacillus reuteri* cells from an abiotic surface way better than agents like phosphate buffer saline.

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APPENDIX

Fig 1: MIC and Kill-time experiment for DMSO against *L. reuteri.*

Fig 2: MIC and Kill-time experiment for Tween 80 against *L. reuteri.*

Fig 3: MIC and Kill-time experiment for Acetic acid against *L. reuteri.*