UCL SCHOOL OF PHARMACY

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PHARMACEUTICAL AND MEDICAL APPLICATIONS OF INKJETS

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Thesis declaration form

I, Cornelius Dodoo, certify that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

Signature: Date:

Dedication

This work is dedicated to the memory of my mother, Mrs. Vida Dodoo.

Acknowledgement

I thank the almighty God for His protection and guidance throughout this PhD. His grace and mercies have brought me this far.

I also thank my supervisors, Professor Simon Gaisford and Dr Paul Stapleton, for their support and pieces of advice. A special mention to Professor Abdul Basit for his immense support as well.

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Abstract

Inkjet printing is a technology that is witnessing many applications across numerous areas of research. This technology is adopted here to address some challenges in medicine and pharmaceutical science. A thermal inkjet (HP 5940) was modified and used for this work; the robustness of the modified printer was confirmed afterwards. With printing of bacteria as a theme in this thesis, an initial assessment of the effect of ink jetting on the viability of bacterial cells was conducted and the damage was found to be negligible.

An analytical application of ink jetting in antimicrobial susceptibility testing was conducted. A model was developed whereby varying antibiotic concentrations were printed onto agar-coated glass slides; a fixed bacterial population was then printed onto the varying antibiotic concentrations. The concentration of printed antibiotic exhibiting no bacterial growth after incubation was then computed based on an initial cartridge characterisation using HPLC. Minimum inhibitory concentrations obtained for antibiotics tested, using the designed model, when compared with standard broth microdilution technique were within an acceptable range, i.e., one doubling dilution apart.

The inkjet printing technology was also applied in formulating probiotics. Probiotics for site-specific delivery into the lower small intestines or colon were formulated by printing the probiotic strain (*Lactobacillus acidophilus* LA 5) onto edible starch paper and encapsulating organisms in Phloral® pre-coated capsules to protect organisms from the harshness of stomach fluids. The encapsulated formulation exhibited over 40% recovery at sites of interest with 78.3% of the administered strain adhering to intestinal cells. The formulation was able to eliminate completely a population of *E. coli* when co-incubated.

The potential of ink jetting in formulating probiotic oro-dispersible films (ODFs) was also explored. ODFs containing *Streptococcus salivarius* and xylitol were prepared and the benefits in managing dental caries were assessed. The probiotic ODFs reduced *Streptococcus mutans* numbers by 2.86 log cycles.

Impact Statement

The use of inkjets despite the increasing interest has generally involved piezoelectric inkjets mainly because of the perceived effect of the high temperature accompanying droplet formation in thermal inkjets. This work, therefore, utilised a thermal inkjet and demonstrated the negligible effect of the process of bubble formation using live cells. It is intended this will dispel such doubts and increase the use of thermal inkjets in research.

Antimicrobial resistance (AMR) is a major public health issue currently; testing the efficacy of antibiotic therapies against infections is one of the ways of addressing this, i.e., via antimicrobial susceptibility testing (AST). Manual determinations of AST are accompanied by a heavy workload, automated determinations are also limited by the expensive nature of the equipment. Considering the global nature of AMR, it is important that such tests could be conducted anywhere without cost being a problem. An inexpensive model for AST using inkjets was developed here to address the heavy workload of manual determinations and the cost element of automated determinations.

A major challenge with current probiotic formulations is the loss of viability of products upon storage. This has resulted in most commercial products having reduced numbers when assessed. With inkjets proved as useful tools for personalising medicines, the concept was adapted in formulating probiotics. An on-demand method of formulating probiotics for targeted delivery to intestines and locally in the mouth was demonstrated in this thesis; this will eliminate the need for long-term storage of probiotics. The ease of manufacture will also aid further research into probiotic science and help verify some of the health claims attributed to probiotics.

With lots of research into the gut microbiota and its role in disease prevention, mapping the presence or absence of certain organisms to diseases will be possible soon. At such a time, this model of printing probiotics would have progressed and be useful in formulating tailored probiotics at the point of care.

Contents

List of Figures

List of Tables

Abbreviations

API – active pharmaceutical ingredient

AST – antimicrobial susceptibility testing

AUC – area under the curve

CBA – Columbia blood agar

CFS – cell-free supernatant

CFU – colony forming unit

CO_2 – carbon dioxide

DMEM – Dulbecco's Modified Eagle Medium

DMSO – dimethylsulphoxide

DNA – deoxyribonucleic acid

EDTA – ethylene diamine tetraacetic acid

EFSA – European Food Safety Authority

EUCAST – European Committee on Antimicrobial Susceptibility Testing

FaSSGF – fasted state simulated gastric fluid

FaSSIF – fasted state simulated intestinal fluid

FBS – fetal bovine serum

FDA – Food and Drugs Authority

FeSSGF – fed state simulated gastric fluid

FeSSIF – fed state simulated intestinal fluid

GIT – gastrointestinal tract

GSK – GlaxoSmithKline

HCl – hydrochloric acid

HME – hot melt extrusion

HP – Hewlett-Packard

HPLC – high-performance liquid chromatography

HPMC – hydroxypropyl methylcellulose

IJP – inkjet printing

IBD – inflammatory bowel disease

MIC – minimum inhibitory concentration

MRS agar – de Man, Rogosa, and Sharpe agar

NASA – National Aeronautics and Space Administration

ODF – oro-dispersible film

PBS – phosphate-buffered saline

PCR – polymerase chain reaction

RPM – revolutions per minute

RSD – relative standard deviation

SEM – Scanning Electron Microscopy

SHIME – Simulator of the Human Intestinal Microbial Ecosystem

SIF – simulated intestinal fluid

SFM – French Society for Microbiology

TAM – thermal activity monitor

USP – United States Pharmacopoeia

UV – ultraviolet

VBNC – viable but non-culturable

WHO – World Health Organisation

Chapter 1 General Introduction

1.1 Gut Microbiota

Microbes colonise all the surfaces of the human body that are exposed to the environment. The gastrointestinal tract (GIT) represents the largest surface area exposed to the external environment and contains most of these microbes (Unger et al., 2015, Clemente et al., 2012). The intestinal microbiota (i.e., microorganisms living in a particular environment) is composed of a complex, dense, and diverse bacterial community of several species with bacterial numbers as high as 10^{14} cells and an approximate weight over 1 kg (Nicholson et al., 2005, Chassard and Lacroix, 2013, Scott et al., 2008). The entire length of the GIT is colonised by bacteria, which increase in number, complexity and diversity down the tract with the colon being the most diverse part (Power et al., 2014). It has been reported that there are ten times more bacterial cells than human cells and 150 times more bacterial genes than the human genome in the human body (Unger et al., 2015). The microbiota plays a major role in gut health and the general well-being of humans such that it has occasionally been referred to as “the forgotten organ” or “the neglected organ” (Scott et al., 2008, Clarke et al., 2014, O'Hara and Shanahan, 2006). It is of such importance that large-scale projects like the US Human Microbiome Project and the European Metagenomics of the Human Intestinal Tract have been initiated to characterise the human microbiota and understand the symbiotic relationship between microbes and their host (Human Microbiome Project, 2012, Qin et al., 2010, Lozupone et al., 2012).

The GIT is a muscular tube that runs from the mouth to the anus and is composed of four main specialised regions; the oesophagus, stomach, small intestine, and large intestine (Figure 1-1). The GIT is approximately 6 m in length with a varying diameter along this length. The oesophagus, the first specialised region, acts as a channel to move materials into the stomach and has a pH between 5 and 6. The stomach serves as a temporary storage for ingested materials and reduces materials into a uniform consistency. The

stomach has a pH between 1 and 3, mainly due to hydrochloric acid secretion. Other secretions into the stomach include pepsin and gastrin. The small intestine, the longest section of the GIT, consists the duodenum and ileum. The small intestine receives Brunner's glands, biliary, and pancreatic secretions with bicarbonate which neutralises the acidic outflow of the stomach resulting in a pH between 5 and 7.5. The small intestine completes digestion and is highly specialised for absorption with a surface area of 200 m^2. The large intestine is important for water and electrolyte exchange as well as storage and compaction of faeces and has a pH between 6 and 7.5 (Betton, 2013, Iannitti and Palmieri, 2010, Jandhyala et al., 2015, Ashford, 2013).

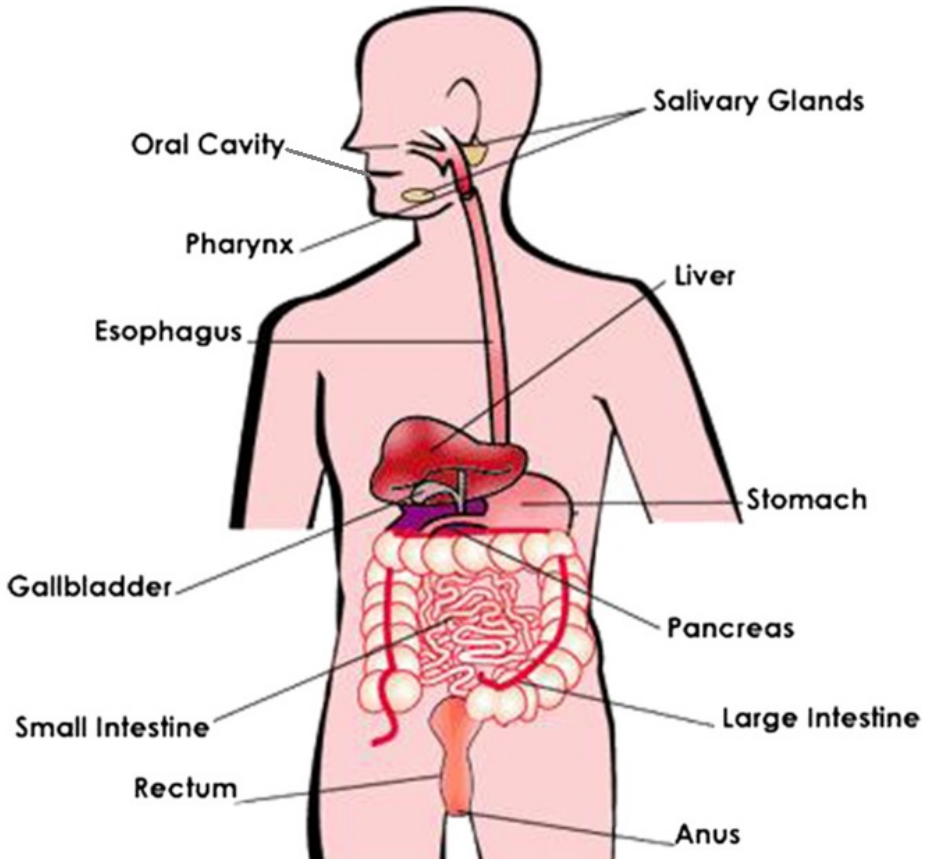

Figure 1-1: Anatomy of the gastrointestinal tract showing various parts and organs (adapted from Iannitti and Palmieri, 2010).

1.1.1 Composition of the gut microbiota

The composition of the intestinal microbiota is very diverse. Some argue that the intestines are colonised at birth whilst others claim colonisation of the intestines starts in-utero since microorganisms have been isolated from meconium (the earliest infant faeces) (Fuller, 1989, Jimenez et al., 2008, Nicholson et al., 2012, Clemente et al., 2012). The infant microbiota is more variable day-to-day in its composition and is less stable over time compared with that of the adult (Unger et al., 2015). Colonisation continues until around 2 - 3 years of age when the gut microbial communities have developed a richness and diversity that is characteristic of a healthy adult gut (Derrien and van Hylckama Vlieg, 2015). The earliest gut colonisers are either aerobes or facultative anaerobes e.g., enterococci (*Enterococcus faecalis*) and enterobacteria (*Escherichia coli*), and then strict anaerobes mainly bifidobacteria as well as clostridia and bacteroides. These arise as a result of eventual oxygen depletion in the gut, with the bifidobacteria population occurring mainly due to breastfeeding (Isolauri, 2012, Walsh et al., 2014).

Bacteria in the gut can be grouped according to their degree of pathogenicity as beneficial, potentially pathogenic (opportunists), and pathogenic. The beneficial organisms (e.g., lactobacilli and bifidobacteria) help maintain the gut health and overall human well-being whereas the pathogenic organisms (e.g. clostridia and klebsiella) result in infections and inflammation. The potentially beneficial organisms (e.g., *Escherichia coli*, streptococci) are part of the normal flora of the gut, however, these become pathogenic once their numbers become high (Westerbeek et al., 2006). More than 1,000 bacterial species have been identified in the adult gut with the most abundant species in the healthy gut being members of the phyla *Firmicutes* and *Bacteroidetes* (Walsh et al., 2014, Unger et al., 2015). The *Firmicutes* are the largest bacterial phylum in the gut with more than 200 genera, including *Lactobacillus, Mycoplasma, Bacillus*, and *Clostridium*; the *Bacteroidetes*, the second most dominant has about 20 genera including *Bacteroides* as shown in Table 1-1.

Table 1-1: An illustration of the dominant genera at different regions in the gastrointestinal tract (adapted from Tappenden and Deutsch, 2007).

Region	**Dominant Genera/ Species**
Stomach	*Candida albicans*
	Helicobacter pylori
	Streptococcus spp.
	Lactobacillus spp.
Duodenum	*Candida albicans*
	Streptococcus spp.
	Lactobacillus spp.
	Bacteroides spp.
Jejunum	*Bacteroides spp.*
	Candida albicans
	Lactobacillus spp.
	Streptococcus spp.
Ileum	*Bacteroides spp.*
	Clostridium spp.
	Lactobacillus spp.
	Veillonella spp.
	Enterococcus spp.
	Enterobacteriacae

Region	Dominant Genera/ Species
Colon	*Bacteroides spp.*
	Peptostreptococcus spp.
	Bacillus spp.
	Clostridium spp.
	Enterococcus spp.
	Bifidobacterium spp.
	Fusobacterium spp.
	Eubacterium spp.
	Streptococcus spp.
	Ruminococcus spp.

Microbial load varies along the GIT (Figure 1-2), with about 10^5 – 10^8 cells per gram in the mouth, 10^1 – 10^3 cells per gram in the stomach and duodenum, and 10^4 – 10^7 cells per gram in the jejunum and ileum. Most of the organisms in the intestinal microbiota are in the large intestine with bacterial numbers as high as 10^{11} – 10^{12} cells per gram in the colon. More than 70% of all the microbes found in the body and gut are in the large intestine (Schippa and Conte, 2014, Jandhyala et al., 2015, Derrien and van Hylckama Vlieg, 2015, Power et al., 2014, Tappenden and Deutsch, 2007, Vrieze et al., 2010).

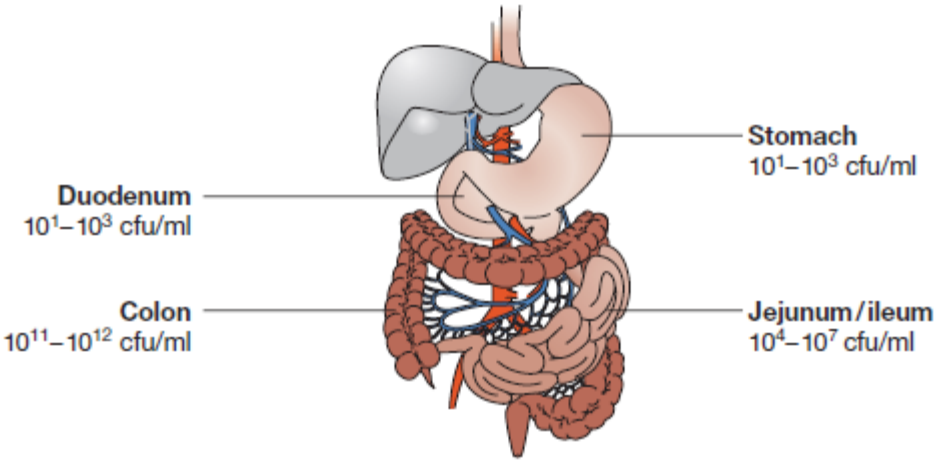

Figure 1-2: An illustration of the bacterial load in the gastrointestinal tract (adapted from O'Hara and Shanahan, 2006).

1.1.2 Factors affecting gut microbiota composition

The composition of the microbiota despite its diversity and complexity is influenced by many factors. These factors affect either the early colonisation of the gut or modify the already colonised gut.

The mode of childbirth is the first factor that affects the gut microbiota. It is known that babies delivered vaginally and via caesarean section have variations in the type of bacteria that colonise their gut. Babies delivered through the vagina are exposed to vaginal and faecal bacteria of maternal origin, mainly lactobacilli, prevotella, and atopobium. Infants born by caesarean section have an initial exposure to environmental bacteria from equipment, air, other infants, and nursing staff with staphylococci being a dominant early member (Nicholson et al., 2012, Fallani et al., 2010).

The type of diet one eats also affects the microbiota composition. Diet provides nutrients for both the host and bacteria in the GIT (Power et al., 2014). In infants, the intestinal microbiota is mainly bifidobacteria when they are fed on breastmilk whereas, the microbiota is more diverse with bifidobacteria, in addition to bacteroides, clostridia, enterobacteria, and streptococci when they

are fed on formula food. This, however, does not imply that formula food is a better alternative since breast milk also contains several bioactive compounds that are not available in formula feeds. Breastmilk also has the advantage of meeting the nutritional and physiological demands of the infant. Breastmilk contains antimicrobial components and growth factors that stimulate the development and maturation of the intestinal mucosa (Jandhyala et al., 2015, Westerbeek et al., 2006, Iannitti and Palmieri, 2010). In adults, diet can have a significant impact on the gut environment such as influencing gut transit time and pH. Also, the intake of carbohydrates, fats, and proteins has a direct impact on gut microbiota (Scott et al., 2013). Intake of a diet rich in fruits, vegetables, and fibres is claimed to result in a higher richness and diversity of the gut microbiota (Jandhyala et al., 2015).

Antibiotics, especially broad-spectrum antibiotics, although intended for their bactericidal and bacteriostatic activity against pathogens, usually have effects on the gut microbiota (Modi et al., 2014, Jandhyala et al., 2015). Antibiotic treatment leads to a reduction in the diversity of the gut ecology (Jandhyala et al., 2015). Some bacterial communities are quite resilient and can revert to a pre-treatment state within weeks after withdrawal. Other alterations, however, persist for a long period following the withdrawal of the treatment; some members of the microbial community fail to return or take several months to return to pre-treatment levels whilst others may even be lost from the community indefinitely (Power et al., 2014, Jandhyala et al., 2015). In these instances, there is reduced resistance to colonisation resulting often in the dominance of foreign microbes that can out-populate commensal and symbiotic bacteria eventually leading to changes in microbiota and diseased states (Clemente et al., 2012, Modi et al., 2014).

Other factors such as the age of gestation and geography, indirectly affect the bacterial community as well. It is not known whether prematurity itself affects the bacterial population, however, most preterm babies have an immature immune system and are vulnerable to several opportunistic infections. These infections generally require antibiotic treatments and could result in changes

in the microbiota (Westerbeek et al., 2006). Also, individuals in different geographical locations have different feeding habits and lifestyles. For instance, lower breastfeeding rates and early weaning are reported in Scotland as compared to Scandinavian countries (Fallani et al., 2010). Western diets have also been reported to be high in fat and simple carbohydrates as compared to African diets that are low in fat and high in complex carbohydrates, and these result in variations in intestinal microbiota geographically (Magnusson et al., 2015, de Filippo et al., 2010).

1.1.3 Role of gut microbiota

The role of the gut microbiota can be broadly categorised under three main headings: metabolic, protective, and structural functions.

The metabolic capacity of the gut microbiota is one that is highly recognised and reported to have an activity similar to the liver (Scheline, 1973). The metabolic activities include the breakdown of non-digestible dietary residue to generate short chain fatty acids – which are sources of energy – in the process (Janssen and Kersten, 2015). Gut microbiota have even been reported to play a role in the metabolism of drugs. Olsalazine, a medication for treating inflammatory bowel disease, has been broken down into two molecules of the locally effective 5-aminosalicylic acid in animal models by gut bacteria (Knoll et al., 2012). Loperamide oxide has also been metabolised by gut bacteria to loperamide (an antidiarrheal) in animal models (Lavrijsen et al., 1995). The synthesis of some vitamins (K and B) has been attributed to gut microbiota as well (Hill, 1997, Tappenden and Deutsch, 2007). The gut microbiota, especially, in the colon has been of interest for some time as a site for drug delivery. The ability of these organisms to metabolise carbohydrates is taken advantage of by drug delivery systems like COLAL-PRED® – made with ethylcellulose and a specific form of amylose – to deliver prednisolone to the colon. Non-drug specific techniques like Phloral® (made with starch and Eudragit S) are also known (Shukla and Tiwari, 2012, Ibekwe et al., 2008).

Gut microbiota exhibit protective functions through the release of antimicrobial materials known as bacteriocins; these are proteinaceous molecules that are synthesised and can interfere with the growth of most pathogenic bacteria (O'Hara and Shanahan, 2006). These have bactericidal action and are selective for prokaryotes. Gut microbiota also compete with pathogens for nutrients and attachment sites resulting in pathogen displacement. Beneficial gut organisms, especially the lactic acid bacteria, are known to utilise both carbohydrate and protein sources for energy production and in effect reduce nutrients available for other potentially harmful organisms in the gut (Isolauri, 2012, Pessione, 2012, Tappenden and Deutsch, 2007, O'Hara and Shanahan, 2006).

The structural function of the gut microbiota is exhibited through barrier fortification and apical tightening of tight junctions. Short chain fatty acids, from the metabolic activities of intestinal microbiota, have been noted to have a role in regulating cell proliferation and apoptosis (Bartholome et al., 2004). The commensal microbiota appears to be very important in maintaining intestinal cell proliferation, differentiation, and function which are necessary for maintaining critical structural attributes. The gut microbiota is also involved in the degradation of mucus glycoproteins produced by the epithelium (Falk et al., 1998, O'Hara and Shanahan, 2006, Tappenden and Deutsch, 2007).

1.1.4 Dysbiosis and restoration

A healthy microbiota is one in which there is a balanced composition of symbionts (organisms with known health-promoting functions), commensals (permanent residents which provide no benefit or detriment to the host), and pathobionts (permanent residents of the microbiota with the potential to induce pathology) (Round and Mazmanian, 2009). Dysbiosis is a condition in which there is a deregulation of the normal homeostasis of the intestinal microbiota usually with a reduction in the number of symbionts or an increase in the number of pathobionts (Figure 1-3). This is often involved in the pathogenesis

of various diseases and characterised by a decrease in bacterial diversity (Louis et al., 2014, Suvorov, 2013). Dysbiosis usually results in a lack of immune regulation and breakdown in tolerance to commensal microorganisms (Ferreira et al., 2014). Dysbiosis has been linked with important human diseases like autoimmune and auto-inflammatory disorders (e.g., allergies), obesity, inflammatory bowel disease, gastric ulcers, and colon cancer. The list of diseases associated with dysbiosis keeps increasing even though the pathogenesis of most of these diseases is not clearly understood (Nicholson et al., 2012, Schippa and Conte, 2014).

A few approaches have been suggested for replenishing the gut microbiota after dysbiosis, one such method is faecal microbial transplant. This involves introducing faecal bacterial communities from a healthy individual to a recipient whose microbiota has been disrupted or altered (Walsh et al., 2014). It has advantages as providing the full spectrum of microbial organisms from a healthy individual.

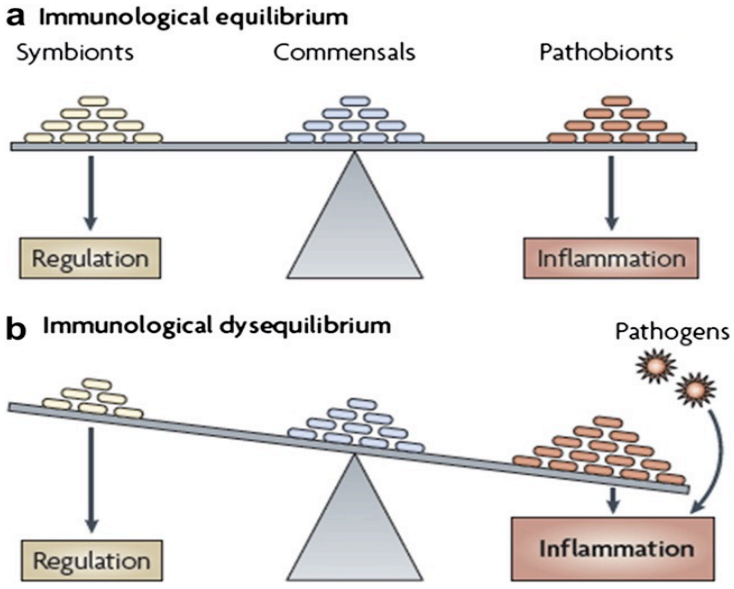

Figure 1-3: Illustration of a healthy gut microbiota showing (a) a balance between symbionts, commensals, and pathobionts and (b) when there is a shift in this system, i.e., dysbiosis (adapted from Round and Mazmanian, 2009).

Challenges with this approach include the introduction of any resistant strains that are present in the donor to the recipient. This approach also appears slightly unattractive to most patients as the mode of delivery could be either oral consumption, nasogastric intubation into stomach or intestine, or the use of enemas into the colon (de Vos, 2013, Borody et al., 2014). Another approach is the introduction of non-digestible food ingredients into the gut e.g. inulin, polydextrose, lactulose, and maltodextrins. These are known as prebiotics and they beneficially affect the host by selectively stimulating the growth and/or the activation of one or a limited number of health-promoting bacteria in the intestinal tract (Collins et al., 1998, Oliveira et al., 2009, Marzorati et al., 2010). The introduction of probiotics, which are live microorganisms that when administered in adequate amounts confer a health benefit on the host, is another approach and elaborated further in the next section (FAO/WHO, 2002).

1.2 Probiotics

The concept of probiotics emerged from the early 20th century when the Russian immunologist Elie Metchnikoff observed that Bulgarian peasant farmers had long life-spans. He suggested this was due to the consumption of large quantities of fermented milk rich in lactobacillus (Dixon, 2002). Metchnikoff suggested that pathogens present in the intestine released toxins which were poisonous to the body and the consumption of fermented milk helped alleviate the effects of these pathogens and their toxins (Fuller, 1991, Vasiljevic and Shah, 2008). With progress in the field, better understanding of the concept has been gained; this can be observed in the modification in the definition of probiotic over the years. In the mid-twentieth century, it was suggested that microbial imbalance arising due to antibiotics could be restored by a probiotic-rich diet (Vergin, 1954). This partly led to the probiotics being referred to as the opposite of antibiotics. The origin of both words; probiotics meaning ‘for life’ and antibiotics, ‘against life’ in Greek also contributed to this

inference (Hamilton-Miller et al., 2007a, Reid et al., 2003). The definition was modified in the 1960's to substances secreted by one organism which stimulate the growth of another (Lilly and Stillwell, 1965). Probiotics were later defined as organisms and substances that contribute to intestinal microbial balance (Parker, 1974).

One common trend in most of the earlier definitions of probiotics was the fact that little attention was placed on the 'live' nature of the cells. However, with further understanding in the field, definitions proposed in the late 1980's and beyond accorded emphasis to the state of the cells. Fuller (1989), defined probiotics as live microbial feed supplements which beneficially affect the host animal by improving its intestinal microbial balance. Salminen also defined probiotics as live microbial culture or cultured dairy products which beneficially influence the health and nutrition of the host (Salminen, 1996). Following heightened interest in probiotics, a joint committee made up of the Food and Agriculture Organisation of the United Nations and the World Health Organisation suggested the definition of probiotics as live microorganisms that when administered in adequate amounts confer a health benefit on the host (FAO/WHO, 2002). This definition was also recommended by the International Scientific Association for Probiotics and Prebiotics (Rijkers et al., 2011).

Probiotics are composed of organisms mainly from the genera *Lactobacillus* and *Bifidobacterium*. *L. acidophilus* is reported to be the first identified probiotic species (Anusha et al., 2015). Other genera like *Streptococcus* and *Enterococcus* and some yeasts (particularly *Saccharomyces boulardii*) have also been documented as probiotics. Lactobacilli and Bifidobacteria are, however, dominant because of their 'generally recognised as safe' (GRAS) status (Shah, 2007, Fuller, 1991).

Not every microorganism can be considered as a probiotic, though research into probiotics is still relatively rudimentary, certain criteria have been reported. A key and very important requirement for an organism to be considered a probiotic is that it must be an organism known to be safe, i.e., have a GRAS

status. Pathogenic organisms do not meet this requirement, hence, cannot be considered as candidates for probiotics (Collins et al., 1998).

The organism must be a normal inhabitant of the intestine. Since most probiotics exert their effect in the GIT, it is prudent to consider only organisms that are known to be normal inhabitants of the intestine as candidates for probiotics intended for delivery into the intestine. Such probiotics exert their effects by attaching to and colonising the intestine (Vasiljevic and Shah, 2008).

For an organism to be considered as a probiotic, it must have been documented to have a health benefit. It is important to clarify that although some live viruses have been used in vaccines and are known to have health benefits they are not considered as probiotics. Vaccines with viruses as active ingredients are classified as drugs and not probiotics (Sanders, 2009). Health claims attributed to probiotics are also strain specific, hence, the selection should not be based only on the species as there are instances that the desired effects of probiotics are not shared by all strains within that particular species (Douglas and Sanders, 2008).

There are no hard and fast rules about the minimum number of cells needed in probiotic formulations. This is mainly because different cells have different properties in terms of colonisation of the gut; probiotics are also efficient at different concentrations (Sanders, 2009). Minimum numbers of 10^6 CFU/g, 10^7 CFU/g, and 10^8 CFU/g have all been recommended to be present at the point of consumption. The FAO/WHO, however, recommends that the minimum viable numbers of each probiotic strain in a product at the end of the product's shelf-life should be indicated on the product label (Shah, 2007, Krishnakumar and Gordon, 2001, Douglas and Sanders, 2008, FAO/WHO, 2002).

A few terms have been associated with probiotics. Probiotics that have been formulated in combination with prebiotics, are known as synbiotics. Others use pharmabiotics, to refer to formulations containing microorganisms with positive health effects (Shah, 2007, Broeckx et al., 2016). Probiotic formulations have also been referred to as neutraceuticals, i.e., dietary substances that deliver a

concentrated form of a bioactive substance in quantities that exceed what can be obtained from food (Cook et al., 2012, Govender et al., 2014).

1.2.1 Clinical uses

The probiotic market and the science supporting probiotics is one that is rapidly expanding; the increasing health benefits attributed to probiotics is a major contributing factor (Sanders, 2009). Probiotic products can be broadly subdivided under foods and dietary supplements based on the carriers. Probiotics delivered as foods are predominantly delivered as yoghurts. The probiotics market has expanded to an extent that other vehicles as smoothies, cheese, chocolates, cereals, and infant formulae have been used (Vasiljevic and Shah, 2008, Sanders et al., 2007). Probiotics packaged as dietary supplements, which is the focus of this work are usually packaged as tablets, capsules, dried powders in sachets or as liquid formulations in bottles (Iannitti and Palmieri, 2010).

The recent increased interest in probiotics also stems from the greater awareness of the human microbiome and its potential applications. Also, an increased realisation of the need for alternatives for antibiotics has contributed to this. Antibiotics have been around for over 50 years, yet, hospital infection rates are not declining and multi-drug resistant bacteria continue to emerge creating a major public health problem as a result (Broeckx et al., 2016, Teughels et al., 2011). There is accumulating clinical data supporting the role of probiotics in human health. Some of the health claims attributed to probiotics are highlighted briefly below.

The prevention of antibiotic-associated diarrhoea is one of the main proposals for the use of probiotics. It has been suggested that antibiotic therapy usually results in the elimination of beneficial gut bacteria and the dominance of other indigenous non-beneficial microbes especially, *Clostridium difficile*. These microbes and the toxins they release usually lead to diarrhoea and other complications. The consumption of probiotics has been proposed to help

stabilise the numbers of these beneficial microbes especially when taken concurrently with antibiotic treatment and continued for a period after the treatment (Tung et al., 2009, Vasiljevic and Shah, 2008).

Probiotics have also been suggested as a remedy for lactose intolerance. Lactose intolerance occurs when lactose is not hydrolysed into glucose and galactose by the enzyme lactase. These enzymes are usually present in breast-fed infants, however, after weaning, the enzyme disappears making hydrolysis of lactose – mainly from consumption of milk and other dairy products – an issue. Symptoms of lactose intolerance include bloating, flatulence, nausea, abdominal pain, and diarrhoea (Vasiljevic and Shah, 2008, Fuller, 1991). The rate of lactose transit into the large intestine is known to have an effect on lactose intolerance; lactose metabolism by lactobacillus strains have also been shown to alleviate lactose malabsorption (Gismondo et al., 1999).

Inflammatory bowel disease (IBD) is a complex condition with unknown aetiology although environmental, immunologic, and genetic factors have been suggested to have a role (Dotan and Rachmilewitz, 2005, Bai and Ouyang, 2006). The terminal ileum and colon of the gut which have the most abundant flora are the sites where IBD usually occurs. The location of the condition in addition to the fact that antibiotic therapy can be used in treatment has led to the assumption that bacteria could have a role in the aetiology of the disease (Ewaschuk et al., 2006). Others suggest IBD occurs as a result of an overly aggressive cell-mediated immune response to commensal enteric bacteria in a genetically susceptible host (Podolsky, 2002, Sartor, 2004). IBD mainly encompasses Crohn's disease and ulcerative colitis (Bai and Ouyang, 2006). Treatment is generally with corticosteroids, aminosalicylates, and antibiotics (Podolsky, 2002, Sartor, 2004, Kato et al., 2004). Remission of disease after treatment is a major clinical challenge. The role of probiotics in maintaining and prolonging remission of the disease has been investigated by some researchers and there are reports of probiotics reducing the recurrence of IBD

after treatment (Guslandi et al., 2003, Mimura et al., 2004, Chermesh and Eliakim, 2006, Vilela et al., 2008).

Studies have been conducted whereby probiotics have been used in the prevention of infectious complications after surgery for digestive organs (Rayes et al., 2002b, Rayes et al., 2002a, Kanazawa et al., 2005). Usually, after such procedures, use of antibiotics at a high dose for long periods is avoided because of the possibility of developing resistance to antibiotics from such actions (Nomoto, 2005). Probiotics, therefore, have a potentially useful role in controlling infections after such procedures. The incidence of infectious complications like sepsis and peritoneal abscess were decreased in individuals who had undergone hepatectomy, extra-hepatic bile duct resection, or reconstruction of the biliary tract in patients with highly invasive biliary tract cancer who were given probiotic and prebiotic combination (Kanazawa et al., 2005). A significantly lower incidence of infection was also noted in patients who had undergone liver transplant and were given probiotics post-operation (Rayes et al., 2002b).

Bacterial vaginosis is a condition arising due to alteration of vaginal flora and microenvironment. This usually arises when faecal microbes come into contact with the vaginal tract. Patients normally complain of a grey, malodorous fishy-smelling vaginal discharge whilst a substantial proportion are asymptomatic (Barrons and Tassone, 2008, Sanders et al., 2007). Bacterial vaginosis is usually a risk for sexually transmitted infections as well as complications in pregnancy (Potter, 1999). Treatment is usually done with oral metronidazole or topical clindamycin, however, recurrence after treatment is common (MacDermott, 1995). Lactobacilli in the vagina of healthy females constitute the normal bacterial flora and have a physiological role in maintaining a low pH (< 4.5) and protecting against invasion by other microorganisms (Eriksson et al., 2005). *Lactobacillus rhamnosus* GR-1 and *Lactobacillus reuteri* RC-14 have been used to some success to prevent recurrence of the infection (Marcone et al., 2010, Anukam et al., 2006a, Anukam et al., 2006b).

Probiotics have also been reported to have a role in immunostimulation. *L. acidophilus* TMC 0356 has been reported to stimulate macrophages to secrete both inflammatory and anti-inflammatory cytokines *in-vitro* (Morita et al., 2002). Probiotics through immunomodulation have also been found to have a role in treating atopic dermatitis (Kim et al., 2014, Michail et al., 2008). This approach appears more convenient as other treatment options like the use of topical steroids and systemic corticosteroids often result in non-adherence due to the unpleasant accompanying side effects of therapy. *In-vitro* production of interleukin-12 and interferon-gamma, which are vital cytokines for antitumor and antimicrobial immunity have also been demonstrated after stimulation with *Lactobacillus casei* strain Shirota (Kato et al., 1999).

Probiotics especially certain strains of *L. acidophilus* and some bifidobacteria have been suggested to have anti-carcinogenic properties. This is exhibited by the removal of pro-carcinogens like nitrosamine, heterocyclic amines, phenolic compounds, and ammonia (Shah, 2007, Goldin and Gorbach, 1984). Some strains of *B. lactis* and *L. gasseri* have also been demonstrated to release biologically active amounts of hydroxycinnamic acids (caffeic, ferulic and p-coumaric acids) which have potential anti-carcinogenic action in the colon (Couteau et al., 2001). Most of these anticancer studies, however, have only been demonstrated in animal models.

Probiotics have been reported to have a role in managing obesity. Obesity results from an excess caloric intake relative to energy expenditure. Obesity can arise due to many factors including environmental and genetic origins (Tennyson and Friedman, 2008). Some animal studies have demonstrated the potential of probiotics in reducing glucose levels in diabetic mouse models after probiotic administration as well as a delay in development of glucose tolerance (Tabuchi et al., 2003, Matsuzaki et al., 1997).

It has been demonstrated that probiotics like *Bifidobacterium longum* BL1, *L. acidophilus* RP 32, *L. brevis* NR1C1684, and *L. acidophilus* ATCC 4962 can deconjugate bile salts resulting in lower cholesterol concentrations (Pereira and Gibson, 2002, Klaver and Meer, 1993). Deconjugated bile salts are not

rapidly absorbed in the intestinal lumen as compard to their conjugated counterparts (Shah, 2007, Klaver and Meer, 1993, Liong and Shah, 2005, Xiao et al., 2003, Pereira and Gibson, 2002). Xiao et al. (2003) found a significant reduction in total serum cholesterol, low-density lipoprotein cholesterol, and triglyceride concentrations after consumption of milk products.

Caglar et al. (2005) highlighted the use of probiotics in aeronautics and space technology. With intestinal problems being a major health problem of astronauts, the National Aeronautics and Space Administration (NASA) has a project named Lacmos ('Lac' is Latin for milk and 'Mos' for 'Cosmos') researching into probiotic food products to address these intestinal problems and enable humans to live in space.

1.2.2 Challenges of probiotic science

Regulation of probiotics is a major challenge in probiotic science. Probiotics are classed under food and nutritional supplements rather than drugs, yet there are various obstacles to overcome with regulation. Cross-border differences in terms of what can be passed as a probiotic is a major obstacle. Regulation in Europe is conducted by the European Food Safety Authority (EFSA), whilst the Food and Drugs Authority (FDA) is the regulatory body in the United States of America. The main challenge arises from how health claims are substantiated. A health claim implies a relationship between a specific food and maintenance of a good health status, or that a given food can reduce the risk of a particular disease (van Loveren et al., 2012). In Europe, EFSA requires all health claims to be supported by trials in a target human population (e.g., pregnant women, the elderly, etc.). The implication is that every new strain or combination of strains should be subjected to costly and lengthy clinical studies in humans to substantiate the health claim (Rijkers et al., 2011, Salminen and van Loveren, 2012). In the USA, claims relating to normal body functioning, i.e., structure/function claims do not require FDA approval. The result is the flooding of the market with probiotic foods and supplements with

various structure or function claims making it difficult to differentiate between products that are scientifically backed from those with little evidence. The downside is that most products on the market are ineffective. In Japan, health claim approval can be granted with animal studies (Reid, 2008, Donovan et al., 2012, van Loveren et al., 2012). Regulatory science needs to bridge the gap between probiotic product development and the science that ensures product safety and functionality to advance probiotic science (Rijkers et al., 2011).

Probiotic science also faces many product development challenges during manufacture/production, storage, and delivery of probiotics. Targeting of probiotics to their main site of action will be ideal in the evaluation of health claims attributed to probiotics. This will enhance consumer and scientific confidence in probiotics. Solid dose probiotics are currently produced in the dried form mainly by microencapsulation via freeze-drying or spray-drying. These processes impart extreme stresses on the microbial cells (Sanders, 2009, Douglas and Sanders, 2008, Chandramouli et al., 2004, Cui et al., 2000). This is elaborated further in the next section.

Another challenge is maintaining probiotic viability and product stability over the shelf life. The physical parameters of the final product matrix play an important role in product stability. Loss of cell viability during storage is noted to be more drastic than during processing in most cases (Jankovic et al., 2010).

Two major biological barriers that probiotics must overcome to reach the lower part of the gastrointestinal tract in a viable state to exert biological activity are gastric acidity and bile secretion in the small intestine. Probiotic microorganisms vary considerably in their tolerance to low pH and bile salts (Chassard et al., 2011).The ability of a probiotic formulation to overcome these barriers contributes to the effectiveness of the formulation.

Oxygen tolerance is another challenge probiotics encounter; especially since most of these beneficial organisms are either microaerophilics or strict anaerobes. Probiotics lack the mechanism to completely convert oxygen to hydrogen peroxide and the catalase to convert hydrogen peroxide to water,

hence, the presence of oxygen results in cell loss. It is important in the production design, storage, and product use to keep exposure to oxygen minimum (Vasiljevic and Shah, 2008, Broeckx et al., 2016, Miller et al., 2002).

1.2.3 Current techniques for formulating probiotics

Formulating probiotics is very challenging since product viability must be maintained during formulation and after consumption by consumers. Formulating products that can withstand the harshness of the GIT and target them to the intestines is currently receiving great interest as are formulation processes that are not deleterious to organisms (Kailasapthy, 2002, Mortazavian et al., 2007). Cryopreservation is one of the standard ways of preserving bacterial viability over long periods. The high energy costs in maintaining sub-zero conditions during transport and storage is a major commercial drawback (Broeckx et al., 2016). Many approaches have been documented in formulating both liquid and solid dosage formulations (Kailasapthy, 2002, Dianawati et al., 2015, Kim et al., 1988, Burgain et al., 2011).

Most consumers, however, find solid dosage forms of probiotics more convenient over liquid formulations (Dianawati et al., 2015). Ideally, a probiotic formulation should be able to withstand the conditions of the GIT, especially, the stomach and be released at its target site often the small and large intestines (Rokka and Rantamäki, 2010, Gilliland, 1989). Approaches for formulating solid dose probiotics will be elaborated upon due to relevance to this work.

Earlier research on probiotic formulation has been centred on immobilising cells in a matrix. This concept has evolved with time and most of the current work is based on microencapsulation of cells (Kailasapthy, 2002). Microencapsulation is a process in which the cells are retained within an encapsulating matrix or membrane (Krasaekoopt et al., 2004). Microencapsulation of probiotic bacteria has been a promising technology to

ensure bacterial stability during the drying process and to preserve their viability during storage. Microencapsulation is reported to impart functional properties like acid and bile tolerance as well as the controlled release of contents based on the excipients added (Dianawati et al., 2015, Poncelet, 2006, Cui et al., 2000).

The two frequently used methods for microencapsulation are freeze-drying and spray-drying, although, other techniques like vacuum desiccation and fluidised bed drying are available (Dianawati et al., 2015, Efiuvwevwere et al., 1999, Kim et al., 1988).

Freeze-drying is a procedure based on sublimation under high vacuum and occurs in three phases, i.e., freezing, primary drying, and secondary drying. It involves cells being first frozen at -196 °C and then dried by sublimation under high vacuum (Meng et al., 2008, Santivarangkna et al., 2007). A schematic overview of a freeze-dryer is shown in Figure 1-4. Freeze-drying has advantages like a relatively mild operational temperature (Dianawati et al., 2015). Freeze-drying produces concentrated cultures that can be stored at ambient temperatures (Palmfeldt et al., 2003). A major drawback with the use of freeze-drying is the cost of equipment, use of freeze-dryers on a commercial scale is expensive and this restricts its use (Peighambardoust et al., 2011). Freeze-drying is also a time-consuming procedure partly because drying via sublimation is a slow process. This prolonged procedure aggravates other adverse conditions like the formation of intracellular ice crystals and macromolecule denaturation which result in loss of viability (Johnson and Etzel, 1995, Zayed and Roos, 2004, Patist and Zoerb, 2005).

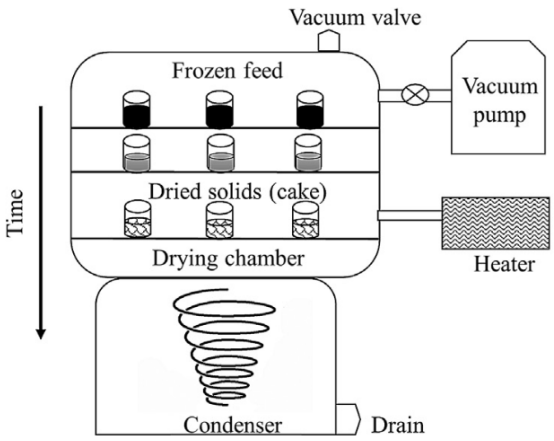

Figure 1-4: A schematic overview of freeze-dryer (adapted from Broeckx et al., 2016).

Spray-drying, on the other hand, is a more economical process (Kailasapthy, 2002). It is rapid and involves the injection of the spray-drying medium at high velocity at temperatures up to 200 °C, which then blasts through a nozzle leading to the formation of granules (Meng et al., 2008). A schematic overview of a spray-dryer is shown in Figure 1-5. The operational temperature is a major drawback; the inlet and outlet temperatures of spray-dryers usually result in reduced viability from simultaneous dehydration and thermal inactivation of microorganisms (Anal and Singh, 2007).

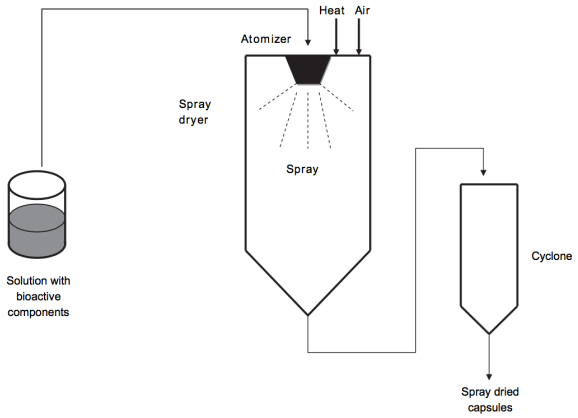

Figure 1-5: A schematic overview of spray-dryer (adapted from de Vos et al., 2010).

1.2.4 Formulating probiotics using inkjet printing

Several reports have highlighted the lack of viable organisms in packaged probiotics; other commercial products have also been noted to contain less than the stated number of strains (Hoa, 2000, Fredua-Agyeman and Gaisford, 2015, Hamilton-Miller, 2002, Temmerman et al., 2003, Hamilton-Miller et al., 2007b, de Vos et al., 2010, Huff, 2004, Masco et al., 2005). Fredua-Agyeman and Gaisford (2015) also observed tolerance issues with solid products to intestinal fluids when analysed relative to liquid formulations. This problem can in part be attributed to the fact that most of these solid formulations were manufactured via freeze-drying and the stress imposed by the manufacturing procedure usually results in the loss of viability. Also, most of these products don't have any protective mechanism against the harshness of some sections of the GIT (Broeckx et al., 2016, de Vos et al., 2010, Burgain et al., 2011).

Most consumers, however, prefer dehydrated probiotic formulations due to convenience (Dianawati et al., 2015). Also, with probiotics being oxygen intolerant, the deleterious effect of oxygen on probiotics is greater on liquid formulations used over a period due to oxygen penetration. An ideal product will, therefore, be a solid formulation with improved viability and delivery mechanism (Vasiljevic and Shah, 2008, Dianawati et al., 2015).

Inkjet printing (IJP) is a promising technique for on-demand production of medicines with many recent applications. With solid dosage forms being more preferred by consumers, this work focusses on exploiting the possibility of ink jetting probiotics on-demand to eliminate the need for long-term storage and accompanying reduction in viability upon storage. Another advantage of using IJP in formulation is the fact that long hours of drying will be eliminated since droplets are generally small and drying can be achieved under more tolerable conditions (Buanz et al., 2015, Preis et al., 2015). This will also boost consumer confidence since formulations will be made on-demand by the local chemist or hospital pharmacy, the current target group for this model.

1.3 Inkjet Printing

Inkjet printing (IJP) – a technique that has been around for over half a century – can be described as a non-contact technique capable of reproducing digital image data on a substrate using picolitre ink droplets. IJP had initially been restricted to paper printing in commercial printing and publishing units, day-to-day printing in offices, and privately in homes. However, inkjet printers are very powerful machines that can serve varied purposes (Ferris et al., 2013, Le, 1998).

IJP can be applied to many procedures that require nano-manipulation but are limited by an inability to position precisely very small drops of liquid, e.g., microdosing (Derby, 2008). In recent years, IJP has been applied in fields like electronics, where the technique has been used in the production of transistor circuits as well as in medicine and drug developments. Interesting work has also been done in ceramics and polymer engineering (Sirringhaus et al., 2000, Mott et al., 1999, Kim et al., 2010).

Advancements in IJP has resulted in the evolution of the usual two-dimensional printing to a three-dimensional (3-D) format. 3-D printing employs an additive manufacturing process whereby products are built on a layer-by-layer basis, through a series of cross-sectional slices (Berman, 2012).

1.3.1 Types of inkjet printing

Continuous inkjets and drop-on-demand inkjets are the two main types of ink jetting. In continuous ink jetting, as the name implies, there is a continuous stream of droplets flowing through a nozzle as a result of fluid instability. These droplets are charged upon generation and are either deflected by an electric field onto a substrate to form an image or recirculated back for reuse. In drop-on-demand ink jetting, the ink is produced only when needed via a pressure pulse generated in a fluid-filled chamber. The risk of contamination associated with the recirculation of inks in continuous inkjets is a major disadvantage in comparison to drop-on-demand inkjets and a reason it is less commonly used in research. Inkjet printers are very accurate in delivery and a positional accuracy of less than 30 μm has been documented (Derby, 2008, Ferris et al., 2013). Drop-on-demand inkjets are classified as either thermal inkjets or piezoelectric inkjets. A third type, solenoid inkjets - utilises gas or hydraulic pressure to aid droplet ejection - was highlighted by Lemmo and colleagues (1998), however, there is very limited information about this technology in literature.

In piezoelectric inkjet printers, voltage mediated actuation of a piezo-crystal creates a pressure pulse that results in droplet ejection as shown in Figure 1-6. In piezoelectric inkjets, the printhead consists of a piezoelectric layer attached to a single crystal silicon substrate which causes ink to be ejected when vibrated. The printhead in piezoelectric printers are located on the printer (Figure 1-7). With piezoelectric printers, there is no temperature change involved in the printing process. Manufacturers of piezoelectric inkjets include Epson and Brother (Allain et al., 2004, Ferris et al., 2013).

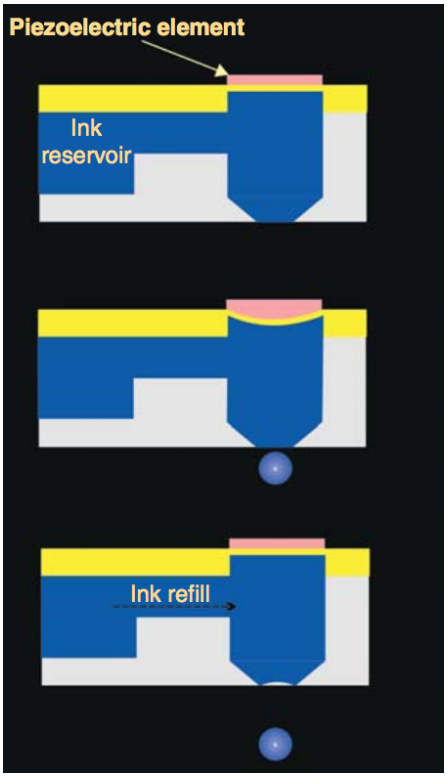

Figure 1-6: A schematic illustration of droplet formation in a piezoelectric inkjet (adapted from Kolakovic et al., 2013).

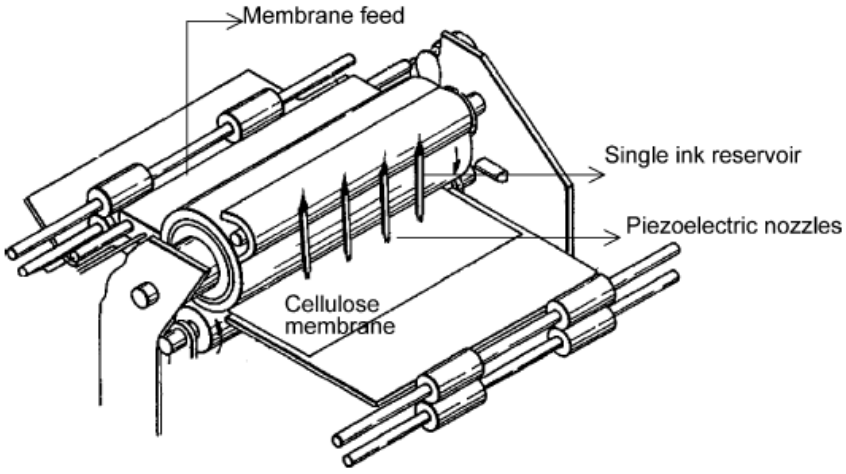

Figure 1-7: A piezoelectric inkjet printer with four printheads (adapted from Allain et al. 2004).

In thermal inkjet printers, also known as bubble-jet printers, rapid localised heating results in bubble formation within the ink chamber and eventually results in droplet formation as shown in Figure 1-8. Thermal inkjets have the printheads located on the cartridge (Figure 1-9), an advantage it has over piezoelectric inkjets. This configuration allows greater ease of cleaning and loading of thermal inkjet cartridges. Thermal inkjets can deliver droplets in the range of 8-95 pL and these can be formed in less than 3 ps. Manufacturers of thermal inkjet printers include Canon, Hewlett-Packard, Lexmark, Compaq, and NEC (Allain et al., 2004, Merrin et al., 2007).

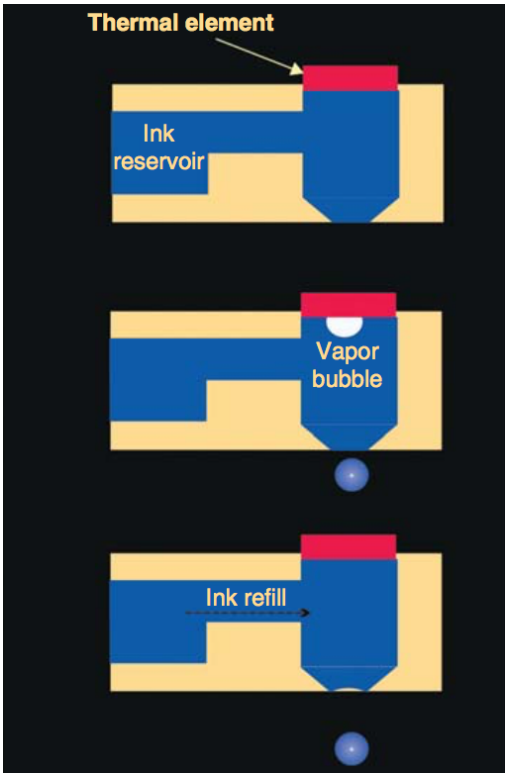

Figure 1-8: A schematic illustration of droplet formation in a thermal inkjet printer (adapted from Kolakovic et al., 2013).

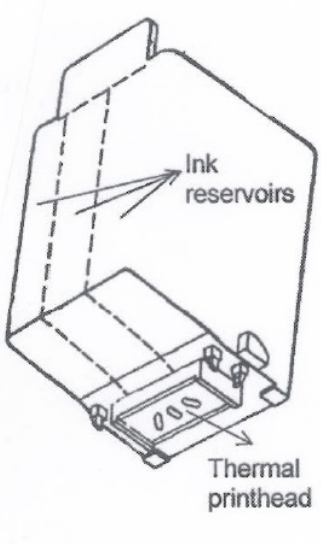

Figure 1-9: A thermal inkjet cartridge showing printhead (adapted from Allain et al., 2004).

1.3.2 Applications of inkjet technology in bio-medicine

New technologies such as IJP are being explored to transform the pharmaceutical production process and the end-to-end supply chain. This is can be partly attributed to the expensive research costs, high-risk, and capital-intensive scale-up in traditional centralised batch manufacturing (Daly et al., 2015).

IJP, as a versatile technique, has many applications in medicine. Mammalian cells have been printed successfully using this technique (Mironov et al., 2006). This technique has a potential use in drug development where it can be applied in drug screening and preparation of pharmaceutical co-crystals (Buanz et al., 2013, Lemmo et al., 1998, Scoutaris et al., 2016b).

Inkjets can also be used in clinical settings in the preparation of personalised-dose medicines for patients. Buanz and co-workers used the technique to prepare personalised doses of salbutamol sulphate on oral films (Buanz et al., 2011).

IJP has applications in biomedical device manufacture. Orthopaedic surgeons have replaced lost limbs with prosthetic limbs made by ink jetting. Medical

devices with anti-bacterial properties have also been fabricated using IJP. These were, however, produced using the more advanced three-dimensional (3D) printing technique (Mertz, 2013, Sandler et al., 2014). In 3D printing, sequential layers of material are deposited and objects of any shape or geometry can be created. A computer-aided design model is used in the production; many 3D printing technologies exist including stereolithography, sintering, melting, and fused deposition modelling. This printing technology has been used in other areas of medicine including fabrication of tablets as well as modified-release drugs (Preis et al., 2015, Goyanes et al., 2014, Goyanes et al., 2015).

IJP has been used in micro-particle printing. Excipient-free salbutamol sulphate and terbutaline sulphate for inhalation have been successfully formulated by ink jetting into liquid nitrogen to produce freeze-dried porous particles. This production process was a relatively simplified approach compared to spray-drying or micronisation (Mueannoom et al., 2012, Sharma et al., 2013).

Thin film coating of medical devices is another application of IJP. Slow-release active pharmaceutical ingredients (API's) can be applied to surfaces accurately. Coronary stents, for example, require coatings to ensure immunosuppression and prevent coagulation and clogging. Microneedles – narrow needles of the order of microns with lengths up to 1 mm which can pierce through the stratum corneum, but will not penetrate deep enough into the skin to stimulate nerve receptors – have also been coated via ink jetting for transdermal delivery of actives. Anticancer agents like 5-fluororacil, curcumin and cisplatin have been demonstrated for transdermal delivery after ink jetting. Insulin microneedles have also been coated via ink jetting (Uddin et al., 2015, Yu et al., 2015, Daly et al., 2015, Scoutaris et al., 2016a, Ross et al., 2015, Gill and Prausnitz, 2007).

1.3.2.1 Bio-printing

Bio-printing, defined as the use of material transfer processes for patterning and assembling biologically relevant materials – molecules, cells, tissues, and biodegradable biomaterials – with a prescribed organisation to accomplish one or more biological functions (Mironov et al., 2006). In bio-printing, it is important that the selected printing method is neither toxic nor irreversibly damaging for cells and their deoxyribonucleic acids (DNA) (Mironov et al., 2006). Biologicals including bovine serum albumin and smooth muscle cells from rats have been successfully printed (Wilson and Boland, 2003, Roth et al., 2004). Okamoto and colleagues (2000) worked on printing DNA and they developed a method for fabricating DNA microarrays using IJP onto a glass surface.

Another area with great potential for clinical application is tissue and organ engineering. Tissue and organ engineering seeks to fabricate viable replacement body parts (Xu et al., 2005). It is documented that on any given day in the United States about 60,000 people are waiting for kidney transplants, 3,000 for heart transplants, and 17,000 for liver transplants (Mironov et al., 2007). It will be a massive advancement in science if organs and tissues can be 3-D printed successfully from an individual's own cells as this will eliminate the current problem of incompatible donors. Most of the 3-D bio-printing work done involve natural hydrogels like alginate, gelatin, and chitosan, however, variations that occur within batches make reproducibility an issue. This is a major challenge with 3-D bio-printing; however, as a relatively new technique, with research on-going, there is the possibility of many applications in future (Fischer, 2013).

One area in which very little work has been done is the printing of bacteria and the microbiological applications of IJP. Bacterial colony arrays have been successfully printed onto substrates and the printing of colony arrays using inkjets offers a relatively cheaper alternative to obtaining these arrays for the construction of genomic and expression libraries (Zheng et al., 2011, Xu et al., 2004).

1.3.3 Advantages of inkjet printing

A fundamental advantage derived from the use of IJP is the inexpensive nature of inkjet printers. The myriad of applications to which these printers can be put and the relatively low cost makes the technology good value for money – some inkjet printers cost as little as £20. The ink jetting technique has been described as cheap, yet, one that affords extremely fine control of liquid droplets (Buanz et al., 2011).

The ability of some inkjet printers to work with as little as 20 µL of 'ink' makes the technique useful in drug discovery. Scientists can obtain outcomes with little material, especially, when materials being investigated at this stage are limited. Also, because materials are delivered exactly where they are needed, there is reduced wastage of materials (Allain et al., 2004, Derby, 2008).

IJP, as a non-contact technique, offers the added advantage of reduced contamination as the printhead and the substrate do not interact physically. This, eventually, leads to more work being done within a given time as a decontamination process is reduced for most procedures (Ferris et al., 2013, Daly et al., 2015, Scoutaris et al., 2016c).

IJP provides a high-throughput option for conducting scientific experiments. The speed of the technique allows a broader range of studies to be conducted within a short period. This is of importance in drug screening and development (Wilson and Boland, 2003, Lemmo et al., 1998).

Reproducibility of sample volume is also guaranteed under controlled environments. With IJP being an automated technique coupled with the robustness of ink jetting, the risk of errors is reduced since the amount deposited is constant, provided the printhead is in a good condition. Inkjets have been described as inexpensive robots that can dependably dispense minuscule amounts of growth factors and other proteins and even whole cells, in any pattern, gradient, or grid that can be drawn (Alper, 2004, Scoutaris et al., 2016c). Inkjets have been reported to deliver picolitre droplets with a positional accuracy less than 30 µm (Derby, 2008).

1.3.4 Challenges

The main challenge associated with the use of thermal inkjet printers is the blocking of the nozzles of the cartridge. The nozzles are vital, especially in thermal inkjet printers, because of the role played in droplet formation. Blockage can occur through drying of the 'ink' at the nozzle leading to either complete or partial blockage of the cartridge. Also, the salts found in culture media and physiological solution can also deposit at the nozzles and result in clogging (Burg et al., 2010). To minimise the effect of this challenge, some researchers include ethylene diamine tetra-acetic acid (EDTA) to their formulations because of its chelating properties. Though this approach extends the lifetime of the cartridge printhead, the impact on cell viability is not known. Sonicating cartridges in water after use also helps clear blockages (Parzel et al., 2009).

Another challenge posed by IJP is the limitation in dose fabrication. Although, IJP enables on-demand manufacturing and the flexibility of tailor-making medicines to suit patients' needs. It works best for very potent and small dosed formulations. Buanz et al. (2011) needed a large surface area (13 – 26 cm^2) to reach the required dosage of salbutamol sulphate at the highest feasible drug loading.

Ensuring repeatable droplet formation is another challenge especially with pharmaceutical printing. A slight change in excipient may have significant effects on droplet formation. This can be checked by optimising print settings and ink formulation. Printing under controlled environments also helps ensure droplet reproducibility since it controls droplet in-flight deviation due to aerodynamic effects which can result in poor placements (Daly et al., 2015).

1.4 Aims and Objectives

The main aims and objectives of this thesis are:

- To modify an inkjet by substituting the paper feeding mechanism with printing onto a stationary stage.
- To evaluate the potential of inkjets as tools in antimicrobial susceptibility testing.
- To evaluate the potential of inkjets as tools in formulating probiotics for targeted delivery.
- To evaluate the potential of inkjets as tools in formulating probiotic oro-dispersible films.

Chapter 2 Modification of an Inkjet Printer and an Evaluation of its Potential in Antimicrobial Susceptibility Testing

2.1 Introduction

Inkjet printers have been used in various applications in recent times. Depending on the intended application, these printers are either modified extensively (Pardo et al., 2003, Wilson and Boland, 2003, Zheng et al., 2011, Mueannoom et al., 2012) or with little or no modification (Genina et al., 2013, Buanz et al., 2011). Inkjets are very sensitive pieces of equipment; hence, alterations can easily result in non–functionality i.e., printer not working. Extensive care is therefore needed in the modification of an inkjet. A Hewlett-Packard (HP) 5940 Deskjet (USA), a thermal inkjet (Figure 2-1) was used for this work. This model was chosen partly because as an older generation of HP printers, it provided greater ease of modification as compared with the relatively newer versions. Also, the cartridge properties of this printer model permit the printer to function with just one cartridge present (i.e., the ink-backup mode). This printer model is also relatively inexpensive, therefore a good model for product development (Hewlett-Packard, 2005).

Figure 2-1: HP 5940 thermal inkjet printer, out-of-box configuration

After any modification process, it is essential to ascertain functionality and precision of the procedure, this was hence done for this printer model. A printer, aside from its complexity and sensitivity, is just a device that deposits

solution accurately based on a designed template. The potential of the modified printer as an automated pipette was therefore explored in antimicrobial susceptibility testing.

2.1.1 Antimicrobial susceptibility testing

Antimicrobial susceptibility testing (AST) is an evaluation of the performance of an antimicrobial against microbes. Minimum inhibitory concentration (MIC) is described as the 'gold standard' for determining the susceptibility of organisms to antimicrobials and is used to judge the performance of all other methods of AST (Lamy et al., 2004). MIC is defined as the lowest concentration of an antimicrobial agent that will inhibit the visible growth of micro-organisms after overnight incubation (this period is extended for anaerobes which generally require longer incubation). MIC differs from minimum bactericidal concentration (MBC), which is the lowest concentration of antimicrobial agent that will prevent the growth of organism after sub-culture onto antibiotic-free medium (Andrews, 2001). Standards for these tests are set by regulatory bodies such as The Clinical and Laboratory Standards Institute (CLSI), The European Committee on Antimicrobial Susceptibility Testing (EUCAST), The British Society for Antimicrobial Chemotherapy (BSAC), and The French Society for Microbiology (SFM) (Galani et al., 2008). MIC tests are performed on disease-causing organisms, particularly if the organism belongs to a species resistant to frequently used antimicrobial agents (Lamy et al., 2004, Galani et al., 2008, Andrews, 2001). MIC determinations are also performed when there are severe infections (e.g., meningitis) and during unexpected treatment failure. When new antibiotics are discovered, MIC determinations are conducted to assess efficacy. These determinations are conducted for uncommon microorganisms as well (Acar and Goldstein, 1996).

MIC can be determined by methods like agar dilution, broth dilution, and broth microdilution. Agar dilution involves the incorporation of doubling concentrations of antimicrobial into molten growth medium then applying a known number of bacteria onto the agar plate (Andrews, 2001). EUCAST describes broth dilution as a technique in which containers holding identical

volumes of broth with an antimicrobial solution in geometrically increasing concentrations are inoculated with a known number of bacteria. Broth microdilution denotes performing broth dilution in microdilution plates with a capacity ≤ 500 µl (EUCAST, 2003).

The difficulty and workload involved in obtaining varying drug concentrations is a major drawback with these techniques resulting in most of these tests not being performed routinely in clinical settings (Smith and Kirby, 2016). Most clinical labs, therefore, resort to reference labs to perform these tests resulting in a delay in obtaining test results. Others use preformulated antimicrobial dilution panels or the E-test strip; however, due to the rapid emergence of antimicrobial resistance and multidrug-resistant bacteria, there is usually the need to test new or combination of antimicrobial therapies. In such instances, where preformulated concentration gradients are not available there is a clinical challenge (Wexler et al., 1991, Smith and Kirby, 2016). Automated approaches like the spiral gradient endpoint technique and HP D300 digital dispenser have been used as alternatives for manual determination of MIC (Smith and Kirby, 2016, Pong et al., 2010, Hill and Schalkowsky, 1990).

MIC determinations via spiral gradient endpoint are done using the spiral plater (Figure 2-2). This instrument was originally designed to replace traditional serial dilution broth methods for bacterial enumeration. A syringe-driven dispensing pen deposits samples onto a rotating agar plate. The pen moves from the near-centre outwards while the plate rotates; this results in a spiral pattern as shown in Figure 2-3. If movements are done with a constant speed, a higher concentration of material is deposited at the centre and a relatively lower concentration at the periphery of the plate. This concept has been modified and is applied in the determination of MICs, whereby an antibiotic solution is deposited in a decreasing gradient from the centre towards the edge of agar in petri dish in a spiral manner (Hedderich et al., 2011, Wallace and Corkill, 1989, James, 1990).

In MIC determination, this instrument has the advantage of being automated, hence easing the obtainment of different drug concentrations and replacing

the workload of serial dilutions in effect. A few challenges are, however, associated with the technique, the first being the restrictive cost of the instrument making it an issue in resource-challenged settings (Wexler, 1991). Also, the difficulty in determination of the final endpoint and the fact that test inocula may have to be applied manually make the technique unappealing to some scientists (Wallace and Corkill, 1989).

Figure 2-2: A model spiral plater (Image obtained from Autoplate user guide).

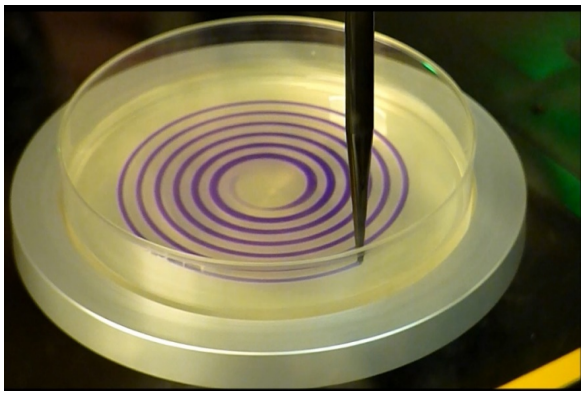

Figure 2-3: An illustration of the spiral plating process (adapted from Wikimedia commons).

The HP D300 digital dispenser (Figure 2-4), however, presents an opportunity for obtaining serial dilutions in well-plates. This has general advantages of

inkjet printers like the accurate and precise dispensing of volumes from picolitres to microliters and the elimination of carryover because it is a non-contact technique. As an automated technique, there is minimal delay and work from manual pipetting. The major drawback here as with the spiral plater is the cost – the digital dispenser costs around $40,000 and a standard microplate reader, $8,000 – which limits such an important technique (Smith and Kirby, 2016, Hewlett-Packard, 2016).

Figure 2-4: HP D300 digital dispenser (Image obtained from HP D300 digital dispenser data sheet).

Due to the challenges of manual susceptibility testing and the benefits of automated AST, the potential of inkjets as inexpensive devices in automated antimicrobial susceptibility testing was explored to facilitate in-house MIC determinations.

2.2 Aims

- To modify a thermal inkjet printer whilst maintaining key functionality.
- To evaluate the robustness of the modified printer.
- To design a sterilisation protocol for the ink jetting technique.
- To determine whether thermal ink jetting reduces viability of bacterial cells.
- To use the modified printer as a tool in antimicrobial susceptibility testing.

2.3 Materials and Methods

2.3.1 Reagents and growth media

Iso-sensitest agar, MRS (de Man, Rogosa and Sharpe) agar and broth from Oxoid were the growth media used. Fast Green dye was purchased from Alfa Aesar, USA. ¼ Ringer's solution tablets, acetonitrile, glycerol, and phosphate-buffered saline tablets were from Fisher Scientific, UK. The antibiotics used, pure amoxicillin, tetracycline, doxycycline, and ampicillin (92.5 – 100.5% purity), were from Sigma-Aldrich, UK.

2.3.2 Printer and cartridge modification

The printer was modified such that rather than the substrate (generally, paper in the unmodified printer) passing through the printer's rollers during operation, printing was done onto a stage mounted underneath the cartridge printhead. The modification process is described briefly. The printer's external paper trays (intake and output trays) located in front of the printer were removed by disengaging the internal hinges (Figure 2-5).

Figure 2-5: Printer with external paper trays removed.

The rear access door of the printer was then removed by means of existing handles (Figure 2-6). The rear access door, together with the intake paper tray provide a pathway for paper and since the paper feeding mechanism was not needed, they were removed.

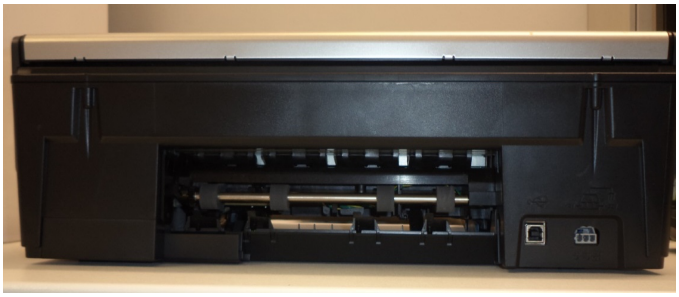

Figure 2-6: Printer with rear access door removed.

The printer lid was opened and surrounding security screws and the plastic case removed (Figure 2-7). Extra attention was paid to avoid damage to the electrical circuits operating the printer.

Figure 2-7: Image showing one of the removed surrounding screws.

The metal protecting case covering the paper guide was unscrewed and removed carefully (Figure 2-8).

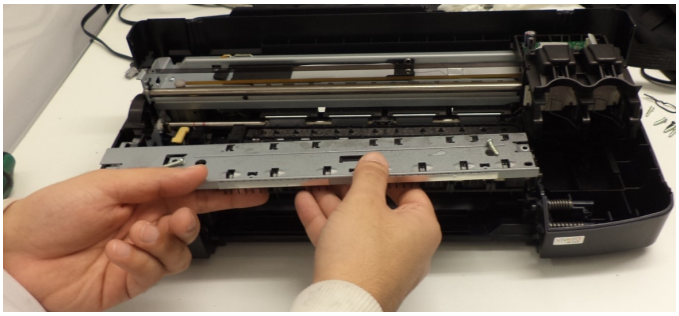

Figure 2-8: Removal of metal protecting case

The secondary rollers attached by gears to the main rollers were disengaged carefully (Figure 2-9) to prevent any damage to the main rollers' sites of interaction with the other mechanical workings of the printer.

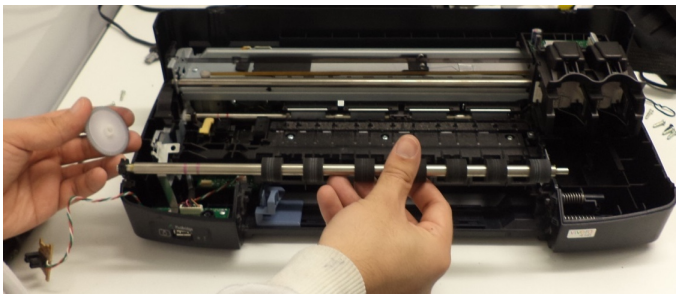

Figure 2-9: Secondary rollers after being disengaged.

The upper plastic paper guide and the foam covering the lower plastic paper guide were then removed (Figure 2-10).

Figure 2-10: Upper paper plastic guide after removal.

The lower plastic paper guide was removed manually with a dremel drill to create an opening for the stage to be mounted (Figure 2-11).

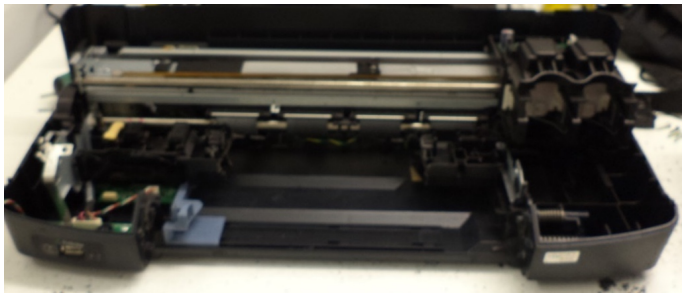

Figure 2-11: Image of printer after cutting through lower paper guide.

An important part of the modification process was the identification of key sensors that needed activation for the printer functionality to be maintained. The lid sensor (Figure 2-12) and the paper feed sensor (Figure 2-14) required manual activation for the modified printer to work. The lid sensor detects when the printer's lid is opened and since this had been removed, manual activation was required to mimic the presence of the lid. This must, however, be disengaged (Figure 2-13) before insertion and removal of cartridges. For the paper feed sensor, since the paper feeding mechanism had been eliminated, manual activation was required to prevent the printer detecting absence of paper.

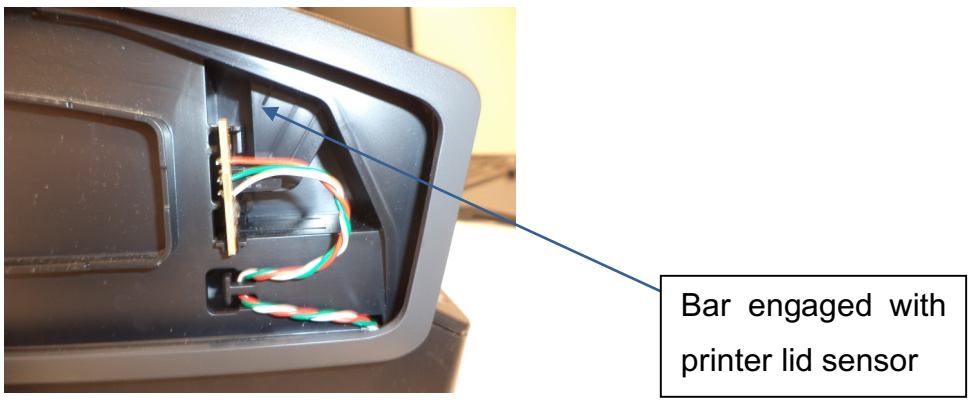

Figure 2-12: Image of printer's lid sensor and the engaged bar when the lid is closed.

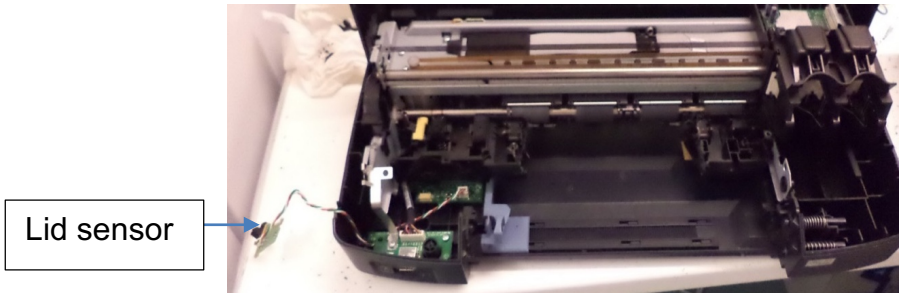

Figure 2-13: The printer's lid sensor extended

The paper feed sensor is close to the rear access door below the main roller axle. Caution was taken when dislodging this sensor and its associated cabling from underneath the roller axis to an accessible area.

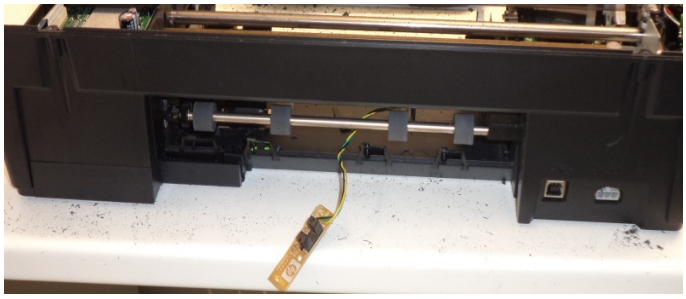

Figure 2-14: The printer's paper feed sensor extended to the back of the printer.

Printing with the modified printer was done onto a stage mounted underneath the cartridge printhead when printing. The stage was positioned as close to the main rollers' edge as possible without touching it; this enabled solutions to be easily deposited on the substrate on the stage (Figure 2-15).

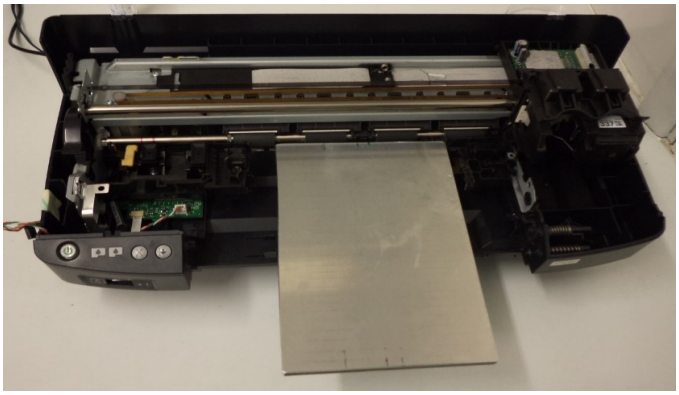

Figure 2-15: The printer with stage positioned.

The distance between the cartridge printhead and the stage was kept fixed with the aid of a height-controllable platform (Figure 2-16).

Figure 2-16: The stage on a height adjustable platform.

The cartridges used were HP black ink cartridges number 337 (Figure 2-17). These cartridges were modified by carefully separating the cartridge cap from the body with a sharp knife. Fragments were removed from the edges of the cap and the body. The plastic tape located on the nozzle plate was kept covering the nozzles to protect them (Figure 2-17).

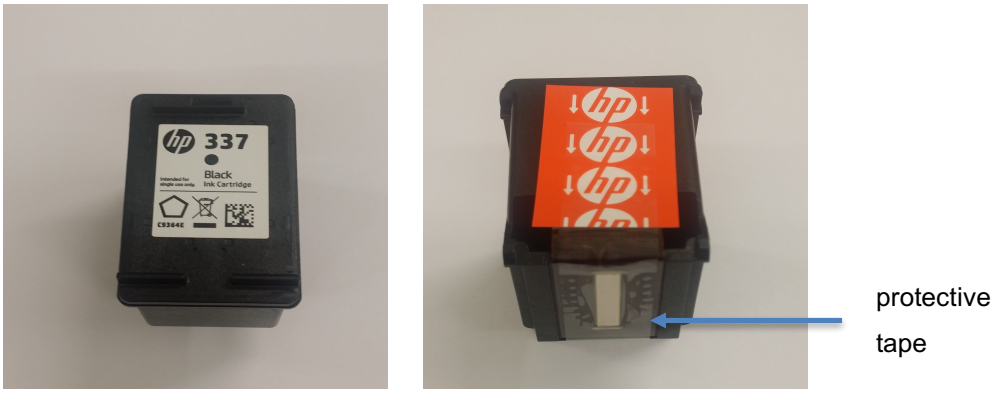

Figure 2-17: Images of HP 337 black cartridge (left) and a highlight of plastic tape covering nozzles (right).

The foam in the cartridge was removed, exposing the ink reservoirs underneath the permeable membrane which was removed with a pair of tweezers. Before cleaning the cartridge, the plastic tape protecting the nozzle plate was removed. The ink solution in the cartridge was flushed out with deionised water several times until it was clear. The nozzles of the cartridge were submerged in a deionised water: ethanol solution (2:1) for 5 minutes. The cartridge was then submerged in deionised water and sonicated for 15 minutes afterwards. The cartridge was finally dried in a convection oven at 40 ºC for 30 minutes and was ready for use (Figure 2-18).

Figure 2-18: Modified cartridge

The software used in issuing commands to the printer was Microsoft Word 2010, part of the Office 2010 suite. Once a printing command has been issued to the printer, the printhead is usually wiped by nozzle plate wipers as a purging mechanism. The cradle then moves toward the stage and rollers become activated at which point the paper feed sensor needs to be activated. The timing of these events is important because should the sensor not become activated, the printer will assume unavailability of paper, trigger a paper jam warning, and stop working until this warning is addressed. This is resolved by manually pressing the resume button. All printing was done using normal print quality settings.

2.3.3 Measuring diameter of cartridge nozzle

Scanning electron microscopy (SEM) was used to measure the diameter of the cartridge nozzles. The cartridge nozzles were prepared by sputter coating with gold for 3 minutes (Quorum model Q150). The images were then obtained with the scanning electron microscope (SEM, Quanta 200 FEG, FEI, Netherlands).

2.3.4 Bacterial culture preparation

Lactobacillus acidophilus (LA - 5) from the Christian Hansen culture collection (Reading, UK) was the micro-organism used. *L. acidophilus* was grown on de Man, Rogosa and Sharpe (MRS) agar and incubated under anaerobic conditions for 48 hours at 37 ºC. A few colonies were taken and used to inoculate 7 ml of MRS broth to create a starter culture and incubated for 24 hours. 99 ml of fresh MRS broth was inoculated with 1 ml of the starter culture to create a 1: 100 dilution. This was incubated for another 24 hours, at which point the bacteria were in the stationary phase. The culture was then mixed using a magnetic stirrer to ensure an even mixture and then dispensed into falcon tubes. Centrifugation was done at 9500 rpm and 4 ºC for 10 minutes using a Sigma 3-16KL centrifuge (Germany) to harvest the cells after which supernatant was removed carefully by suction. The cells were washed with phosphate-buffered saline (PBS) and centrifuged at 9500 rpm and 4 ºC for 10 minutes. The supernatant was removed by suction and the washing process repeated. The cells were resuspended in ¼ Ringer's solution made up with 15% $^{v}/_{v}$ glycerol, acting as a cryoprotectant. The bacterial cultures were mixed continuously using a magnetic stirrer and 1.8 ml of the culture was rapidly dispensed aseptically into 2 ml cryovials (Nunc). Sealed vials were immersed gently into liquid nitrogen for 10 minutes after which the frozen vials were removed and stored in a freezer at – 80 ºC. Post-freezing enumeration was approximately 10^7 CFU/ml representing 97% recovery.

2.3.5 Print substrate (bio-paper) preparation

Agar-coated glass slides (Figure 2-19) were chosen as the print substrate. Unless otherwise stated, this was the substrate used in this chapter. This was chosen to provide nutrients for cell growth and to enable work to be carried out with a minimum distance between stage and printhead. Microscope glass slides (Thermo Scientific, UK) frosted at one end were used to prepare print substrates. This type of slide was chosen to make room for holding the slide without touching the agar. Before use, the glass slides were sterilised by rinsing with 70% ethanol and dried. 1 ml of sterilised agar (50 ºC) was pipetted onto the end of the sterilised glass slide closer to the frosted end. The glass slide was held at an acute angle during the process to allow the agar to flow gently towards the other end. This created an even thin film of agar on the slide. The agar was allowed to set and the procedure was repeated such that each slide had two 1 ml MRS agar films on the surface. The agar-coated glass slides were then stored in sterile petri dishes.

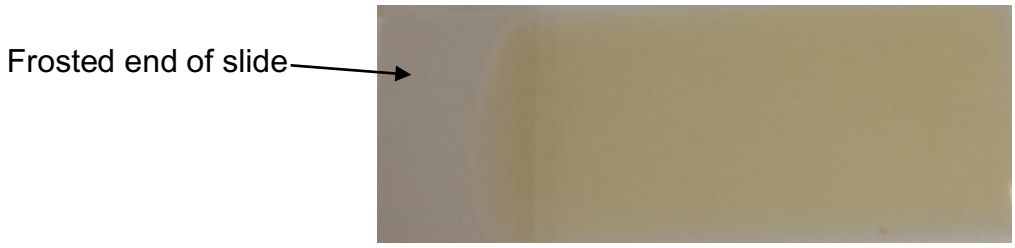

Figure 2-19: Agar-coated glass slide

2.3.6 Protocol for cartridge sterilisation

Autoclaving - saturated steam under pressure - was one of the sterilisation techniques investigated. Modified cartridges were wrapped in aluminium foil prior to autoclaving at 121 ºC and 100 KPa for 15 minutes. The effectiveness of the procedure was then determined. 1 ml of sterile deionised water was loaded into the ink channel of the sterilised cartridge and used to deposit a 2

cm x 2 cm square template onto Iso-sensitest agar–coated glass slides. A control was also run by pipetting 20 µl of the sterilised water directly onto the slide. The experiment was done in triplicate and the slides were incubated overnight at 37 ºC.

70% ethanol was evaluated as an alternative sterilisation technique. The inner chamber and outer part of cartridges were initially rinsed repeatedly with sterile deionised water, then sprayed with 70% ethanol with greater attention given to the nozzles. Cartridges were dried completely under aseptic conditions and effectiveness determined as above.

2.3.7 Evaluating robustness of modified printer

These series of experiments were conducted to evaluate any potential inter- or intra-cartridge variations due to the modification. Three modified HP 337 black cartridges were used for this experiment. 1 mg/ml solution of Fast Green dye was used as the “ink” for printing. 1 cm x 1 cm squares were printed in triplicate for each cartridge onto the clear acetate sheets. The print-outs were then carefully cut and immersed in 1 ml deionised water to dissolve dye. The dye solutions were vortexed to ensure complete dissolution after which high-performance liquid chromatography (HPLC) analysis was conducted using the conditions described below. Statistical analyses were conducted afterwards using a Student's t-test with a significance level of 0.05.

2.3.7.1 High-performance liquid chromatography

The liquid chromatographic system used was Agilent Technologies 1200 series with quaternary pump and degasser. The column used was a Phenomenex C18 column (150 mm x 3.90 mm, 5 µm). A gradient system (Table 2-1) was adopted with acetonitrile HPLC grade as the organic phase and 55 mM acetate buffer (pH = 5 ± 0.02) as the aqueous phase. A flow rate

of 1 ml/min was used for 10 minutes and the injection volume was 10 µl. A wavelength of 600 nm was used and the column temperature set at 30 ºC.

Table 2-1: Gradient system used in HPLC

Mobile phase	**Time (minutes)**			
	0	5	6	7
Acetonitrile	15%	60%	15%	15%
55 mM Acetate buffer	85%	40%	85%	85%

2.3.8 Effect of autoclaving on cartridge function

This experiment was conducted to evaluate the impact of one autoclaving cycle on the efficiency of cartridges. Three modified HP 337 cartridges were used for this experiment. 1 mg/ml solution of Fast Green dye was used as the “ink” for printing.

1 cm x 1 cm squares were printed in triplicate on clear acetate sheets for each cartridge. The print-outs were then carefully cut and dissolved in 1 ml deionised water. The solutions were vortexed to ensure complete dissolution and HPLC analyses conducted using the conditions described in section 2.3.7.1. The cartridges were then autoclaved and the procedure repeated. Statistical analyses were conducted afterwards using a Student's t-test with a significance level of 0.05.

2.3.9 Effect of thermal ink jetting on bacterial cell

Three tests were conducted to evaluate the effect of thermal ink jetting on bacterial cells, i.e., colony plate counting, measuring the absorbance of bacterial suspension, and Gram-staining.

2.3.9.1 Colony plate counts

0.5 ml of *L. acidophilus* (10^7 CFU/ml) - from the bacterial stock prepared - was added to 4.5 ml of PBS to create a 10^6 CFU/ml bacterial culture. 1 ml was then put into the ink chamber of a sterilised cartridge for printing. The printing template used was a 2 cm x 20 cm rectangle. Printing was done repeatedly into a sterile petri dish until the volume of liquid in the petri dish was enough to enable withdrawal of 50 µl. This was then serially diluted (1 in 10), plated out onto MRS agar, and incubated for 48 hours under anaerobic conditions. Two cartridges were used and the experiment was done in triplicate. The initial number of bacteria in the sample was also determined simultaneously for each experiment.

2.3.9.2 Measuring absorbance of bacterial suspension

0.5 ml of *L. acidophilus* (10^8 CFU/ml) was put into the ink chamber of a sterilised cartridge and used for printing. Printing was done as above (2.3.9.1). 50 µl was then diluted to 500 µl using PBS. A further 1 in 10 dilution was done (300 µl to 3 ml using PBS as diluent).

200 µl was then transferred to 8 bijous and each diluted to 2 ml using MRS broth. A pipetted control experiment was also run. Both control and test bijous were kept at 37 °C. 1 ml from both control and test bijous was pipetted hourly from corresponding bijou into clean cuvettes and examined using a spectrophotometer (Thermo Scientific, USA) to measure the absorbance. The experiment was conducted in triplicate.

2.3.9.3 Gram-staining

20 µl of *L. acidophilus* was pipetted onto a sterile glass slide and used as a control. 2 cm x 10 cm template was also used to deposit bacterial solution (10^7 CFU/ml) onto sterile glass slides. Both samples were heat-fixed and gram-staining experiments performed (McClelland, 2001). Slides were viewed afterwards using Zeiss light microscope (Germany) fitted with a camera.

2.3.10 Optimising printing by modified printer

A series of experiments were conducted to optimise the ink jetting technique. The bacterial concentrations used were 10^7, 10^6, 10^5, 10^4, and 10^3 CFU/ml. The print templates used were a set of 1 cm x 1 cm squares, greyscale, 2 cm x 2 cm square, and two rows of dots (Figure 2-20 - Figure 2-23). To further test the precision and to highlight controlled delivery, a text (DODOO) using Calibri (font size 36) (Figure 2-24) was printed using 10^7, 10^6, 10^5, and 10^4 CFU/ml. 1 ml of each bacterial concentration was put into a sterilised black cartridge and used to print the templates onto separate MRS agar-coated slides. After printing, the slides were incubated for 48 hours under anaerobic conditions.

Figure 2-20: Image of template for set of 1 cm x 1 cm squares.

Figure 2-21: Image of template for 2 cm x 2 cm square.

Figure 2-22: Image of template for greyscale.

Figure 2-23: Image of template for two rows of dots.

DODOO

Figure 2-24: Image of template for text.

2.3.11 Use of thermal ink jetting in antimicrobial susceptibility testing

With the concept of MIC determination as discussed in the introduction (2.1.1), a model was designed whereby varying amounts of antibiotics could be applied to a standard bacterial population such that the lowest amount of the antibiotic that inhibits the visible growth of microorganisms after incubation could be identified. To achieve varying concentrations using IJP, a concept known as 'Y-value' was introduced.

The term 'Y-value' was used to represent a series of rectangles of fixed width but varying height (conventional Y-axis). An example of the effect of varying the Y-value is shown in Figure 2-25, where three rectangles have the same shade of black (100%) and width (0.5 cm) but the Y-value (i.e., height) changes from 0.5 cm to 1.5 cm. Printing these templates onto a fixed area results in a linear increment in the volume of solution deposited and the amount of material per unit area.

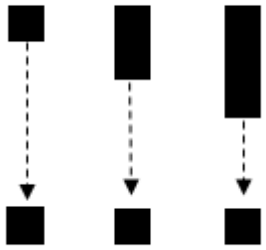

Figure 2-25: Illustration of Y-value concept

2.3.11.1 Standardisation of cartridges

The objective of standardising cartridges was to know the quantity of “ink” the cartridges jetted out for each Y-value. Y-values with a height of 0.5, 1, 2, and 3 cm and a constant width of 0.2 cm were printed individually onto clean acetate sheets using 1 mg/ml Fast Green dye in the modified HP 337 black cartridges. The acetate sheets were carefully cut into bijous and 1 ml of deionised water added to dissolve the dye. The resulting solutions were then transferred into HPLC vials for analysis using the HPLC conditions detailed in Table 2-1. A calibration curve for Fast Green dye in the range 0.0625 – 2 µg/ml was also obtained.

2.3.12 Protocol for determining minimum inhibitory concentrations

After standardising cartridges, a model was developed whereby each print template had two strips with different Y-values (0.5 and 1 cm), (1.5 and 2 cm), and (2.5 and 3 cm) and a constant width of 0.2 cm as demonstrated in Figure 2-26. An interval of 2.5 cm between the different Y-values was used to prevent cross-interaction between different Y-values. *L. acidophilus* was used as test organism and ampicillin, tetracycline, doxycycline, and amoxicillin were the antibiotics used.

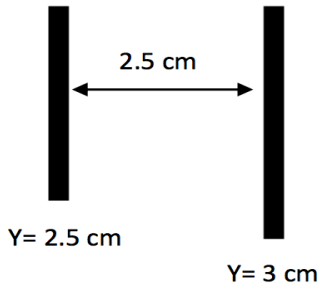

Figure 2-26: Illustration of MIC determination template for printing antibiotics

2.3.12.1 Procedure

1 ml of known antibiotic concentration was put into the ink compartment of a sterilised black cartridge. This was used to print the Y-value set (0.5 and 1 cm), (1.5 and 2 cm), and (2.5 and 3 cm) onto MRS agar-coated slides as shown in Figure 2-27 on a stage, 2 mm from cartridge head. Each Y-value set was duplicated. The cartridge was then removed and the main parts of the printer that encountered the cartridge were sprayed with 70% ethanol and allowed to dry. 1 ml of *L. acidophilus* (10^5 CFU/ml) was put into another sterile cartridge and used to print an even film (1 cm x 5 cm rectangle) of bacteria across all the slides. A control was also set up which had just bacterial film with no drug. The experiment was repeated with varying antibiotic concentrations and each concentration triplicated. The slides were then incubated under anaerobic conditions for 48 hours. Broth microdilution was simultaneously performed using 96-well microtitre plates and results used to validate IJP results (Andrews, 2001). All triplicates were conducted on separate days with freshly prepared antimicrobial dilutions.

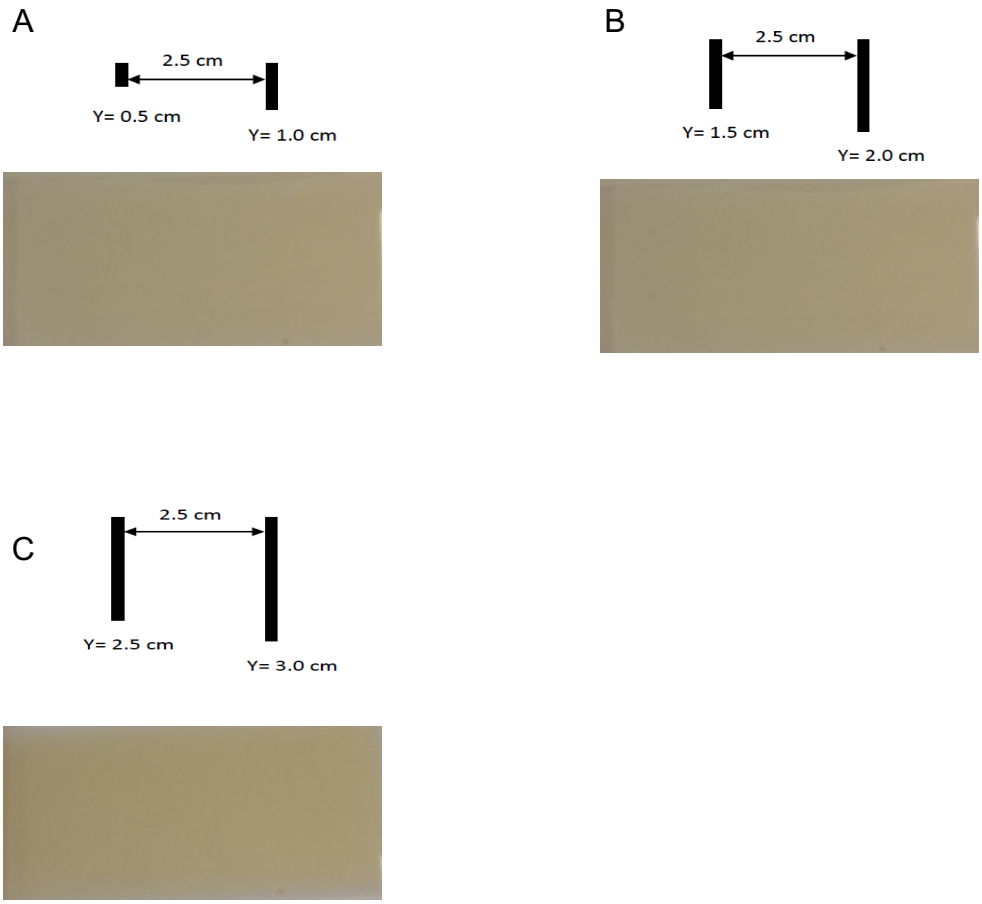

Figure 2-27: Illustration of designed MIC determination model, 0.5 cm and 1 cm Y-value (A), 1.5 cm and 2 cm Y-value (B), 2.5 cm and 3 cm Y-value (C).

2.3.13 Effect of varying contact time between bacteria and drug on MIC

In the model designed, the bacteria and drug were placed into separate black cartridges and investigations were carried out to determine whether the time involved in changing cartridges and disinfecting parts of the printer in contact with cartridge influenced the MIC. Three different sets of experiments were conducted using the protocol (2.3.12.1) with tetracycline as the test drug. In the first set, tetracycline was printed first and a period of 20 minutes allowed before the bacteria were printed. 20 minutes was chosen because it was sufficient time for cartridge change and complete drying of 70% ethanol. In the second set, the bacteria were printed first then tetracycline immediately afterwards. In this instance, the jetted antibiotics encounter bacteria right after

printing. A third set was conducted whereby an interval of 48 hours (duration of incubation) was allowed between drug deposition and bacterial printing.

2.3.14 Effect of varying inoculum density

The protocol for MIC determination as detailed in section 2.3.12.1 was followed with one set having an inoculum density of 10^5 CFU/ml and another set having an inoculum density of 10^6 CFU/ml. The experiment was carried out using ampicillin and tetracycline against *L. acidophilus.*

2.4 Results and Discussion

The printer was functional after modification, however, with the substrate being stationary, templates approximately over 1 cm in length were continuously jetted onto a fixed area. These templates were printed onto an area with the length limited by the length of the nozzle plate of the cartridge. Print-outs were also laterally inverted. Black cartridges were used and not tricolour cartridges because tricolour cartridges produce droplets of different sizes from the different compartments of the tricolour cartridge. These droplet sizes are not user controllable, however, with black cartridges, droplets of the same size are produced and therefore presents a better option in assay development (Buanz et al., 2013).

2.4.1 Measuring diameter of cartridge nozzle

SEM images highlighted unblocked nozzles; with an average diameter of 19.17 $\pm$ 0.1 µm (Figure 2-28). This diameter confirmed the possibility of ink jetting bacteria which generally have diameters in the range 0.5 – 2 µm (Jiang and Chen, 2016).

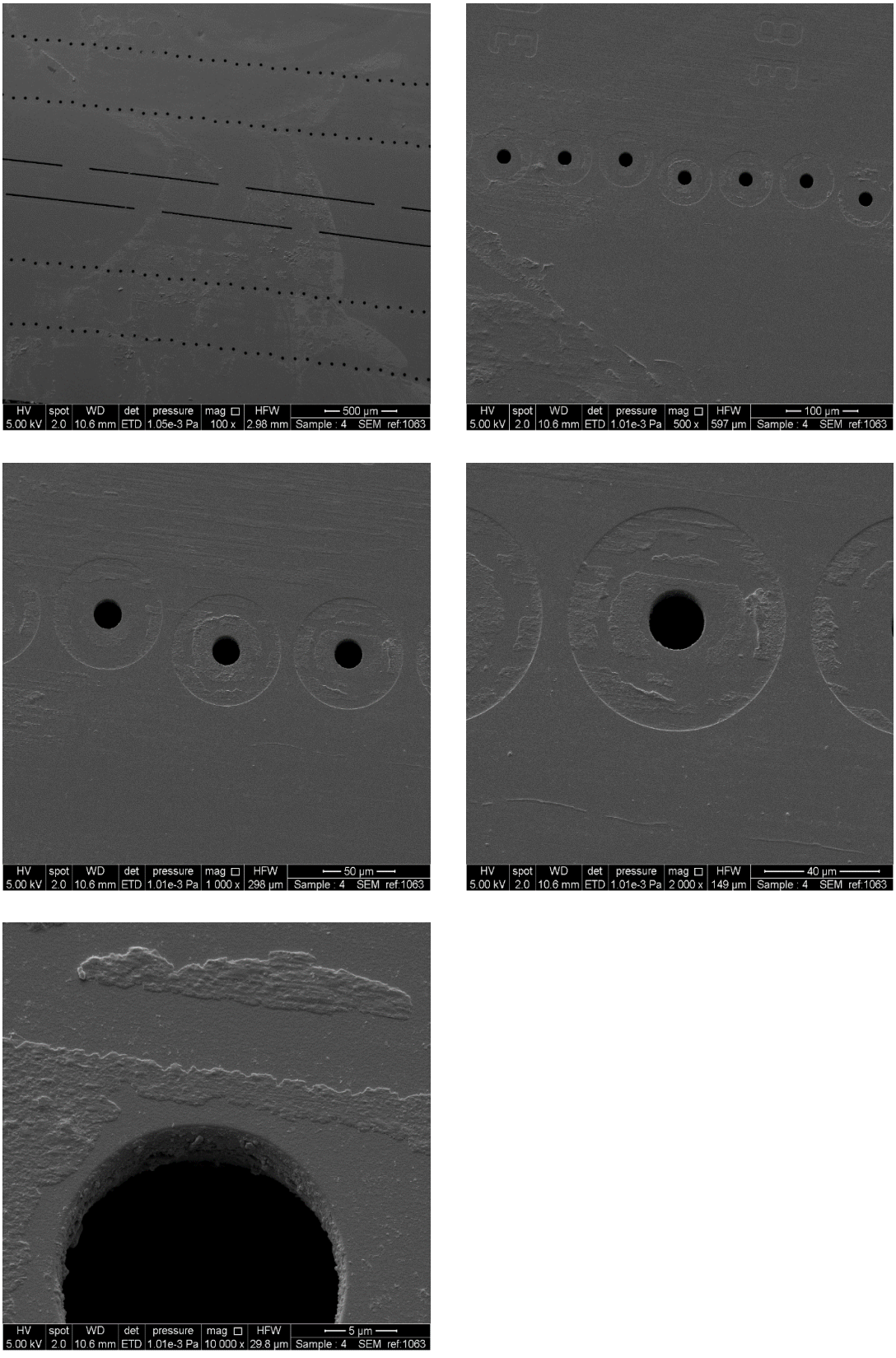

Figure 2-28: Electron micrographs of HP 337 cartridge nozzles at different magnifications

2.4.2 Sterilisation technique

Sterilisation by autoclaving and the alternative sterilisation procedure, i.e. using 70% ethanol both proved to be effective. Most of the cartridges used could withstand one autoclaving cycle, however, the efficiency of some cartridges started declining after three cycles of autoclaving and some failed completely after five cycles. Thus, an alternative sterilisation protocol needed to be adopted. It is worth noting that cartridges are manufactured to be used as disposable consumables; however, in this work as with most research and developments, they had to be re-used a few times (Ihalainen et al., 2015). Autoclaving is a reliable and easily controlled process for decontamination. It was appropriate to evaluate the impact of an autoclaving cycle on cartridges whilst maintaining a milder, yet, effective alternative protocol for sterilisation of cartridges (Lauer et al., 1982). The results of evaluating the impact of an autoclaving cycle on cartridge efficiency can be found in next section. None of the agar-coated slides had bacterial colonies growing on them after overnight incubation. There was no growth on the agar-coated slides for the control as well, confirming sterility of the deionised water used.

2.4.3 Evaluating robustness of modified printer and effect of an autoclaving cycle

Printers, by their design, are robust devices, however, it was imperative to find the impact of the modification process on this robustness. The area under the curve (AUC) obtained by HPLC analysis of Fast Green dye ink jetted before and after autoclaving is indicated in Table 2-2.

Table 2-2: Area under curve obtained via HPLC before and after autoclaving (n=3).

	Cartridge	Average Area Under Curve	Standard Deviation	Relative Standard Deviation
Before Autoclaving	1	232.33	5.03	2.17 %
	2	232.33	0.58	0.25 %
	3	239.33	2.08	0.87 %
After Autoclaving	1	242.67	2.08	0.86 %
	2	238.00	1.00	0.42 %
	3	246.67	1.53	0.62 %

The relative standard deviation (RSD) was used as a means of quantifying the robustness of the modified printer. Prior to autoclaving, RSD values of 2.17%, 0.25%, and 0.87% were obtained for the three cartridges and after autoclaving, RSD values of 0.86%, 0.42% and 0.62% respectively were obtained. These low values (less than 5% RSD) indicated the repeatability and precision of the procedure, highlighting the robust nature of the modified inkjet. Statistical analyses performed with a Student's t-test showed the average AUC values before and after autoclaving were not significantly different ($p > 0.05$); confirming that a single autoclaving cycle did not have a negative impact on the cartridges. In instances where the experimenter prefers to use autoclaving as a sterilisation technique, it will be prudent to perform just one autoclaving cycle.

2.4.4 Effect of thermal ink jetting on bacterial cell

Colony plate counting – a conventional bacterial enumeration technique – was used to evaluate bacterial viability. Analysis of bacterial cells after thermal ink jetting showed the cells were intact and viability was comparable to the control. This evaluation revealed that the printing procedure did not have any deleterious effect on bacterial viability. The bacterial population after ink jetting was similar to that of control (pipetted); approximately 10^7 CFU/ml in all instances (Figure 2-29).

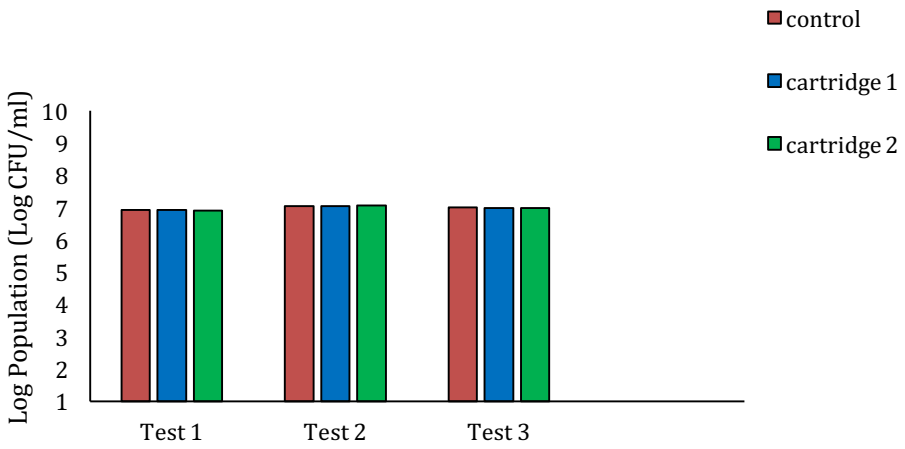

Figure 2-29: Effect of thermal ink jetting on bacterial viability (y-axis, cell number expressed as Log CFU/ml; x-axis, a triplicate sampling of two independent cartridges and control which represents the original cell number before printing).

Bacterial absorbance at 600 nm is a technique used for bacterial enumeration. Absorbance at this wavelength is directly proportional to bacterial population as it gives an indication of the turbidity of the medium. Bacterial growth can be categorised into lag, log, and stationary phases (Braissant et al., 2013). These are characteristic of any bacterial species. The lag phase characteristics of thermal ink jetted *L. acidophilus* was, therefore, monitored using absorbance at 600 nm. The absorbance of both the thermal ink jetted and pipetted (control) bacterial species were almost superimposable in the lag and early log phases of bacterial growth, (Figure 2-30), indicating no significant change in growth

characteristics. This further highlighted the negligible effect of the thermal ink jetting procedure on bacterial viability.

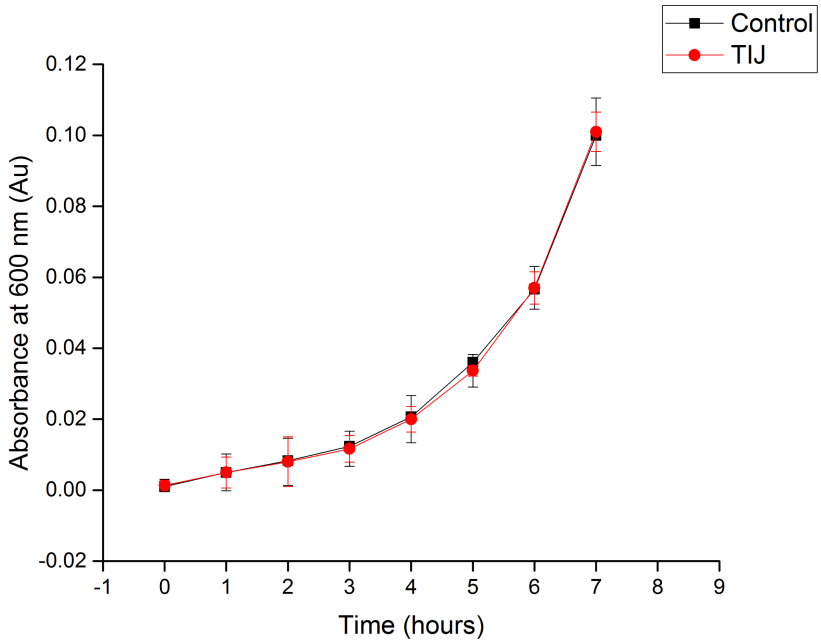

Figure 2-30: Bacterial absorbance at 600 nm comparing thermal ink jetted (TIJ) and pipetted (control) bacteria, showing almost superimposable values with error bars representing standard deviation from mean values.

The final assessment conducted was the Gram-staining experiment. This is a simple albeit useful technique to differentiate bacteria into two large groups, Gram-positive and Gram-negative. The basis for this differentiation is the physical and chemical composition of the cell wall of bacteria which helps to retain and release stains (McClelland, 2001, Bartholomew and Mittwer, 1952). Gram-staining was key in evaluating any potential damage the thermal ink jetting procedure may have caused to bacterial cell wall integrity. Visualisation by light microscopy after Gram-staining revealed that both the ink jetted and pipetted bacteria gave the same outcome, i.e., adsorbed primary dye (Figure 2-31). This implied the cell wall integrity of bacterial species were still intact with no damage to the cell wall incurred during droplet formation. A live/dead staining technique could also have been performed to ascertain the viability of the ink jetted cells (Green et al., 2011).

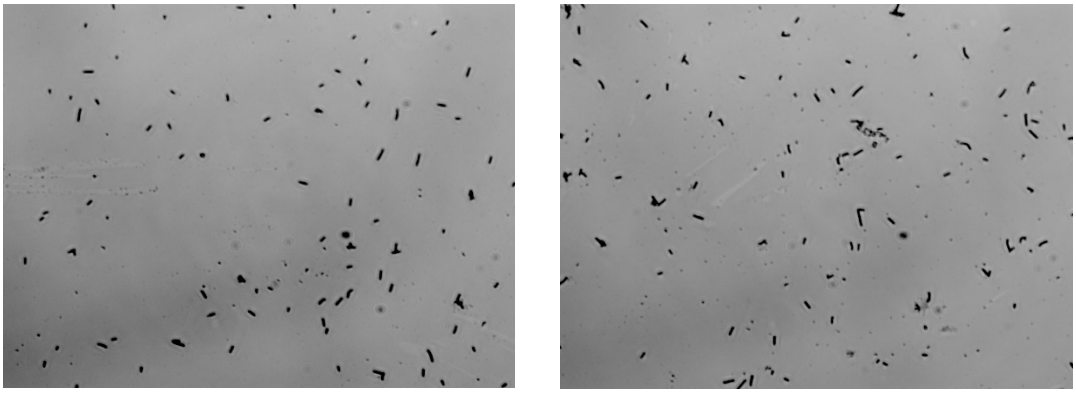

Figure 2-31: Light microscope images after Gram-staining pipetted (left) and ink jetted (right) *L. acidophilus.*

In bio-printing, it is important that the selected printing method is neither toxic nor irreversibly damaging for cells and their DNA (Mironov et al., 2006). The primary concern when using thermal inkjets to print bacteria is whether cell viability will be maintained after transit through the printhead because the heating elements can reach a significantly high temperature (about 200 ºC) during droplet formation. This is a major reason why most bioprinting work is usually conducted with piezoelectric printers (Allain et al., 2004). Although the temperature at the surface of the heating element increases during droplet formation, the actual temperature rise in the printing solution is significantly smaller; the heat acts to create expanding gas bubbles that drive the droplet from the nozzle and the bubbles effectively insulate the solution. Xu et al. (2005) showed that viable Chinese hamster ovary cells (mammalian cells) could be printed with a viability of 90% and that the effective temperature of the solution (bio-ink), does not rise more than 10 ºC above ambient conditions. Since mammalian cells can be successfully printed, bacterial cells should in principle be unaffected by the process of inkjet printing since these are relatively tougher organisms as demonstrated here. Merrin et al. (2007) have demonstrated bacterial viability after printing with piezoelectric printers. Zheng et al. (2011) have used fluorescence to ascertain viability after ink jetting.

2.4.5 Optimising ink jetting by the modified printer

Post-incubation images when a set of 1 cm x 1 cm squares was the template for ink jetting 10^7, 10^6, 10^5, 10^4 and 10^3 CFU/ml bacterial concentrations are shown in Figure 2-32. 10^7 and 10^6 CFU/ml (A and B), showed clearly defined squares of bacterial colonies, however, there were some colonies observed outside the designed template (satellite growths). 10^5 CFU/ml (C) resulted in bacterial growth with a defined outline representative of the template with no satellite growth. For 10^4 CFU/ml (D), although there were no satellite growths, a lower bacterial density was visible relative to 10^5 CFU/ml. Colonies observed for 10^3 CFU/ml (E) had no resemblance to the print template used.

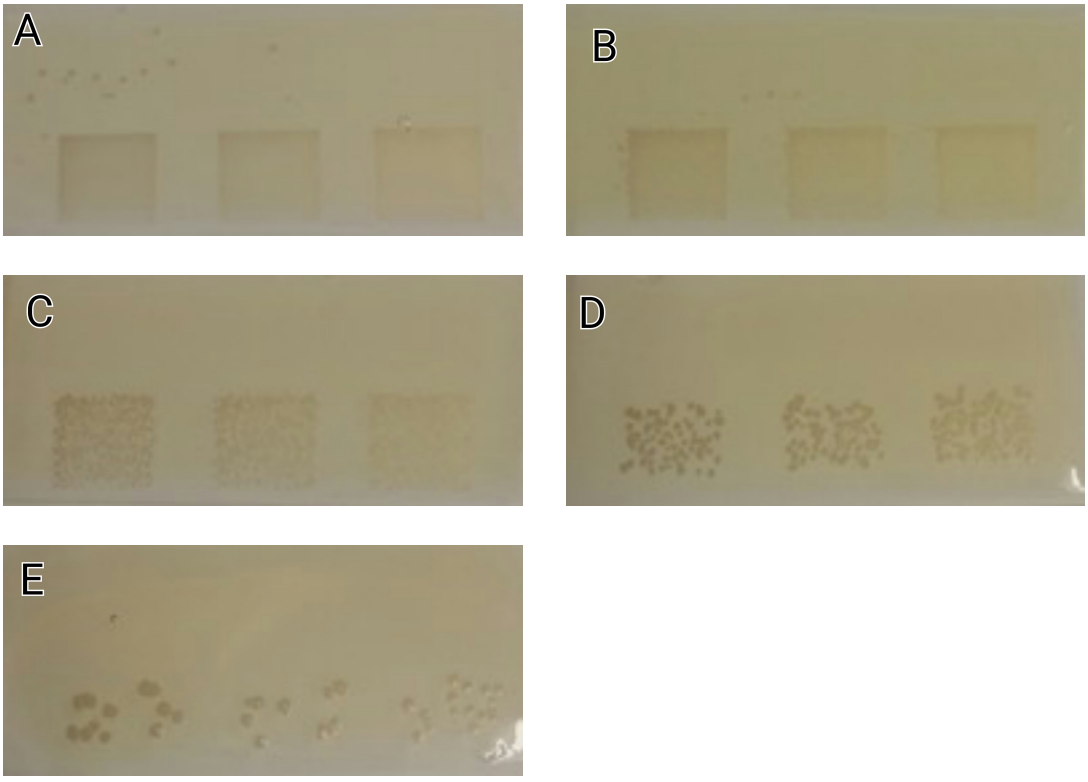

Figure 2-32: Post-incubation images for the set of 1cm x 1cm squares (10^7 CFU/ml (A), 10^6 CFU/ml (B), 10^5 CFU/ml (C), 10^4 CFU/ml (D), and 10^3 CFU/ml (E)).

When ink jetting was done with a 2 cm x 2 cm square, the print-outs had the horizontal element of the square being longer than the vertical (Figure 2-33). This was expected as the modification of the printer had restricted print-outs to a maximum vertical element equivalent to the length of cartridge nozzle hence, any shape with a vertical element greater than the length of the cartridge nozzle will be printed repeatedly onto the same area. The trends of the print-outs were like the earlier images (Figure 2-32). 10^7, 10^6, 10^5, and 10^4 CFU/ml showed clearly defined shapes of bacterial colonies (A-D), with 10^7 and 10^6 CFU/ml having satellite growths. 10^3 CFU/ml showed colonies with no resemblance to the template (E).

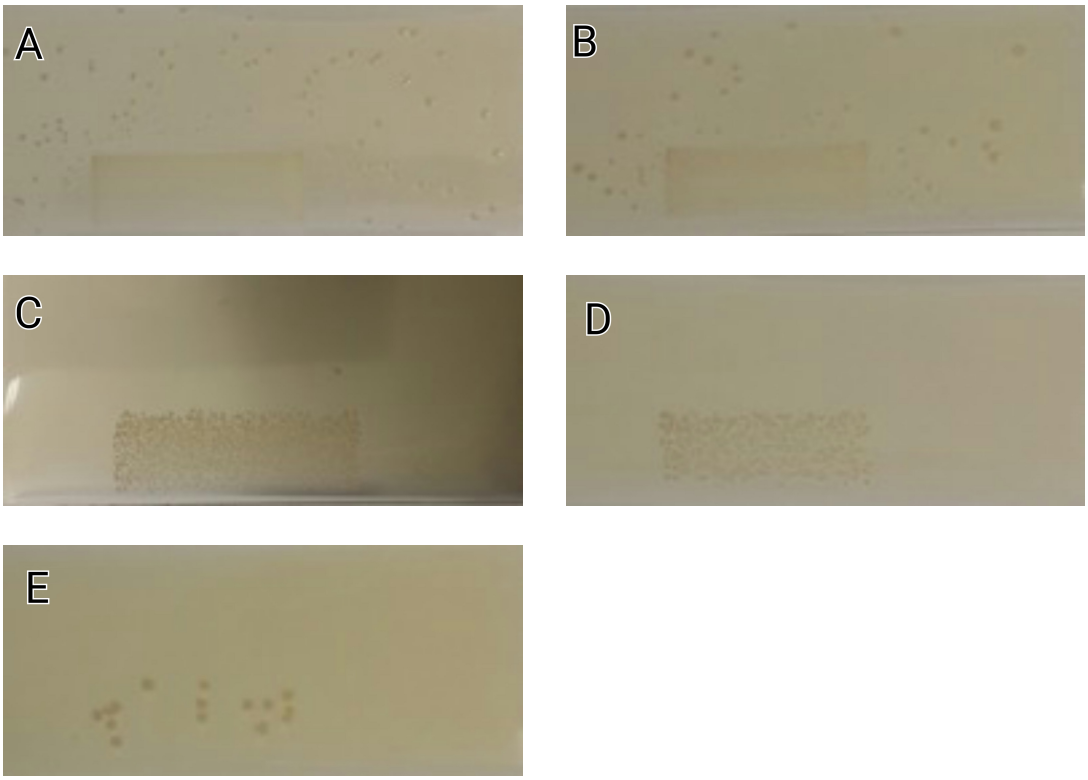

Figure 2-33: Post-incubation images of 2 cm x 2 cm square (10^7 CFU/ml (A), 10^6 CFU/ml (B), 10^5 CFU/ml (C), 10^4 CFU/ml (D), and 10^3 CFU/ml (E)).

A greyscale template with black at one end decreasing gradually in intensity to white at the other end was used. It was expected that after incubation, a densely populated bacterial growth will be observed at the end representing black with the bacterial density decreasing gradually to no bacterial growth at the end representing white (Figure 2-34). 10^7 CFU/ml showed a very densely populated strip and the gradual reduction in bacterial density was not clearly observed; satellite growths were also present (A). 10^6 and 10^5 CFU/ml showed well-defined strips with no satellite growths (B and C), the gradual decline in bacterial density was, however, more visible in 10^5 CFU/ml. 10^4 CFU/ml showed a strip with a decrease in bacterial density across the slide, however, the decrease in density was not consistent and representative of the template (D). 10^3 CFU/ml had colonies not consistent with the template (E).

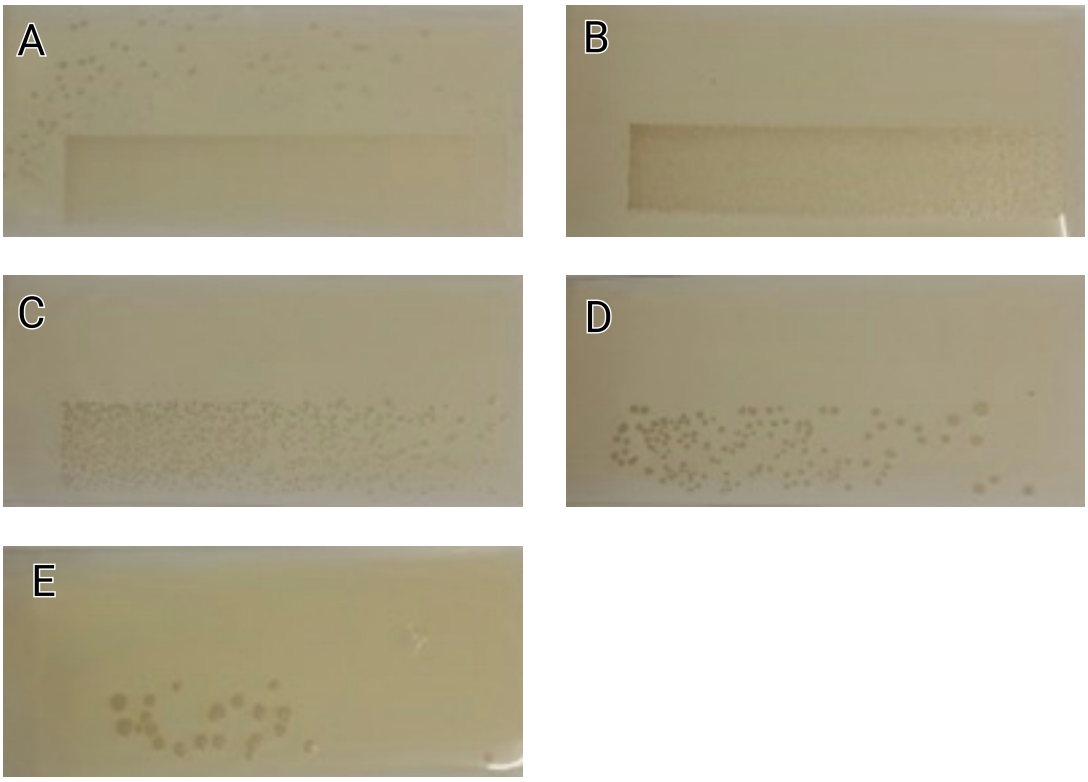

Figure 2-34: Post-incubation images of greyscale (10^7 CFU/ml (A), 10^6 CFU/ml (B), 10^5 CFU/ml (C), 10^4 CFU/ml (D), and 10^3 CFU/ml (E)).

Printing was also done with two rows of twenty dots to ascertain the ability of the modified printer to transfer images in detail (Figure 2-35). 10^7, 10^6, and 10^5 CFU/ml showed clearly defined dots with 10^7 and 10^6 CFU/ml having satellite growths (A and B). 10^4 CFU/ml had colonies across the length of the agar-coated slide representative of the entire length of the dots, however, these dots were not clearly defined (D). 10^3 CFU/ml had a few colonies on the slide (E).

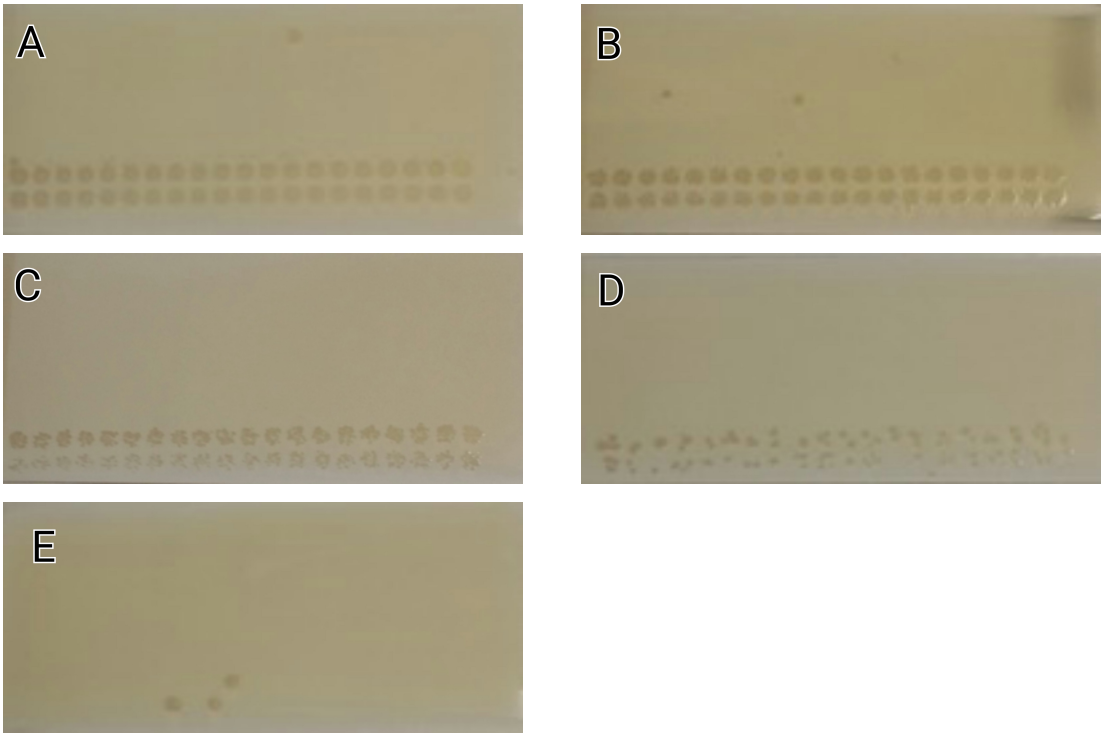

Figure 2-35: Post-incubation images of two rows of dots (10^7 CFU/ml (A), 10^6 CFU/ml (B), 10^5 CFU/ml (C), 10^4 CFU/ml (D), and 10^3 CFU/ml (E)).

To further test the ability of the modified inkjet printer to print in detail, a text (DODOO) was used as the template. Post-incubation images for concentrations used (10^7, 10^6, 10^5 and 10^4 CFU/ml) are shown in Figure 2-36. Bacterial growth was visible with letters separated; 10^5 CFU/ml, however, produced the most well-defined image without satellite growth (C).

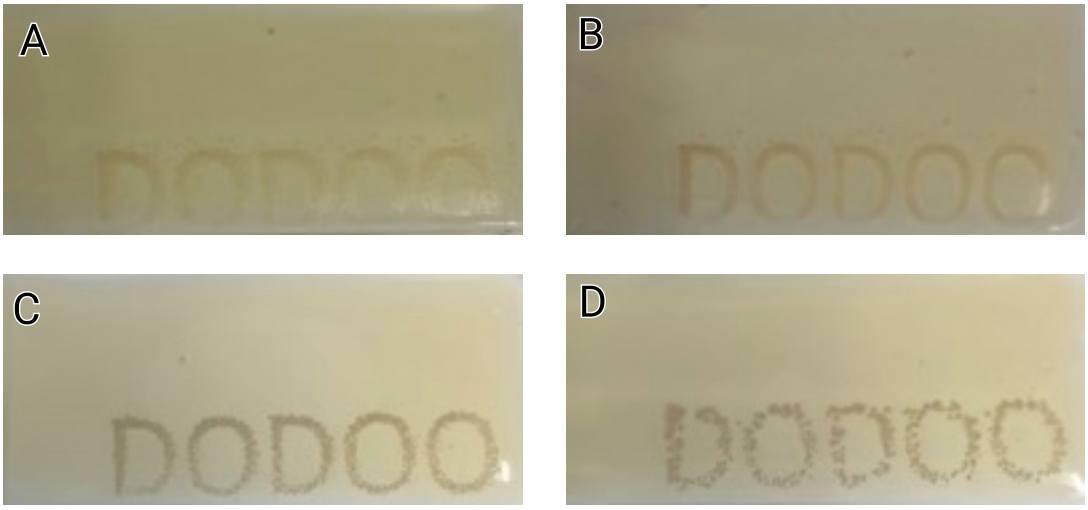

Figure 2-36: Post-incubation images of text (10^7 CFU/ml (A), 10^6 CFU/ml (B), 10^5 CFU/ml (C), and 10^4 CFU/ml (D)).

The satellite growths observed for the various templates can be attributed to the aerosols accompanying droplet formation during ink jetting (Iida et al., 2014, Udey et al., 2013). Since the distance between the printhead and the substrate was kept at a permitted minimum, it was inferred that for controlled printing, 10^7 and 10^6 CFU/ml were not the optimum concentrations to use, due to bacterial colonies growing at unexpected regions. 10^5 CFU/ml produced results typical of the print templates used and had no satellite growths. 10^4 and 10^3 CFU/ml produced results with no satellite growths, however, the nature of the images produced after incubation were usually not representative of the print templates and this can be attributed to the fact that the low bacterial concentration resulted in some of the printed droplets not having bacterial cells in them. From the above, 10^5 CFU/ml was chosen as the optimum concentration of *L. acidophilus* to use for controlled delivery under the conditions used.

2.4.6 Use of thermal ink jetting in antimicrobial susceptibility testing

Prior to AST, it was imperative to standardise the cartridges. This gave information about the jetting characteristics of the cartridge and helped in determining the amount of material jetted with a given set of templates. Fast green dye was used in the standardisation. Any dye can be used for standardisation so long as quantitative analysis can be conducted afterwards. Fast Green dye was selected based on availability and the possibility of quantifying deposited amounts analytically. HPLC was used to ascertain the quantity jetted out as Fast Green absorbs light at 600 nm. Y- values with height 0.5, 1, 2, and 3 cm with a constant width of 0.2 cm were the dimensions of the template used in standardisation. It was necessary to standardise using the same Y-values to be used in MIC determination. A calibration curve (Figure 2-37) for Fast Green dye was obtained with a good linearity (r^2 = 0.9999) between 0.0625 and 2 µg/ml. The graph of Y-value against area under the curve (AUC) (Figure 2-38) was also linear (r^2 = 0.9992) validating the Y-value concept. The volume of liquid deposited by inkjets usually contains a solute which is a fraction of the total solute in the cartridge. This fraction is fixed per template; this is denoted here as the characteristic ratio per template. This was obtained by expressing the amount of Fast Green dye per Y-value as a fraction of the cartridge concentration used in printing Y-values (Equation 2-1). A plot of the characteristic ratio against the Y-value for the templates used is shown in Figure 2-39.

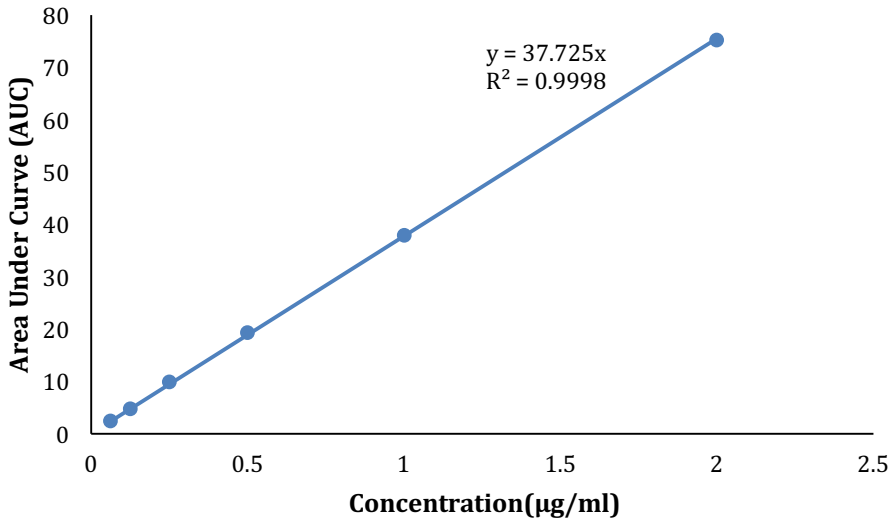

Figure 2-37: Calibration curve for Fast Green dye between 0.0625 – 2 µg/ml.

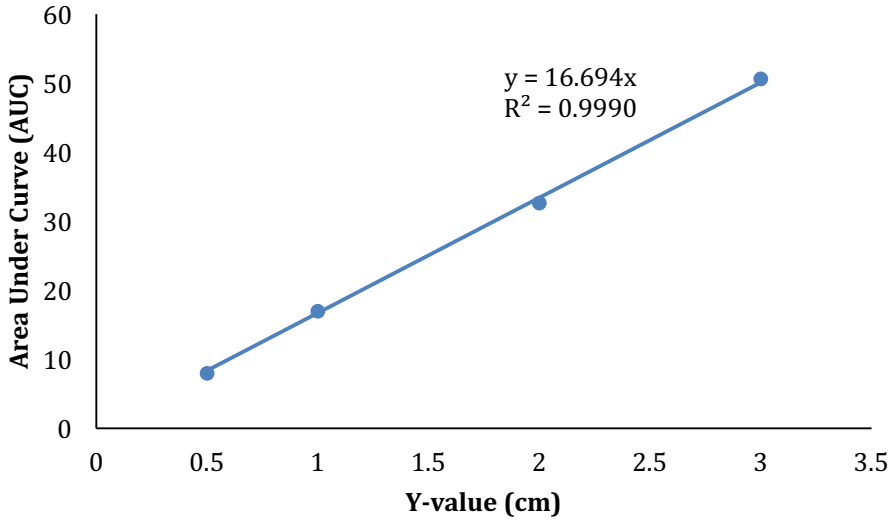

Figure 2-38: Y-value against area under curve (AUC)

Equation 2-1

$$characteristic\ ratio = \frac{equivalent\ concentration}{cartridge\ concentration}$$

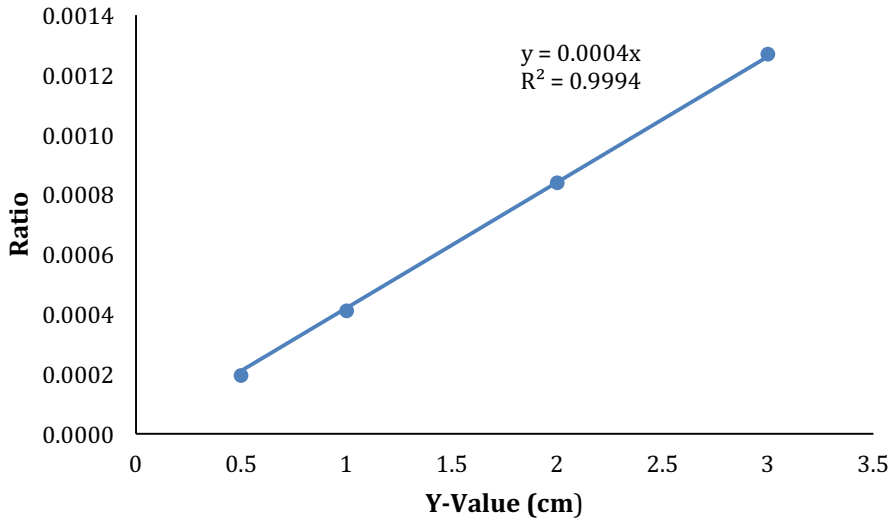

Figure 2-39: Y-value against characteristic ratio

Once the cartridges had been standardised, susceptibility testing could in principle be performed with the MIC being the least Y-value at which growth was inhibited. Figure 2-40 shows images from MIC determination for ampicillin using a concentration of 0.2 mg/ml in the cartridge. Figure 2-40A shows an even strip of bacterial colony after incubation, i.e., the control. Figure 2-40B shows a similar colony pattern to the control even though an antibiotic solution was deposited, indicating that the concentration of antibiotic present was not enough to inhibit the growth of *L. acidophilus*. Two distinct zones were seen in Figure 2-40C, representative of growth inhibition, in the areas where the antibiotic solution was deposited. Figure 2-40D showed almost no growth on the slide. The MIC in this instance was the concentration of drug at a Y-value of 1.5 cm; this was the least concentration of antibiotic that inhibited bacterial growth after incubation. Figure 2-40C also represents an ideal image for MIC determination since the two different concentrations are clearly visible. Assuming Figure 2-40D was the first slide to show inhibition, a higher cartridge drug concentration would have been used for observation. In Figure 2-40D, it is difficult to infer if there had been any interaction between the two Y-values due to growth inhibition across the whole slide.

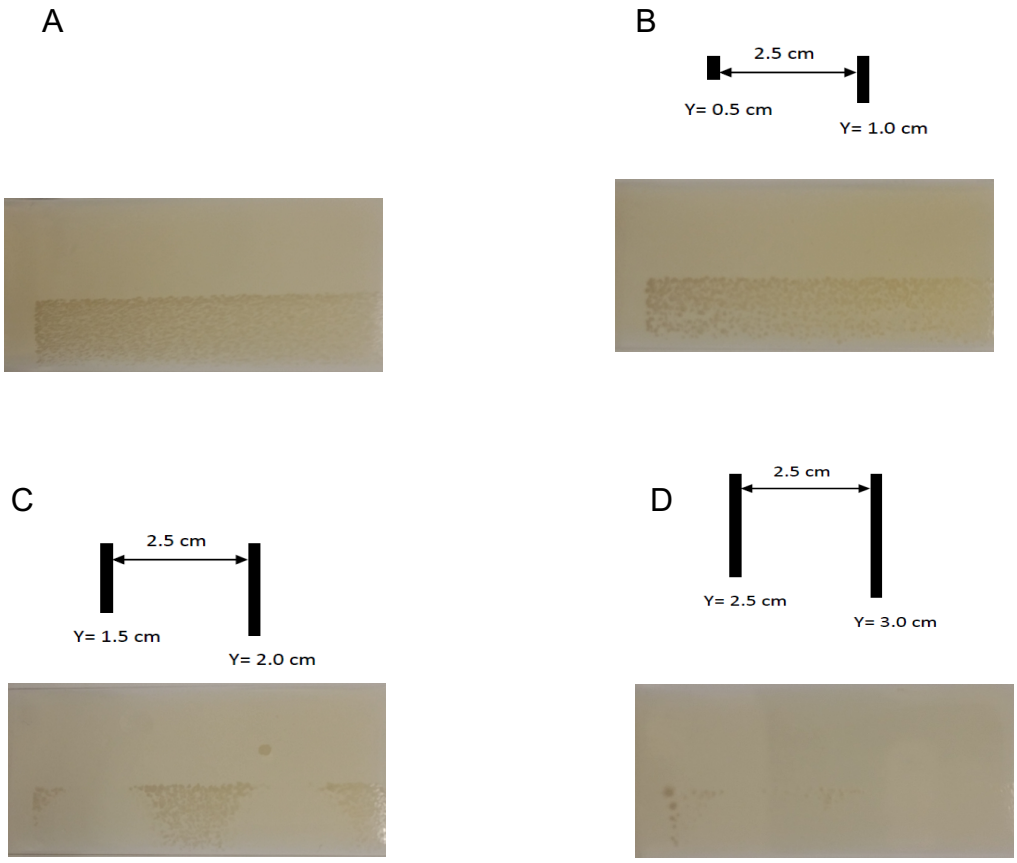

Figure 2-40: Illustration of MIC on bio-paper post-incubation; Control (A), 0.5 cm and 1 cm y-value (B), 1.5 cm and 2 cm y-value (C), 2.5 cm and 3 cm y-value (D).

Table 2-3 represents results after incubation for the various cartridge concentrations evaluated, with '+' indicating growth and '-' representing no growth for the indicated Y-values.

Table 2-3: Results for MICs determined via ink jetting.

Drug	Cartridge concentration (mg/ml)	Y-value (cm)					
		0.5	1.0	1.5	2.0	2.5	3.0
Tetracycline	0.1	+	+	+	+	+	+
	0.2	+	+	+	+	+	+
	0.3	+	+	+	+	+	+
	0.6	+	+	+	+	-	-
	0.7	+	+	+	-	-	-
Ampicillin	0.1	+	+	+	+	+	-
	0.2	+	+	-	-	-	-
	0.25	+	+	-	-	-	-
Doxycycline	0.3	+	+	+	+	-	-
	0.4	+	+	+	-	-	-
	0.5	+	+	-	-	-	-
	0.6	+	+	-	-	-	-
Amoxicillin	0.2	+	+	+	+	-	-
	0.3	+	+	+	-	-	-
	0.4	+	-	-	-	-	-
	0.5	+	-	-	-	-	-

The MIC obtained by the broth microdilution (Table 2-4) was recorded in the standard manner; i.e., if growth was observed at 0.5 µg/ml and no growth was observed at 1 µg/ml, the MIC was documented as 1 µg/ml. However, with the ink jetting technique because the concentration terms were in linear increments rather than doubling increments, MIC's were rounded off to the next incremental doubling dilution for comparisons with broth microdilution (Table 2-5). A similar approach was adopted when comparing spiral gradient endpoints with standard techniques and since the ink jetting model being designed is similar to the SGE technique, the same approach of comparison was used (Hill and Schalkowsky, 1990).

Table 2-4: Results for MICs determined via broth microdilution.

Drug	**MIC**
Tetracycline	2 µg/ml
Ampicillin	0.25 µg/ml
Doxycycline	1 µg/ml
Amoxicillin	0.5 µg/ml

A sample calculation for the corresponding MIC value using 0.6 mg/ml tetracycline is shown below:

$$MIC\ at\ 2.5cm = 0.6\ x\ (characteristic\ ratio\ at\ 2.5\ cm)$$

From graph (Figure 2-39), 2.5 cm ≡ 0.001

$$MIC = 0.6\ x\ 0.001 = \ 6\ \text{x}\ 10^{-4}\ \text{mg/ml}\ (0.6\ \mu\text{g/ml})$$

Table 2-5: Results for MICs determined via ink jetting (IJP) and broth microdilution.

Antibiotic	Cartridge Concentration (mg/ml)	Inkjet printing (MIC) (µg/ml)	Corrected IJP MIC (µg/ml)	Broth microdilution (MIC) (µg/ml)
Tetracycline	0.60	0.60	1	2
	0.70	0.56	1	2
Ampicillin	0.10	0.12	0.125	0.25
	0.20	0.12	0.125	0.25
	0.25	0.15	0.25	0.25
Doxycycline	0.3	0.30	0.5	1
	0.4	0.32	0.5	1
	0.5	0.30	0.5	1
	0.6	0.36	0.5	1
Amoxicillin	0.2	0.20	0.25	0.5
	0.3	0.24	0.25	0.5
	0.4	0.16	0.25	0.5
	0.5	0.20	0.25	0.5

The corrected MIC values from the test concentrations used were all within one doubling dilution from the broth microdilution MIC, which were within the range of acceptable MIC values when comparing with a standard technique (Pong et al., 2010, Wexler et al., 1996, Paton et al., 1990, Andrews, 2001, Jorgensen and Ferraro, 2009). As a model still in developmental stages, this outcome was considered very promising and a good foundation for further optimisation.

Zheng et al. (2011) attempted to use ink jetting in assessing the susceptibility of bacteria to antimicrobial agents. In their approach, a rectangular gradient grey strip ranging from pure white at one end to pure black at the other end was used to print antibiotic solutions onto the bacterial coating. The gradient grey scale was used as a means of delivering antibiotics with a continuous concentration gradient. The results obtained were compared with MIC values calculated from zones of growth inhibition.

A major criticism identified with their technique is the possible diffusion of drug solution on agar-coated slides which could result in difficulty identifying accurately the lowest inhibitory concentration since the concentration variations were continuous. The cup plate method used as a standard for comparison was also not the best option to use in quantitative MIC determinations (Amsterdam, 1996). We consider the use of the Y-value in this work as a better approach, as it offers discrete concentrations.

Smith and Kirby, (2016), demonstrated a more accurate technique in AST via ink jetting. With the aid of a commercial digital dispenser, HP D300, they deposited antimicrobial stock solutions, in 2-fold serial dilutions, directly into a 384-well plate for broth microdilution testing. After addition of bacteria and incubation, MICs were determined by plate absorbance readings and automated data analysis. The cost element of the digital dispenser and plate reader is a major limitation with this approach especially, in resource-challenged regions.

Investigations conducted to ascertain whether the time of contact between antibiotics and bacteria played a role in MIC determinations are shown in Table

2-6. Comparable results were obtained when the antibiotic (tetracycline) was printed immediately onto bacteria and when an interval of 20 minutes – to make up for time used to change cartridges and disinfect vital parts of printer – was allowed between antibiotic deposition and bacterial prints. In both instances, a cartridge concentration of 0.3 mg/ml yielded no growth inhibition whilst 0.7 mg/ml cartridge concentration resulted in growth inhibition at a Y-value of 2.0 cm equivalent to 0.56 µg/ml. Growth was observed for 0.3 mg/ml in both instances and this could possibly be that the effective concentrations on the slides were sub-inhibitory. When an interval of 48 hours was allowed between printing of drug and antibiotic, growth was observed and the nature of bacterial growth on all slides for both 0.3 mg/ml and 0.7 mg/ml cartridge concentrations were similar to that of control. This could also be that the effective drug concentration on the slides decreased over the duration highlighting a possible time dependence nature of this model.

This implies that unlike other susceptibility testing techniques (agar dilution) whereby media with antibiotics incorporated can be stored for future use, it will be inappropriate with this technique. This observation could be because the volume of antibiotic solution incorporated onto agar-coated slides was very small. Hill and Schalkowsky (1990), revealed that MICs don't vary significantly when an interval of interaction up to 7 hours was allowed between drug and organism. However, since relatively smaller quantities of antibiotics were used here in comparison to the SGE technique, the impact of time on the MIC is very significant. Also, in instances where anaerobic species are being investigated, it is better to keep the interval of interaction to a minimum as these organisms require a longer generation time. It is, therefore, recommended that for consistency in MIC results with this model, it is best to perform tests within a time frame of 20 minutes.

Table 2-6: Results showing the effect of contact time on MIC values

Time	Cartridge concentration (mg/ml)	Y-value (cm)					
		0.5	**1.0**	**1.5**	**2.0**	**2.5**	**3.0**
0 mins	0.3	+	+	+	+	+	+
	0.7	+	+	+	-	-	-
20 mins	0.3	+	+	+	+	+	+
	0.7	+	+	+	-	-	-
48 hours	0.3	+	+	+	+	+	+
	0.7	+	+	+	+	+	+

When the inoculum density was increased from 10^5 to 10^6 CFU/ml, an increment of 0.5 cm y-value was observed for all MICs except for 0.7 mg/ml tetracycline (Table 2-7). The relatively higher equivalent MIC values for the 10^6 CFU/ml inoculum was expected because a slightly greater bacterial population was being acted upon by the same amount of antibiotic as used for 10^5 CFU/ml. However, when these MIC values were corrected by rounding up to the next doubling dilution for comparison with standard techniques; the values obtained for ampicillin using 10^5 CFU/ml inoculum (0.125 µg/ml) and 10^6 CFU/ml inoculum (0.25 µg/ml) increased by one doubling dilution. The MIC obtained for 10^6 CFU/ml inoculum was, therefore, equal to that for the broth microdilution as seen in Table 2-4. For tetracycline, however, the same MIC value was observed for both inoculum densities i.e., 1 µg/ml for both 10^5 CFU/ml and 10^6 CFU/ml inoculum densities. A plausible explanation for this is that as the MIC values increased, the range between successive doubling concentrations increased correspondingly, resulting in a greater likelihood for slightly higher MIC values obtained via ink jetting to be similar when corrected to the next doubling concentration irrespective of the change in inoculum density. A major drawback with the use of 10^6 CFU/ml inoculum was the satellite drops on the agar- coated slides, hence, 10^5 CFU/ml would still be used for subsequent fine tuning of the model.

Table 2-7: Results showing effect of inoculum density on MIC.

Antibiotic	Cartridge concentration (mg/ml)	10^5 CFU/ml inoculum density		10^6 CFU/ml inoculum density	
		MIC Y-value (cm)	Equivalent concentration (µg/ml)	MIC Y-value (cm)	Equivalent concentration (µg/ml)
Ampicillin	0.10	3.0	0.12	No MIC	N/A
	0.20	1.5	0.12	2.0	0.16
	0.25	1.5	0.15	2.0	0.20
Tetracycline	0.60	2.5	0.60	3.0	0.72
	0.70	2.0	0.56	3.0	0.84

2.5 Conclusion

A thermal inkjet (HP 5940) was successfully modified, substituting the paper feeding mechanism with printing onto a stationary stage, without inhibiting its key functionality. An effective sterilisation protocol, whereby, cartridges can be sterilised using autoclaving was evaluated. This method is appropriate when cartridges are to be used as disposable consumables. When cartridges are intended for multiple use, sterilisation with 70% ethanol is a milder and effective alternative. The modified printer was also found to maintain its robustness, exhibiting insignificant inter- and intra-cartridge variability.

A model for the determination of MICs using ink jetting was designed and used to determine the MIC of ampicillin, tetracycline, amoxicillin, and doxycycline against *Lactobacillus acidophilus*. The MIC values obtained were all within one doubling dilution from MIC obtained via a broth microdilution standard. This range is considered acceptable when comparing MICs obtained by a different technique with a standard technique.

With antimicrobial resistance being a global issue and the rapid emergence of multidrug-resistant bacteria, there is often a need to perform susceptibility testing for less commonly used or newer antimicrobial agents. The automated susceptibility testing model described here, aims to address the heavy workload of manual testing and to bridge the cost element of automated susceptibility testing to make this accessible irrespective of the availability of funds.

Chapter 3 Formulating Probiotics for Oral Delivery using Thermal Ink Jetting

3.1 Introduction

Dairy products supplemented with probiotics are a natural means of probiotics administration. However, for the purposes of prevention or treatment of diseases, specifically targeted applications, formulae, devices, or carriers with a slow release of probiotics might be needed (Meurman and Stamatova, 2007). As detailed in the chapter 1, current techniques for the formulation of probiotics mainly involve microencapsulation via spray-drying or freeze-drying. There are, however, contrasting reports on the effect of microencapsulation on bacterial cell viability upon storage and protection from harsh conditions. Some researchers have found the process to have significant effects on bacterial viability and tolerance to gastric acids; others have reported no significant improvement after microencapsulation (Guerin et al., 2003, Iyer and Kailasapathy, 2005, Sun and Griffiths, 2000, Sultana et al., 2000).

Due to the harshness of these manufacturing process, excipients are added. Pharmaceutical formulations are generally made up of two main parts, the active ingredient and the excipients. Excipients are added to enhance product performance by providing a more efficacious product as well as improve stability and patient compliance. An ideal excipient aids in maintaining product uniformity and stability throughout the manufacturing process to administration by the consumer (Elder et al., 2016, Abrantes et al., 2016).

In a probiotic formulation, excipients are mainly needed to maintain the viability of cells throughout the formulation process and upon storage. Most probiotic formulations are made by drying the bacterial cells as such the excipients often used are mainly to enable cells to withstand elevated temperatures and maintain viability during storage. These excipients are generally known as protectants. Microorganisms usually survive better in low water activity, however, over-drying results in severe often lethal stress; protectants tend to

create a balance with optimum water activity to maintain viability (Carvalho et al., 2004, Desmond et al., 2001, Wang et al., 2004, Potts, 2001).

A variety of protectants have been used including carbohydrates (especially the sugars, e.g., trehalose, sucrose, lactose, and glucose), amino acids (sodium glutamate), polymers (dextran and polyethylene glycol), polyols (glycerol and mannitol) as well as complex mixtures like non-skimmed fat milk (Efiuvwevwere et al., 1999, Costa et al., 2000).

Due to the challenges encountered with current formulation strategies, the potential of ink jetting – which has been demonstrated in the previous chapter not to have any damaging effect on bacterial cells – in formulating probiotics was investigated. *L. acidophilus* (LA 5), an aero-tolerant organism, was chosen as the model probiotic species rather than a *Bifidobacterium spp.* since it presents greater flexibility in these initial stages of formulation development. Lactobacilli are also the most common probiotic bacteria associated with the human GIT (Caglar et al., 2005). The tolerance of the formulated probiotic to gastrointestinal fluid was evaluated. To target these formulated probiotics to the intestines, the ink jetted formulation was encapsulated into ready-to-use capsules coated with Phloral®. Phloral® is a coating technology – developed at University College London – that targets the release of drugs to the colon. It consists of a blend of bacteria-activated (resistant starch) and pH-activated (Eudragit® S) components. The independent triggers of a bacterially-triggered component within a pH-responsive polymer are effective, complementary, and act as failsafe mechanisms for each other in drug delivery (Ibekwe et al., 2008, McConnell et al., 2008). The ability of the formulated probiotic to adhere to intestinal cells was also ascertained *in vitro* using Caco-2 cells. The antibacterial property of the formulation was finally assessed against *E. coli*. A major analytical technique, isothermal calorimetry, which was used is elaborated further upon.

3.1.1 Isothermal microcalorimetry

Microcalorimetry is a non-invasive and non-destructive technique which works on the basic principle that all physical and chemical processes are accompanied by heat exchange between a system and the surrounding. The resulting heat flow is measured as a function of time. Isothermal microcalorimetry (IMC) involves measurement under isothermal conditions in the microwatt range. Other types of calorimeters are differential scanning, adiabatic, constant-volume, and constant-pressure calorimeters (Zhao et al., 2013, Braissant et al., 2010c, von Ah et al., 2009, Phipps and Mackin, 2000).

All living systems produce heat; therefore, physiological activity can be monitored by calorimetry with measurements being proportional to the rate at which a given chemical or physical process takes place. Physiological studies using IMC is not a new concept; studies date back to the 18th century where scientists used an ice calorimeter to monitor heat released by small animals (Braissant et al., 2010a).

Isothermal microcalorimeters (Figure 3-1) are often employed in thermodynamic investigations; they are also useful as general analytical instruments, for example, in the characterisation of stability and incompatibility of materials of pharmaceutical and technical importance (Johansson and Wadso, 1999).

IMC possesses varied advantages, one of which is the inherent sensitivity of the technique. With sensitivity as low as 0.2 μW, a wide range of processes can be monitored. Isothermal microcalorimeter thermostats can be set at any temperature within an instrument's performance range (e.g. 15 - 300°C) with a high accuracy, typically within 0.02 °C. IMC being a passive and non-destructive method does not require much sample preparation and allows subsequent evaluations of any kind on the undisturbed sample. IMC also imposes no requirements on the physical nature of the sample under investigation (the sample can be solid, liquid, or inhomogeneous). In addition, rate processes involving samples that are difficult to monitor in real-time using other methods - e.g., those taking place on opaque solids or in porous samples

- can be monitored easily using microcalorimetry (Said et al., 2015, Braissant et al., 2010a, Braissant et al., 2010c, Koenigbauer, 1994).

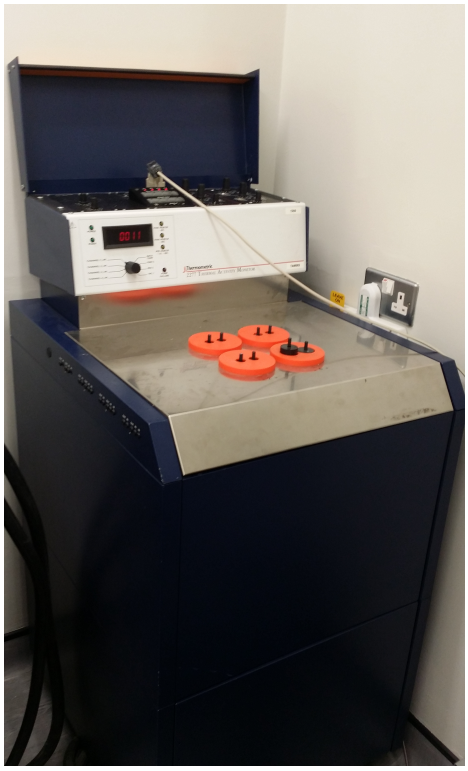

Figure 3-1: Image of thermal activity monitor (TAM 2270)

IMC, notwithstanding its advantages, has some limitations and challenges. A major limitation with IMC is the non-specific nature of the heat signal. Heat arises as a result of physical or chemical processes in the system. A calorimetric signal will not identify a measured process or its different phases. Another drawback with the use of IMC especially when biological systems are involved is oxygen depletion and the build-up of metabolic waste due to a closed system being employed. These challenges must be considered during data analysis. Also, most IMC experiments require a period of equilibration during which the sample and reference attain the same temperature. In instantaneous processes, data are lost during this period; however, this can be reduced by using flow systems. It has also been noted that IMC offers lower throughput as compared to other analytical techniques (Wadso, 2002, Johansson and Wadso, 1999, Braissant et al., 2010c).

IMC is an extremely versatile technique and has been applied in many fields including clinical and pharmacological analyses, ecology, agriculture, forestry, and biotechnology (Johansson and Wadso, 1999). Some medical and pharmaceutical applications are highlighted below.

IMC can be used as an appropriate tool for detection of infections or microbial contamination of clinical products. Bacterial contamination of platelet products remains one of the most important causes of transfusion-related morbidity and mortality (Trampuz et al., 2007). IMC has been used for the rapid detection of platelet contamination by microorganisms. IMC is also effective in monitoring in real-time slow growing organisms. Contamination by *Mycobacterium tuberculosis*, for instance, can be detected using this technique within hours to a few days (Braissant et al., 2010b).

IMC is a useful tool with potential in AST. With bacterial metabolism being accompanied by heat, the susceptibility of microorganisms can be determined readily as the rate of metabolism is directly proportional to the number of viable organisms. von Ah et al. (2009) used IMC to determine minimum inhibitory concentrations of antibiotics against microorganisms. IMC has been used to evaluate the efficacy of silver wound dressings against common wound bacteria (Said et al., 2014b).

IMC has been used in solid state characterisation to investigate properties like crystallisation. IMC can detect very low levels of amorphicity which is not possible with conventional techniques like x-ray powder diffraction. This makes it a useful analytical instrument after processes (e.g., milling, compaction) that may impart degrees of amorphicity to crystalline products (Phipps and Mackin, 2000).

IMC can be applied in the study of dissolution properties of solids in simple and, more importantly, complex media (Buckton et al., 1991, Ashby et al., 1989). Buckton and colleagues (1989) used this technique to study the dissolution of tetracycline hydrochloride from a commercial sustained-release product and from a dispersion of the drug in a semi-solid matrix.

Microcalorimetry being a direct measurement of reaction processes occurring within a sample can be used to investigate physical and chemical stability of materials. This has been used in emulsion technology to study the physical stability of components and the surfactants (Fubini et al., 1988, Fubini et al., 1989).

Just as IMC can be useful in studying antimicrobial susceptibility, it can be used in investigating probiotics, where real-time data can be obtained. This technique can be used to evaluate the behaviour of microbes to varying environmental conditions in GIT as well as the comparison of formulations. With the rise in health claims of probiotics, IMC can be used to evaluate interactions between probiotics and potentially pathogenic bacteria that exist within the microbiota. The technique has been used to compare some probiotic products on the market (Fredua-Agyeman and Gaisford, 2015, Fredua-Agyeman et al., 2017).

3.2 Aims

- To design an appropriate probiotic bio-ink for ink jetting.
- To evaluate the recovery of ink jetted probiotics from a substrate.
- To assess the viability and stability of the probiotic formulation with time.
- To evaluate the tolerance of the probiotic formulation to gastrointestinal fluid.
- To overcome any potential barriers to delivering viable strains to the intestines.
- To evaluate adhesion of *L. acidophilus* to intestinal cells.
- To evaluate the antibacterial properties of the probiotic formulation.

3.3 Materials and Methods

3.3.1 Print substrate

Edible starch paper (Lakeland, UK) was the print substrate used in this chapter.

3.3.2 Growth media and reagents

Sodium hydroxide pellets, sodium chloride, and glacial acetic acid were purchased from Fisher Scientific, UK. Sodium dihydrogen phosphate dihydrate and hydrochloric acid were purchased from VWR, UK. The simulated intestinal fluid powder used was from Biorelevant, UK. Pepsin, dimethylsulphoxide, trehalose, xylitol, sucrose, glucose, and triton X-100 were purchased from Sigma-Aldrich, UK. Dulbecco's Modified Eagle Medium, heat-inactivated foetal bovine serum, 1% non-essential amino acid, trypsin-ethylene diamine tetraacetic acid, and trypan blue dye were from Gibco, UK. Cooked meat medium, nutrient agar, and MRS agar were from Oxoid, UK.

3.3.3 Bio-ink formulation

The bacterial stock prepared in section 2.3.4 was used. This was to prevent batch-to-batch variability observed with freshly prepared daily cultures. Depending on the intended cell density, a few frozen vials were centrifuged and concentrated. Frozen cells were thawed in a water bath at 40 °C for 3 minutes (Said et al., 2014a). The bio-ink was then made by suspending bacteria in phosphate-buffered saline (PBS).

3.3.4 Evaluating recovery from substrate

Calorimetry and colony plate counting were the techniques used for this evaluation. The bio-ink was composed of 10^7 CFU/ml bacterial population suspended in PBS. A 1 cm x 2 cm rectangular template was used for ink jetting onto edible starch paper.

Calorimetric readings were conducted by carefully inserting the imprinted starch paper into sterile 3 ml calorimetric ampoules filled with 3 ml of pre-warmed (37 °C) MRS broth. The ampoules were then hermetically sealed as shown in Figure 3-2 by crimping and vortexed for 10 seconds. Calorimetric readings were then taken over a period of 40 hours.

Figure 3-2: A hermetically sealed glass ampoule filled with MRS broth

All calorimetric experiments were conducted using a 2277 Thermal Activity Monitor (TAM, TA Instruments Ltd., UK) operated at 37 ± 0.1 °C. Prior to data recordings, samples were placed into the thermal equilibration position of the calorimeter for 29 minutes then lowered into calorimeter and data collection started exactly 30 minutes after inoculation. A software package, Digitam 4.1, which records 1 data point every 10 seconds was used to record data. A purity plate was set up for each ampoule after the experiment by streaking a loopful of culture onto MRS agar plate. Subsequent growth of colonies of a single type and morphology confirmed purity of the culture. As a control, calorimetric data of pipetted *L. acidophilus* concentrations was also taken. Final ampoule concentrations of 10^5 CFU/ml, 10^4 CFU/ml, and 10^3 CFU/ml *L. acidophilus*

were used. To ensure accuracy in calorimetric results, the calorimeter was calibrated periodically.

Colony plate counts were conducted by suspending imprinted templates in 2 ml PBS for 20 minutes. Two sets of 30-second vortexing were done at 0 and 20 minutes. 1 in 10 serial dilutions were then conducted after which plating out onto MRS agar was done. The agar plates were then incubated under anaerobic conditions for 48 hours.

3.3.5 Evaluating effect of protectants on bacterial viability

Bio-inks were composed of 10^7 CFU/ml bacterial concentration in 10% sucrose, 10% trehalose, or PBS (control). The three bacterial solutions were printed onto edible starch paper using a 1 cm x 2 cm template. These were then inserted into a 3 ml calorimetric glass ampoule and 3 ml of pre-warmed (37 °C) MRS broth added. The ampoules were hermetically sealed and vortexed afterwards. Calorimetric readings were taken as above (section 3.3.4) for a period of 40 hours. Another set of imprinted starch paper was kept at 2 - 8 °C and on the 7th day, calorimetric readings repeated.

For colony plate counts, the same template dimensions (1 cm x 2 cm) were used to print the three bacterial solutions onto edible starch paper. The imprinted starch paper was then stored at 2 - 8 °C for a period of 7 days and daily enumeration conducted as above (section 3.3.4).

3.3.6 Evaluating tolerance of imprinted *Lactobacillus acidophilus* to simulated gastrointestinal fluids

Five different types of simulated gastrointestinal fluids were investigated; fasted state simulated gastric fluid (FaSSGF), fed state simulated gastric fluid (FeSSGF), fasted state simulated intestinal fluid (FaSSIF), fed state simulated intestinal fluid (FeSSIF), and pig small intestinal fluid (PIF) (Fredua-Agyeman and Gaisford, 2015, Wang et al., 2015, Aburub et al., 2008).

3.3.6.1 Sourcing and preparation of simulated gastrointestinal fluids

The composition of the artificial fluids used is indicated in Table 3-1.

Table 3-1: Composition of artificial gastrointestinal fluids in 100 ml medium.

Property	**FaSSGF**	**FeSSGF**	**FaSSIF**	**FeSSIF**
pH	1.2	4.0	6.5	5.0
Sodium chloride	0.2g	1.187g	0.62g	1.187g
Sodium hydroxide pellets	-	0.404g	0.04g	0.404g
Sodium dihydrogen phosphate dihydrate	-	-	0.45g	-
Glacial acetic acid	-	0.865g	-	0.865g
Biorelevant simulated intestinal fluid powder	-	-	0.224g	1.12g
Pepsin	0.32g	0.32g	-	-

3.3.6.1.1 Fasted State Simulated Gastric fluid (FaSSGF)

100 ml of sodium chloride and hydrochloric acid (HCl) mixture was first prepared by dissolving 0.2 g of sodium chloride in 90 ml purified water. The pH was adjusted to 1.2 with HCl and volume made up to 100 ml. 0.32 g of pepsin was then added to 50 ml of the sodium chloride and HCl solution and stirred until complete dissolution. The volume was adjusted to 100 ml afterwards. The solution was filter-sterilised and used within 48 hours of preparation.

3.3.6.1.2 Fed State Simulated Gastric Fluid (FeSSGF)

100 ml of buffer was first prepared by dissolving 0.404 g of sodium hydroxide (pellets), 0.865 g of glacial acetic acid, and 1.187 g of sodium chloride in 90 ml of purified water. The pH was adjusted to 4.0 and volume increased to 100 ml with purified water at room temperature. 0.32 g of pepsin was then added to 50 ml of buffer and stirred until complete dissolution. The volume was then adjusted to 100 ml. The solution was sterilised by filtration and used within 48 hours of preparation.

3.3.6.1.3 Fasted State Simulated Intestinal Fluid (FaSSIF)

100 ml of buffer was first prepared by dissolving 0.04 g of sodium hydroxide pellets, 0.45 g of sodium dihydrogen phosphate dihydrate, and 0.62 g of sodium chloride in 90 ml of deionised water. The pH was adjusted to 6.5 and volume was increased to 100 ml with deionised water at room temperature.
0.224 g of simulated intestinal fluid powder was then added to 50 ml of buffer and stirred until complete dissolution; additional buffer solution was added to 100 ml. The solution was left for 2 hours at which point the solution became slightly opalescent and was ready to use. The solution was sterilised by filtration using a membrane filter of 0.22 µm pore size (Millex, UK) and used within 48 hours of preparation.

3.3.6.1.4 Fed State Simulated Intestinal Fluid (FeSSIF).

100 ml of buffer was first prepared by dissolving 0.404 g of sodium hydroxide (pellets), 0.865 g of glacial acetic acid, and 1.187 g of sodium chloride in 90 ml of deionised water. The pH was adjusted to 5.0 and volume increased to 100 ml with deionised water at room temperature.
1.12 g of simulated intestinal fluid powder was then added to 50 ml of buffer and stirred until complete dissolution; additional buffer solution was added to 100 ml. The solution was sterilised by filtration using a membrane filter with 0.22 µm pore size and used within 48 hours of preparation.

3.3.6.1.5 Pig Small Intestinal Fluid

Pig gastrointestinal tracts were obtained from a cross-breed of large white and landrace pigs (n = 3) at an abattoir (Cheale Meats, Essex, United Kingdom). The gastrointestinal tracts from pigs were then dissected to collect the fluid from the small intestine. The fluid was collected within 5 hours at 4 °C following sacrifice into multiple aliquots and immediately stored at −80 °C. Prior to use, the fluid was centrifuged at 10,000 rpm (Eppendorf Mini spin, Germany) for 10 minutes at 25 °C. The supernatant obtained was sterilised by filtration afterwards. The pH of the small intestinal supernatants was 6.6 ± 0.2.

3.3.6.2 Tolerance testing

Three different bio-inks were prepared using the bacterial stock suspended in 10% sucrose, 10% trehalose, and PBS (control) to produce a 10^7 CFU/ml bacterial population. A 1 cm x 2 cm template was used to deposit solutions onto edible starch paper and suspended correspondingly into simulated intestinal fluid (FaSSGF, FeSSGF, FaSSIF, or FeSSIF) for 2 hours at 37 °C; vortexing was done for 30 seconds before and after incubating. Sterile ampoules were filled with 2970 µl of pre-warmed (37 °C) MRS broth and 30 µl of resultant simulated fluid was added. The ampoules were then hermetically sealed and vortexed. Calorimetric readings were taken over a period of 40 hours. A purity plate was set up for each experiment by taking a loopful of culture and streaking out onto an agar plate. The experiment was also done using filtered supernatant from PIF and bio-ink composed of 10% trehalose. The sterility of the filtered supernatant was also confirmed by plating out.

Colony plate counting was also conducted to further support calorimetry determinations using the same bio-inks. A 1 cm x 2 cm template was used to deposit bio-inks onto starch paper and suspended correspondingly in FaSSGF, FeSSGF, FaSSIF, FeSSIF, and PIF (only 10% trehalose) for 2 hours at 37 °C. Vortexing was done for 30 seconds before and after incubating. Serial dilutions and subsequent plating out onto MRS agar were done. The agar plates were then incubated under anaerobic conditions for 48 hours. The experiment was repeated for FaSSGF and FeSSGF with enumeration at 0, 5, 10, 20, 30, 60, 90 and 120 minutes with a higher dosing of 15 cm x 2 cm.

3.3.7 Overcoming the gastric environment

Capsule coating was used as a means of protection against the gastric environment. An evaluation of the effect of pre-coating capsules before filling with active (opened coating) and then filling with active before coating (closed coating) was conducted first.

3.3.7.1 Capsule coating

Capsules were coated using Phloral®. The coating medium was prepared using the ingredients in Table 3-2.

Table 3-2: Recipe for Phloral® coating

Ingredients	Quantity (mg/cap)
Eudragit® S	28
Starch	12
Triethyl citrate	8
Glycerol monostearate	2
Butanol	27.5
Tween 80	0.8
Deionised water	357.14
Ethanol 96% (w/w)	421.12
Total solids	50.8

The coating was performed with a Caleva Mini Coater (UK), a fluidised bed coater. The parameters used for coating are indicated in Table 3-3. The duration of the coating varied based on the target weight gain.

Table 3-3: Coating conditions

Parameter	
Spray rate	0.4 g/min
Temperature	25 ºC
Atomising pressure	0.2 bar
Pump	35 %
Fan speed (m/s)	9 - 11 m/s

3.3.7.2 Effect of opened and closed capsule coating techniques

Paracetamol was used as the active ingredient to evaluate the differences between opened and closed capsule coating techniques. Two types of capsules were used; gelatin and hydroxypropyl methylcellulose (HPMC) capsules (size 0) (Qualicaps®, USA).

For the closed capsule coating, capsules (total surface area of 500 mm^2) were filled with 30 ± 2 mg paracetamol powder and closed tightly. The capsules were then coated with Phloral® with a target weight gain of 5 mg/cm^2. The coated capsules were dried at room temperature afterwards.

For the opened capsule coating, the capsule head and body were coated separately with Phloral®; the number of caps or bodies to be coated was chosen to equal the total outer surface as used in the closed coating process. Coating was done with a target weight gain of 5 mg/cm^2 and the capsules were then dried at room temperature. 30 ± 2 mg of paracetamol powder was loaded into capsules and closed tightly. To determine the pH dissolution profile of the coated capsules, dissolution testing was conducted.

3.3.7.3 Dissolution testing

Dynamic dissolution testing method using USP-II apparatus (Model PTWS, Pharmatest, Hainburg, Germany) was carried out as detailed in Goyanes et al., (2015b). Capsules were initially put into 750 ml of 0.1 M HCl for 2 hours to mimic fasted gastric conditions and residence time. The capsules were then transferred into 950 ml of modified Hank's buffer (Table 3-4) for 35 minutes. 50 ml of pre-Kreb's solution (400.7 mM sodium bicarbonate and 6.9 mM potassium hydrogen phosphate) was added to produce a modified Kreb's buffer *in situ*. The pH was then modified with time to mimic intestinal transit in the fasted state; sink conditions was attained in all instances. Modifications used as indicated in Table 3-5 were achieved with the aid an Auto pH System®. The paddle speed was set at 50 rpm and the tests were conducted at 37 ± 0.5 ºC (n = 6). Paracetamol release was determined using an in-line UV spectrophotometer (Cecil 2020, Cecil Instruments Ltd., Cambridge, UK) at the wavelength of 243 nm. Data were processed using Icalis software (Icalis Data Systems Ltd., Berkshire, UK).

Table 3-4: Composition of modified Hank's buffer

Ingredient	**Ionic composition (mM)**
Sodium chloride	136.9
Potassium chloride	5.37
Magnesium sulphate heptahydrate	0.812
Calcium chloride	1.26
Sodium hydrogen phosphate dihydrate	0.337
Potassium dihydrogen phosphate	0.441
Sodium bicarbonate	4.17

Table 3-5: pH scheme mimicking intestinal transit

Time	**pH**
0 min	5.6
5 min	6.0
10 min	6.5
20 min	6.8
At 35 min	Add 50 ml of pre-Krebs solution to each vessel. This will produce a litre of Krebs buffer in each vessel.
36 min	7.0
50 min	7.2
80 min	7.4
210 min	6.5 (a higher carbon dioxide, CO_2, flow is necessary to drop the pH rapidly; it will take about 15 min to attain the set point).

3.3.7.4 Encapsulating ink jetted probiotics

Based on the outcome of the dissolution testing, probiotics were formulated using the opened capsule coating method. Both gelatin and HPMC capsules were used. Different target weight gain was investigated initially (5, 8, 10 mg/cm^2) to find the maximum permissible coating that did not hinder capsule closure. 5 mg/cm^2 was chosen as suitable capsule weight gain afterwards. *L. acidophilus* (10^7 CFU/ml) suspended in 10% trehalose was used as bio-ink. Probiotics were formulated by ink jetting 2 cm x 15 cm black rectangular template onto starch paper. This was then inserted into the capsule body which was then closed with a cap before dissolution testing was conducted.

3.3.7.4.1 Dissolution testing

With this formulation intended for site-specific delivery, an improvised dissolution testing system was used, mimicking the United States Pharmacopoeia's (USP) standard dissolution testing for such formulations as closely as possible. Capsules were immersed in three consecutive media for 2 hours each i.e., 0.1 M HCl, phosphate buffer at pH 6.8, and phosphate buffer at pH 7.4. All the media used were pre-warmed to 37 ºC prior to use. Bacterial enumeration was conducted at hourly intervals in all media. To ensure the process was carried out at 37 ºC with 50 rpm paddle movement as observed in USP dissolution testing; a modified dissolution testing was carried out in an incubator shaker at 37 ºC with shaking set to 50 rpm. 5 ml media were used and capsules were completely immersed with the aid of sinkers.

Another set of capsules were immersed into above media separately, i.e., either 0.1 M HCl, phosphate buffer (pH 6.8), or phosphate buffer (pH 7.4) for 2 hours and enumeration conducted for all three media afterwards. Based on the outcome, the dissolution testing was modified with the duration in 0.1 M HCl reduced to 30 mins whilst all other tests and conditions maintained.

3.3.8 Evaluating adhesion of *L. acidophilus* to intestinal cells *in-vitro*

To evaluate the ability of probiotics to adhere to intestinal cells, an *in-vitro* test was conducted using Caco-2 intestinal cells. Caco-2 intestinal cells were obtained as a gift from Department of Pharmaceutical & Biological Chemistry in University College London.

3.3.8.1 Cell culture

3.3.8.1.1 Growth medium

The growth medium used was Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% of heat-inactivated foetal bovine serum (FBS) and 1% non-essential amino acid. The prepared medium was used to routinely grow Caco-2 cell lines in cell culture flasks (Nunc) with surface area 75 cm^2 or to seed cells in a well-plate (Nunclon). All cells were incubated at 37 ºC in a humidified atmosphere of 5% CO_2 (NuAire™ Autoflow CO_2 Air-Jacketed Incubator, NuAir Inc.).

3.3.8.1.2 Cell sub-culturing

Caco-2 cells were sub-cultured every 2-3 days when the cells were approximately 70 - 80% confluent. The spent culture medium was aspirated and discarded. The cells were rinsed by gently adding 10 ml PBS to the side of the vessel opposite the attached cells (to prevent disturbance to cells) and the vessel rocked back and forth several times to remove traces of serum, calcium, or magnesium that can potentially inhibit the dissociation reagent. The wash solution was then aspirated and discarded after which 1.2 ml of pre-warmed 0.25% trypsin-ethylene diamine tetraacetic acid (trypsin-EDTA) was added as a dissociation reagent. The vessel was then rocked gently to completely cover cell monolayer. The flask containing the cell lines was incubated for 5 minutes at 37 ºC in 5% CO_2. The cells were then observed with an inverted microscope (Evos FL, UK) for confirmation of cell detachment. 3 ml of pre-warmed growth medium was added to halt the activity of trypsin-EDTA. The cells were then transferred into 15 ml centrifuge tubes and

centrifuged at 1000 rpm for 10 minutes at 20 ºC. The cell pellets were resuspended in 3 ml of pre-warmed medium. Using a split ratio of 1:3, the cells were resuspended in fresh 20 ml pre-warmed medium in cell culture flask. The flask was then labelled with the cell name, passage number and date and placed in an incubator with humidified atmosphere of 5% CO_2 at 37 ºC.

3.3.8.1.3 Cell cryopreservation

The Caco-2 cells were cryopreserved for long-term storage. The freezing medium was composed of the growth medium with 10% dimethylsulphoxide (DMSO) kept at 2 – 8 ºC.

Cryopreservation of cells was preceded by steps similar to the cell sub-culturing (section 3.3.8.1.2). The adhered cells were dissociated with trypsin-EDTA and resuspended in growth medium. The total number of cells and viability was determined. A viability over 90% was obtained prior to cryo-freezing. The suspension was centrifuged and cell pellets resuspended in 3 ml freezing medium. 1.2 ml was then aliquoted into sterile cryovials. The cryovials were labelled and transferred into a cryo-freezing container, Mr. Frosty™ Freezing Container (Thermo Scientific, UK), containing 250 ml isopropanol and stored at -80 ºC. The cryo-freezing container reduces the temperature at approximately 1 ºC per minute. The vials were then transferred into a liquid nitrogen dewar and stored until needed.

3.3.8.1.4 Cell recovery

A cryovial was removed from liquid nitrogen and immediately thawed in a water bath at 37 ºC with gentle swirling for about 1 minute. The thawed content was then transferred into an appropriate volume of pre-warmed medium and centrifuged at 1000 rpm for 10 minutes at 20 ºC. The supernatant was then aspirated and the cell pellet resuspended in 3 ml growth medium. Using a split ratio of 1: 3, 20 ml of pre-warmed growth medium in cell culture flask was inoculated with cells. The flask was then labelled and incubated at 37 ºC with humidified atmosphere of 5% CO_2. The cells were then sub-cultured accordingly.

3.3.8.1.5 Enumeration of cells and viability

Cell enumeration and viability were determined with the aid of a haemocytometer and the trypan blue dye exclusion test. 50 µl of trypan blue dye was added to 50 µl of Caco-2 cell suspension and mixed thoroughly. This was then loaded onto a haemocytometer (Fortuna, Germany) with a clean cover slip placed on. The haemocytometer was mounted on an inverted light microscope and cells counted with magnification (x 10). Dead cells were stained as these picked up the colour of dye whereas live cells remained unstained. Cells in the outer 4 quadrants were counted and an average determined. Total cell count and percentage viability were computed as shown in equations below.

Equation 3-1

$$cells/ml = average\ count * dilution\ factor * 10^4$$

Equation 3-2

$$total\ count = cells/ml * total\ volume$$

Equation 3-3

$$percentage\ viability = \frac{number\ of\ unstained\ cells}{total\ count\ (stained\ and\ unstained)} * 100$$

3.3.8.2 *In vitro* adhesion testing

The evaluation done here was similar to work carried out by Forestier et al. (2001). A bacterial suspension of known concentration in 10% trehalose was ink jetted onto edible starch paper using a 1 cm x 2 cm template. This was then suspended in 2 ml of PBS at 37 ºC to disintegrate starch paper.

Caco–2 monolayers were seeded at a concentration of 2 x 10^5 cells per well in 12-well plates and incubated at 37 ºC with humidified atmosphere of 5% CO_2. Caco–2 cells were used in the late post-confluence state with passage numbers between 39 and 54. Prior to adhesion testing, Caco–2 monolayers were washed twice with 1 ml PBS and 1 ml of the cell culture growth medium added to each well. 1 ml of the disintegrated ink jetted starch paper in PBS was also added. The plate was then incubated at 37 ºC for 1 hour after which medium was aspirated. The monolayers were then washed three times with 1 ml PBS to remove any non-adhered bacterial cells. The cells were lysed by addition of 1 ml 0.1% triton X-100 solution and the number of viable adhering bacteria determined by plating out serial dilutions onto MRS agar. The number of adhered cells were then expressed as a percentage of the initial number. An estimate of Caco–2 cells in wells was conducted each time adhesion tests was done. This was to ensure the available Caco-2 cells was not less than bacterial cells being added. The Caco-2 cells used for these tests were between 2.9 x 10^5 – 1 x 10^6 cells per well.

3.3.9 Evaluating the antibacterial properties of Ink jetted formulation

The antibacterial property of probiotic formulation was investigated against *Escherichia coli* (NCTC 10418). Antibacterial activity was monitored using calorimetry.

Cooked meat medium supplemented with 2% glucose (CMMg) was used as the growth medium. A power-time curve of *E. coli* (10^4 CFU/ml) co-incubated with ink jetted *L. acidophilus* formulation (equivalent to 2.8 x 10^3 CFU per template) was obtained. Plate counts for both organisms were conducted at 24 and 48 hours. As a control, power-time curves were also obtained for individual species and plate counts conducted at 24 and 48 hours. *E. coli* enumeration was conducted on nutrient agar plates and *L. acidophilus* enumeration on MRS agar plates.

3.4 Results and Discussion

3.4.1 Evaluating recovery from substrate

Ink jetting of pharmaceutical actives has received much attention recently, however, relatively limited information on ink jetting of live bio-actives exists in the literature. Biological materials are naturally delicate with viability easily subject to change; hence, it was vital to assess recovery of ink jetted formulation from the substrate. 1 cm x 2 cm template dimension albeit small was used as a dimension in this developmental phase. Plate counting to enumerate microorganisms in the deposited template indicated bacterial numbers of 2.8 x 10^3 CFU per template. Calorimetry was used to monitor bacterial growth because it gives real-time data on bacterial growth (Fredua-Agyeman et al., 2017). Use of isothermal calorimetry to study *L. acidophilus* dates as far back as 1978, when Fujita and colleagues studied pure lactic acid species under limiting substrate conditions. In recent times, a few studies have been done using this technique to monitor lactic acid bacteria (Fredua-Agyeman and Gaisford, 2015, Stulova et al., 2015, Kabanova et al., 2009, Fujita et al., 2009).

Data from calorimetry are usually represented as power against time, known as a thermogram (Braissant et al., 2010a). The power signal in this instance arises due to metabolic heat in the system which is directly proportional to the bacterial population in the system. The area under the curve (AUC), hence, represents heat energy (Joules) and gives an indication of the number of organisms in the system. The thermogram, as indicated in Figure 3-3, showed an onset of activity at approximately 15 hours and a peak intensity between 0.35 mW and 0.45 mW at 10 hours after onset. Such peaks (Figure 3-3) are typical of microbial growth in static ampoules, i.e., an initial rise to a maximum then drop to baseline after some time (Braissant et al., 2013, Wadso, 2001, Kabanova et al., 2009).

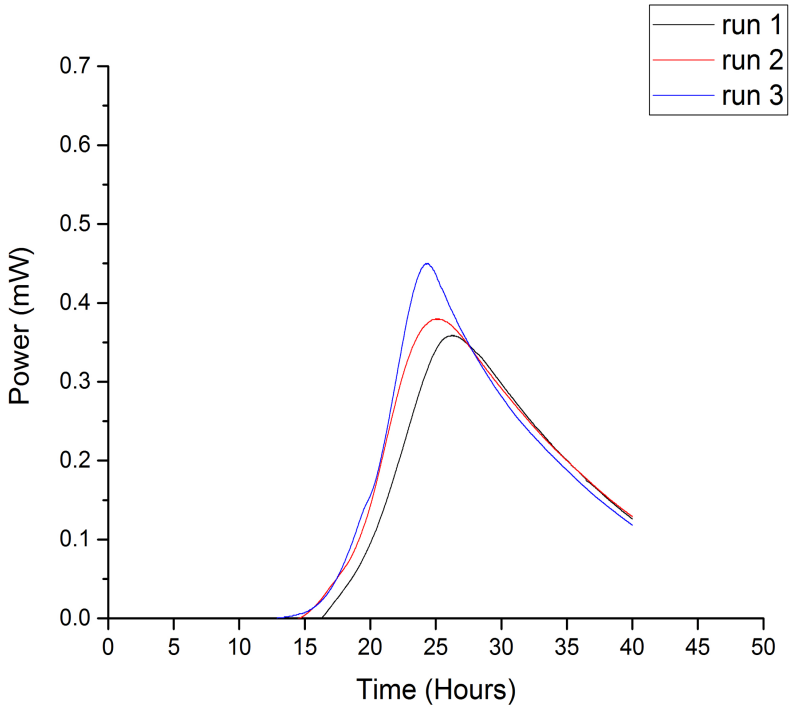

Figure 3-3: A thermogram illustrating growth of ink jetted *L. acidophilus* from substrate.

When microbes are inoculated into an ampoule with an appropriate culture medium, there is an initial lag period which is dependent on the cell density and age of cells (Belaich, 1980). The calorimeter although very sensitive has a limit of detection of about 10^5 - 10^6 active cells; any cell density below this limit contributes to the lag time (Gaisford et al., 2009, Braissant et al., 2010c, Kabanova et al., 2009). With continuous bacterial metabolism and utilisation of the nutrients in the medium for growth, cell density increases and a corresponding heat production results in signals being obtained. These signals increase proportionally to metabolic activity in the medium to the maximum after which the signal returns to baseline. This drop in signal occurs because of utilisation of all nutrients in the medium. A return to baseline, however, does not correspond to death of cells but rather a halt in metabolic activity as cells are usually in the stationary phase of growth. Microbes have been shown to be viable over long hours even after the stationary phase of growth and this has been confirmed by viable counts being obtained after plating out at such

periods. This is demonstrated further in subsequent sections in this chapter (Braissant et al., 2013, Hrenovic, 2009, Stulova et al., 2015).

The initial 15-hour lag phase was, therefore, mainly attributable to the fact that the cell population introduced was below the limit of detection and cells were also adapting to their new environment (Stulova et al., 2015). This is visible in the reduction of lag time when a higher inoculum was used to inoculate (Figure 3-4).

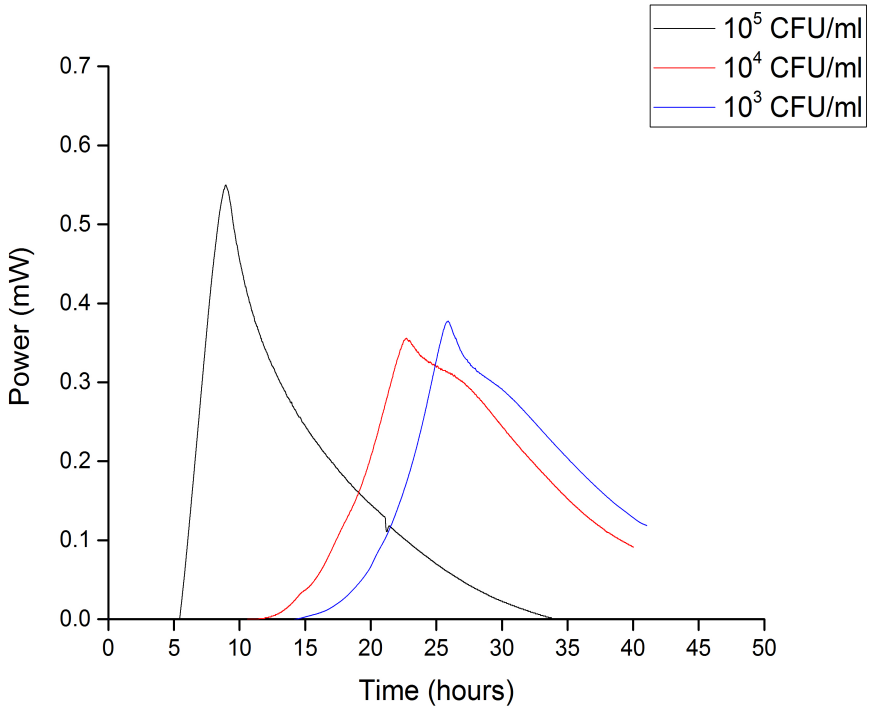

Figure 3-4: A thermogram comparing different concentrations of *L. acidophilus*.

Incubation of lactobacilli was done between 40 – 48 hours rather than overnight since these, like most probiotic species, are slow growing anaerobic organisms (Juturu and Wu, 2015). To further highlight the precision of the ink jetting procedure and its null effect on bacterial viability, thermograms were obtained for pipetted 10^3 and 10^4 CFU/ml bacteria and compared with ink jetted bacteria. The initial rise in the signal obtained for the ink jetted template

(2.8 x 10^3 CFU per template) was between that of 10^3 and 10^4 CFU/ml (Figure 3-5).

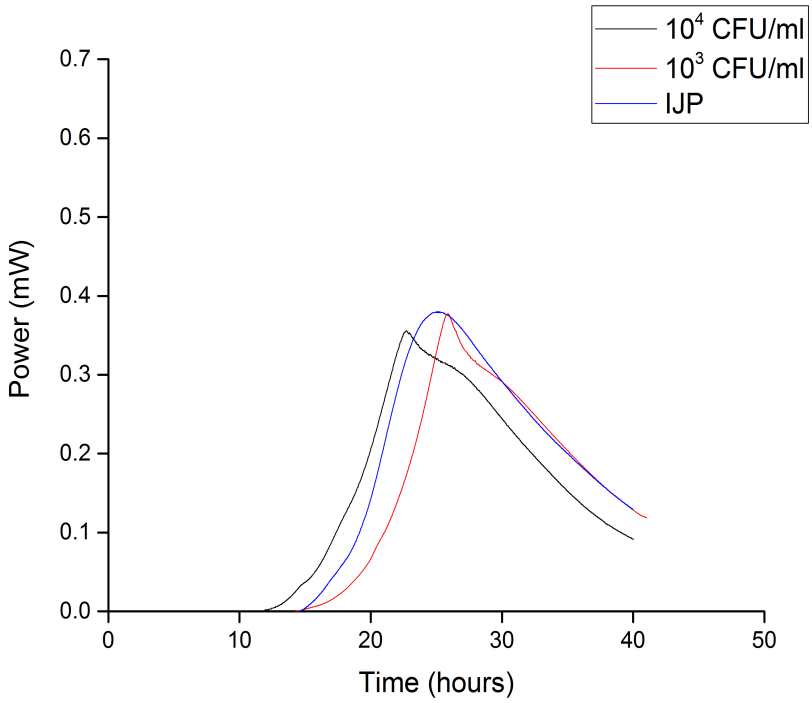

Figure 3-5: A thermogram comparing (2.8 x 10^3 CFU per template) ink jetted (IJP) and pipetted (10^3 and 10^4 CFU/ml) *L. acidophilus*.

Integration of power-time plots resulted in graphs representative of typical bacterial growth curve highlighting more clearly the various phases (i.e., lag, log, and stationary phases) of bacterial growth. These integrals are also representative of cumulative heat energy at any given time point (Braissant et al., 2010c, Stulova et al., 2015, Belaich, 1980). The graph comparing integrals of 10^3,10^4 CFU/ml, and ink jetted population (2.8 x 10^3 CFU per template), Figure 3-6, translates the same set of data as observed in Figure 3-5, with an onset of bacterial activity earlier for 10^4 CFU/ml followed by the ink jetted then 10^3 CFU/ml. It can, however, be noticed that the IJP integral (19.36 ± 1.27) was slightly higher than that for 10^4 CFU/ml (18.86 ± 1.54) at the time of stopping the experiment (Figure 3-7).

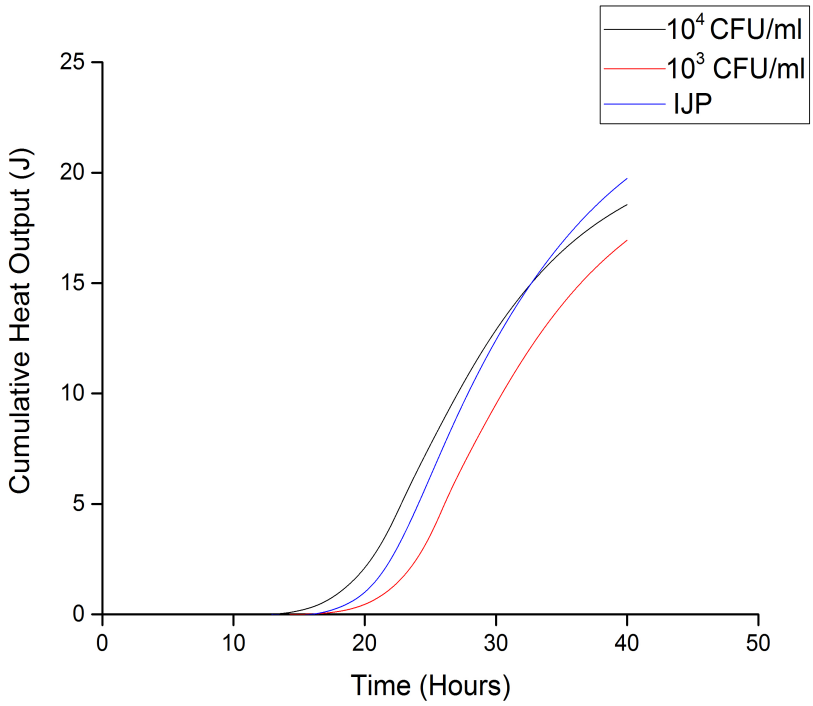

Figure 3-6: An illustration of cumulative heat output comparing (2.8 x 10^3 CFU per template) ink jetted (IJP) and pipetted (10^3 and 10^4 CFU/ml) *L. acidophilus*.

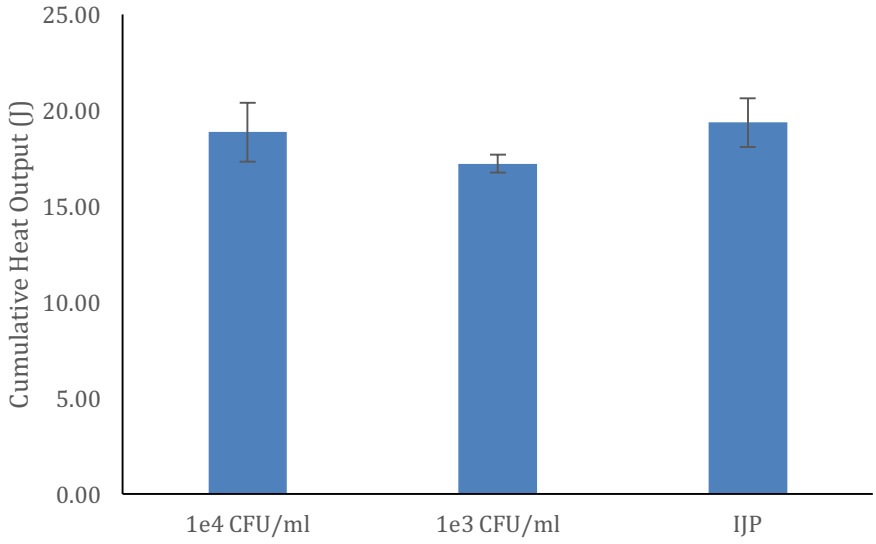

Figure 3-7: A bar chart of the cumulative heat output comparing (2.8 x 10^3 CFU per template) ink jetted (IJP) and pipetted (10^3 and 10^4 CFU/ml) *L. acidophilus*.

A plausible reason could be that with the ink jetted bacteria, the substrate (starch paper) on which the bacteria were deposited albeit small, could be serving as an additional nutrient source as compared to the 10^4 CFU/ml hence, the slightly higher cumulative metabolic heat energy. This implies that in addition to depositing live bacterial cells, ink jetting bacteria onto starch paper could potentially be serving as an additional nutrient supply to the organisms.

3.4.2 Evaluating effect of protectants on bacterial viability

With knowledge of the fact that inkjet droplets are deposited in the picolitre (10^{-12} L) range (Allain et al., 2004), it was imperative to evaluate how best to optimise as well as preserve bacterial viability upon storage. The ink jetted formulation was rehydrated in PBS; other media like deionised water and skimmed milk have been used for rehydration, with no significant differences being observed between these and PBS (Teixeira et al., 1995). Dehydration is known to be a major stressor when probiotics are dried under 45 °C (Broeckx et al., 2016). It was, therefore, important to incorporate an excipient to alleviate this. Trehalose and sucrose have previously been shown to offer good protection during the storage of lactic acid bacteria, as such, these were chosen as additives (Strasser et al., 2009, Leslie et al., 1995, Sun and Davidson, 1998).

When the bio-ink was composed of *L. acidophilus* suspended in 10% trehalose or 10% sucrose, a higher loading, 3.96 ± 0.06 and 4.01 ± 0.18 Log CFU per template (i.e., 9.1 x 10^3 and 1.0 x 10^4 CFU per template) respectively, was observed relative to control, 3.45 ± 0.1 Log CFU per template (2.8 x 10^3 CFU per template) (Figure 3-8). This was an interesting observation as the initial aim for sugar addition was to help organisms survive dehydration over time. Also, the sugars were added to modify the viscosity of the bio-ink to prevent dripping of the bio-ink from the cartridge nozzles. An ideal viscosity range of 2 – 20 mPas is recommended for ink jetting. The addition of 10% sucrose or trehalose produced bio-inks with viscosities of 3.7 ± 0.3 and 3.8 ± 0.4 mPas

respectively, which were within the ideal range. High viscosities lead to decreased speed and accuracy of the expelled droplet, printing fails at extremely high viscosities if the ink cannot flow into the liquid chamber rapidly enough to refill it (Daly et al., 2015, Ihalainen et al., 2015). A student's t-test revealed a significant difference ($p < 0.05$) between the effect of both sugars on bacterial loading and the control. When the effect of 10% sucrose and 10% trehalose on bacterial loading was compared no significant difference ($p > 0.05$) was obtained. This highlighted the importance of the sugars on the formulation and the similarity in activity between them. Trehalose and sucrose have been shown to have similar protectant effects during drying; a superiority of trehalose over sucrose during storage over long periods has, however, been reported (Tymczyszyn et al., 2007b, Crowe et al., 2001, Leslie et al., 1995, Sun and Davidson, 1998).

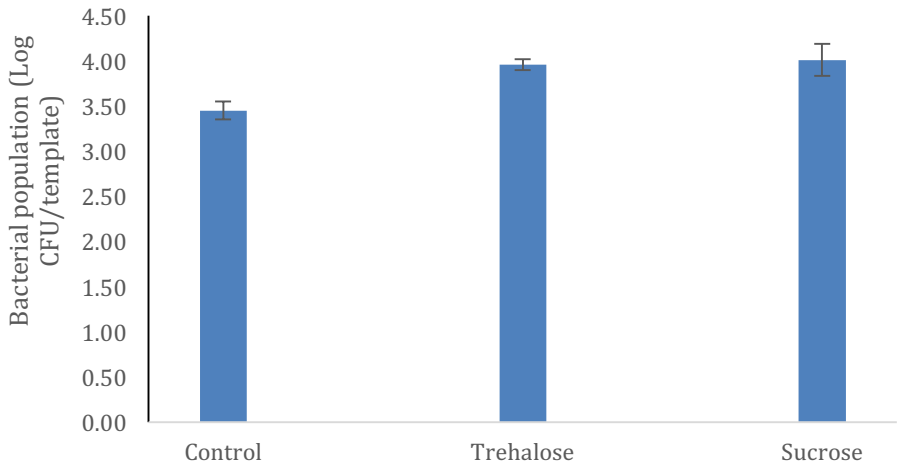

Figure 3-8: An illustration of *L. acidophilus* enumeration after ink jetting formulation composed of 10% trehalose, 10% sucrose, or control (no sugar, i.e., PBS).

In this case, considering how delicate and fundamental this model for probiotic formulation was, the only excipient introduced was a sugar, i.e., 10% sucrose or 10% trehalose. The major stress anticipated here was osmotic stress as a result dehydration of cells (Desmond et al., 2001, Potts, 2001). Both trehalose

and sucrose have been previously documented to offer protection to bacterial cells under high temperatures and other stresses (Strauss and Hauser, 1986, Costa et al., 2000, Zhao and Zhang, 2005, Rumian et al., 1993, Sun and Davidson, 1998, Strasser et al., 2009). A few hypotheses have been proposed for the mechanism of sugar protection, the most popular of these hypotheses is the water replacement hypothesis. This suggests that the replacement of water by sugars between the lipid headgroups of membranes via the formation of hydrogen bonds helps stabilise native structures in a dry state (Crowe et al., 1987, Palmfeldt et al., 2003, Crowe et al., 2001, Tymczyszyn et al., 2007a).

Thermograms obtained indicated a similar trend whereby the formulation with 10% sucrose had a faster onset of activity relative to 10% trehalose and control (Figure 3-9). The AUCs were 23.00 $\pm$ 0.85 J, 21.73 $\pm$ 2.22 J, and 19.36 $\pm$ 1.27 J for sucrose, trehalose, and control respectively (Figure 3-10 and Figure 3-14). This could lead to the assumption that these sugars were being metabolised hence, the resulting increased population. However, considering the short time (less than 30 minutes) between preparation and ink jetting any sugar entering the bacterial cells may not be lost in metabolism thereby making this assumption null (Zayed and Roos, 2004). This phenomenon could be attributed to the fact that these sugars have the potential to form hydrogen bonds with the polar head groups in phospholipids in the cell membrane of bacterial cells (Crowe et al., 1987). It is, therefore, likely that for each droplet being jetted out, sugar molecules could have interacted with bacterial cells to have more bacterial cells together resulting in the differences noted for the loading. Another possible explanation is that, the removal of water from bacterial cells causes these to contract, thereby, reducing the cell volume and drawing the bilayers close together to form a gel-like phase and in the process more cells being entrapped within each droplet (Broeckx et al., 2016, Garvey et al., 2013).

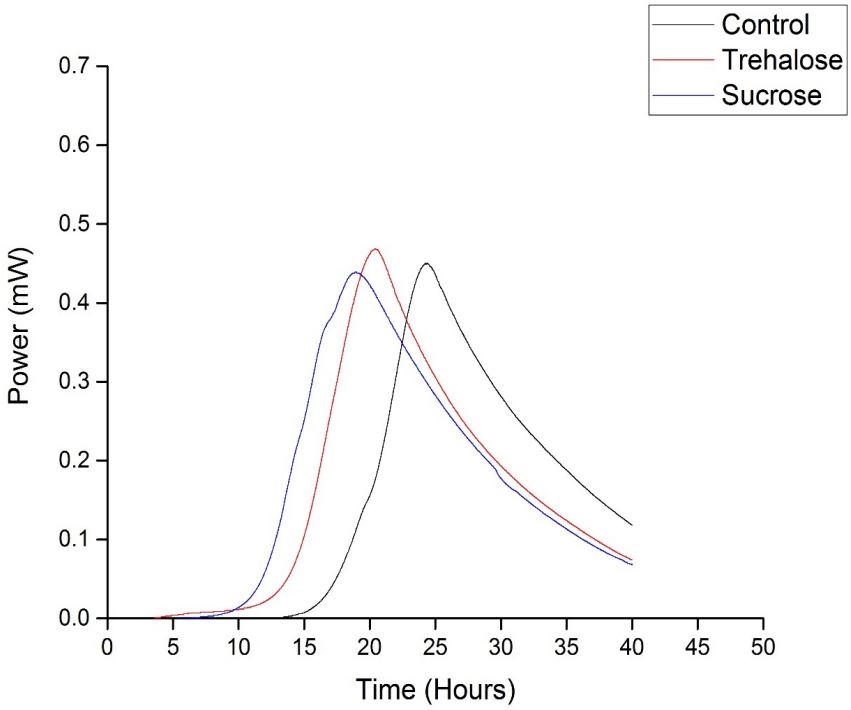

Figure 3-9: A thermogram showing effect of protectant on *L. acidophilus* viability.

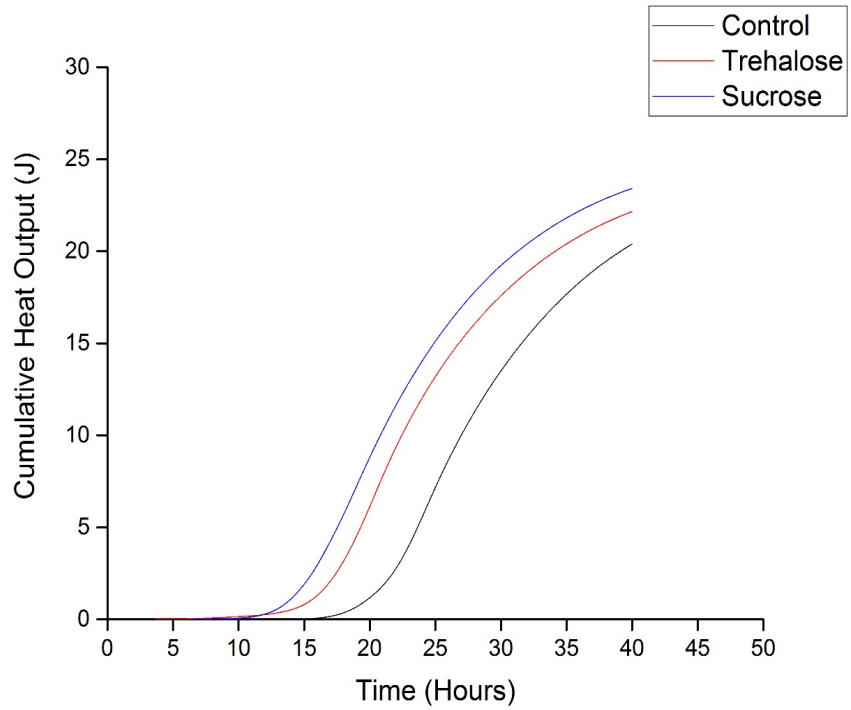

Figure 3-10: Cumulative heat output illustrating effect of protectant on *L. acidophilus* viability.

The immediate application for such a formulation will be as an extemporaneous formulation at point of care (i.e., under hospital settings or in the local pharmacy); the stability was therefore studied over a 7-day period at 2 - 8 ºC during which daily enumeration of the formulation was conducted. This temperature range was chosen as the stability of dried samples decreases during storage with higher survival rates usually being recorded at lower storage temperatures (Carvalho et al., 2004, Wang et al., 2004, Meng et al., 2008). It was during this period that the effect of the sugars was more evident. From the graph, Figure 3-11, it can be observed that there was a reduction in *L. acidophilus* numbers in all formulations, however, this reduction was greatest for the control (1.27 Log cycle) with reductions of 0.86 and 0.88 Log cycles obtained for sucrose and trehalose respectively.

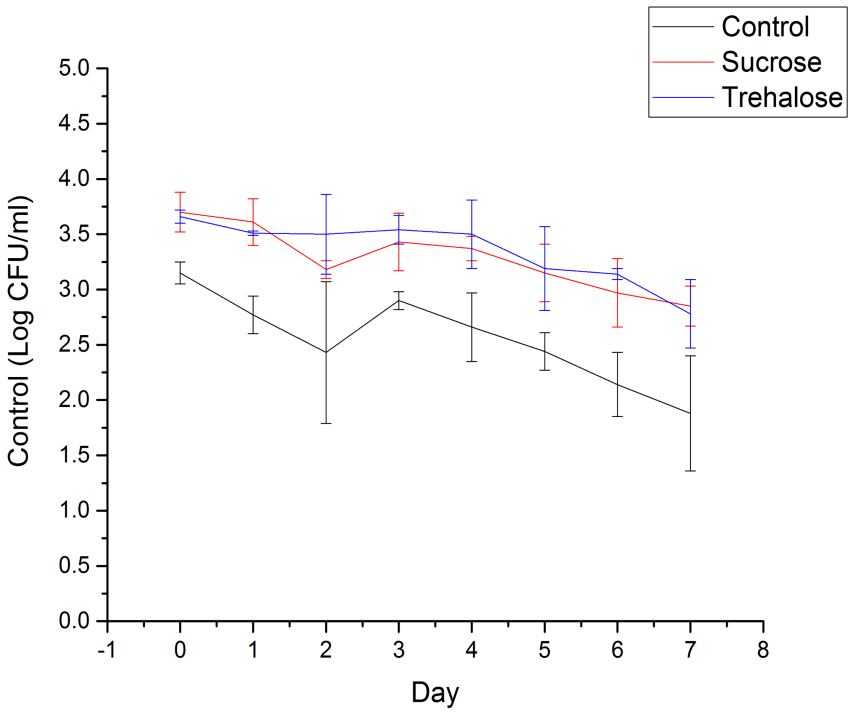

Figure 3-11: An illustration of *L. acidophilus* colony plate counts showing effect of protectants over 7 days.

Thermograms obtained on Day 7 of storage (Figure 3-12), correlated with data obtained for plate counts with trehalose and sucrose almost superimposable and an onset of activity around 15 hours. Integration of these showed growth curves highlighting a similarity between sucrose and trehalose as protectants (Figure 3-13).

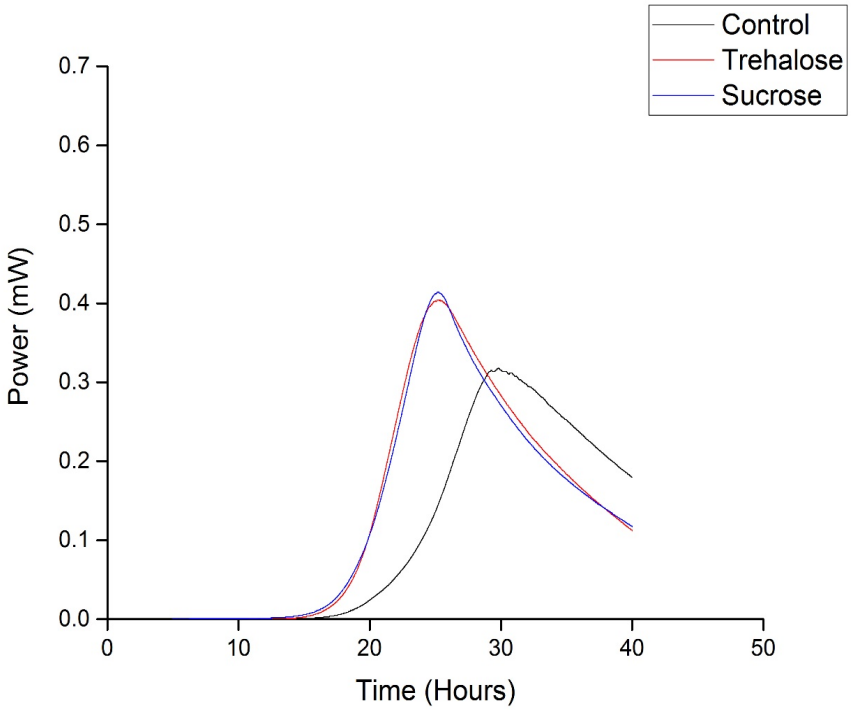

Figure 3-12: A Thermogram for *L. acidophilus* formulated with 10% trehalose, 10% sucrose, and control on day 7.

Comparing the cumulative heat outputs on Days 0 and 7, Figure 3-14, shows relatively higher values for sugars as compared to the control, a similar trend to the colony plate counts observed in Figure 3-11. Further exploration to optimise viability upon storage is required despite the relatively higher numbers when sugars were added to the formulation. By using a higher printing concentration, the impact of this decrease in numbers upon storage can be minimised as long as relatively higher numbers are still available at the time of consumption.

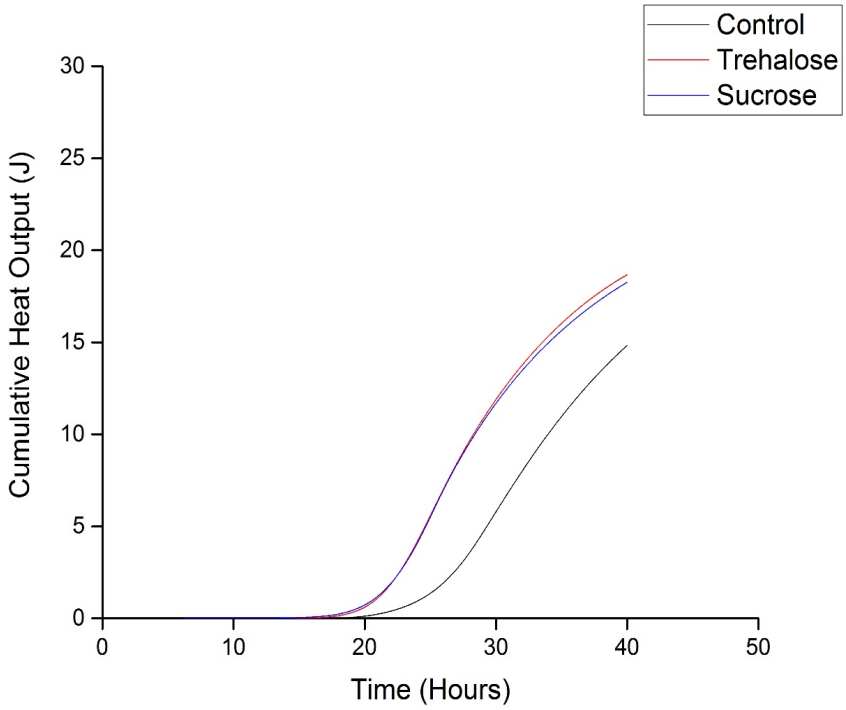

Figure 3-13: An illustration of cumulative heat output for *L. acidophilus* formulated with 10% trehalose, 10% sucrose, and control on day 7.

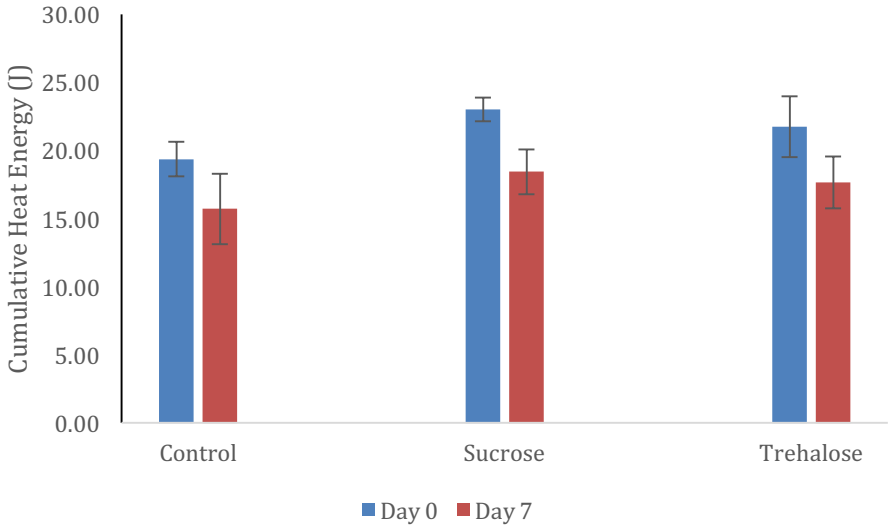

Figure 3-14: Bar graph comparing cumulative heat output on days 0 and 7 for *L. acidophilus* formulated with 10% trehalose, 10% sucrose, and control.

3.4.3 Evaluating tolerance of imprinted *Lactobacillus acidophilus* to simulated gastrointestinal and pig small intestinal fluids

In conventional drug design with chemotherapeutic agents, dissolution tests are conducted to evaluate product release at target sites. The formulation design in this instance involved live cells; it was, therefore, imperative to ascertain the tolerance of this formulation to gastrointestinal fluids. Relevant gastrointestinal media (i.e., media with physiological pH and salt concentrations) were used to mimic gastrointestinal conditions (Aburub et al., 2008). Once ingested, probiotics descend into the stomach for a period depending on the presence or absence of food and then into the upper small intestine and eventually into the lower small intestine and colon where most probiotic activity originates. These different parts of the GIT have different characteristics and interact differently with the probiotics. Gastrointestinal tolerance testing is a common assay in probiotic formulations to assess any influence of gastrointestinal fluid on cell viability (Teoh et al., 2011, Liong and Shah, 2005, Fredua-Agyeman and Gaisford, 2015, Charteris et al., 1998).

Simulated gastro-intestinal media were all freshly prepared to prevent any degradation, especially, pepsin in simulated gastric fluid (Aburub et al., 2008). A baseline signal observed after the formulation was exposed to FaSSGF highlighted the absence of any viable cells (Figure 3-15). This implied that the cells after being exposed to FaSSGF for 2 hours were non-viable. FaSSGF has a pH of 1.2, typical of a strong acid and kills most organisms when in contact (Klayraung et al., 2009). It is usually assumed that because these probiotics are acid-producing organisms, they should be able to withstand the gastric conditions. Probiotic organisms, however, produce lactic acid, which is a weak acid with a pH that is mild thus allowing the probiotic strains to tolerate them. Govender et al., (2014) reported that bacterial viability can drop by about 60% after exposure to gastric fluids.

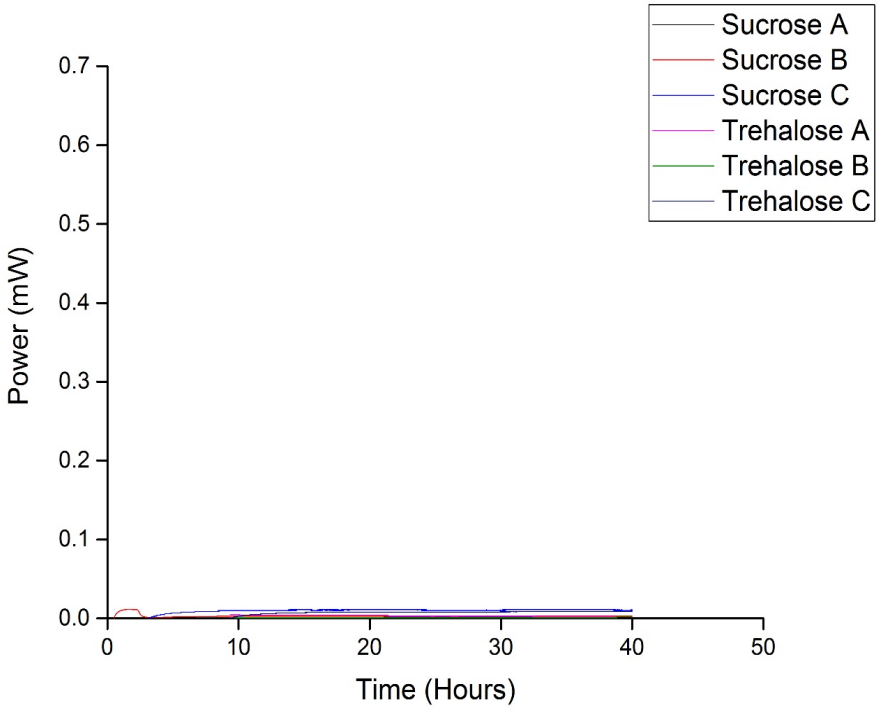

Figure 3-15: Thermogram obtained after exposing *L. acidophilus* to FaSSGF.

When the formulation was exposed to FeSSGF, FaSSIF, and FeSSIF, signals were, however, obtained (Figure 3-16 - Figure 3-18). One attribute of these media is their relatively higher pHs as compared to FaSSGF. The pHs of the FeSSGF, FaSSIF, and FeSSIF used were 4 ± 0.1, 6.5 ± 0.1, and 5 ± 0.1 respectively. It was noted that the peaks for the FeSSGF had a longer lag phase relative to FeSSIF and FaSSIF highlighting the delay in adapting to the growth medium. Despite these organisms being acid-producing they clearly preferred the close-to-neutral pH values. This observation was noted during the development of a general non-selective growth medium to support lactobacilli with an ideal pH between 6.2 and 6.5 being suggested (de Man et al., 1960).

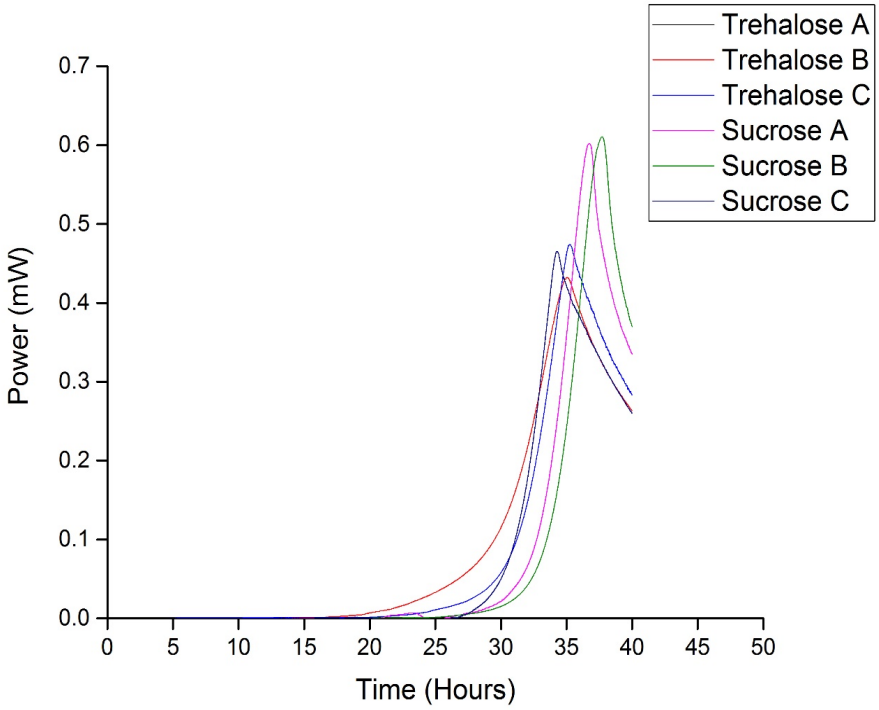

Figure 3-16: Thermogram obtained after exposing *L. acidophilus* to FeSSGF.

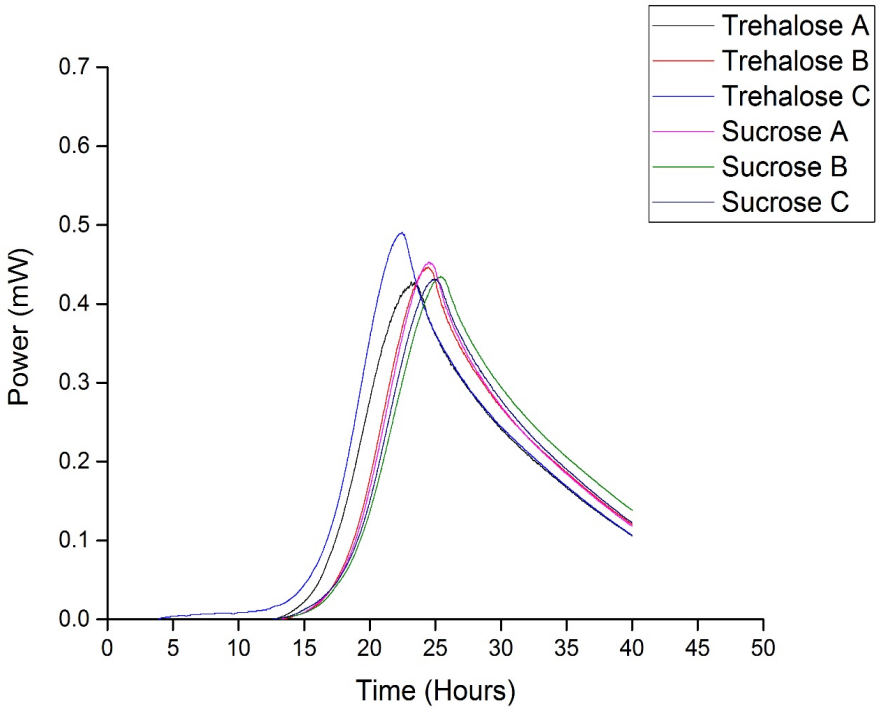

Figure 3-17: Thermogram obtained after exposing *L. acidophilus* to FaSSIF.

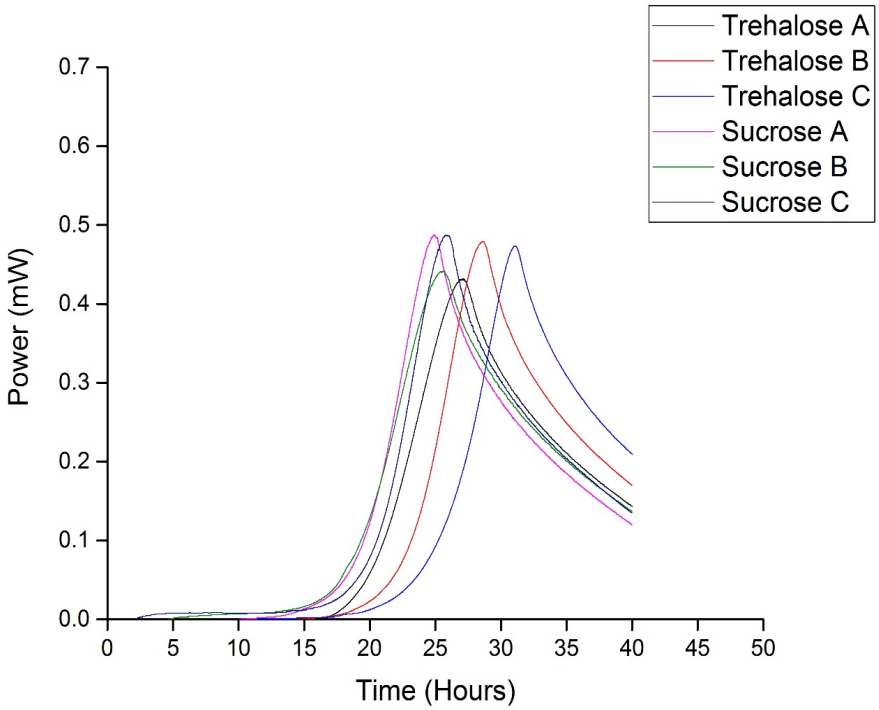

Figure 3-18: Thermogram obtained after exposing *L. acidophilus* to FeSSIF.

The data obtained for the FaSSIF and FeSSIF were not surprising. Colonisation of the small intestines by gut microbiota is known; hence, *L. acidophilus* was expected to withstand exposure to simulated intestinal fluids better than stomach fluids (O'Hara and Shanahan, 2006). Closer mimicking of *in-vivo* conditions using PIF (Figure 3-19) revealed probiotic species being even more tolerant of this medium relative to the biorelevant intestinal fluids. PIF was a more natural medium and could possibly have contained nutrients from food ingested by the pig prior to sacrifice. This nutrient-rich medium could have supported bacterial metabolism and growth compared to the biorelevant media which were composed mainly of salts and surfactants.

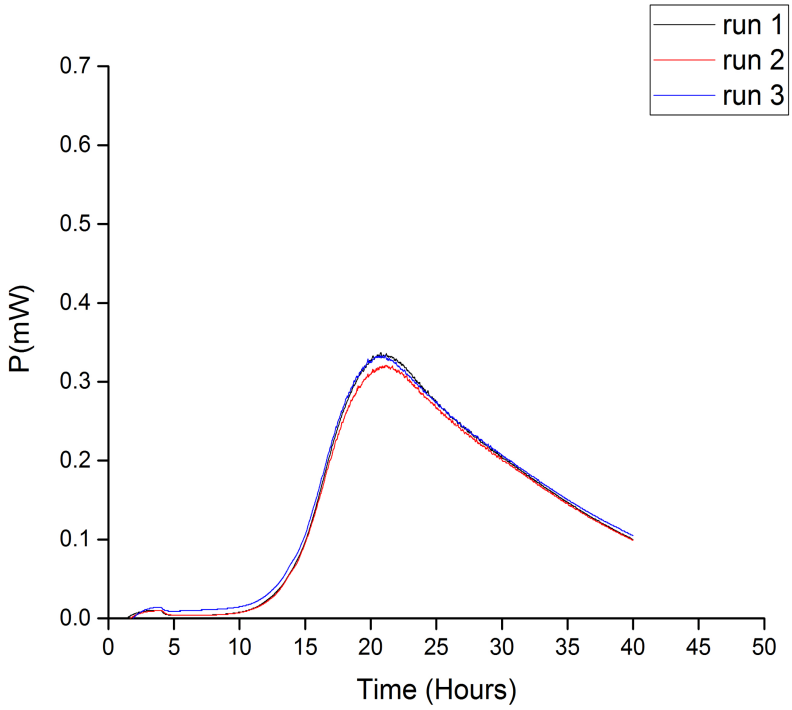

Figure 3-19: Thermogram obtained after exposing *L. acidophilus* to PIF.

Another phenomenon observed from the thermograms was the lower intensity of the PIF signal as compared to the biorelevant fluids. The signal for the PIF had a relatively earlier onset and prolonged signal as compared to biorelevant fluids resulting in a comparable AUC to FeSSIF and FaSSIF as shown in Figure 3-20. Computing the AUC for the signals of other simulated gastrointestinal fluids confirmed the greater tolerance to the biorelevant intestinal and pig small intestinal fluids relative to the biorelevant gastric fluids (Figure 3-20). A similar trend was obtained for the plate counts (Figure 3-21), with the intestinal fluids having higher numbers relative to FeSSGF, with no bacterial growth being observed in FaSSGF.

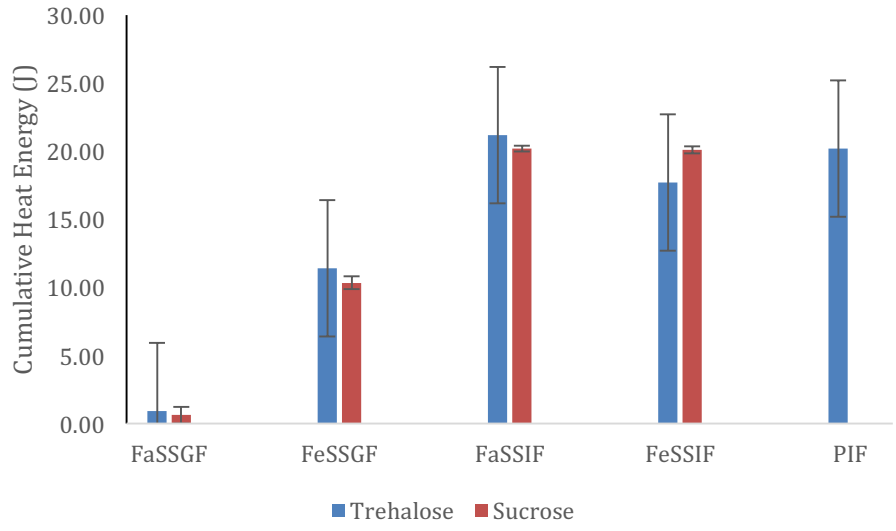

Figure 3-20: An illustration of cumulative heat output for *L. acidophilus* after exposure to simulated gastrointestinal fluids.

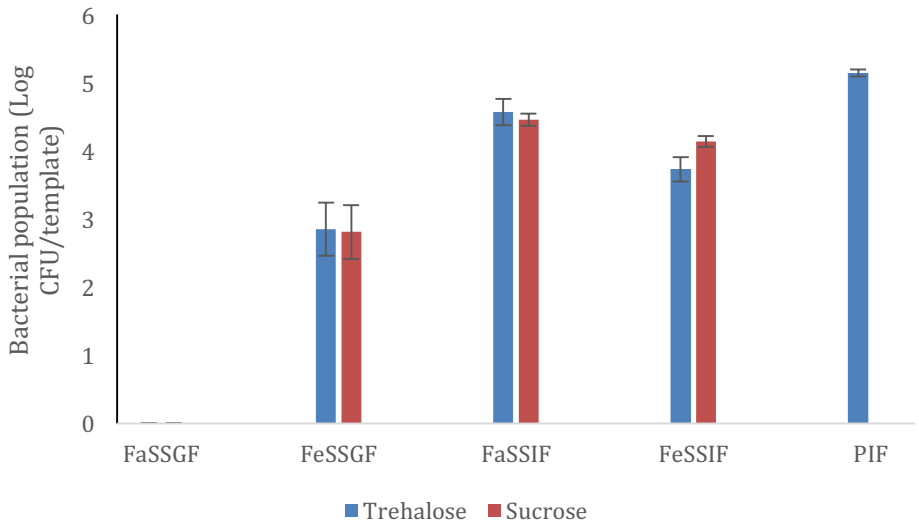

Figure 3-21: An illustration of colony plate counts for *L. acidophilus* after exposure to simulated gastrointestinal fluids.

The comparable plate count data with the calorimetry data further highlight the importance of calorimetry as a useful tool in pre-formulation and formulation. With these data, it was evident that to deliver the formulation in a viable form into the lower small intestines there was the need to overcome the gastric environment. Strategies to overcome this are explored in the next section.

The loading dose was increased by modifying the template dimensions (2 cm x 15 cm) to evaluate any improvement in gastric tolerance over 2 hours. There was no improvement in tolerance to FaSSGF with a loss of viability within 5 minutes (Figure 3-22).

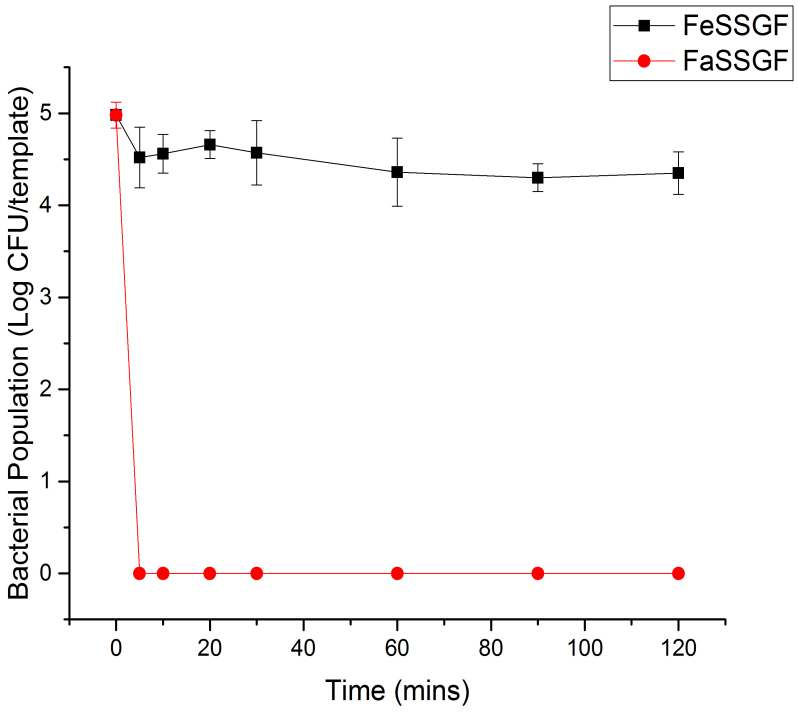

Figure 3-22: An illustration of bacterial enumeration at different times over 2 hours after exposure to FaSSGF and FeSSGF.

Most of the commercial probiotic formulations available are released in the stomach after intake and expected to travel down to the small intestines or colon with gastrointestinal motility. Few products have 'to be taken with meals' indicated on their packages. The science behind this is understandable as the pH of the stomach after a meal can rise to between 4 and 5. This pH is tolerable to probiotics hence, viability can be maintained; however, there is the possibility of these organisms staying in the stomach for a longer time and being exposed to fasted state pH. Gastric emptying after food intake is a complex process which can be further complicated by consumption of snacks in between meals. Gastric emptying is of fundamental value to ensuring that the supply of nutrients and fluid to the body is maintained (Varum et al., 2013, Newton, 2010). Other probiotic products don't have the inscription 'to be taken with meals' on their packages and the claim of probiotics especially, lactobacilli being gastric acid resistant is usually cited. This claim, as demonstrated here and by other researchers, is not entirely true. There is the possibility of certain strains having greater tolerance, but this cannot be applied as a generalisation to all lactobacilli (Douglas and Sanders, 2008).

3.4.4 Overcoming the gastric environment

The Phloral® coating technology could theoretically be adapted to any drug and used for a variety of disease states. With the main components, Eudragit® S which is a synthetic polymer that dissolves at pH > 7 and resistant starch which is not digested by mammalian amylase enzymes secreted by the pancreas but by colonic bacterial enzymes, this technology is useful in drug targeting (Ibekwe et al., 2008, McConnell et al., 2008). The interest in dosage forms, where the release of the active substance can be controlled and modulated – prolonged-release or site-specific delivery – to optimise its therapeutic effect, has increased steadily during the last 50 years (Conti et al., 2013, Tonnesen and Karlsen, 2002). The need to protect probiotics was emphasised recently by Caillard and Lapointe when they evaluated some

commercial probiotics and found that only the enteric coated formulations had resistance after exposure to gastric acid at fasting and all the unprotected formulations had high drops in numbers after exposure to gastric media (Caillard and Lapointe, 2017).

Based on the results from the tolerance testing, it was necessary to overcome the barrier created by gastric fluid. The process of coating capsules can be a major challenge when heat and moisture sensitive materials are involved (Huyghebaert et al., 2004). Since viability of probiotic formulation was key, open capsule coating was the intended coating technique but the effect of coating technique on dissolution testing needed to be verified; this was done using paracetamol. The dissolution profile of paracetamol was similar for both opened and closed capsule coating techniques for gelatin and HPMC (Figure 3-23 and Figure 3-24). Both capsules showed no release after 2 hrs in acid in agreement with the United States and European Pharmacopoeia requirements for such formulations (i.e., less than 10% release after 2 h in 0.1 M HCl). A sharp burst of release was then observed from approximately 150 minutes to 175 minutes. It was during this period that the pH increased above 7, the threshold pH for dissolution of Eudragit® S (Ibekwe et al., 2008). This agreed to work done by Huyghebaert et al. (2004) whereby a similar evaluation was conducted with no difference in the coating techniques being observed.

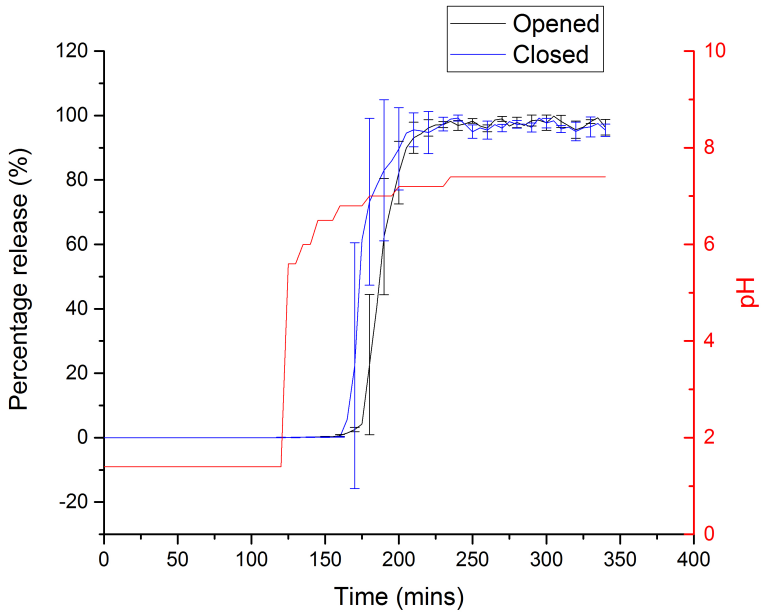

Figure 3-23: Percentage release of paracetamol using Phloral®-coated gelatin capsules comparing opened and closed capsule coating.

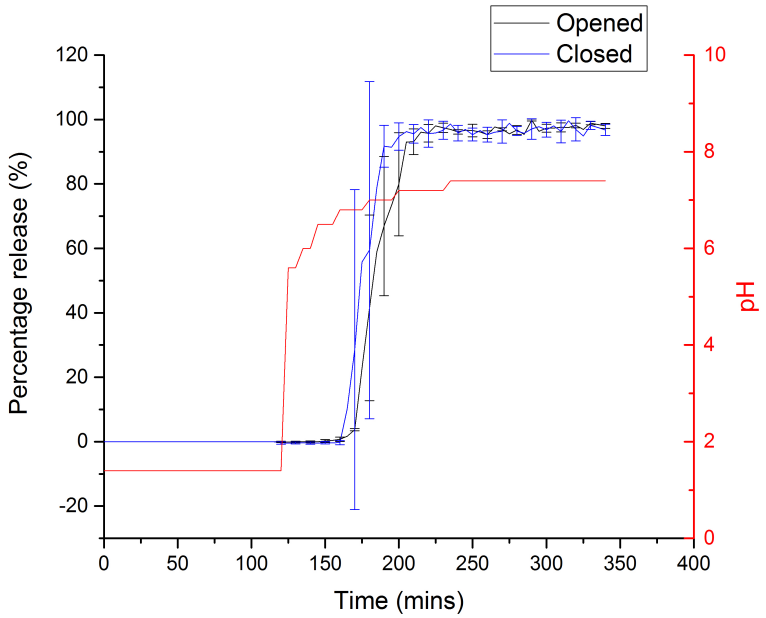

Figure 3-24: Percentage release of paracetamol using Phloral®-coated HPMC capsules comparing opened and closed capsule coating.

Based on the results obtained, pre-coated capsules were used for the probiotic formulation, capsule coating is generally independent of capsule content. This form of capsule coating has potential applications in other drug development and formulation, especially, for heat and moisture sensitive materials. This technique can also be used in retail or hospital pharmacy, and research and development sections of the pharmaceutical industry where enteric coating of capsules offers a problem due to lack of appropriate equipment and training (Huyghebaert et al., 2004, Cole et al., 2002).

The dissolution properties of the encapsulated probiotics after 2 hours in 0.1 M HCl and subsequent transfer into pH 6.8, then 7.4 did not yield promising results (Figure 3-25). No viable bacteria were obtained after enumeration at various time points. No viable counts were expected in pH 1.2 and 6.8 as the coating properties implied an intact polymer since threshold pH for dissolution was not reached, but after pH 7 when capsules had disintegrated, viability was expected. Due to the same reason, an intact capsule, similar outcomes were expected when the capsules were placed individually in these media without subsequent transfer (Figure 3-26 and Figure 3-27). However, when the capsules were placed in pH 7.4, the viable bacteria released were about 90% (Figure 3-28). Of the three media used, based on the tolerance testing and literature, 0.1 M HCl is noted to be the main challenging medium (Govender et al., 2014, Klayraung et al., 2009).

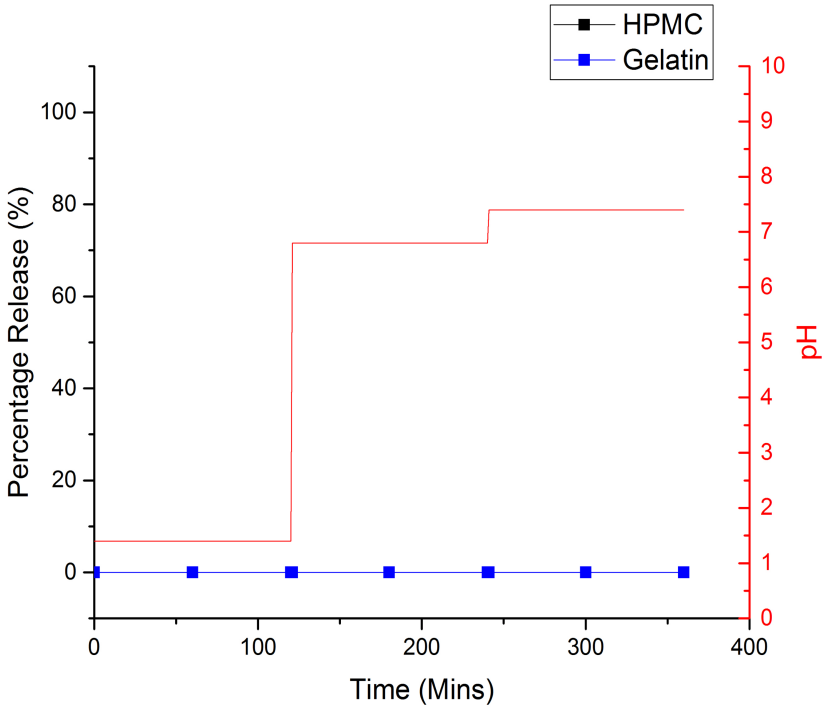

Figure 3-25: Graph illustrating no viable counts after capsules were immersed consecutively for 2 hours in 0.1 M HCl, pH 6.8, and pH 7.4.

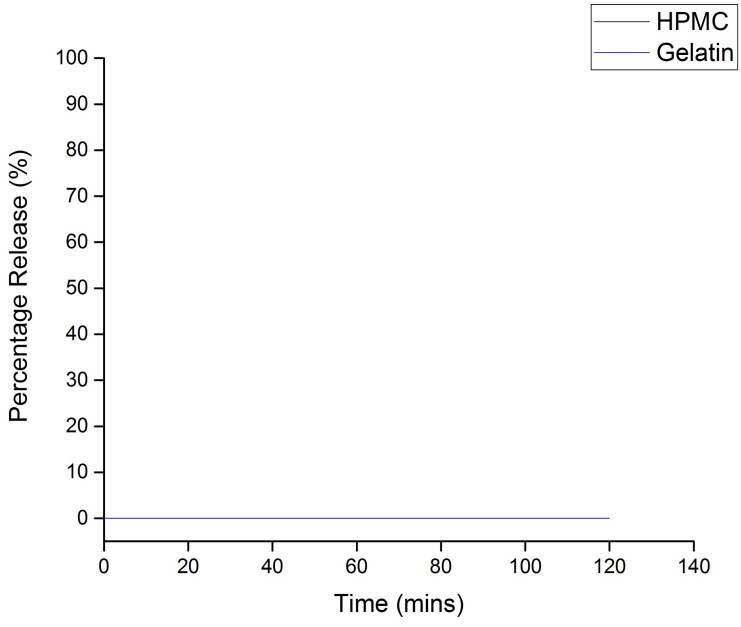

Figure 3-26: Graph illustrating no viable counts after capsules were immersed in 0.1 M HCl for 2 hours.

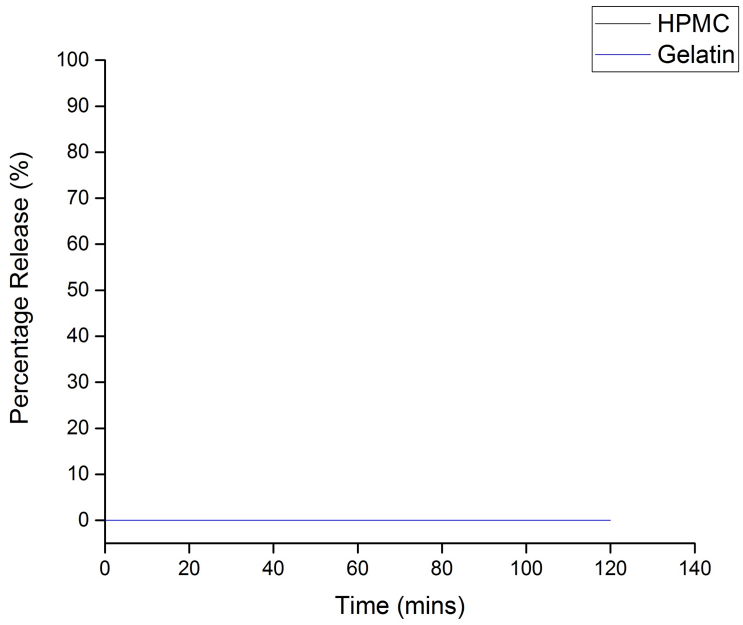

Figure 3-27: Graph illustrating no viable counts after capsules were immersed in pH 6.8 for 2 hours.

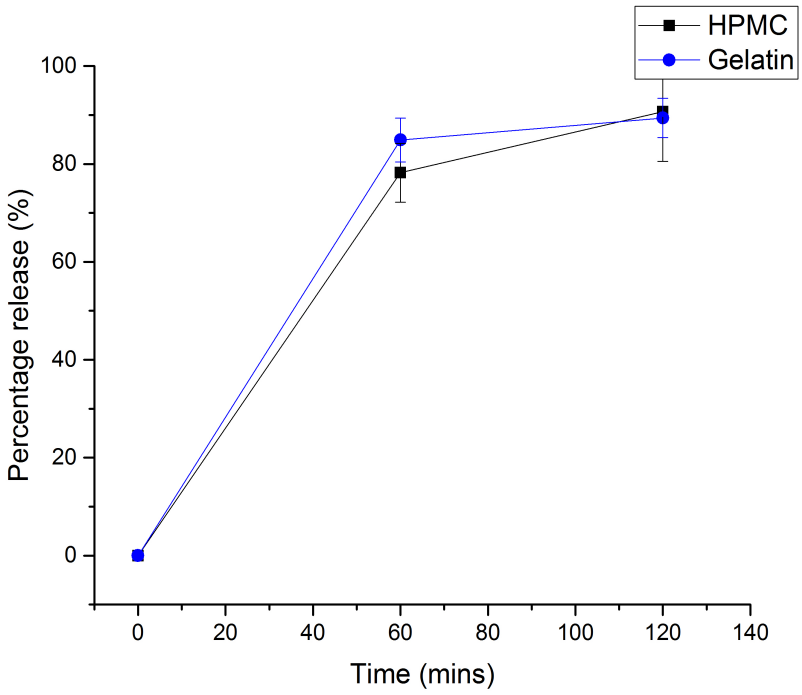

Figure 3-28: Graph illustrating percentage viability after capsules were immersed in pH 7.4 for 2 hours.

With the gastric environment known to be the harshest of the three media, the 2-hour duration in the simulated gastric medium was, therefore, reduced to 30 minutes as this is a reasonable *in vivo* gastric residence time whilst maintaining the duration for the subsequent media (Troncon et al., 1994). This yielded some positive results, 100% and 44.58% recoveries were obtained for gelatin and HPMC respectively (Figure 3-29).

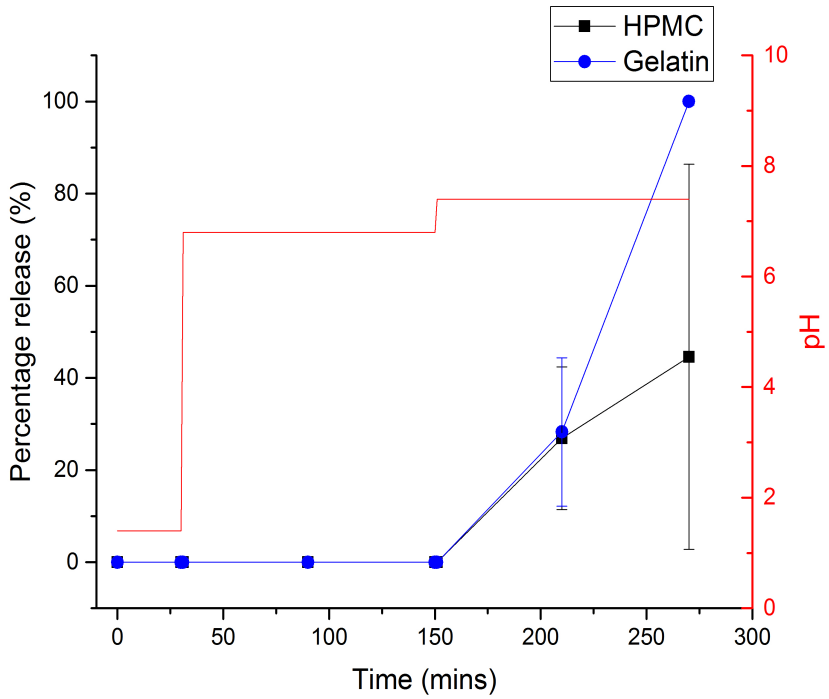

Figure 3-29: Graph illustrating percentage viability after capsules were immersed consecutively for 30 mins in 0.1 M HCl, then for 2 hours each in pH 6.8 and pH 7.4.

There is no standard dissolution test for probiotics in the literature. However, there is the likelihood of conducting dissolution testing for modified-release formulations using the USP standard dissolution testing. With the possibility of estimating bacterial numbers using UV spectrophotometers, the opacity of the medium containing bacterial cells released from capsules can, in theory, be estimated. However, the challenge of contamination and cross-contamination or a very stringent sterilisation process must be in place. Also, the line used for liquid transfer must be disposable since bacterial biofilms could potentially

build up in these (Said et al., 2015). These challenges do not make such an approach commercially attractive.

With gradual advancements in probiotic science, hopefully standards similar to dissolution testing for probiotic formulations will be a reality in future. The dissolution testing method used here was to simulate as closely as possible the USP standard of dissolution testing for modified release dosage forms. The use of the incubator shaker to mimic temperature conditions as well rotor speed has been demonstrated previously (Liserre et al., 2007).

In this instance, however, phosphate buffers were used albeit the advantages of bicarbonate buffers due to the challenge of keeping carbon dioxide evolution under control. The volume of medium used was 5 ml because the objective was to completely immerse capsules which was achieved using sinkers. Secondly, considering the number of organisms deposited on the substrate, a volume that would permit detection of any organisms released was needed. Although large volumes have been recommended for dissolution testing in the pharmacopoeia, in reality volumes as low as 20 ml have been observed in the small intestine after feeding (Schiller et al., 2005). The same group also noted a mean fasted stomach volume of 45 ± 18 ml for 12 individuals. These volumes are very variable, however, with further optimisation of this formulation, greater volumes would be used in analyses.

Protection of these organisms is challenging; in the current formulation using ink jetting, the starch paper used was very hygroscopic, therefore, it could attract fluid upon the least contact. The loss of viability after 2 hours in gastric pH could potentially be due to penetration of the gastric fluid into capsules through the bridge where capsule cap meets the body. In probiotic encapsulation, the objective is not only to protect the cells against adverse environment, but also to allow their release in a viable and metabolically active state in the intestine (Picot and Lacroix, 2004). The results obtained upon reduction of time in the gastric pH clearly indicated the duration of exposure as a critical factor here. Poelvoorde et al. (2008) also demonstrated the release of probiotics from an enteric coated formulation, whereby, probiotics were

loaded onto pellets using skimmed milk as a protectant and subsequently enteric coated the formulation. After 2 hours in 0.1 M HCl, only 1% viable bacteria were obtained. The difficulty of maintaining the viability of gastro-resistant coated probiotics after successive transfer from simulated gastric fluid to intestinal fluids has been observed by other researchers (Huyghebaert et al., 2005, Poelvoorde et al., 2008).

Although USP standards indicate a 2-hour gastric acid immersion, a median gastric transit time of 37 minutes in the fasted state has been reported in human studies (Ibekwe et al., 2008). The improved viability obtained after capsules had been immersed in the gastric medium for 30 minutes was quite promising although improvements to formulation are still needed to meet the 2-hour USP standard. HPMC is usually preferred to gelatin as pre-coating material due to its rough surface which promotes adhesion of coating material (Cole et al 2002). Both materials were successfully coated with Phloral® and all coated capsules exhibited good integrity. The difference in viability obtained was because all the gelatin capsules used demonstrated good bacterial viability when capsules coating dissolved; however, with the HPMC capsules, some exhibited very low viability which resulted in the relatively lower average obtained. It must be noted that comparing individual capsules, some HPMC capsules had similar bacteria released as gelatin capsules.

3.4.5 Testing *in vitro* adhesion of *Lactobacillus acidophilus* to intestinal cells

The Caco-2 cell density available for adhesion was 2.9 x 10^5 – 1 x 10^6 cells per well. Bacterial enumeration after adhesion to Caco-2 cells was 78.3% of the administered bacteria (Figure 3-30).

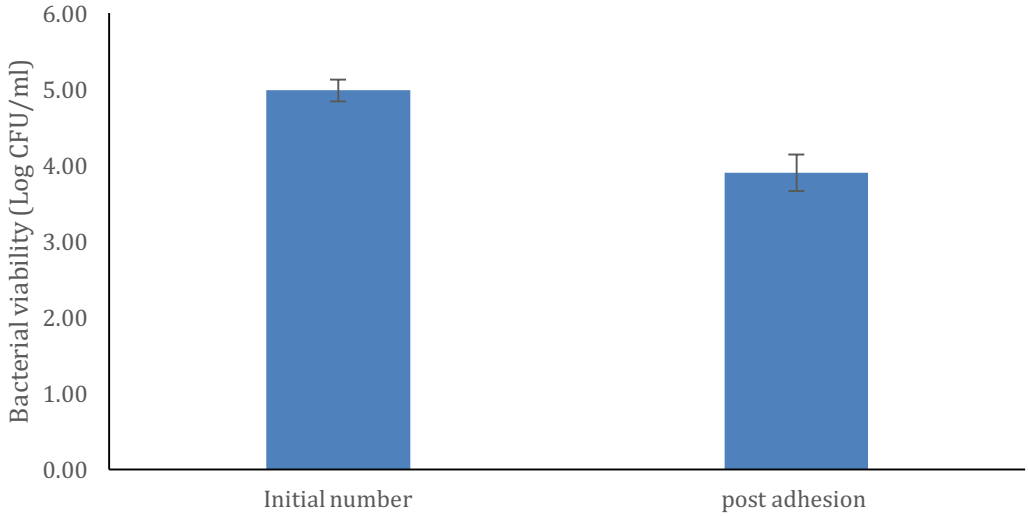

Figure 3-30: An illustration of bacterial population post-adhesion in comparison to initial numbers, error bars represent the standard deviation from mean.

The ability of probiotic strains to adhere to the mucosal surfaces of the intestine and the subsequent long or short-term colonisation is an important criterion for the selection of probiotic strains. Non-adhered bacterial cells usually travel through the intestinal system and are subsequently eliminated from the body before any health benefits are imparted to the individual (Govender et al., 2014, Maragkoudakis et al., 2006, Forestier et al., 2001). One of the proposed mechanisms of probiotic action is the ability to compete with pathogens for adhesion sites in the GIT. A probiotic strain demonstrating good adhesion has the propensity to compete for adhesion sites with pathogenic species (Sanders, 2009, Verna and Lucak, 2010, O'Hara and Shanahan, 2006). Dissolution analysis data are unable to give any information about the effectiveness of probiotic bacteria to adhere to the intestinal wall and to simulate competition with the natural intestinal flora of the human intestinal

system (Govender et al., 2014). Many *in vivo* studies use probiotic cells in faecal matter as an indication of adhesion with the assumption that once viable cells have been excreted, then a proportion would have adhered to the intestines in the process. Cells in faecal matter are cells that have not adhered to the intestinal wall; hence, they are not ideal to use as an indication of adhesion (Govender et al., 2014). The difficulties in performing adhesion testing *in vivo,* especially in humans, have led to the development of *in vitro* models (Greene and Klaenhammer, 1994). Simple models have been developed to estimate probiotic adhesion whereby probiotic numbers after addition to intestinal cells are estimated *in vitro* (Tuomola and Salminen, 1998, Forestier et al., 2001, Maragkoudakis et al., 2006, Gopal et al., 2001). The adhesion of the *L. acidophilus* was performed using Caco-2 intestinal cells, a human colon carcinoma cell line that expresses several markers characteristic of normal small intestinal villous cells (Greene and Klaenhammer, 1994).

Caco-2 cells were sub-cultured every 2-3 days when the cells were approximately 70 - 80% confluent and in the exponential phase of growth. The 78.3% adhesion obtained was promising. Maragkoudakis et al. (2006), using a similar technique for *L. plantarum* ACA-DC 146, obtained a maximum adhesion of 25.5%. The adhesion obtained here was almost three times that obtained Maragkoudakis and colleagues. A possible reason could be the differences in bacterial species used. Some species and specifically strains naturally adhere better than others (Tuomola and Salminen, 1998, Maragkoudakis et al., 2006, Forestier et al., 2001, Gopal et al., 2001). The adhesion obtained highlights the high adhesion propensity of the strain of probiotic used. It also further confirms the absence of loss of cell properties after ink jetting.

3.4.6 Evaluating the antibacterial properties of Ink jetted formulation.

Thermograms for *E. coli* and IJP *L. acidophilus* were obtained individually as well as when co-incubated. Signals for *E. coli* control were seen relatively earlier (less than 2 hours) due to its high rate growth rate; that for the ink jetted *L. acidophilus* was, however, seen after about 10 hours. When these organisms were co-incubated, two independent peaks were obtained, the first peak was almost superimposable to the *E. coli* control. The second peak, however, was not similar to the ink jetted *L. acidophilus* formulation (Figure 3-31). With just two organisms present in the growth medium, the second signal was most likely a growth signal from *L. acidophilus* metabolism. The difference in onset and peak height for *L. acidophilus* could be due to reduced nutrient availability from initial metabolism by *E. coli.* The plate counts were quite interesting; the numbers obtained at 24 hours for *E. coli* when co-incubated with the formulation were very similar to that of *E. coli* control at 24 hours. However, when the same enumeration was conducted at 48 hours, during which time growth of *L. acidophilus* would also be in stationary phase, no viable cells were obtained for *E. coli* in the co-incubated test as compared to the over 7 Log CFU/ml (10^7 CFU/ml) counts in the control. Plate counts for *L. acidophilus* over 48 hours was similar i.e., over 8.5 Log CFU/ml (3.2×10^8 CFU/ml) in both the co-incubated and control test (Figure 3-32).

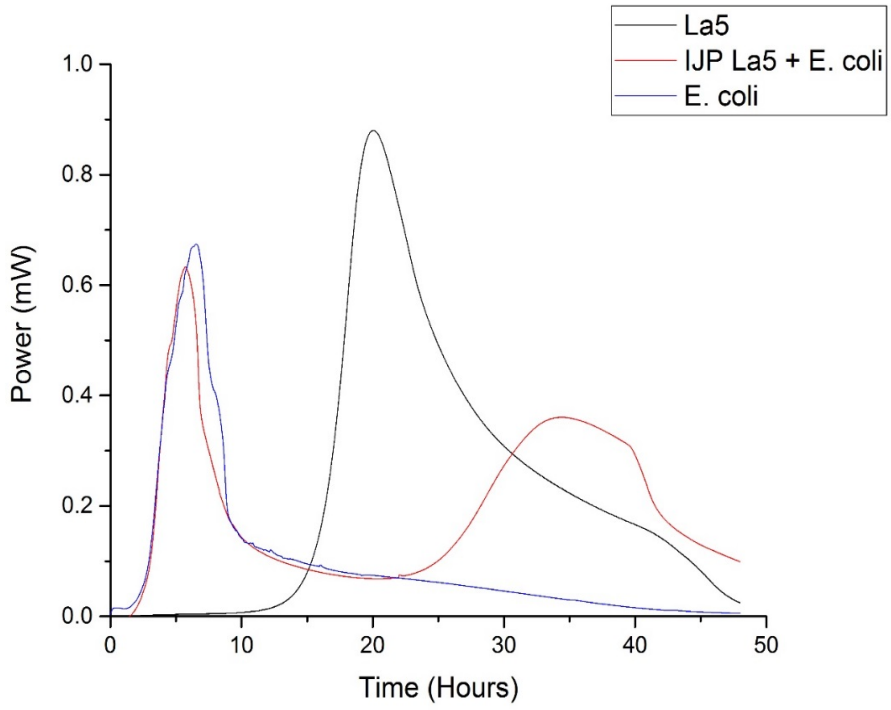

Figure 3-31: Thermograms after co-incubation of ink jetted (IJP) *L. acidophilus* and *E. coli* and their respective controls.

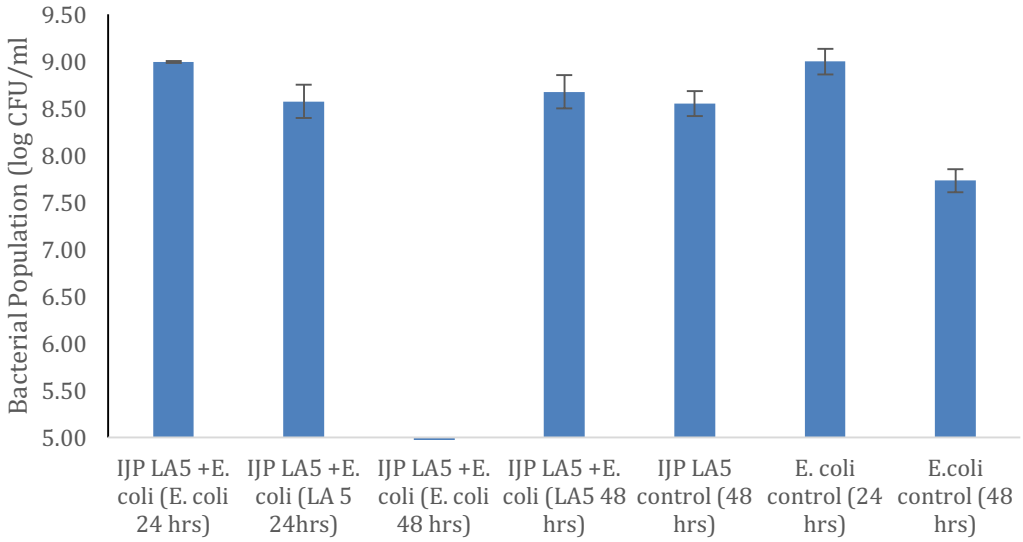

Figure 3-32: Plate counts at 24 and 48 hours after the co-incubation of ink jetted (IJP) *L. acidophilus* LA 5 and *E. coli* and their respective controls.

Intestinal Infectious diseases caused by pathogenic organisms are major causes of mortality in both developed and developing countries. Organisms like *Shigella spp., Vibrio cholerae*, pathogenic *E. coli*, *Campylobacter spp.,* and rotavirus are the usual organisms involved in such cases (Nomoto, 2005). *E. coli* was therefore chosen as the test organism for the probiotic formulation. CMMg, a growth medium that supports the growth of both organisms was used for analyses. Calorimetry was used to monitor in real-time the interactions between these organisms. Other complicated methods are available which mimic closely the current commensal microbiota in the intestinal system for studying interactions between an organism and the microbiota. An example of such a model is the Simulator of the Human Intestinal Microbial Ecosystem (SHIME) (Molly et al., 1993, Alander et al., 1999).

The growth rate and the generation time are often used to indicate how fast a bacterial culture grows. A change in these two parameters can indicate a positive or negative effect of a treatment on cultures and can be useful when investigating new antimicrobial compounds (Braissant et al., 2013). Among the proposed mechanisms of probiotic activity include the release of chemicals or substances with antibacterial activity, competition for adhesion sites and available nutrients, and production of acids which make the milieu unfavourable for pathogenic bacterial growth (Sanders, 2009, Govender et al., 2014, Verna and Lucak, 2010). In this instance, however, since growth occurred in a liquid medium, no competition for adhesion sites occurred. Competition for nutrients can be a major factor here; *L. acidophilus,* being a slow growing organism, attained its exponential growth phase when the *E. coli* was already in its stationary phase. This scenario makes competition for nutrients a very difficult mechanism for *L. acidophilus* to antagonise *E. coli* growth. Microorganisms are known to stay in the stationary for a very long time even without nutrients (Juturu and Wu, 2015). It was, therefore, expected that *E. coli* numbers would be maintained over the 48-hour period as obtained with plate counts for the *E. coli* control. This was not the case when the *L. acidophilus* growth was complete; no *E. coli* colonies were obtained from the medium after enumeration. This could be that *L. acidophilus* may have

released antibacterial substances into the growth medium during its growth and/or produced a very unfavourable medium for *E. coli* from acid released during growth (Forestier et al., 2001). *E. coli* generally adapts well to acidic environments; as such release of acid by *L. acidophilus* did not cause the complete elimination of *E. coli* population (Gomez Zavaglia et al., 2003, Benjamin and Datta, 1995). The release of antibacterial substances by *L. acidophilus* could, therefore, be the major reason for the elimination of *E. coli* population. Another possibility could be that the *E. coli* organisms had entered a viable but non-culturable (VBNC) state which could have resulted in no colonies being obtained after enumeration (Bogosian and Bourneuf, 2001, Su et al., 2013). Polymerase chain reaction assays could be useful in deducing whether the *E. coli* cells were dead or in a VBNC state. A live/dead staining technique could also have helped ascertain the state of *E. coli* cells (Willenburg and Divol, 2012, Wu et al., 2015, Green et al., 2011). The scenario observed here, whereby, pathogen growth was established before that of the probiotics is the exact opposite of what was observed by Fredua-Agyeman and colleagues evaluating the activity of probiotics against *Clostridium difficile*. The inhibition of *C. difficile* growth by probiotics that they observed was attributed to the fact that probiotic population was well established before pathogenic species. Hence, in addition to reduced nutrients being available for growth of *C. difficile*, production of growth-inhibitory substances from probiotic metabolism could have contributed to the inability of *C. difficile* to establish (Fredua-Agyeman et al., 2017). In this scenario, the pathogenic species, *E. coli,* had an established population before probiotic growth began yet, total elimination of *E. coli* was observed after 48 hours. This brings a new dimension to probiotic activity in that, most of the recommendations of probiotics are as preventive therapies, however, in instances where an infection is already established there could be a potential of using probiotics as effective means of eliminating some pathogenic species.

3.5 Conclusion

After demonstrating the null effect of ink jetting on bacterial cell viability in the previous chapter, the potential of ink jetting in formulating probiotics on-demand was explored in this chapter. With several studies confirming reduced viability in some commercial products and even total absence of viable organisms in certain products; this model will allow clinicians to formulate their own probiotics for trials. The ease of formulation will also promote further research and development in probiotic science and aid verification of some of the health claims attributed to probiotics.

The bio-inks used in ink jetting were formulated using 10% trehalose or 10% sucrose as excipients to protect the bacterial cells from dehydration. Both sugars proved beneficial, with higher bacterial loading and bacterial viability over 7 days relative to a control with no sugar. These cells were ink jetted onto starch paper as a substrate which was also found to serve as an additional nutrient source for these probiotics.

An evaluation of the ink jetted formulation to gastrointestinal fluids revealed a lack of tolerance to gastric acid despite, the model probiotic organism being a lactobacillus. To overcome this, the probiotic species were encapsulated in Phloral® coated capsules for delivery into the lower small intestines and colon. The Phloral® -encapsulated probiotics could withstand 30 minutes in 0.1 M hydrochloric acid and subsequent 2-hour transfers into pH 6.8 and 7.4 with viable bacterial obtained after capsule dissolution. To further evaluate probiotic properties of the formulation, the ability of ink jetted probiotic to adhere to intestinal cells was demonstrated with 78.3% of delivered organisms adhering to Caco-2 cells.

The antibacterial property of the probiotic formulation was evaluated against *E. coli*. It was observed that although *E. coli* was a relatively faster growing organism compared to *L. acidophilus,* when co-incubated, no *E. coli* colonies were obtained upon plate counting. This demonstrated the potential of using probiotics not only as preventive remedies but the possibility in treatment.

Chapter 4 Probiotic Oral Films

4.1 Introduction

The oral cavity is anatomically connected to the nasopharynx, the larynx, the tonsils, the middle ear through the Eustachian tube, and the gastrointestinal tract. Therefore, it influences, and is influenced by, general human health (Teughels et al., 2011). The mouth is continuously washed with saliva, producing warm (35 – 36 °C) and moist conditions with a pH range of 6.75 – 7.25, which favours microbial growth (Marsh, 2003). The oral cavity is a complex ecosystem of rich and diverse microbiota. These organisms have differing nutritional, atmospheric, and physicochemical (pH) requirements which influence the composition of the microbiota. Other parameters like shedding and non-shedding surfaces, salivary, and crevicular fluids also influence composition; homeostasis is, however, often attained in the healthy host. These organisms are usually present either in the planktonic state or integrated into biofilms (Caglar et al., 2005, Bizzini et al., 2012). Various lactobacilli and a few bifidobacteria and *Streptococcus* strains have been shown to inhabit the healthy mouth (Hojo et al., 2007, Rastogi et al., 2011).

The normal microbiota is recognised to protect the oral cavity from infections (Haukioja, 2010). Infections and diseases of the oral cavity constitute some of the commonest and costly forms of human infections. In the UK, the National Health Service spends over £1.6 billion per annum on dental treatment (Marsh, 2003). These usually occur due to changes in the resident oral microbiota resulting in the dominance of potential pathogens. Dental caries and periodontal diseases are globally considered the most important oral health problems (Caglar et al., 2005, Bizzini et al., 2012). Dental plaque – a biofilm of microorganisms on the tooth surface – is an aetiological factor in the development of these oral diseases (Dhawan and Dhawan, 2013).

Dental caries is one of the most prevalent chronic diseases worldwide and individuals are susceptible to this throughout their lifetime. *Streptococcus mutans* has been identified as the main causative organism; *Streptococcus*

sobrinus is also known to be a causative agent in some instances. In dental caries, the production of organic acid together with rapid sugar metabolism results in a low pH environment and eventual demineralisation of the tooth structure. Inhibiting colonisation of *S. mutans* is reported to have a role in preventing the formation of dental plaque and development of dental caries (Dhawan and Dhawan, 2013, Yao and Fine, 2014, Bizzini et al., 2012, Cagetti et al., 2013).

Periodontal disease is a chronic, inflammatory disease affecting the supporting structures of the teeth. It results from bacterial infections and may cause bone loss, bleeding and erythema of the gingival tissue, and mobility of the teeth. Periodontal disease may at times be preceded by inflammation of the gums (gingivitis). Periodontal diseases in comparison with caries has less information available regarding the effects of beverages/foods on disease aetiology (Marsh, 2003, Spratt et al., 2012, Yao and Fine, 2014, Yanine et al., 2013).

Another condition arising due to bacterial imbalance is halitosis, an unpleasant odour emanating from the breath which can significantly impact social interactions. Halitosis is not a disease but a discomfort, although some oral diseases including periodontitis may be the underlying cause. Oral microorganisms, especially those on the tongue and in periodontal pockets, are suspected to be the primary cause of halitosis and it can occur in all ages. Volatile sulphur compounds (e.g. hydrogen sulphide and dimethyl sulphide) which are by-products of metabolism by certain oral microorganisms are among the common malodourous substances released (Haukioja, 2010, Bizzini et al., 2012, Burton et al., 2005).

The impact of these on individuals is significant, the pain and impairment of function accompanying dental caries and periodontal disease is considerable (Bizzini et al., 2012). The best approach to managing oral infections is to prevent initial occurrence. Good oral hygiene, a reduction in intake of sugary foods, and a stop to habits like smoking are ways to prevent the initial occurrence (Cagetti et al., 2013, Spratt et al., 2012). Traditional treatments of

oral diseases are considered very expensive (Bizzini et al., 2012). Other methods of treatment often involve mechanical removal (brushing, tongue cleaner, dental floss) of the bacteria/plaque with or without antibiotics (Yao and Fine, 2014). However, it is difficult to achieve complete eradication with most of these organisms repopulating quickly and recolonising the oral surfaces once antimicrobial use is stopped. These agents also stay in the mouth at effective doses only for a short period (minutes) and are mainly below MIC levels for prolonged periods (hours) (Cagetti et al., 2013, Marsh, 2003, Burton et al., 2005, Heinemann et al., 2013). Fluoride is also known to reduce occurrence in dental caries, although, it has limited ability when there is an infection (Caglar et al., 2005). Plant and fungal extracts like mushroom and chicory homogenates, and quinic acid (also found in mushrooms) have shown promising results as anti-caries or anti-gingivitis agents but require further studies to evaluate effectiveness (Spratt et al., 2012, Conti et al., 2013).

An ecological approach to treatment has been proposed involving the administration of non-virulent bacteria (i.e. probiotics) to prevent recolonisation of these pathogenic species after antimicrobial therapy. These can also be adopted for use after rinsing with oral antiseptics, such as chlorhexidine. Treating oral infections by reducing the pathogenic bacterial population and facilitating a favourable environment that allows tooth remineralisation has been suggested to halt disease progression (Bizzini et al., 2012, Dhawan and Dhawan, 2013, Wescombe et al., 2012, Tagg and Dierksen, 2003). There are numerous commercial products that have probiotics incorporated in different formulations for promoting oral health. ProBiora3® from Oragenics – a probiotic mouthwash which contains *Streptococcus oralis*, *Streptococcus uberis,* and *Streptococcus rattus* – is reported to promote fresh breath, whiter teeth, and gum and tooth health. PerioBalance® lozenges (which are reported to promote healthy teeth and gums, reduce plaque, and fight bad breath); KForce Breath Guard® mouthwash and lozenges; and various probiotic forms of BLIS K12 utilise *Streptococcus salivarius* as the main probiotic organism (Yao and Fine, 2014, Bizzini et al., 2012, Tagg and Dierksen, 2003).

The presence of sugars (e.g. sucrose and glucose) increases the risk of dental diseases like dental caries. Sugar alcohols especially xylitol, on the other hand, have been reported to suppress growth of *S. mutans* and in effect reduce risk of dental caries. Xylitol is a natural sweetener that can be found in foods as berries, fruits, vegetables and mushrooms. It, however, has minimal effects on the non-pathogenic species like *S. salivarius* and *L. rhamnosus* (Ohshima et al., 2016, Cagetti et al., 2013, Marsh, 2003). Xylitol has been considered a prebiotic since it is non-fermentable by most oral bacteria. It also stimulates saliva secretion thereby aiding in the mineralisation or remineralisation of tooth structure (Kojima et al., 2016, Leepel et al., 2009, Soderling, 2009).

4.1.1 Probiotics and oral health

Consumer interest in the possible preventive and health maintenance benefits of oral probiotics, as well as research into oral health claims, have led to an increased interest in the use of probiotics in oral health (Bowen, 2012, Marsh, 2003). A balance between the beneficial and pathogenic bacteria determines the oral health of an individual. The mechanism by which probiotics act in the oral cavity is not well understood; it is assumed to be a result of immunomodulation as well as non-immunologic defence mechanisms that influence the development and stability of the microflora and inhibit colonisation by pathogens. Modification of the environmental conditions of the oral cavity via pH and/or oxidation-reduction potential is reported to prevent stain and plaque formation (Dhawan and Dhawan, 2013). Co-aggregation of certain lactobacilli has also been noted to prevent the formation of dental plaque. Development of strains which can colonise the oral cavity and stay there long enough to produce an effect is paramount in tackling oral infections (Twetman et al., 2009, Bizzini et al., 2012, Rastogi et al., 2011, Caglar et al., 2005, Yao and Fine, 2014). The potential use of probiotics in the management of oral diseases may reduce the cost of conventional treatment. The idea of replacing pathogenic organisms in the mouth with non-harmful variants is considered attractive (Dhawan and Dhawan, 2013).

A few organisms have been used experimentally in oral probiotics evaluations. Chewing gums made with strains of *L. reuteri* ATCC 55730/ATCC PTA 5289 have been shown to significantly reduce levels of *S. mutans* (Caglar et al., 2009). *L. rhamnosus* GG and *S. salivarius* K12 are also common candidates used in oral probiotic formulations. *L. rhamnosus* strains are reported to offer oral health benefits. These have been used extensively as probiotics for improving the GIT. These are suggested to antagonise activities of cariogenic streptococcus species. *L. rhamnosus* GG is the most widely used strain (Meurman, 2005, Caglar et al., 2005). *S. salivarius* strains are among the early colonisers of the oral cavity and are usually present in high numbers throughout the lifetime of the healthy host, with various benefits at any given age as shown in Figure 4-1. Although *S. salivarius* is not normally considered a food supplement like other probiotics for improving the GIT; it has a safe profile (GRAS) and has been used mainly for consumer oral health products. Strain K12 is the most popularly used and thought to produce bacteriocins for protection against oral pathogens and throat infections (Wescombe et al., 2012, Hale et al., 2012, Burton et al., 2005, Haukioja, 2010, Devine and Marsh, 2009).

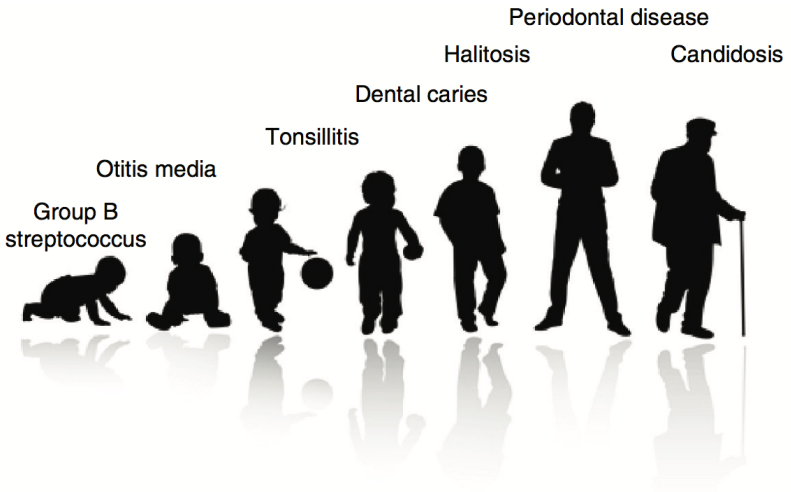

Figure 4-1: Diseases that may be alleviated by *S. salivarius* probiotics and the ages at which they generally tend to manifest (adapted from Hale et al., 2012).

A potential risk from probiotic use is the production of acid from metabolism of dietary carbohydrates which could increase the risk of caries formation;

however, with the right strain selection, this can be overcome. Also, consumption of probiotics with milk as a vehicle is suggested to reduce acidogenic properties of probiotics by the buffering action of milk (Meurman, 2005). Benefits are also known to be derived from the calcium in the milk in strengthening teeth (Meurman, 2005, Cagetti et al., 2013, Bizzini et al., 2012). Xylitol has been used as a sweetener in chewing gums to prevent plaque formation. It has also proved beneficial in preventing biofilm formation. Although xylitol has proved to be effective in plaque control, in individuals with poor oral hygiene, there is reduced plaque control. Similarly, some strains of *S. mutans* have been reported to be resistant to otherwise effective xylitol amounts (Soderling, 2009, Badet et al., 2008).

An ideal approach in the treatment of oral diseases is to deliver or apply formulation directly into the mouth and stay there for an extended period. Oro-dispersible films are a delivery mechanism that can be used to achieve this.

4.1.1.1 Probiotic oral films

The use of oro-dispersible films (ODFs) for oral drug delivery is not a new concept. Drugs like rasagline mesylate and salbutamol sulphate, have been successfully formulated for oral delivery (Genina et al., 2013, Buanz et al., 2011). There are several commercial products including Listerine® Pocket Pack/Breath Strips (mouth refresher containing essential oils) by Pfizer/Johnson & Johnson, Triaminic Thin Strips® (phenylephrine and diphenhydramine) by Novartis, prescription medications like Suboxone® sublingual films (buprenorphine and naloxone) by Reckitt Benckiser Pharmaceuticals, and Breakyl® Buccal films (fentanyl citrate) by Meda Pharma (Preis et al., 2015).

ODFs allow for suitable delivery of actives in people with swallowing difficulties. ODFs, as a drug delivery mechanism, are attractive because they involve a cost-effective manufacturing process and are easy to use as a dosage form. It is also an innovative way of personalising medicines extemporaneously for

both local and systemic delivery. A major drawback is the low drug loading which makes ODFs suitable for low-dose and highly potent drugs (Krampe et al., 2016, Preis et al., 2013, Buanz et al., 2015, Heinemann et al., 2013).

Due to its impact on probiotic viability, it is necessary to impart as little stress as possible to these organisms during probiotic formulation. Ideally, a probiotic vehicle should be suitable for all ages since early exposure, in the case of babies, may facilitate permanent colonisation of health promoting strains (Singh et al., 2011).

Solvent casting and hot melt extrusion (HME) are the main formulation approaches adopted; both require the drug to be mixed into the polymer matrix. Flexography, a contact printing method that uses rotating rollers to deposit the printing solution onto the substrate has also been explored (Krampe et al., 2016, Buanz et al., 2015). A very high temperature accompanies HME, this can lead to a reduction in cell viability when used in formulating probiotics (Scarpa et al., 2017, Maniruzzaman et al., 2012). The contact with rotating rollers in flexography also has implications of potentially contaminating the ODFs. There is also a challenge of cleaning rollers after formulating which adds to making flexography an unattractive technique. The impact of organic solvents used in solvent casting on bacterial cells is a challenge with this technique in formulating probiotics. Although the solvents evaporate, residual solvent has been reported to be present (Krampe et al., 2016, Maniruzzaman et al., 2012).

The possibility of formulating probiotic oral films using IJP for the treatment of oral infections was, therefore, explored. In addition to this method being a non-contact technique, it has been proved not to damage bacterial cells in earlier chapters. This method has the advantage of tailoring ODF to patients' needs, whether a combination of strains is needed or a probiotic-prebiotic combination. The phosphate ion solubilisation by probiotic strains was also evaluated. Phosphate plays a significant role in the susceptibility of teeth to caries progression and is needed for teeth mineralisation and remineralisation (Abou Neel et al., 2016).

4.2 Aims

- To evaluate the suitability of starch paper as an oro-dispersible film substrate for printed medicines.
- To evaluate the phosphate ion solubilising potential of selected probiotic strains.
- To formulate oro-dispersible films of probiotic strains and xylitol.
- To evaluate the potential of the ink jetted probiotic strains and xylitol in treating oral infections.

4.3 Materials and Method

4.3.1 Growth media and reagents used

Columbia blood agar, MRS (de Man, Rogosa, Sharpe) agar, nutrient agar, cooked meat medium, anaerobe basal broth, defibrinated horse blood, and mitis salivarius agar were from Oxoid, UK. Phosphate-buffered saline tablets, ¼ Ringer's solution tablets, and glycerol were purchased from Fisher Scientific, UK. Trehalose, xylitol, and glucose were from Sigma-Aldrich, UK. Bacitracin was from the Wellcome Trust, UK.

4.3.2 Organisms

The microorganisms used were *Streptococcus salivarius* NCTC 8618, *Streptococcus mutans* NCTC 10449, and *Lactobacillus rhamnosus* NCIMB 30174.

4.3.3 Culture stock preparation

S. salivarius and *S. mutans* were grown on Columbia blood agar supplemented with 5% defibrinated horse blood (CBA_{hb}) and incubated under anaerobic conditions for 48 hours at 37 ºC. A few colonies were taken and used to inoculate 7 ml of anaerobe basal broth supplemented with 5% horse blood to create a starter culture and incubated for 24 hours. 99 ml of fresh anaerobe basal broth supplemented with 5% horse blood was inoculated with 1 ml of the starter culture to create a 1: 100 dilution and this was incubated for 24 hours. The culture was then mixed using a magnetic stirrer to ensure homogeneity and dispensed into falcon tubes. Centrifugation was done at 9500 rpm and 4 ºC for 10 minutes to harvest the cells after which the supernatant was removed carefully by suction. The cells were washed with phosphate-buffered saline and centrifuged at 9500 rpm and 4 ºC for 10 minutes. The supernatant was

removed by suction and the washing process repeated. The cells were resuspended in ¼ Ringer's solution made up with 15%$^{v}/_{v}$ glycerol acting as a cryoprotectant. The bacterial cultures were mixed continuously using a magnetic stirrer and 1.8 ml of the culture was rapidly dispensed aseptically into 2 ml cryovials (Nunc). Sealed vials were immersed gently into liquid nitrogen for 10 minutes after which the frozen vials were removed and stored in a freezer at – 80 ºC. Post-freezing enumeration was 10^6 CFU/ml for both species.

L. rhamnosus was prepared for storage using same media and procedure for *L. acidophilus* as documented in section 2.3.4.

4.3.4 Substrate characterisation

Potato starch paper (Lakeland, UK) was used as the substrate. Substrate characterisation involved determining its thickness, disintegration, and topography using scanning electron microscopy.

4.3.4.1 Substrate thickness

The thickness of substrates (n=5) was measured using a digital micrometer (Fowler, USA). 2 cm x 2 cm substrates were used and the readings taken from the four corners and the centre.

4.3.4.2 Disintegration testing

A method used by Garsuch and Breitkreutz, (2010) was adopted here. The substrate (2 cm x 2 cm) was placed in a petri dish containing 2 ml of distilled water. The gelation times of the films (n=5) were recorded as the disintegration time.

4.3.4.3 Scanning electron microscopy (SEM)

Scanning electron microscopy (SEM) was used to observe the topography of the starch paper. The samples were prepared by sputter coating with gold for 3 minutes (Quorum model Q150). The images were then obtained with the scanning electron microscope (SEM, Quanta 200 FEG, FEI, Netherlands).

4.3.5 Phosphate ion solubilisation assay

Phosphate ion solubilisation was assessed using in-house prepared Pikovskaya agar plates. 10 µl of exponentially grown culture was spot-inoculated on plates and incubated at 37 °C for 14 days. *L. rhamnosus* and *S. salivarius* were the organisms tested and *S. mutans* was used as a positive control. The composition of the prepared Pikovskaya agar is indicated in Table 4-1.

Table 4-1: Pikovskaya medium composition in 1L

Ingredient	Quantity (g)
Glucose	10
Tricalcium Phosphate	5
Ammonium sulphate	0.5
Yeast extract	0.5
Sodium chloride	0.2
Potassium chloride	0.2
Magnesium sulphate heptahydrate	0.1
Manganese sulphate monohydrate	0.002
Ferrous sulphate heptahydrate	0.002
Agar	20

4.3.6 Optimum concentration of xylitol against *S. mutans*

A stock solution of xylitol was prepared. Different volumes were added to cooked meat medium supplemented with 2% w/v glucose (CMMg) to give final xylitol concentrations of (0.1, 0.5, 1, 5% $^{w}/_{v}$) in sterile 3 ml calorimetric glass ampoules. Thawed culture of microorganisms (*S. salivarius* and *S. mutans*) were then inoculated separately – 30 µl of 10^6 CFU/ml – into these ampoules. The ampoules were sealed with crimped caps and vortexed for 10 seconds. Calorimetric readings were taken afterwards.

4.3.6.1 Ink jetting optimum xylitol concentration

Based on the results from section 4.3.6, 0.5% xylitol solution was chosen as the optimum xylitol concentration that inhibited *S. mutans* but was tolerated by *S. salivarius*. The amount of xylitol that had to be ink jetted onto acetate paper to give a concentration of xylitol equivalent to 0.5% xylitol solution in a 3 ml ampoule was determined. A calibration curve between 0.5 mg/ml and 30 mg/ml was obtained using HPLC. A stock solution of xylitol (0.5 g/ml) was prepared and used to deposit templates of varying dimensions onto acetate paper. The print-outs were then carefully cut and immersed in 1 ml deionised water to dissolve the xylitol. The xylitol solution was vortexed to ensure complete dissolution after which HPLC analysis for xylitol as described in the USP was conducted.

The liquid chromatographic system used was Agilent Technologies 1200 series with quaternary pump and degasser. The column used was a Phenomenex SCX column (250 mm x 4.60 mm, 5 µm). The mobile phase consisted of water: acetonitrile mixture (80:20) with a flow rate of 0.5 ml/min. A column temperature of 80 ºC and wavelength of 192 nm were used for detection. Each assay was run for 10 mins.

4.3.7 Evaluating recovery of *S. salivarius* from oral films

Frozen cells were thawed in a water bath at 40 °C for 3 minutes. 3 vials were concentrated together to give 10^7 CFU/ml. The bio-ink was then made by suspending this population (1 in 10) in either PBS (control) or 10% trehalose. A rectangular template (2 cm x 15 cm) was used for ink jetting onto edible starch paper.

Calorimetry was conducted by carefully inserting the imprinted starch paper into sterile 3 ml calorimetric ampoules filled with 3 ml of pre-warmed (37°C) CMMg. The ampoules were then hermetically sealed by crimping and vortexed for 10 seconds. Calorimetric readings were then taken for 20 hours.

4.3.8 Evaluating antibacterial properties of *S. salivarius* and xylitol formulation

The antibacterial property of the probiotic formulation was investigated against *S. mutans* and monitored using calorimetry with CMMg used as the growth medium. A power-time curve of *S. mutans* (10^4 CFU/ml) co-incubated with ink jetted *L. acidophilus* (2 cm x 15 cm) and xylitol (2 cm x 16 cm) formulation was obtained. Post-calorimetry plating was conducted after 24 hours using mitis salivarius agar and mitis salivarius agar supplemented with 15% sucrose and 0.2 i.u of bacitracin per ml were used to differentiate between the streptococci. As a reference, pure cultures of both species were streaked onto both media to confirm selectiveness.

A cell-free supernatant (CFS) of *S. salivarius* was obtained and used in the antibacterial evaluation. CFS was obtained by incubating ink jetted *S. salivarius* in CMMg for 5 days. This was then centrifuged at 9500 rpm and 4 ºC for 10 mins, the supernatant was then decanted and filtered using 0.22 µm diameter filters to obtain the CFS. Varying volumes of the CFS were added to ampoules containing CMMg inoculated with *S. mutans* and the calorimeter used to monitor growth.

4.3.9 Evaluating tolerance of *S. salivarius* to artificial saliva

Artificial saliva was prepared using the recipe for GlaxoSmithKline's (GSK) standard in-house saliva formulation (Table 4-2). The pH was adjusted to 7 and sterilised by filtration using a membrane filter of 0.22 µm pore size.
A 2 cm x 15 cm template was used to deposit *S. salivarius* (10^7 CFU/ml) onto potato starch paper and suspended into 5 ml artificial saliva for 2 hours at 37 °C. Vortexing was done for 30 seconds before and after incubating. 30 µl of the resultant artificial saliva mix was added into 3 ml sterile glass ampoule containing CMMg. The ampoules were then hermetically sealed and vortexed. Calorimetric readings were taken afterwards for 20 hours. A purity plate was set up for each experiment by taking a loopful of culture and streaking out onto an CBA_{hb} plate.

Table 4-2: Recipe for artificial saliva (GSK's standard in-house formulation)

Ingredient	Quantity (g)
Sodium chloride	0.175
Potassium chloride	0.100
Calcium Chloride	0.100
Distilled water	100

4.4 Results and Discussion

4.4.1 Substrate characterisation

The ODFs presented a mean thickness of 160 ± 3 µm and were very porous as shown in Figure 4-2. This value, however, did not meet the suggested 70 µm thickness reported to be suitable for consumers (Garsuch and Breitkreutz, 2009). The starch paper was chosen based on it being edible and to demonstrate the recovery of organisms after ink jetting onto a substrate.

For the 5 films tested, the gelation time ranged between 35 to 80 seconds. As there is no official test for evaluating disintegration of films, the method used by Garsuch and Breitkreuz, (2010) was adopted. Observation of gelation time was, however, found to be quite subjective since films did not dissolve. This approach will be most suited for fast dissolving films.

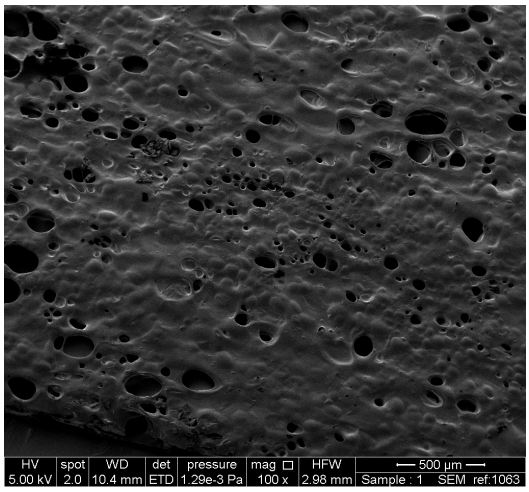

Figure 4-2: Electron micrograph of starch paper used, showing its porous nature.

All the tests were done with the plain potato starch with no organisms deposited. Since the organisms will be deposited onto substrate rather than incorporated into the film forming matrix the properties of the plain substrate will be similar to that of the ink jetted substrate (Buanz et al., 2015).

4.4.2 Phosphate solubilisation assay

After incubating organisms on Pikovskaya agar plates under anaerobic conditions for 14 days, a phosphate-solubilising organism presents a halo/clear zone around the site of inoculum. This was observed for both *L. rhamnosus* and *S. mutans* whilst no halo was observed for *S. salivarius* (Figure 4-3 - Figure 4-5). Pikovskaya medium is used in soil microbiology to evaluate phosphate solubilisation of by soil microorganisms (Goteti et al., 2014, Kumar Meena et al., 2015).

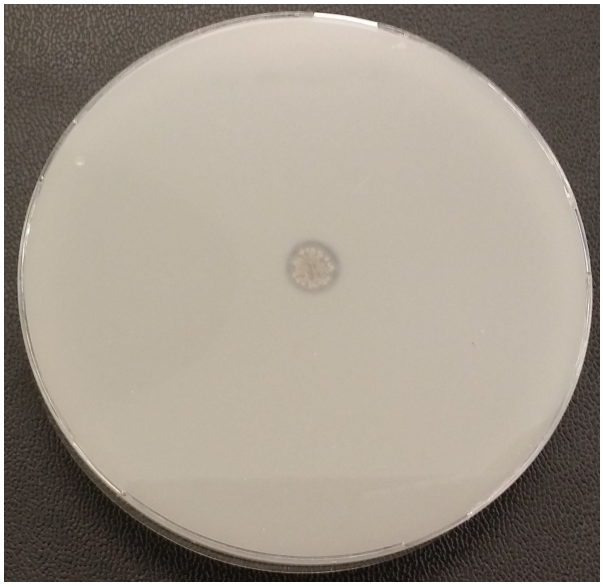

Figure 4-3: Image after incubating *S. salivarius* on pikovskaya agar with no visible halo after incubation.

Figure 4-4: Image after incubating *L. rhamnosus* on pikovskaya agar showing a visible halo after incubation.

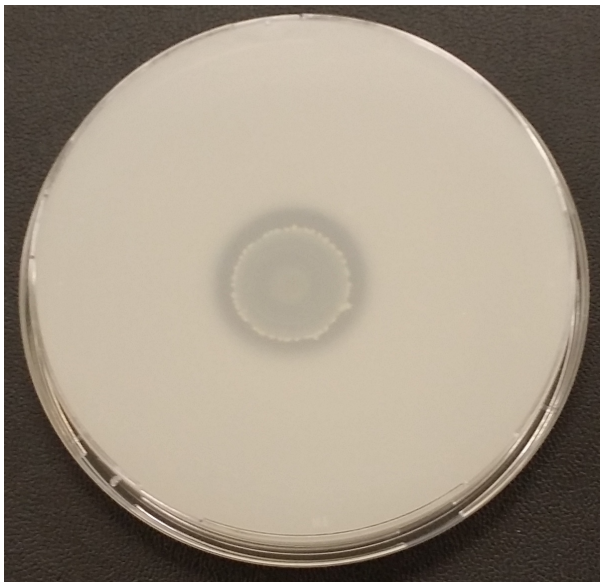

Figure 4-5: Image after incubating *S. mutans* on pikovskaya agar showing a visible halo after incubation.

A very important, yet omitted, test in most oral probiotics evaluation is the phosphate ion solubilising potential of probiotic organisms. Phosphate is an important ion needed for teeth mineralisation and remineralisation, and plays a significant role in the susceptibility of teeth to caries progression. The teeth are also comprised of a phosphate-based mineral, apatite, in the enamel and dentine (Abou Neel et al., 2016). Any activity that results in the decrease in phosphate amounts has implications on teeth integrity and should not be used for oral treatment or be used with caution.

S. mutans, the primary organism that causes dental caries is a known phosphate-solubilising organism. This was, therefore, used as a positive control in the evaluation of the two probiotic candidates being evaluated. Incubation was done for 14 days since maximum solubilisation would have occured during this period (Goteti et al., 2014). After incubation, it was observed that both *S. mutans* and *L. rhamnosus* had halos indicative of phosphate solubilisation whilst *S. salivarius* did not. The *L. rhamnosus* strain was, as a result, not used for the further formulation of ODFs. This, however, does not mean *L. rhamnosus* is not a suitable candidate for oral formulations. As with most probiotics, this activity may be limited to the NCIMB 30174 strain.

4.4.3 Optimum concentration of xylitol against *S. mutans*

The effect of varying xylitol concentration on growth and metabolism of *S. salivarius* and *S. mutans* are shown in Figure 4-6 and Figure 4-7 respectively. The AUCs which give an indication of the cumulative heat output are also represented in Figure 4-8 and Figure 4-9 for *S. salivarius* and *S. mutans* respectively. It was observed that increasing xylitol concentrations to 1% did not have any significant impact on *S. salivarius* metabolism. Peaks of similar characteristics were obtained for concentrations used except 5% xylitol. A different scenario was observed for *S. mutans*, xylitol concentrations as low 0.1% were enough to result in delayed metabolism, concentrations of 0.5% resulting in the very inhibited onset of metabolism.

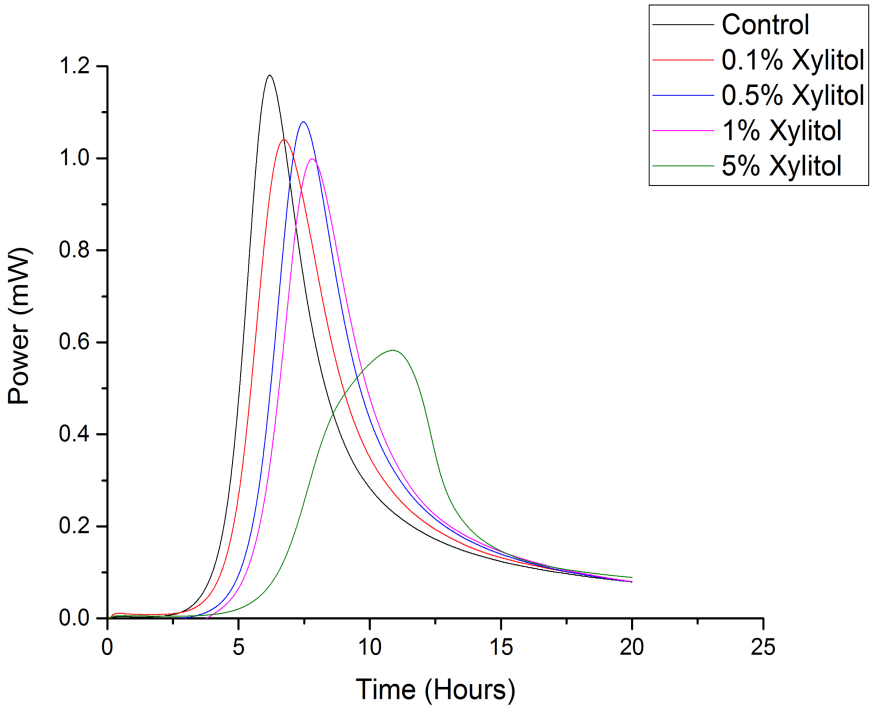

Figure 4-6: Effect of varying xylitol concentrations on *S. salivarius* metabolism.

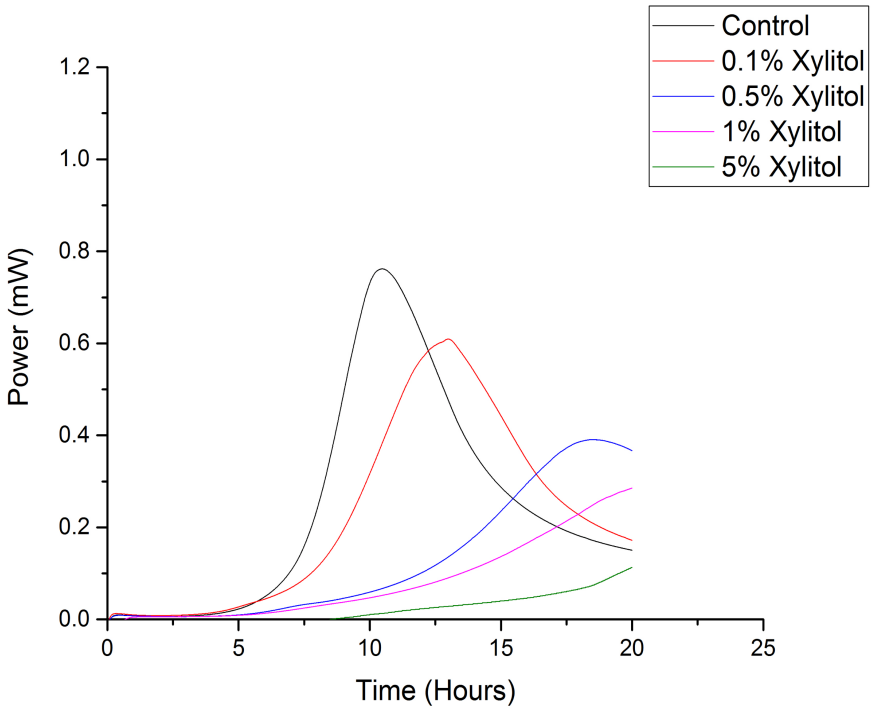

Figure 4-7: Effect of varying xylitol concentrations on *S. mutans* growth.

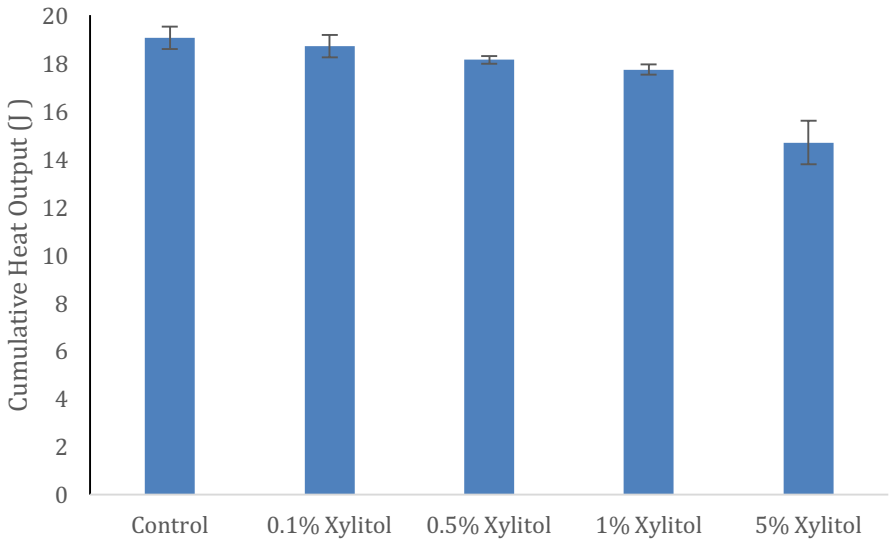

Figure 4-8: Cumulative heat output representative of total AUC for *S. salivarius* under varying xylitol concentrations.

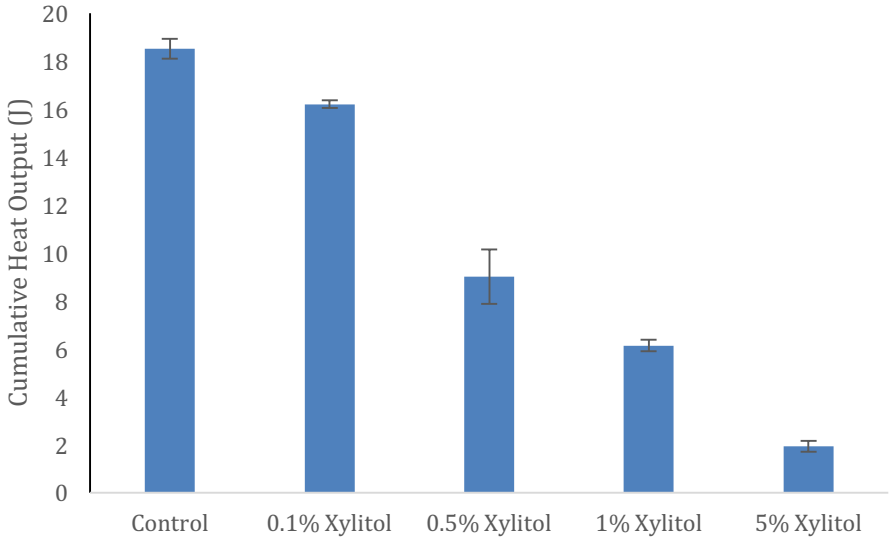

Figure 4-9: Cumulative heat output representative of total AUC for *S. mutans* under varying xylitol concentrations.

Based on these results, investigations were carried out to print an amount of xylitol onto a substrate that will present the same effect as 0.5% xylitol concentration in a 3 ml ampoule. With 0.5 % being equivalent to 15 mg xylitol in 3 ml. A calibration curve, Figure 4-10, was obtained for xylitol in the range

0.5 to 32 mg/ml using HPLC. Varying template dimensions as indicated in Table 4-3 were used to deposit the xylitol concentrations. These dimensions were chosen as the amount deposited with these dimensions still maintained substrate integrity with no break in the substrate. After obtaining the AUCs as indicated in Table 4-3, the AUC that was approximately similar to that of 15 mg/ml as computed in Equation 4-1 was determined. It was observed that 2 cm x 16 cm was the dimension with AUC (2830.20 ± 16.46) closest to 2369.70 representing 15 mg/ml. Hence, this dimension was used to deposit xylitol solution for co-incubation with *S. mutans* in the calorimeter.

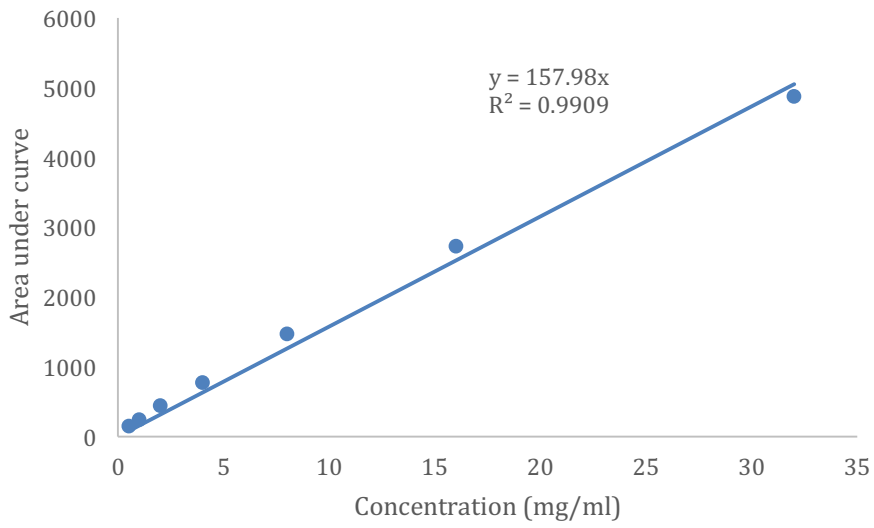

Figure 4-10: Calibration curve for xylitol in the range 0.5 to 32 mg/ml

Table 4-3: Varying template dimensions used for ink jetting xylitol and corresponding AUC (n=3)

Template Dimension (cm x cm)	AUC
1 x 1	149.10 ± 19.98
1 x 2	277.00 ± 29.09
1 x 4	434.87 ± 20.46
1 x 8	802.13 ± 2.42
1 x 16	1540.40 ± 14.68
2 x 1	275.20 ± 7.88
2 x 2	495.00 ± 12.13
2 x 4	820.83 ± 17.29
2 x 8	1574.60 ± 41.58
2 x 16	2830.20 ± 16.46

Equation 4-1: Expected AUC using calibration curve equation

$$y = 157.98x$$

$$when\ x = 15$$

$$y = 2369.7$$

A signal similar to 0.5% xylitol concentration was obtained when the ink jetted xylitol was co-incubated with *S. mutans* (Figure 4-11). The AUC (Figure 4-12) obtained for *S. mutans* in the presence of the ink jetted xylitol was higher than in the presence 0.5% xylitol. Considering that the ink jetted xylitol had slightly more than 0.5% xylitol, it was expected to produce greater inhibition with a more delayed onset of *S. mutans* growth or slightly reduced peak intensity. This anomaly can be attributed to the fact that with the ink jetted xylitol, the

amount of xylitol available was based on release from the substrate as compared to the already available 0.5% pipetted xylitol control. Hence, although an amount equivalent to 0.5% xylitol was ink jetted, this amount was not available until substrate was completely disintegrated.

Ink jetting of actives onto starch paper for ODF formulation is not a new concept. This substrate was used by Buanz et al. (2011) for personalising salbutamol. Xylitol is a sugar that has been reported to inhibit the growth of pathogenic organisms in the mouth whilst having minimal effects on the beneficial organisms. Xylitol, unlike most sugars (sorbitol, fructose, glucose) is a 5- carbon sugar and inhibits glycolysis in *S. mutans* because it is a non-fermentable sugar (Ohshima et al., 2016, Cagetti et al., 2013, Marsh, 2003). Badet et al. (2008) reported on the importance of xylitol in preventing the formation of a multispecies biofilm and the relevance of using it for the prevention of oral diseases caused by dental plaque.

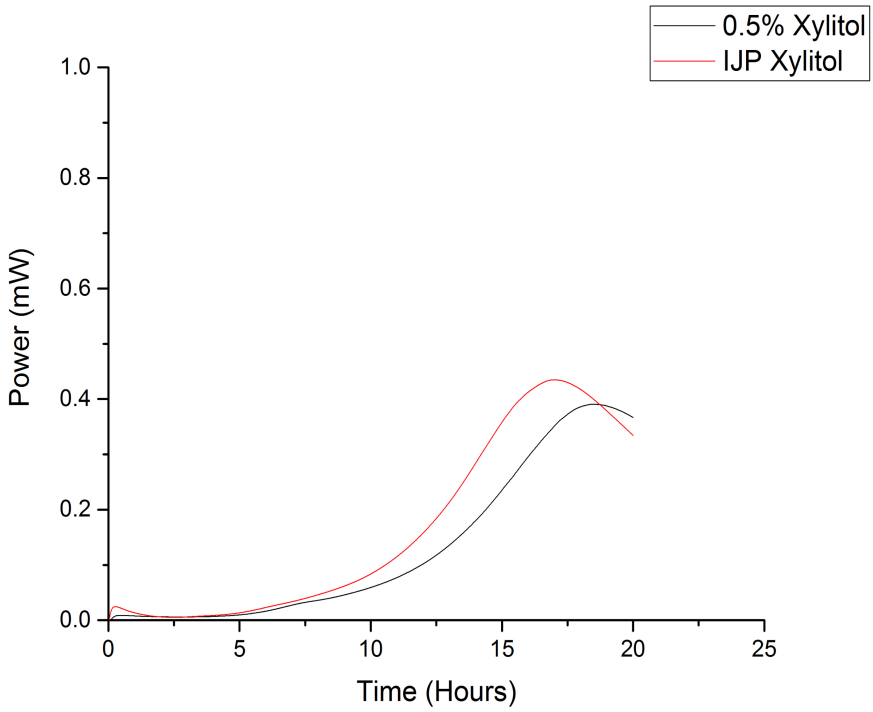

Figure 4-11: Thermogram comparing metabolism of *S. mutans* in the presence of 0.5 % xylitol and ink jetted (IJP) equivalent.

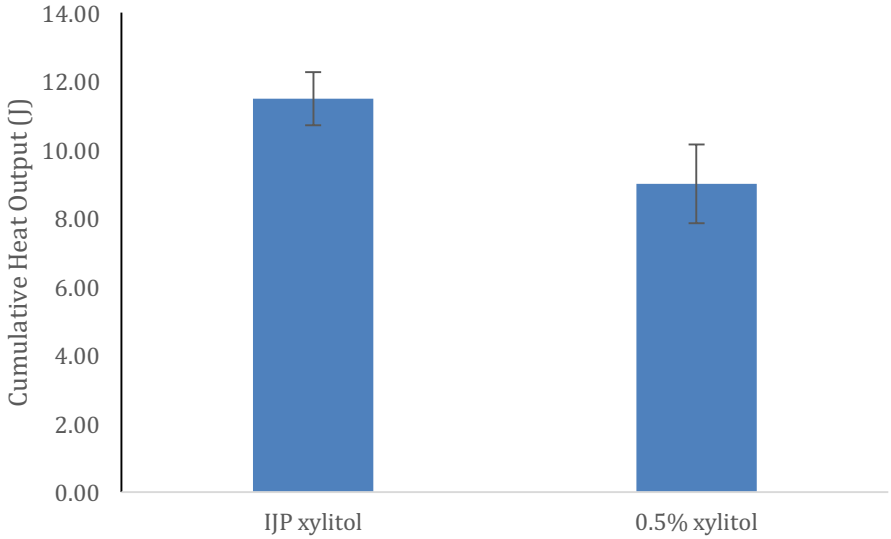

Figure 4-12: Cumulative heat output of *S. mutans* in the presence of 0.5 % xylitol and ink jetted (IJP) equivalent.

Aside its antibacterial properties xylitol is also known to stimulate secretion of saliva which helps in teeth mineralisation and remineralisation. Mineralisation is the process in which an inorganic substance precipitates onto an organic matrix, e.g., biological processes like the formation of hard connective tissues, such as bone, dentin, and cementum, in which collagen fibrils form a scaffold for a highly-organised arrangement of calcium phosphate crystals. Remineralisation is the restoration of minerals after removal from hard tissues. This makes xylitol a good additive in the formulation of ODFs for treating dental caries. Secretion of saliva can also dilute, neutralise, and buffer organic acids formed by biofilm microorganisms (Abou Neel et al., 2016, Hara and Zero, 2010).

4.4.4 Evaluating recovery of *S. salivarius* from oral films

To evaluate the recovery of *S. salivarius* using 10% trehalose as the excipient, thermograms, as shown in Figure 4-13, were obtained. The signal obtained had a slightly higher albeit similar shape of signal as control.

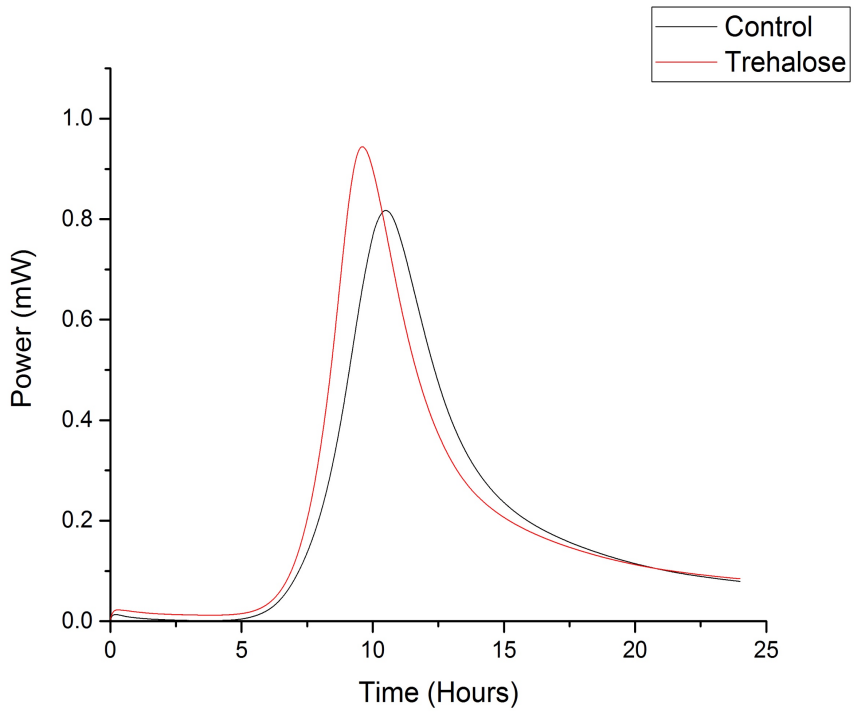

Figure 4-13: Recovery of *S. salivarius* from starch paper using trehalose as protectant.

The use of alternative non-cariogenic sweeteners is recommended for oral formulations. Trehalose is a natural sweetening agent with good taste and thermostable. As demonstrated in chapter 3, trehalose offers advantages to bacterial viability after ink jetting. In this scenario, sucrose was not used as a protectant due to its role in causing dental caries. Trehalose, however, is reported to prevent oral dryness and not to promote the occurrence of dental caries (Mori et al., 2010, Neta et al., 2000). Good recovery was obtained for the ink jetted *S. salivarius* as revealed by the thermograms. This suggested a high probability that the formulation will exhibit activity when administered.

Heinemann et al., (2013) demonstrated recovery of *L. acidophilus* or *Bifidobacterium animalis* subsp. Lactis from ODFs prepared by casting. With the potential of recovery demonstrated, ODF formulation consisting of both ink jetted *S. salivarius* and xylitol was the next objective.

4.4.5 Antibacterial properties of *S. salivarius* and xylitol formulation

To evaluate the usefulness of probiotic formulation – ODFs composed of ink jetted *S. salivarius* and xylitol – in treating dental caries, it was co-incubated with *S. mutans*. Thermograms obtained, (Figure 4-14), showed that the signal obtained after co-incubating ODF had a relatively delayed onset and intensity in comparison to *S. mutans* and ink jetted *S. salivarius*. Plate counting post-calorimetry (Figure 4-15) revealed a drop in *S. mutans* population albeit the relatively smaller peak.

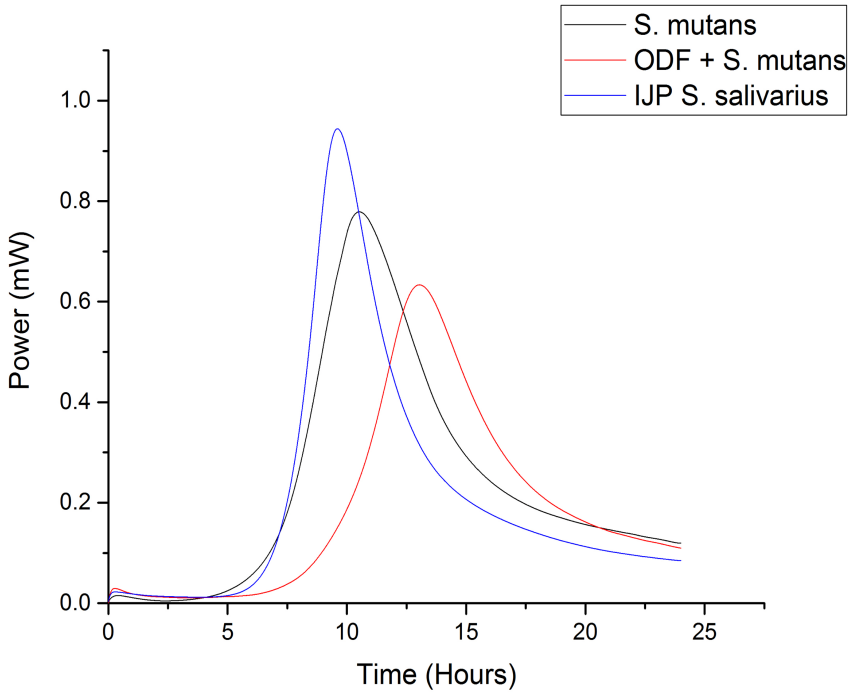

Figure 4-14: Thermograms of S. mutans (control), ink jetted (IJP) S. salivarius, and co-incubation of probiotic oro-dispersible film (ODF) and S. mutans.

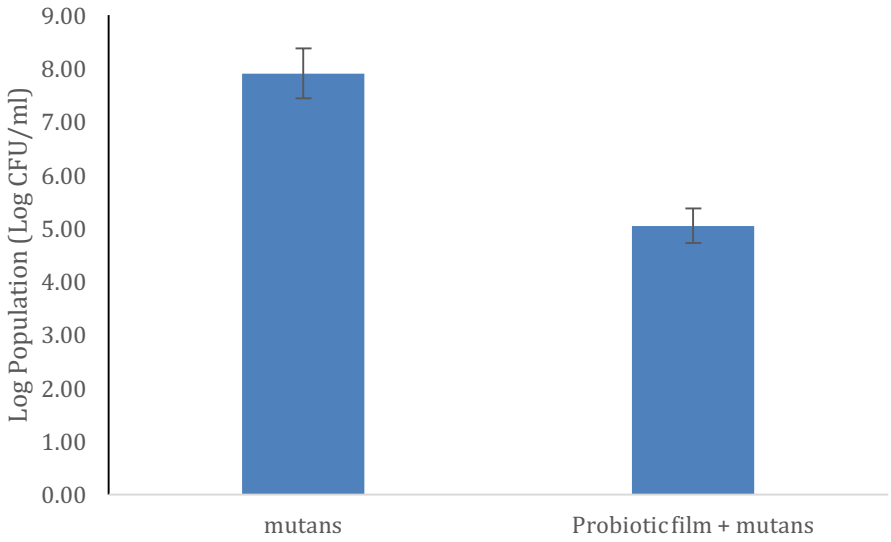

Figure 4-15: Colony plate counts of *S. mutans* control and *S. mutans* after co-incubating with probiotic ODF.

The ODF was composed of two agents suggested to provide oral health benefits. Xylitol, as demonstrated in the earlier section, showed inhibition to the activity of *S. mutans* with little effects on *S. salivarius*. Hence, using an effective medium concentration of 0.5% xylitol (ink jetted) with *S. salivarius,* it was expected that a complemented antibacterial activity will be obtained against *S. mutans*. The signals obtained indicated reduced metabolic activity in the ampoule. Due to the similarities between individual signals for *S. mutans* and *S. salivarius*, it was difficult to match the nature of the peak obtained after co-incubation to either of the pure cultures. This highlighted a drawback with microbial calorimetry, especially, when species under investigation are closely related.

Plate counting data was, therefore, used to ascertain nature of reaction occurring in the co-incubation tests. In this instance, a medium that differentiates between *S. salivarius* and *S. mutans* had to be used. Mitis salivarius (MS) agar supports the growth of streptococci, however, when supplemented with 15% sucrose and 0.2 i.u of bacitracin per ml (MSBS) it selects for growth of *S. mutans* (Liljemark et al., 1976, Staat, 1976, Kurasz et al., 1986). Pure cultures of both species were streaked and/or spread on MS

and MSBS agar as references for comparison. *S. salivarius* on MS agar showed large, pale-blue, mucoid colonies that were glistening in appearance (Figure 4-16). *S. mutans* on MS agar showed raised, convex, undulate, opaque, pale-blue colonies that were granular in appearance (Figure 4-17) (Hamada et al., 1979, Chapman, 1944). When pure cultures of both species were streaked on MSBS, no growth was observed for *S. salivarius,* however, characteristic colonies typical of *S. mutans* was obtained (Figure 4-18).

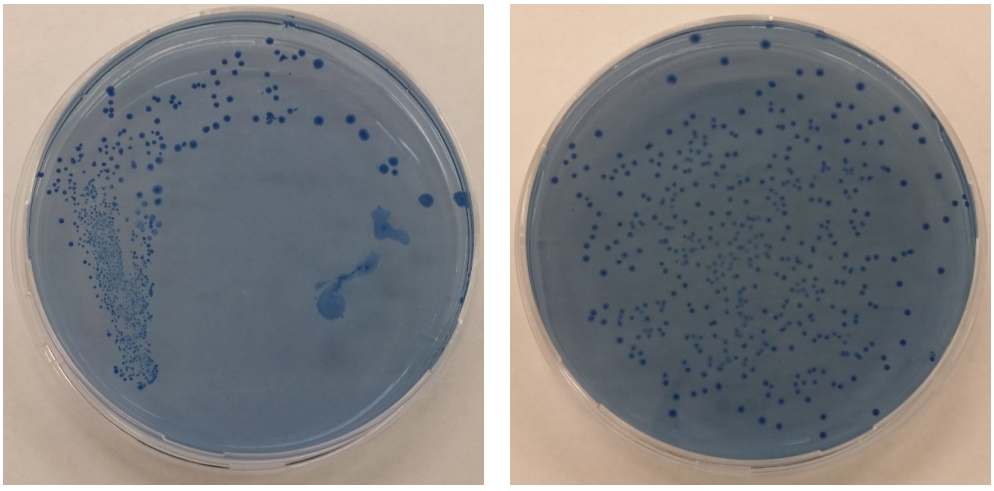

Figure 4-16: Images of pure colonies of *S. salivarius* colonies after streaking (left) and plating (right) onto mitis salivarius agar.

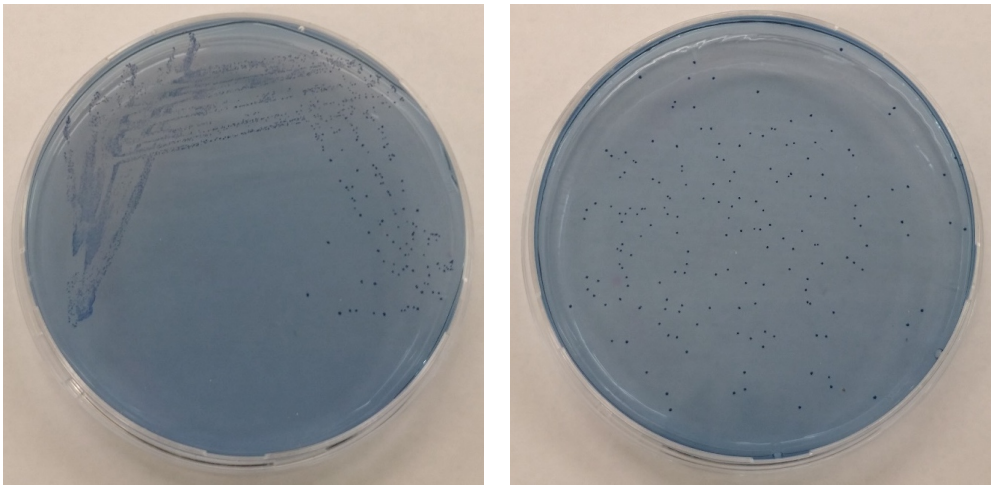

Figure 4-17: Images of pure colonies of *S. mutans* colonies after streaking (left) and plating (right) onto mitis salivarius agar.

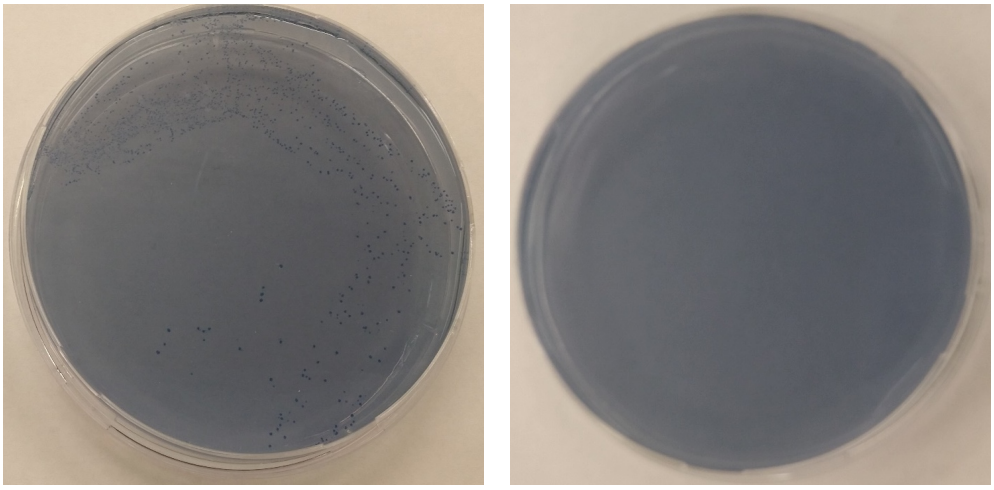

Figure 4-18: Images after streaking pure colonies of *S. mutans* (left) and *S. salivarius* (right) onto MSBS agar.

With these references, enumeration of *S. mutans* post-calorimetry was conducted on MSBS. A drop in numbers from 7.9 to 5.04 Log CFU/ml was obtained post-calorimetry for *S. mutans* co-incubated with ODF in comparison to *S. mutans* control. Although there was not complete elimination of the *S. mutans* population, this was promising as it represented a 2.86 Log reduction. Generally, when comparing the bactericidal activity of antimicrobials against bacteria, a 3 Log reduction is considered to be a significant; the 2.86 Log reduction obtained here was, therefore, considered a positive outcome (Usacheva et al., 2014, Heffernan et al., 2013, Koh et al., 2013, Sun et al., 2014).

A streak out post-calorimetry onto MS agar as shown in Figure 4-19 revealed some large, pale-blue, glistening mucoid colonies characteristic of *S. salivarius* also present, however, enumeration was not possible due inability to select *S. salivarius* growth. This implied that *S. salivarius* was also present in the ampoule at the end of the experiment.

S. mutans and *S. salivarius* could also have been differentiated using species specific PCR assays (Goh et al., 1997, Blaiotta et al., 2005). Species specific PCR assays of sodium dismutase genes was used by Blaiotta et al. (2005) to differentiate *Staphylococcus carnosus* and *Staphylococcus simulans.* The

same group used the technique to identify *Staphylococcus equorum* from 26 different species of Staphylococcus (Blaiotta et al., 2004). Other approaches like the chaperonin 60 gene identification method and reverse checkerboard hybridisation were used by Goh et al. (1997) to identify bacterial isolates.

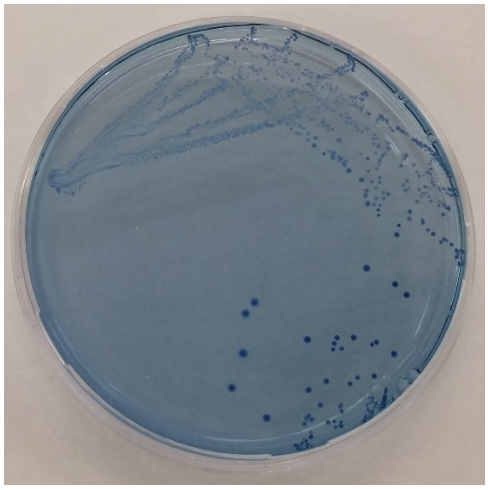

Figure 4-19: Image after streaking out ampoule content post-calorimetry onto mitis salivarius, showing colonies representative of both *S. mutans* and *S. salivarius*.

To further evaluate antibacterial properties, varying amounts of cell-free supernatant (CFS) from *S. salivarius* was incubated with *S. mutans*. Thermograms obtained, (Figure 4-20), showed a reduction in peak intensity with increasing amounts of CFS. This highlighted a reduction in the metabolism of *S. mutans* in the presence CFS from *S. salivarius*. It must be noted that increasing the volume of CFS did not imply a reduction in the growth medium available. The growth medium used here, cooked meat medium, unlike other media does not dissolve when formulated. Hence, the same quantity of meat pellets was weighed into each ampoule for tests to ensure equal nutrient availability.

Plate counts when conducted at 11 hours, representative of highest signal intensity and metabolic activity is showed in Figure 4-21. A gradual decrease in *S. mutans* population was observed with increasing CFS volume.

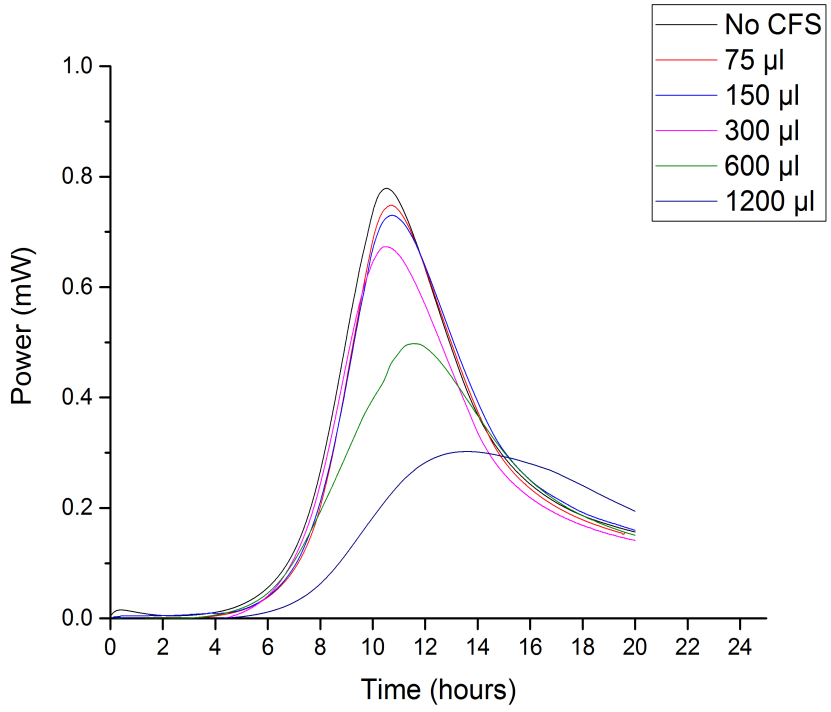

Figure 4-20 Thermogram of *S. mutans* in varying amounts of *S. salivarius* cell-free supernatant.

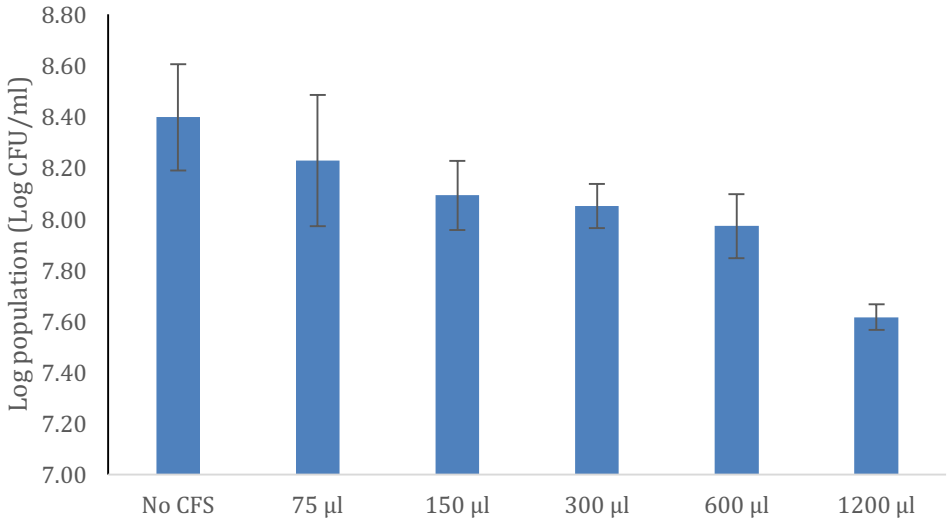

Figure 4-21: Enumeration of *S. mutans* population at the highest signal intensity.

With probiotics reported to release materials with the ability to inhibit pathogenic species during metabolism, the motive of using CFS was to investigate any bacteriocin-like activity by the CFS from *S. salivarius*. Increasing the CFS volume was to serve as a means of increasing amounts of any bacteriocin-like substance that may be present then monitoring decrease in *S. mutans* numbers and metabolism with time. CFS from *S. salivarius* have been demonstrated to inhibit *S. mutans* activity and to play a role in preventing biofilm formation by *S. mutans* (Ogawa et al., 2011). In a similar approach, Fredua-Ageman et al., (2017) used varying amounts of freeze-dried CFS from *L. acidophilus* and *Bifidobacterium lactis* against *Clostridium difficile*. Amounts as high as 20-fold freeze-dried CFS from *L. acidophilus* and 5-fold freeze-dried CFS from *B. lactis* were needed to obtain complete inhibition. The quantity of CFS in this work was relatively minimal compared to the high amounts of CFS in their work which could be a reason complete inhibition was not obtained here.

4.4.6 Evaluating tolerance of *S. salivarius* to saliva

Thermograms obtained after exposing ink jetted *S. salivarius* to saliva are shown in Figure 4-22, highlighting the tolerance demonstrated by these organisms to the artificial saliva.

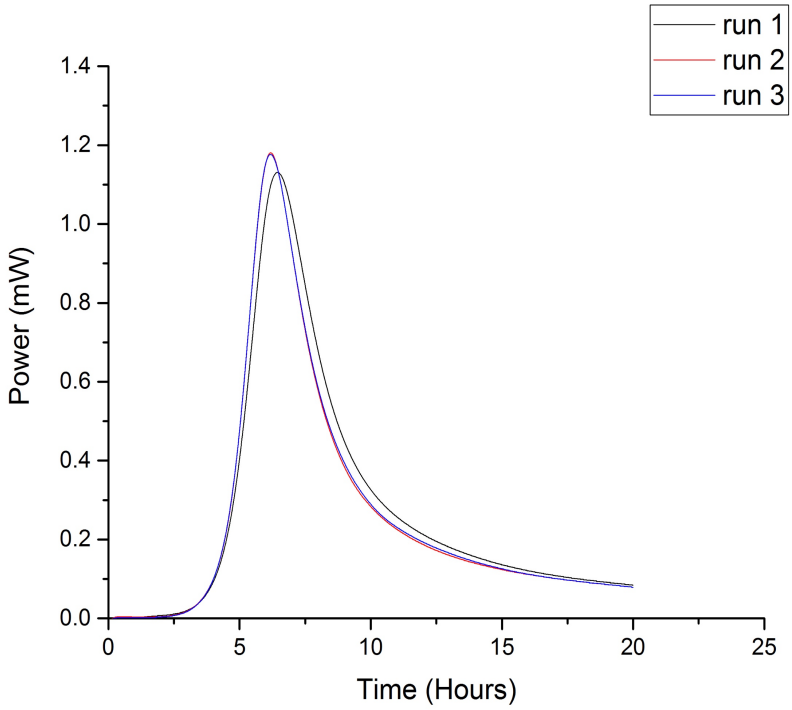

Figure 4-22: Thermogram obtain evaluating tolerance of *S. salivarius* to saliva.

GSK's recipe for artificial fluid was used in this instance. The European and US Pharmacopoeias do not describe artificial salivary fluids. Calls have been made for standardisation of artificial saliva that will guarantee a uniform release medium to enable comparative studies at different labs. Phosphate buffer at pH 6.0 has been used in the pharmacopoeias for dissolution tests of medicated chewing gums (Garsuch and Breitkreutz, 2009, Preis et al., 2013). This medium, as observed from the thermogram, was not expected to have any deleterious effects on *S. salivarius*. These organisms are naturally resident in the mouth constantly bathed with saliva as such the signals obtained were expected. The artificial saliva used also had a neutral pH, such pH is well tolerated by most organisms.

4.5 Conclusion

The possibility of formulating ODFs using starch paper was evaluated in this chapter. Although this substrate served as a good material for deposition of probiotics, it had a thickness that is considered unsuitable for consumers. Starch paper has, however, been used as substrate in personalising a number of medicines. The use of probiotics in oral care applications is increasing, however, with information on oral probiotic formulation and supporting science being limited, it is paramount that any candidate chosen for such formulations should help alleviate dental diseases rather than contribute to them. The phosphate solubilisation test used here revealed *L. rhamnosus* (NCIMB 30174) as a phosphate solubilising strain and should not be considered for oral probiotic formulation.

ODFs made of *S. salivarius* and xylitol were prepared and this exhibited some potential in the management of dental caries. A reduction in *S. mutans* numbers was observed after co-incubating *S. mutans* together with ODFs made of *S. salivarius* and xylitol. The challenge in *in-vitro* evaluation evident here was due to these species belonging to the same genera limiting options available for differentiating. The model, however, can be adapted in formulating probiotic ODFs using a variety of probiotic organisms to investigate of other conditions like periodontal disease and halitosis.

Chapter 5 General conclusion and future work

Inkjet printing is a technology anticipated to revolutionise personalised medicine and push the innovation portfolio by offering new paths in patient-specific treatments. With the gradual shift in the paradigm of the pharmaceutical and healthcare industries from mass manufacture towards personalised medicine, 2D- or 3D inkjet printing could become an integral manufacturing platform. This can benefit the consumer by dispensing tailored medicines and give solutions to the pharmaceutical industry and pharmacies to meet the future needs of making customised medicines optimally at the point of care. Aside from customising medicines for consumers, printing technologies offer realistic opportunities for manufacturing APIs with poor solubility, which is an increasing challenge in the pharmaceutical industry. Good quality control and characterisation of printed systems as well as regulatory perspectives of flexible manufacturing, however, need to be outlined (Preis et al., 2015, Kolakovic et al., 2013, Scoutaris et al., 2016c).

The printing technology as highlighted in this thesis is useful in research and product developments. Printer models especially, relatively older models can be easily modified and used for research. The HP 5940 inkjet used was modified with the robustness of the modified printer intact. With the right expertise, this printer can be further modified such that cartridge motion will not be limited to left-right motion but also a front-back motion can be incorporated. This will make this printer model useful in various areas of research. An assumption with thermal inkjet printers that was addressed was the fact that the high temperature involved in droplet formation in these printers can lead to damage of materials (both chemical and biological). With ink jetting of bacterial cells as a major theme throughout this work, bacterial viability after ink jetting was maintained after ink jetting was determined and found to be optimal.

The potential of the modified printer in antimicrobial susceptibility testing (AST) was evaluated. With the WHO recently publishing a list of diseases for which new antibiotics are needed, it is important to preserve current classes of

antibiotics (WHO, 2017). One way of controlling antibiotic resistance is by rapid and easy means of performing antimicrobial susceptibility testing, which the model described in this work intends to address.

The minimum inhibitory concentration values obtained using ink jetting were comparable to values obtained using standard broth microdilution methods. Promising results were obtained after treating *L. acidophilus* with some antibiotics. *L. acidophilus* was used here due to its GRAS status and the model being in a developmental stage. With basic functional parameters now defined, this model will be tried with other organisms that have the potential of causing diseases. Antibiotics from different classes with different modes of action must be tried as well. These tests will serve to further validate the designed model. Once validated, this model will offer a cost-effective automated means of performing antimicrobial susceptibility testing.

The potential of the ink jetting technology in probiotic formulation was also explored. The potential of the ink jetting technology in aiding the formulation of probiotics for targeted delivery and probiotic oral films were explored. Interests in probiotics have been growing for some time now, several health benefits have been attributed to probiotics. One of such is directly linked to the earlier application the ink jet technology was put to, i.e., reducing antimicrobial resistance. With antibiotics having been in existence for over half a century, and bacteria continuously developing resistance to these; other options of addressing this menace are being explored with the use of probiotics as one of the alternatives. Probiotic science, however, falls short of adequate evidence-based results. Two major reasons for this is the difficulty in maintaining product viability during formulation and a lack of adequate numbers reaching the intestines. An on-demand approach of formulation was, therefore, demonstrated whereby probiotics can be ink jetted and encapsulated using site-specific coating materials for delivery. The site-specific encapsulated ink jetted probiotic could withstand an exposure of 30 minutes in 0.1 M HCl with a relatively higher viability as compared to complete loss of viability within 5 minutes for the unprotected organisms. Another

outcome from these sets of experiments was the fact that the assumption of lactic acid bacteria, especially the lactobacilli, being acid resistant may not be entirely true for all strains. The ease of this formulation approach will help propel probiotic science and increase consumer confidence in probiotics. Some areas that need further work include the exploration of other excipients that will optimise bacterial loading after ink jetting. Fat-based excipients need to be explored as these have been reported be beneficial in probiotic viability and transit through the GIT (Efiuvwevwere et al., 1999, Costa et al., 2000). The impact of these on improving the 30-minute tolerance to 0.1 M HCl need to be investigated. Although this is a model intended for extemporaneous product preparation with a short shelf-life, longer stability studies need to be conducted, the outcome of which might be beneficial in using this model in mainstream probiotic manufacture. Finally, rat models need to be used to evaluate the ability of this formulation approach in treating diseases and ascertain some of the health claims of probiotics.

The use of probiotics in oral health is another area with great consumer interest. With inkjets having been utilised in the preparation of oro-dispersible films, the potential of formulating probiotic oral films was explored (Buanz et al., 2011). Probiotic ODFs made with *S. salivarius* and xylitol were used against *S. mutans* to ascertain the potential of inkjets in formulating probiotic ODFs and the ability of such a formulation in managing dental caries. An area that needs to be explored further in this work is the ability of *S. salivarius* to colonise the mouth. There are lots of uncertainty about the ability of probiotics to colonise the mouth. This has led to some researchers suggesting oral probiotic be incorporated in products as toothpaste which are used daily. *In-vitro* colonisation needs to be evaluated using adhesion to hydroxyapatite beads; rat models can then be used to evaluate potential colonisation in animals. Animal models will also help understand the mechanisms of action of probiotics in the mouth, especially, the suggested role of probiotics in oral immunity. The ability of probiotic ODFs to disrupt pre-existing biofilms of the target organisms is worth investigating (Cagetti et al., 2013, Spratt et al., 2012, Twetman et al., 2009).

An important assay highlighted in this work is the phosphate solubilisation ability of the probiotic organism. This helps assess the risks and benefits of potential probiotic organisms and should be recommended for all probiotic ODFs. With the increasing evidence that the use of probiotics can deliver oral health benefits, it is important to emphasise that the production of ODFs that contain probiotic bacteria is a viable technology – a view shared by Heinemann et al., (2013). Other commercial film forming materials need to be investigated and inkjets can be useful equipment in the production of probiotic ODFs.

Probiotics, in general, have massive potential in medicine, care must, however, be taken in subjects who are immunocompromised as there have been some cases of bacteraemia associated with probiotic use in such individuals (Devine and Marsh, 2009).

The ink jetting technology has been demonstrated both as an analytical and formulation tool in this thesis. With further research using inkjets, many potential applications will be discovered that will aid the advancement of science and quality of life.

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Presentations

Oral Presentation at UKPharmsci 2016, Glasgow, UK. A novel way of formulating probiotics using inkjet printing.

Oral Presentation at Printing for Fabrication 2016, Manchester, UK. Effect on thermal ink-jet printing on bacterial cells.

Oral Presentation at North American Thermal Analysis Society 2016, Florida, USA. Isothermal microcalorimetry: a vital technique in the preformulation and formulation of probiotics.

Oral Presentation at International Society for Biological Calorimetry 2016, Basel, Switzerland. Evaluating the gastrointestinal fluid tolerance of personalised probiotics using calorimetry.

Poster presentation at AAPS 2016, Denver, USA. Colon Targeted Delivery of Probiotics.

Poster Presentation at Printing for Fabrication 2015, Oregon, USA. Determination of minimum inhibitory concentrations using thermal inkjet printing.