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Antibacterial activity of garlic (*Allium sativum)* against probiotic *Bifidobacterium* species

BY

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Submitted in partial fulfilment of the requirements for the degree

Magister Scientiae

In the Faculty of Natural and Agricultural Sciences, Department of Microbiology and Plant Pathology, University of Pretoria, Pretoria, South Africa

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Declaration

I declare that the dissertation "Antibacterial activity of garlic (*Allium sativum)* against probiotic *Bifidobacterium* species", which I hereby submit for the degree Magister Scientiae at the University of Pretoria, is my own work and has not previously been submitted by me for a degree at this or any other tertiary institution.

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PUBLICATIONS AND CONFERENCE CONTRIBUTIONS

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LIST OF ABBREVIATIONS

4-MP: 4-mercaptopyridine

AAD: Antibiotic-associated diarrhea

AGE: Aged garlic extract

AMP: Ampicillin

ARDRA: Amplified ribosomal DNA restriction analysis

cF: Carboxyfluorescein

cFDA: Carboxyfluorescein diacetate

cfu: Colony forming units

CSLM: Confocal scanning laser microscopy

DAS: Diallyl sulphide

DD: Disk diffusion

DNA: Deoxyribonucleic acid

EPS: Extracellular polysaccharides

ESEM: Environmental scanning electron microscope

FAO-WHO: Food and Agriculture Organization of the United Nations - World Health Organization

F6PPK: Fructose-6-phosphate phosphoketolase enzyme

FS: Forward scatter

FT-IR: Fourier-transform infrared

GC: Garlic clove

GIT: Gastrointestinal tract

GP: Garlic paste

Gp: Garlic powder

GRAS: Generally regarded as safe

GS: Garlic spice

HDL: High density lipoproteins

HIV: Human immunodeficiency virus

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IBS: Irritable bowel syndrome

IgA: Immunoglobulin A

IR: Infrared

ISA: Iso-Sensitest agar

ISAPP: International Scientific Association for Probiotics and Prebiotics

LAB: Lactic acid bacteria

LDL: Low density lipoproteins

LSM + cysteine: LAB susceptibility test medium supplemented with cysteine

MBC: Minimum bactericidal concentration

MIC: Minimum inhibitory concentration

MRS: De Man Rogosa Sharpe

MSA: Multiplicative scatter correction

NASBA: Nucleic acid sequence-based amplification

NK: Natural killer

NO: Nitric oxide

PCA: Principle component analysis

PCR: Polymerase chain reaction

PI: Propidium iodide

rep-PCR: repetitive sequence-based PCR

RT-PCR: Reverse transcriptase PCR

RNA: Ribonucleic acid

rRNA: Ribosomal RNA

RT-SDA: Reverse transcriptase-strand displacement amplification

SAC: S-allyl cysteine

SEM: Scanning electron microscopy

SSC: Side scatter

TLRs: Toll-like receptors

VBNC: Viable but not culturable

VLDL: Very low density lipoproteins

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SUMMARY

Antibacterial activity of garlic (*Allium sativum)* against probiotic *Bifidobacterium* species

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Supervisor **:** Dr. M.S. Thantsha
Department **:** Microbiology and Plant Pathology
Degree **:** MSc (Microbiology)

During the past decade there has been an explosion in the probiotic industry due to an increase in concern for health. It is well known that these probiotic products offer consumers numerous health benefits and that viability of cultures in these products need to be maintained at high levels. It is therefore important to test for antimicrobial compounds or substances that may come into contact with probiotics and thereby negatively affect and decrease their viability. Garlic (*Allium sativum*) has been used as a natural medicinal remedy for thousands of years and research has shown that it has antimicrobial activity against a wide variety of microorganisms. Although it has been tested against numerous pathogenic microorganisms, there have been few studies on its effect on beneficial bacteria, specifically probiotic *Bifidobacterium* species. A great amount of work and money is put into preparing probiotic products with sufficient numbers of viable bacterial cells. All these are devoted to ensure that the consumers seize the optimal purported health benefits from probiotic cultures incorporated within the different products. Hence it is necessary to recognize any compound or substance that poses a threat to viability of these probiotic cells, thereby rendering them ineffective.

Therefore, the current study aimed at determining whether garlic had any antibacterial activity towards selected *Bifidobacterium* spp. *In vitro* studies revealed that garlic has an inhibitory effect on these specific probiotic bacteria. The disk diffusion assay revealed antibacterial activity of garlic preparations characterized by inhibition zones ranging from 13.0 ± 1.7 to 36.7 ± 1.2 mm. Minimum inhibitory concentration (MIC) values for garlic clove extract ranged from 75.9 to 303.5 mg/ml (estimated to contain 24.84 to 99.37 µg/ml allicin) while the minimum bactericidal concentration (MBC) ranged from 10.24 to 198.74 µg/ml

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allicin. Susceptibility of the tested *Bifidobacterium* species to garlic varied between species as well as between strains even within a small numbers of the tested bifidobacteria. Among the tested *Bifidobacterium* spp., *B. bifidum* LMG 11041 was most susceptible to garlic, whereas *B. lactis* Bi-07 300B was the most resistant. These results were contrary to what has been generally published in literature, that garlic selectively kills pathogens without negatively affecting beneficial bacteria. Garlic clove, garlic powder, garlic paste and garlic spice showed varying degrees of potency, with fresh garlic clove extract and garlic paste extract having the highest and lowest antibifidobacterial activity, respectively.

It became necessary to investigate the actual antibacterial mechanism of action of garlic on *Bifidobacterium* spp., upon realization that its extracts inhibits growth of or kills some of these bacteria, whose contribution to health and well being of consumers is to a large extent dependent on their viability. This was determined by using scanning electron microscopy (SEM) and Fourier-transform infrared (FT-IR) spectroscopy. Scanning electron microscopy was used to investigate the effect of garlic on the morphology and cell surface properties of the tested strains while FT-IR spectroscopy was used to determine any biochemical changes taking place in garlic-treated bifidobacteria. Scanning electron microscopy showed various morphological changes such as cell elongation, distorted cells with bulbous ends and cocci-shaped cells. Behavioural changes were also observed such as swarming of cells was also observed. FT-IR spectra confirmed that garlic damaged *Bifidobacterium* cells by inducing biochemical changes within the cells. It identified some of the main targets sites of garlic on bifidobacteria, mainly, the nucleic acids and fatty acids (lipids) in the cell membrane.

Flow cytometry analysis was used to determine the level at which the garlic decreased the viability of *Bifidobacterium* cells as well as the extent of damage induced by the garlic. Results revealed a drop in viability with associated decrease in stainability of some the cells, for all strains upon treatment with garlic clove extract. The inability of cells to be stained by nucleic acid stains, hence presence of cells referred to as ‘ghost cells’, has been associated with extensive damage and lysis of cellular membranes resulting in loss nucleic acids. Interestingly, re-inoculation of the cells analysed by flow cytometry into a fresh growth medium and their subsequent reanalysis using the same technique showed an increase in percentage of viable cells and a decrease in percentages of damaged, unstained and dead cells. This suggested that injured cells were able to recover and regress to their active state.

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Therefore, *Bifidobacterium* cells exposed to sub lethal amounts of garlic can repair any damage and regrow. However, it was not determined how long active compounds of garlic remain stable within the gastrointestinal tract.

This study is the first, according to our knowledge, to show that garlic exhibits antibacterial activity against beneficial bacteria specifically, probiotic bifidobacteria. Furthermore, the results revealed that the mechanism of action of garlic towards bifidobacteria is similar to that which was reported for pathogenic bacteria. Bacterial death and growth inhibition occurs due to damage to the fatty acids/lipids in the cell membrane, modification of the nucleic acids (DNA and RNA).

This study is of significant importance to consumers, medical practitioners as well as to the probiotic industry. It suggests that if garlic comes into contact with probiotic bifidobacteria, they die and thus become unable to deliver the promised health benefits to the consumers. Therefore, consumers should be advised against ingestion of probiotic products and garlic simultaneously, as this study reveals that garlic does indeed inhibit some probiotic *Bifidobacterium* spp. The probiotic industry should also consider including this information on their product labels to make consumers aware of this fact. Failure to include this information may lead to market deterioration due to loss of interest in the products as soon as consumers realize they do not get their money's worth from the products. Lastly, medical practitioners should also be made aware of this as they also prescribe probiotics to patients for various health reasons. The effect of food matrices on the antibacterial effects, as well as determination of how long the active compounds of garlic remain within the gastrointestinal tract, in relation to levels of garlic ingested will confirm whether indeed there is concern. But for now, in light of results of the current study, caution needs to be taken in simultaneous use of probiotics and garlic, until further testing indicates otherwise.

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INTRODUCTION

The conception and evolution of probiotics began in 1907 when Metchnikoff proposed that ingested bacteria, such as lactobacilli, are able to modify gut flora by replacing harmful microbes with useful microbes (Dobrogosz *et al*., 2010; Amalaradjou and Bhunia, 2012). In 2002 the Food and Agriculture Organization of the United Nations - World Health Organization (FAO-WHO) defined probiotics as "live microorganisms which, when administered in adequate amounts confer health benefits on the host. These health benefits include among others, assisting in food digestion and lactose intolerance, control of enteric infections such as antibiotic-associated diarrhea (AAD), irritable bowel syndrome (IBS), cholesterol-lowering, anticarcinogenic and antimycotic effects, treatment of allergies, eczema and urinary tract infections (Kolida *et al*., 2006; Amalaradjou and Bhunia, 2012). The probiotic industry has encountered many disbeliefs and scepticisms concerning probiotics' health benefits but numerous studies showing scientific evidence have proven this "probiotic concept" correct (Lacroix and Yildirim, 2007; Dobrogosz *et al*., 2010; Aureli *et al*., 2011). The most commonly used probiotics include strains of the genus *Bifidobacterium*, *Lactobacillus*, *Enterococcus*, *Streptococcus*, *Leuconostoc* and *Saccharomyces* (Amalaradjou and Bhunia, 2012).

Probiotics are available in conventional and fermented products for nutritional purpose such as yoghurt, cheese, fruit juice and ice-cream (Fasoli *et al*., 2003; Champagne *et al*., 2011). They are also found in food supplements or formulations and are available as dietary supplements in the form of capsules or tablets, powders and even sprays (Fooks *et al*., 1999; Fasoli *et al*., 2003; Kramer *et al*., 2009). Viability is an important requirement for optimal probiotic functionality and products containing probiotics should contain 10^6-10^8 CFU/ g of viable cells in order to confer health benefits (Kramer *et al*., 2009; Champagne *et al*., 2011). Probiotics have a short shelf life as they are very sensitive to high temperatures, light, high oxygen levels and moisture (Saxelin *et al*., 1999; O'Riordan *et al*., 2001). Viability studies are therefore critical in evaluating the quality of probiotic-carrying products not only to ensure health benefits but also for economic reasons (Lacroix and Yildirim, 2007; Champagne *et al*., 2011).

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Interest in the use of natural remedies such as extracts from medicinal plants has also increased. Recently, these extracts have been suggested as an alternative to antibiotics due to the ever increasing resistance of pathogens to antibiotics (Nazir and Latif, 2012). Garlic (*Allium sativum*) is one such plant that is used as a medicinal herb for its numerous health benefits, and as a flavour enhancing agent. Health benefits for which garlic is used include among others its antimicrobial, anti-inflammatory, antioxidant, antithrombotic and anticarcinogenic effects (Capasso, 2013).

Numerous studies have demonstrated garlic's antibacterial activity against both Gram-positive and Gram-negative bacteria including, among many others, species of *Bacillus*, *Clostridium*, *Klebsiella*, *Proteus* and *Streptococcus*. Pathogenic bacteria such as *Escherichia coli*, *Helicobacter pylori*, *Salmonella typhi* as well as methicillin-resistant *Staphylococcus aureus* (MRSA) and *Salmonella goldcoast* are also inhibited by garlic (Gull *et al*., 2012; Hannan *et al*., 2012; Gaekwad and Trivedi, 2013). In addition, it also possesses antifungal and antiviral activities (Ankri and Mirelman, 1999; Goncagul and Ayaz, 2010). These antimicrobial activities are mainly due to organosulfur compounds, such as allicin, ajoene and diallylsulfides, which are released upon crushing of garlic clove tissue. Allicin is the main active compound among these compounds. It is easily taken up by bacterial cells causing damage by altering their membrane and in turn causing cell lysis and eventually death (Lu *et al*., 2011). It affects bacterial cells by a number of different mechanisms, including total inhibition of RNA synthesis and partially inhibition of DNA and protein synthesis (Al-Waili *et al*., 2007; Deresse, 2010).

The similarities between the health effects of garlic and probiotics suggest that both of them can be recommended to consumers for alleviation of diseases. This is especially possible because it has been documented that garlic exerts differential inhibition between beneficial and potentially harmful bacteria (Rees *et al*., 1993; Hayes, 1996). However, contrary to the availability of extensive literature to indicate the effect of garlic on pathogens, there are few studies on demonstrating its inability to harm beneficial bacteria. A few studies that are available tested the susceptibility of lactic acid bacteria, specifically *Lactobacillus acidophilus* and *Enterobacter* (Rees *et al*., 1993; Naganawa *et al*., 1996; O'Gara *et al*., 2000; Ross *et al*., 2001; Banerjee and Sarkar, 2003; Ruddock *et al*., 2005).

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This study is significant as there is a need to know whether garlic will inhibit and decrease the viability of probiotic bifidobacteria. Firstly, the cost of probiotics to the consumer is relatively high due to commercialization of probiotic products requiring significant time and costly research. Secondly, viability of the product is a very important aspect, not only in terms of making sure the consumer gets what they are paying for, but also ensuring that they get the optimal benefits promised from the products. Another reason to test if garlic inhibits bifidobacteria is because medical practitioners prescribe probiotic products containing bifidobacteria to patients for various reasons. If these patients were to ingest the probiotics and garlic simultaneously, it would be a waste of money for the consumer as the probiotic product would not provide the promised health effects, should garlic decrease viability of the bifidobacteria in the probiotic product. As a result, testing probiotic products against various compounds that may decrease their effectivity is very important.

To the best of our knowledge, there are no studies showing the effect of garlic on *Bifidobacterium* species. Therefore, the main of this study was to determine the effects of garlic on selected strains of probiotic *Bifidobacterium* species. The specific objectives were to:

- Determine whether different garlic preparations have an antimicrobial activity on selected probiotic *Bifidobacterium* species.
- Determine the mechanism of action of allicin on bifidobacteria using scanning electron microscopy.
- Determine internal biochemical changes on bifidobacteria treated with garlic clove extract.
- Determine the effect on the viability as well as the extent of damage of *Bifidobacterium* spp. treated with garlic clove extract and possible recovery and regrowth of injured/damaged cells.

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CHAPTER 1

Literature review

Part of this work is published as a book chapter: M. S. Thantsha, C. I. Mamvura and **J. Booyens**. Probiotics: What they are, Their Benefits and Challenges. New Advances in the Basic and Clinical Gastroenterology'. Editor Tomasz Brzozowski. ISBN 978-953-51-0521-3, InTech, April 4, 2012.

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1.1 THE HUMAN GASTROINTESTINAL TRACT (GIT)

The gastrointestinal tract (GIT) is a complex and diverse ecosystem which consists of over 500 different bacterial species including both beneficial and pathogenic bacteria. The GIT is sterile at birth but immediately after birth becomes colonized by environmental microbes and remains relatively stable throughout life. This collection of microorganisms, known as the intestinal microflora, is mostly located in the colon or large bowel (Tannock, 1997; Gill, 2003; Kolida *et al*., 2006). Bifidobacteria and lactobaclli account for up to one third of the colon flora and are beneficial to human health (Reyed, 2007). Although most members of the indigenous flora are beneficial, there are also pathogenic bacteria present. In a healthy individual there is usually a balance of the two groups. This balance may be altered by numerous disturbances such as antibiotic and radiation therapy, stress, changes in bile secretion, diet, bowel movements and colonization by pathogens (Collins *et al*., 1998; Gill, 2003). It is therefore critical to keep this ecosystem in good balance. Numerous studies have shown that supplementation with probiotics, specifically bifidobacteria can be used to restore and optimize the microbial balance and maintain good health (Gill, 2003; Reyed, 2007).

1.2 PROBIOTICS

1.2.1 History

Live bacterial cultures have been consumed by humans for centuries in the form of fermented milk. In the Old Testament it is written that ‘Abraham owed his longevity to the consumption of sour milk’ (Fioramonti *et al*., 2003; Kolida *et al*., 2006). In 76 B.C. Plinius, a Roman historian also described its use in warding off gastro-intestinal infections (Jankovic *et al*., 2010). The concept of probiotics was first proposed by Metchnikoff in 1905 when he attributed the long life of Bulgarian peasants to the ingestion of fermented milk products. He hypothesised that ingested bacteria, such as lactobacilli, had a positive effect on the normal gut flora of the GIT (Rolfe, 2000; Fioramonti *et al*., 2003; Kolida *et al*., 2006). During this time he isolated Bulgarian bacillus, which is currently known as *Lactobacillus delbreukii* species (spp.) bulgaricus. During World War I, the physician Nissle isolated a strain of *E. coli*, now known as *E. coli* Nissle 1917, from the only remaining soldier in a group suffering from diarrhoea. He proposed that this strain was responsible for preventing diarrhoea because

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it suppressed the responsible pathogens. The foundation upon which the probiotic concept was based was provided by the above mentioned observations (Kolida *et al*., 2006).

1.2.2 Definition

In the past there have been many attempts to define the term 'probiotic', one of the first being described by Lilly and Stillwell in 1965. They defined probiotics as "substances secreted by one microorganism, which stimulates the growth of another". Subsequently, in 1974, Parker defined them as "organisms and substances which contribute to intestinal microbial balance" (Schrezenmier and de Vrese, 2001). In 1989, Fuller tried to improve on Parker's definition and he proposed the following definition: "Probiotics are live microbial feed supplements which beneficially affect the host by improving its intestinal microbial balance" (Salminen *et al*., 1999). Many other descriptions followed until the Food and Agriculture Organization of the United Nations - World Health Organization (FAO-WHO) officially defined probiotics as: "live microorganisms that when administered in adequate amounts confer a significant health benefit on the host". It was later on endorsed by the International Scientific Association for Probiotics and Prebiotics (ISAPP) and is now widely accepted (Reid, 2006).

There has been much scepticism in the probiotic industry concerning the proposed health benefits of probiotics but scientific evidence in recent years has proven this true (Dobrogosz *et al*., 2010). It is estimated that the probiotic industry holds a 10% share of the global functional food market and products containing probiotics are fast becoming a considerable and growing economic importance (Champagne *et al*., 2011).

1.2.3 Microorganisms used as probiotics

Many microorganisms have been used and considered as probiotics. The most commonly used probiotic strains that are commercially available include those of *Bifidobacterium, Lactobacillus* and *Streptococcus* species. The list of probiotic microorganisms extends to include *Bacillus* spp., *Bacteriodes* spp., *Enterococcus* spp., *Escherichia coli*, *Leuconostoc* spp., *Pediococcus acidilactici*, *Propionibacterium* spp., as well as various fungi such as *Saccharomyces boulardii*. Probiotic preparations may include one or more strains of these microorganisms (Amalaradjou and Bhunia, 2012; Collins *et al*., 1998; Rolfe, 2000; Kolida *et*

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al., 2006). However, the most commonly used are species of *Lactobacillus* and *Bifidobacterium* including; *L. brevis*, *L. casei*, the yoghurt strain *L. delbrueckii* subsp. *bulgaricus*, *L. johnsonii* (*acidophilus*), *L. plantarum*, *L. reuteri*, *L. rhamnosus*, *L. salivarius*, and *B. adolescentis*, *B. bifidum*, *B. breve*, *B. cereus*, *B. infantis*, *B. lactis*, *B. longum* among others (Collins *et al*., 1998; Gismondo *et al*., 1999; Saarela *et al*., 2000; Ouwehand *et al*., 2002).

The lactic acid bacteria (LAB) strains have been used since ancient times and incorporated into a wide range of foods without having a negative effect on human health (Collins *et al*., 1998). LAB have many properties which make them prime candidates for their use as probiotics. They can withstand the conditions in the GIT; they resist bile salts, gastric acid and pancreatic enzymes and adhere to the intestinal mucosal surface allowing them to colonize the intestinal tract. They are an important part of the gastrointestinal (GI) flora and inhibit the growth of pathogens such as *Staphylococcus aureus*, *Escherichia coli*, *Salmonella typhimurium*, *Clostridium perfringens* and *Clostridium difficile* (Rolfe, 2000).

1.2.4 Selection criteria

The general microbiological criteria for the selection of probiotics include safety, performance, technology and health benefits (Gibson and Fuller, 2000). With regards to safety, probiotic strains must be of human origin and must be isolated from healthy human GIT's. They should also have an accurate taxonomic identification. They should possess GRAS (generally regarded as safe) status, be non-pathogenic, and have no history of association with diseases such as infective endocarditis or GI disorders. Probiotic strains must not deconjugate bile salts and they should be genetically stable and carry no antibiotic resistance genes that can be transferred to pathogens (Collins *et al*., 1998; Saarela *et al*., 2000; Amalaradjou and Bhunia, 2012).

With respect to their performance, potential probiotic strains should be acid-tolerant and therefore survive human gastric juice and bile. They must be able to survive in sufficient numbers and adhere to the intestinal mucosal surface in order to endure the GIT. Lastly, they should also have antagonistic activity against pathogens such as *Salmonella* spp., *Clostridium*

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difficile and *Listeria monocytogenes* that adhere to mucosal surfaces (Biavati *et al*., 2000; Saarela *et al*., 2000; Mattila-Sandholm *et al*., 2002; Kolida *et al*., 2006).

Technological aspects must also be taken into account before selecting a probiotic strain. Strains should be capable of being prepared on a large scale and should be able to multiply rapidly, with good viability and stability in the product during storage. The strains must not produce off flavours or textures once incorporated into foods. They should be metabolically active within the GIT and biologically active against their identified target. Probiotic strains must be resistant to phages and have good sensory properties (Collins *et al*., 1998; Saarela *et al*., 2000; Mattila-Sandholm *et al*., 2002; Kolida *et al*., 2006)). Therefore probiotic containing foods and products need to be of good quality and must have high enough numbers of viable probiotic cells to ensure consumers get the optimal benefits from the product (Alakomi *et al*., 2005).

1.2.5 Health benefits of probiotics

1.2.5.1 Prevention and treatment of gastrointestinal disorders

Probiotic bacteria are incorporated into foods, especially dairy products such as yoghurt, cheese and fermented milk, due to their exceptional health benefits (Mattila-Sandholm *et al*., 2002). These health benefits include their ability to prevent and treat many GI disorders such as antibiotic-associated diarrhea (AAD) and *Clostridium difficile*-associated intestinal disease, which is the most common cause of AAD. Examples of probiotic strains effective in this regard include *Bifidobacterium, Lactobacillus, Streptococcus* and *Saccharomyces* spp. However, only *S. boulardii, E. faecium* and *Lactobacillus* are clinically effective in preventing AAD. It has also been demonstrated that *S. boulardii* is most effective and safe, in combination with standard antibiotics, for treating *C. difficile*-associated intestinal disease (Rolfe, 2000; Ouwehand *et al*., 2002; Kolida *et al*., 2006).

It has been proven that probiotics prevent traveller's diarrhoea and treat Rotavirus diarrhoea as well as diarrhoea caused by human immunodeficiency virus (HIV) infection. For HIV patients suffering with chronic diarrhoea, the probiotic *S. boulardii* has proven an effective treatment. A few probiotics have been examined for their ability to prevent Traveller's diarrhoea, including *Lactobacillus, Bifidobacterium, Streptococcus* and *Saccharomyces* spp.

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(Rolfe, 2000). Studies have also shown that certain probiotics such as *L. rhamnosus* GG, *L. Reuteri, L. Casei* and *L. lactis* can shorten the duration of Rotavirus diarrhoea in children and infants (Ouwehand *et al*., 2002).

1.2.5.2 Immunomodulation effects

Probiotic bacteria also have immunomodulatory properties and human studies have shown that they can have a positive effect on the immune system of the host. Adhesive probiotic strains such as *L. casei*, *L. rhamnosus* and *L. plantarum* adhere to the gut associated lymphoid tissue and this enhances both systemic and mucosal immunity, stimulating phagocytic cells more efficiently than other bacteria (Saarela *et al*., 2000; Galdeano and Perdigón, 2004). *L. johnsonii* and *L. salivarus* are able to stimulate a mucosal immunoglobulin A (IgA) response, which also increases phagocytic activity. In addition, *L. rhamnosus* GG and *B. lactis* were shown to have immunomodulation effects in a trial involving children with severe eczema resulting from food allergy. Children fed both *L. rhamnosus* and *B. lactis* showed a significant improvement (Saarela *et al*., 2000). Isolauri and colleagues (1992) determined that infants supplemented with a strain of *L. casei* had enhanced concentrations of circulating IgA, which correlated with a shortened duration of rotavirus-induced diarrhoea (Roberfroid, 2000). In other studies oral introduction of *B. bifidum* and *B. breve* was shown to enhance antibody response to ovalbumin and to stimulate IgA response to cholera toxin in mice, respectively (Isolauri *et al*., 2001). Consumption of *B. bifidum* and *L. acidophilus* greatly enhances the non-specific immune phagocytic activity of circulating blood granulocytes (Roberfroid, 2000).

1.2.5.3 Antimutagenic and anticarcinogenic effects

Antimutagenic and anticarcinogenic properties of probiotics have been studied and more and more evidence that these normal intestinal bacteria can influence carcinogenesis, especially with regards to colorectal cancer, is accumulating (Rolfe, 2000; Saarela *et al*., 2000). There are three postulated mechanisms that allow protection from these probiotics. Firstly, the probiotic may inhibit the bacteria responsible for converting procarcinogens into carcinogens. Lactobacilli and bifidobacteria decrease the quantity of faecal microbial enzymes, such as β-glucuronidase, β-glucosidase, azoreductase, nitroreductase and urease, which are also responsible for transforming procarcinogens into active carcinogens, which are harmful and cause cancer (Roberfroid, 2000; Rolfe, 2000; Saarela *et al*., 2000, Schrezenmeir and de Vrese, 2001). Secondly, animal studies have shown that some probiotics inhibit tumor cell

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formation directly, and lastly some probiotic bacteria have been shown to bind and inactivate carcinogens (Rolfe, 2000). Antigenotoxic and antitumor activities of some *Bifidobacterium* and *Lactobacillus* strains have been shown by *in vitro* studies using rat and mice models. *L. acidophilus, L. casei, L. gasseri* and *L. rhamnosus* consumption was shown to decrease faecal and urinary mutagenicity in humans (Saarela *et al*., 2000).

1.2.5.4 Irritable bowel syndrome (IBS)

There is evidence showing that probiotics reduce IBS (Ouwehand *et al*., 2002; Aureli *et al*., 2011). This syndrome usually occurs during childhood and is associated with diarrhea, constipation or both, and symptoms which include abdominal bloating and chronic, recurring pain. There is no specific treatment but studies done by Niedzielin and Kordecki in 1996 demonstrated that there was a reduction in severity of abdominal pain in individuals that consumed *L. plantarum* (Rolfe, 2000; Schrezenmeir and de Vrese, 2001; Kolida *et al*., 2006). Bifidobacteria have also been suggested to relieve constipation due to an observed reduction of their levels in individuals experiencing constipation (Schrezenmeir and de Vrese, 2001; Ouwehand *et al*., 2002).

1.2.5.5 Hypocholesterolemic effects

The hypocholesterolemic effects of probiotics are controversial due to inconsistency of results of studies published in the 1970s, 1980's and those of the 1990's. Earlier studies reported a decrease in cholesterol levels in patients consuming fermented milk products for 2-4 weeks, while the latter reported no significant effect in cholesterol reduction due to probiotc consumption (Roberfroid, 2000). In 1984, Massey showed that initially, the consumption of yoghurt significantly reduced cholesterol in human adult males, but 2 weeks later concentrations returned to control values even with continuous consumption of yoghurt. Similar conflicting results were obtained in 1989 when Lin and colleagues fed experimental animals milk and fermented dairy products (Taranto *et al*., 1998). More recent studies show that some probiotics do indeed have hypocholesterolemic effects. *In vitro* studies performed by Pereira and colleagues (2004), showed that *L fermentum* has cholesterol-lowering properties in humans. *L. plantarum* has also been tested for cholesterol-lowering properties in mice and results indicated a reduction in cholesterol in mice that were fed *L. plantarum* (Nguyen *et al*., 2007). In addition, the *Lactobacillus* cultures fed to chickens also showed significant reduction in total serum cholesterol, low-density lipoproteins and triglycerides (Kalavathy *et al*., 2008).

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1.2.5.6 Treatment of lactose intolerance

Lactose intolerance occurs when individuals have a congenital deficiency of the enzyme β-galactosidase and are therefore unable to digest and absorb significant amounts of lactose. This then causes abdominal cramping, bloating, diarrhoea and nausea. Some probiotics, such as *Lactobacillus*, *Bifidobacterium* and *Streptococcus* spp. are commonly added to dairy products to improve lactose digestion and help alleviate the symptoms in people suffering from lactose intolerance (Roberfroid, 2000; Rolfe, 2000; Ouwehand *et al*., 2002; Kolida *et al*., 2006).

1.2.5.7 Prevention of hepatic encephalopathy

Probiotics have also been proven to decrease the occurrence of hepatic encephalopathy which is a neurological disorder caused by increased levels of ammonia in the blood. The probiotic *L. acidophilus* has proven effective in decreasing fecal urease activity together with neomycin. *Lactobacillus* has also been shown to be antagonistic to *Helicobacter pylori* thereby reducing *H. pylori* gastroenteritis (Rolfe, 2000; Schrezenmeir and de Vrese, 2001).

1.2.5.8 Additional health benefits

Probiotics have also recently been implicated in the treatment of urogenital infections, the most studied and effective strain being *L. rhamnosus* GR-1 (Kolida *et al*., 2006). In addition, probiotics have a potential for application in vaccine development and are being used more frequently as an alternative to antibiotic therapy for treating GIT disorders as there is a huge concern that effective antibiotics are no longer being developed fast enough to compete with the development of microbial resistance to old antibiotics (Rolfe, 2000; Alvares-Olmos and Oberhelman, 2001).

1.2.6 Mechanism of action

1.2.6.1 Antimicrobial activity

Probiotics are able to modify the gut microbiota composition by means of a number of different factors, one being the reduction of luminal pH. This is achieved through their production of lactic and acetic acids, which inhibit the growth of pathogenic bacteria (Ng *et al*., 2009; Wohlgemuth *et al*., 2010). They exert their antimicrobial effects on their host intestinal epithelium directly by production of antimicrobial factors and indirectly through the

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increase in expression of host cell antimicrobial peptides. Host intestinal cells produce defensins, which kill susceptible bacteria, fungi and viruses, and cathelicidins which aid in protection of the host against pathogens. Most probiotics themselves also produce antimicrobial peptides such as bacteriocins (e.g. nisin and lactacin B) (Wohlgemuth *et al*., 2010; Amalaradjou and Bhunia, 2012). Probiotics may also compete with pathogenic bacteria for epithelial binding sites thereby inhibiting pathogen invasion and blocking their adhesion to these sites (Ng *et al*., 2009; Wohlgemuth *et al*., 2010).

1.2.6.2 Improved barrier function

The intestinal barrier serves as a defence mechanism, by maintaining epithelial integrity. Disruption of its integrity turns the intestines into a site where pathogenic bacteria and food antigens enter and reach the submucosa causing intestinal disorders. Probiotics are able to repair and maintain this barrier after damage by stimulating mucus secretion, chloride and water secretion and the binding together of these submucosal cells by tight junctional proteins (Ng *et al*., 2009; Wohlgemuth *et al*., 2010).

1.2.6.3 Enhancement of host immune functions

Probiotics utilize specific effects on different cells involved in innate and adaptive immune responses. These cells include epithelial and dendritic cells, macrophages, B cells, T cells and natural killer (NK) cells (Ng *et al*., 2009). Intestinal microorganisms are recognized by the immune system in the gut through pattern-recognition molecules called Toll-like receptors (TLRs), which results in complex intracellular signal transduction cascades and enhancement of pro- and anti-inflammatory cytokine production (Wohlgemuth *et al*., 2010).

1.2.7 Concerns about the use of probiotics

Although there are numerous advantages and health benefits associated with probiotics, there are certain risks associated with probiotic therapy. These risks are mainly concerned with respect to safety in vulnerable target groups such as immunocompromised individuals (pregnant women, babies and the elderly) or critically ill or hospitalized patients (Boyle *et al*., 2006; Jankovic *et al*., 2010).

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Probiotic cultures are resistant to some antibiotics. There is concern about the possible transfer of antimicrobial resistance from probiotic strains to pathogenic bacteria in the gut. For example, many *Lactobacillus* strains are naturally resistant to vancomycin, which poses a potential threat of transfer of this resistance to other pathogenic bacteria such as *Staphylococcus aureus*. However, these vancomycin-resistant genes in lactobacilli are chromosomal and not readily transferred to other species (Boyle *et al*., 2006).

Another important area of concern is the risk of sepsis. There have been several reports of cases of *Lactobacillus* sepsis and other bacterial sepsis due to the intake of probiotic supplements (Boyle *et al*., 2006). One case included a 67 year-old man who took probiotic capsules daily for mitral regurgitation and developed *L. rhamnosus* endocarditis after a dental procedure (Mackay *et al*., 1999; Borriello *et al*., 2003). In another case, a 4-month old infant with AAD, who was given *L. rhamnosus* after cardiac surgery, developed *Lactobacillus* endocarditis 3 weeks after *L. rhamnosus* treatment (Kunz *et al*., 2004; Boyle *et al*., 2006). However, there have been no reports to date on the occurrence of *Bifidobacterium* sepsis. All cases of bacterial sepsis from the use of probiotics (*Lactobacillus* spp.) have occurred in immunocompromised individuals or patients who have a chronic disease or debilitation. No cases have been reported in healthy individuals. There is also a small risk of adverse metabolic effects from manipulation of the microbiota with the use of probiotics, although probiotic studies to date have not shown significant adverse effects on growth or nutrition (Boyle *et al*., 2006).

1.2.8 Prebiotics and synbiotics

1.2.8.1 Prebiotics

Prebiotics are defined as ‘nondigestible food ingredients that selectively stimulate the growth and/or activity of one or a limited number of gut microflora which in turn beneficially affects the host’s well-being and health’ (Tuohy *et al*., 2003; Davis and Milner, 2009; Wang, 2009). The most important GI microbiota targeted for selective stimulation are lactobacilli and bifidobacteria (Wang, 2009). Non-digestible oligosaccharides such as inulin, other oligosaccharides, lactulose, resistant starch and dietary fibre are common prebiotics (Davis and Milner, 2009). Fruit and vegetables such as leek, asparagus, banana, garlic, onion,

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chicory, artichoke, as well as wheat, oats and soybeans naturally contain the prebiotic inulin (Manning and Gibson, 2004; Davis and Milner, 2009).

Three criteria are required for a dietary substance to be classified as a prebiotic. The substance must not be absorbed in the stomach or small intestine, it must be selectively utilized by the beneficial gut microflora and beneficial luminal or systemic effects should be induced within the host upon its fermentation. An individual needs to consume 4-8g/day of fructooligosaccharide in order to significantly increase levels of bifidobacteria in the gut (Manning and Gibson, 2004).

In the last decade there have been numerous investigations on the health-promoting effects of prebiotics. Some of these effects include better mineral absorption, alleviation of constipation and IBS, protection against colon cancer, enhancement of the immune system, anticarcinogenic effects and lowering of cholesterol (Tuohy *et al*., 2003; Manning and Gibson, 2004; Venter, 2007; Davis and Milner, 2009).

1.2.8.2 Synbiotics

Synbiotics have been defined as ‘a combination of probiotics and prebiotics which improves the survival, establishment and activity of the probiotic in the GIT, thereby having a beneficial effect on the host. Synbiotics aim to improve the viability of the proven probiotic *in vivo* as well as stimulate the indigenous gut microflora such as bifidobacteria. A well-known benefit of synbiotics is that they increase the persistence of the probiotic in the GIT (Tuohy *et al*., 2003; Davis and Milner, 2009).

1.3 BIFIDOBACTERIA

Although *Lactobacillus* spp., which have been extensively studied for many years, are used most often in probiotic products due to their exceptional tolerance to the harsh conditions in the gut, there has been a rapid increase in the use of *Bifidobacterium* spp. as probiotics. Bifidobacteria were first discovered in 1899 by Tissier, who isolated a Y-shaped bacterium from infant faeces and named it *Bacillus bifidus* (Husain *et al*., 1972; Biavati *et al*., 2000). There was not much progress made relating to facts and information on these bacteria in the 50 years after their isolation. Then in 1957, Dehnart recognized numerous biotypes of

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Bifidobacterium and proposed a method to differentiate these bacteria based on their carbohydrate fermentation patterns. By 1963 seven spp. of *Bifidobacterium*, including *B. bifidum* were recognised by Reuter. In the first edition of Bergey's Manual Systematic Bacteriology, 24 species were reported. Since then there are now 32 known species within the genus (Biavati *et al*., 2000).

1.3.1. Morphology and physiology

Bifidobacteria belong to the class *Actinobacteria* and are Gram-positive, anaerobic and fermentative rods typically Y or V-shaped (Moubareck *et al*., 2005; Mättő *et al*., 2007). This rod-shape is highly variable and is influenced by nutritional or other conditions (Boylston *et al*., 2004). Bifidobacteria were previously classified as LAB. Their sensitivity to oxygen varies between species and different strains within a species. Bifidobacteria of human origin have an optimum growth temperature of 37°C- 38°C, whereas animal species grow at slightly higher temperatures of 41°C- 43°C. No growth occurs below 20°C and above 46°C (Biavati *et al*., 2000; Boylston *et al*., 2004). They are acid-tolerant bacteria and their optimum pH is 6.5 to 7.0 with little or no growth below the pH range of 4.5 to 5.0 and above 8.0 to 8.5 (Biavati *et al*., 2000; Roy, 2001; Reyed, 2007). They do not produce spores and promote good intestinal health in humans and animals (Domig *et al*., 2007; Mättő *et al*., 2007). Bifidobacteria produce acetic and lactic acid in a molar ratio 3:2 and are able to degrade glucose (Tannock, 1997; Mättő *et al*., 2007). They are distinguished from *Lactobacillus* species by the presence of fructose-6-phosphate phosphoketolase enzyme (F6PPK) (Doleyres and Lacroix, 2005).

Due to the production of acetic and lactic acid, bifidobacteria have an antagonistic activity against a wide variety of microorganisms such as *Campylobacter*, *Clostridium perfringens*, *Escherichia coli*, *Salmonella*, *Shigella dysentriae*, *Vibrio cholerae*, *Yersinia enterocolitica* (Biavati *et al*., 2000; Reyed, 2007). These acids lower the intestinal pH which then prohibits the growth of pathogens. Acetic acid has a greater antimicrobial effect compared to lactic acid due to an increased amount of undissociated acetic acid in the intestine and because it is produces almost two-fold more than lactic acid. In addition, some bifidobacterial species produce bacteriocins with a broad antimicrobial spectrum of activity towards both Gram-positive and Gram-negative bacteria (De Vuyst and Leroy, 2007).

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1.3.2 Identification

The most practical and common phenotypic tests used to identify or assign a bacterial strain to the genus *Bifidobacterium* is the phosphoketolase assay. This assay makes use of the F6PPK which is intracellular and can be extracted by sonification of the harvested bifidobacterial cells. Cultures can be identified as belonging to the *Bifidobacterium* genus if a reddish-violet colour is observed on completion of the assay (Biavati *et al*., 2000; Orban and Patterson, 2000). Gas chromatography of the fermentation products can also be performed for identification at genus level (Biavati *et al*., 2000).

There are many molecular techniques that have been developed for identification of bifidobacteria. In 1995, Langendijk and colleagues developed three 16S rRNA hybridization probes that detected *Bifidobacterium* species in human fecal microflora using whole-cell hybridization. In 1996, Mangin and colleagues used ribosomal DNA polymorphism to demonstrate between intra- and interspecies differentiation of *Bifidobacterium* strains (Mangin *et al*., 1996). A year later, by designing an oligonucleotide probe specific for this genus, Kaufmann and colleagues developed a practical identification system for bifidobacterial strains isolated from food and faeces. The % GC of bifidobacteria DNA is 55-67% and one of the most reliable identification methods for this species is the DNA-DNA homology technique. The main disadvantage of this technique is that it is time-consuming and laborious and thus not commonly applied nowadays. An additional genotypic tool used for the identification of a wide range of bifidobacteria, isolated from any environment, at spp., subspecies and strain level is repetitive sequence-based PCR (rep-PCR) fingerprinting (Masco *et al*., 2003). Amplified ribosomal DNA restriction analysis (ARDRA) can also be used to differentiate between the strains (Křížová *et al*., 2006).

1.3.3 Ecology

Bifidobacteria coexist with a large number of different bacteria in the intestinal tract of both animals and humans. Studies have found over 7000 strains isolated from different habitats, such as from faeces of human infants, adults and animals, the vagina and in dental caries (Biavati *et al*., 2000). Bifidobacteria are among the first bacteria to colonize the GIT of new-borns and predominate in breast-fed infants until weaning (Schell *et al*., 2002; Reyed, 2007).

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The main spp. isolated from the colon of humans and used in probiotic products are *B. adolescentis*, *B. bifidum*, *B. infantis*, *B. lactis*, *B. breve* and *B. longum* (Collins *et al*., 1998; Ouwehand *et al*., 2002).

Bifidobacteria are very sensitive microbes and have a low survival rate due to stresses occurring during their production, storage and consumption. Although they occur in a wide range of food products such as fermented milks, drinking and frozen yoghurts, ice-cream, probiotic cheeses and fermented soya products, they are sensitive to low pH, hydrogen peroxide and have low viability at a low temperature in refrigerated products (Doleyres and Lecroix, 2005). Therefore it is important to find ways and means to increase their survival and viability as well to know what they are susceptible to.

There are many ways to protect bifidobacteria, such as microencapsulation, which provides a physical barrier against the external environment and keeps cells viable. Microencapsulation is defined as a technology for packing solids, liquids or gaseous materials in miniature sealed capsules, which then release their contents at controlled rates under specific conditions (Anal *et al*., 2006). Microencapsulation in protein or polysaccharide gel beads is a means of protecting sensitive bifidobacteria against acid environments, high oxygen levels and cold temperatures for freezing or refrigeration. Immobilised cell technology and long term continuous culture also produce bifidobacterial cells with enhanced viability and tolerance to harsh environmental conditions (Doleyres and Lacroix, 2005).

1.3.4 Susceptibility to antimicrobials

Bifidobacteria are susceptible to various antimicrobials. There have been several studies and publications on the susceptibility of *Bifidobacterium* spp. to different antimicrobial agents, especially antibiotics (Moubareck *et al*, 2005; Masco *et al*., 2006; Domig *et al*., 2007; Mättő *et al*, 2007). Table 1.1 shows what antibiotics bifidobacteria are susceptible and resistant to. Since there is a continuous rise in the consumption of probiotics, it is important that those designed especially for consumers' health are well documented with regards to antibiotic resistance. There is a concern that antibiotic resistance genes may be transferred to potential pathogens in the gut, rendering them resistant to certain antibiotics, creating an even bigger problem (Moubareck *et al*., 2005)

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Table 1.1: Antibiotics to which *Bifidobacterium* spp. are resistant and susceptible to, as well as those antibiotics which have variable effectivity (Reyed, 2007).

Resistant	Variable effectiveness	Susceptible
Nalidixic acid	Tetracycline	Penicillins
Neomycin	Moxalactam	Vancomycin
Kanamycin	Cloxacillin	Macrolides
Streptomycin	Oxacillin	Clindamycin
Metronidazole	Fusidic acid	Chloroamphenicol
Gentamicin	Furazolidone	Cephalosporins
Polymoxin B	Nitrofurazone	Erythromycin
Colistin		Spiramycin
Sulphonamide		Bacitracin
Aminoglycoside		Oleandomycin
		Imipenam
		Nitrofurantoin
		Ampicillin
		Amoxicillin
		Carbenicillin
		Novobiocin

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The tet(W) gene is known to be responsible for acquired tetracycline resistance in *B. longum*, *B. bifidum*, *B. adolescentis* and *B. pseudocatenulatum* (Moubareck *et al*., 2005; Masco *et al*., 2006).

The antimicrobial activity of essential oils and structurally related food additives has also been tested against *B. longum* and *B. breve* (Si *et al*., 2006). This study found that some essential oils or compounds could be used *in vitro* as substitutes for antibiotics. It is also important to test the susceptibility of probiotics to certain foods or compounds that have antimicrobial activity, as it would be useless to use probiotics together with certain foods that may inhibit their effect or activity.

1.3.4.1 Antimicrobial susceptibility testing methods

The determination of antimicrobial susceptibility of anaerobic organisms is generally more complicated than for aerobic organisms due to the difficulty in isolating and identifying them, and the necessity to test them in an anaerobic environment (Stalons and Thornsberry, 1975). There have been a large number of different methods and protocols which have been described for antimicrobial susceptibility testing of bifidobacteria. These include, among others, the agar dilution method, the disk diffusion (DD) assay, broth-dilution and the E-test (Arora and Kaur, 1999; Masco *et al*., 2006; Domig *et al*., 2007; Mättő *et al*., 2007).

1.3.4.1.1 Agar dilution method

The agar dilution susceptibility-testing method is used for determining the minimum inhibitory concentration (MIC) of an antimicrobial agent required to inhibit the growth of a microorganism. MIC is the lowest concentration needed to inhibit the growth of a microorganism. The agar dilution method provides a quantitative result in the form of an MIC. It is accepted worldwide and is the most well-established method for susceptibility testing and commonly used as the standard method for evaluation of new antimicrobial agents and susceptibility test methods (Schwalbe *et al*., 2007).

This method has various advantages such as the ability to test a large number of organisms at the same time, the ability to detect mixed cultures and flexibility in the antimicrobial selection as well as concentration range to be tested (Stalons and Thornsberry, 1975; Schwalbe *et al*., 2007). When compared to other methods such as the disk diffusion method and broth microdilution, the agar dilution test performs better and has fewer errors (Steward

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et al., 1999). Despite these advantages there are also some problems associated with this method. A major disadvantage is that the organisms tested are likely to be subjected to oxygen when they are spread on the surface of the agar and their growth is therefore likely to be inhibited. The addition of agar to broth also adds to the complexity of the medium and therefore may interfere with the accuracy of the test (Stalons and Thornsberry, 1975).

1.3.4.1.2 Disk diffusion (DD) method

The disk diffusion susceptibility test is where a paper disk is impregnated with a known amount of antimicrobial agent and placed on the surface of a solid medium which has previously been inoculated with the bacterial suspension to be tested. The antimicrobial agent then diffuses out of the disk into the medium, producing a concentration gradient. Bacterial growth in the vicinity of the disk only occurs when the concentration diffusing from the disk is no longer sufficient to inhibit bacterial replication or when the bacteria is resistant to the antimicrobial. If the concentration of the antimicrobial is sufficient and there is no bacterial growth, a zone of inhibition will form (Schwalbe *et al*., 2007).

The advantages of this method are that it is simple and easy to perform; it is also convenient and cost effective. Despite these advantages bifidobacteria are subjected to oxygen when they are spread onto the surface of the agar which may inhibit their growth and may therefore interfere with the results. Another disadvantage is that it only gives qualitative results (Stalons and Thornsberry, 1975). Because quantitative MIC information is preferred above classification into resistant, intermediate and susceptible phenotypes for evaluation of the biosafety of industrial LAB cultures (probiotics), dilution methods and the E-test are favoured above the disk diffusion test (Mayrhofer *et al*., 2008).

1.3.4.1.3 Broth-dilution method

The broth-dilution method is performed by making serial dilutions of the antimicrobial agent in a liquid medium, which is then inoculated with a standardized number of organisms and incubated for a certain period of time. The lowest concentration/highest dilution of antimicrobial preventing the appearance of turbidity is considered to be the MIC. In addition, the minimum bactericidal concentration (MBC) can be determined by sub culturing the contents of the tubes onto antibiotic free medium and examining bacterial growth (Stalons and Thornsberry, 1975).

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Advantages of the broth-dilution method are that the use of broth minimizes exposure of the anaerobic organisms to oxygen, thereby increasing the growth response and reducing the time needed to reach a high amount of growth in the culture. It is a very precise method and can be used to determine both MIC and MBCs. It can also be used to test several antibiotics in synergism studies. This method also gives both quantitative and qualitative results. It is efficient for routine use in laboratories where only a small amount of anaerobic isolates are tested at a time. Disadvantages associated with this method include having fewer sources for variation in testing anaerobic bacteria compared to the agar dilution and disk diffusion methods. There is also a high risk of contamination and this method allows testing of only one organism per series of test tubes (Stalons and Thornsberry, 1975).

1.3.4.1.4 The E-test

The E-test combines the principle of the agar diffusion test with the determination of the MIC value. It is based on the diffusion of a continuous concentration gradient of an antimicrobial agent from a plastic strip into an agar medium. For bacteria sensitive to the antimicrobial, an oval zone of inhibition will result. The point at which the border of the zone of inhibition crosses the strip is the MIC. There have been many studies evaluating the E-test as a routine susceptibility testing method and comparing it with other methods such as the broth micro-dilution, agar dilution and the disk diffusion method (Hughes *et al*., 1993).

In general, the E-test-determined MIC's were favourable when compared to those of the above-mentioned methods (Hughes *et al*., 1993). This method is reasonably fast, accurate and reproducible (Akcali *et al*., 2005). It is also easy to perform, easy to interpret and allows simultaneous testing of several antimicrobials (Hughes *et al*., 1993). This technique is also less labour-intensive than the standard agar and micro-dilution methods (Kelly *et al*., 1999). Despite these advantages there is also a possibility that the anaerobic test organism may be exposed to oxygen and their growth inhibited when they are spread onto the plates. E-tests are also very expensive to perform (Akcali *et al*., 2005).

1.3.5 Isolation and enumeration

In 1990, Rasic and Sad confirmed the two factors important in detecting and enumerating bifidobacteria. They are: an adequate culture medium and anaerobic conditions. The success

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of detection of bifidobacteria in optimal culture media is dependent on the following factors: firstly, if the culture media has no selective effect, then non-bifidobacteria may outgrow bifidobacteria. Secondly, if bifidobacteria colonies are to be identified macroscopically, differential colouring may be helpful. The freshness of the ingredients of the media is also very important for optimal growth. Lastly, the composition of the culture medium allows the growth of different biotypes present in the medium to be investigated (Roy, 2001).

In 1983, Rasic and Kurmann divided the culture media for bifidobacteria into commercial, complex, selective, synthetic and semi-synthetic. When selecting a culture medium for bifidobacteria the following factors must be considered; supply of nutrients and growth material, low oxidation-reduction potential, maintenance of the pH value during growth, the buffering capacity and final pH of the medium. De Man Rogosa Sharpe (MRS) supplemented with cysteine is the medium of choice for industrial quality control laboratories. From the large number of selective media available, it can be said that there is no standard medium for the detection of bifidobacteria (Roy, 2001).

Various growth media have also been specifically used for susceptibility testing. Recently, the LAB susceptibility test medium supplemented with cysteine (LSM + cysteine), was proposed for susceptibility testing of bifidobacteria. The Iso-Sensitest agar (ISA) (BSAC) and Mueller-Hinton agar (CLSI) are the universally applied test media which are generally used for bifidobacteria susceptibility testing but they do not always support growth of any given LAB food strain. The LSM media supplemented with cysteine provides sufficient growth support of all bifidobacterial strains and minimizes antagonistic effects between antimicrobial agents and growth medium components (Masco *et al*., 2006). Schaedler broth and Mueller-Hilton broth are used when broth dilution susceptibility tests are used as they support the growth of the majority of anaerobic organisms (Stalons and Thornsberry, 1975).

1.4. VIABILITY ASSESMENT OF BIFIDOBACTERIA

Assessment of cell viability is one of the main requirements in many areas of microbiology, from environmental research to industrial applications (López-Amorós *et al*., 1997). Bacterial viability is the reproductive capacity of the bacterium and survival is the maintenance of the viability (Bunthof *et al*., 2001). In other words, a microbial cell is considered “viable” if it

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has all the elements and mechanisms needed for continued reproduction. Probiotic-containing foods and products need to contain a high number of viable probiotic cells in order to be beneficial to the consumers (Alakomi *et al*., 2005). Probiotic products should contain at least 10^7 colony forming units (cfu) per ml or g of product (Auty *et al*., 2001). The viability of probiotic bifidobacteria depends on the bacterial strain; fermentation conditions such as composition of growth medium and toxic by-products; downstream processes such as mechanical stress, extreme temperature conditions and oxygen stress; storage and preservation conditions such as temperature, moisture and acid content; and finally conditions in the GIT such as bile salt, acid, and enzymatic processes (Roy, 2001; Lacroix and Yildrimin, 2007).

Cell viability in probiotic products is traditionally assessed by the plate count method, where cells actively grow and form visible colonies on solid media (Keer and Birch, 2003; Alakomi *et al*., 2005). There are many disadvantages associated with this approach. The number of viable microorganisms may be underestimated as this method excludes sub lethally damaged and fastidious uncultivable bacteria, and viable cells that have lost the ability to form colonies (Keer and Birch, 2003). The microorganisms may also be unevenly distributed in the product and may also be in clumps or chains (Auty *et al*., 2001). This method is also time-consuming as it requires an incubation period of 2-3 days before the colonies can be counted. In addition to this, for many species a good growth medium is yet to be found (Auty *et al*., 2001; Bunthof *et al*., 2001). Bifidobacteria are anaerobic organisms and they are likely to be subjected to oxygen when they are spread on the surface of the agar. Growth is therefore likely to be inhibited, which could result in an underestimation of the number of viable cells (Auty *et al*., 2001).

Cultivation-based methods provide only a limited view of the physiological status of cells. There are alternative culture-independent methods that can be used instead for testing the viability of probiotic products. These methods provide a means of detecting the cells that may not been detected (dormant cells) by the culture-based methods (Lahtinen *et al*., 2006).

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1.4.1 Molecular techniques

Nowadays, molecular methods are more popular for assessing bacterial viability. Initially, the presence of intact DNA sequences was used as an indicator of cell viability, with the assumption that dead cells' DNA would be degraded more rapidly than other cellular components. Ribosomal RNA (rRNA) has also been used as an indicator of viability. Molecular methods include the PCR, hybridisation as well as reverse transcriptase PCR (RT-PCR) to detect mRNA which increases the speed and specificity of assessing viability of bacterial species in a sample. Nucleic acid sequence-based amplification (NASBA) and reverse transcriptase-strand displacement amplification (RT-SDA) have also been used as indicators of bacterial viability (Keer *et al*., 2003).

These molecular methods are indirect methods and the persistence of nucleic acids is highly dependent on both the manner of cell death and environmental conditions and therefore, there may be a poor correlation between the results obtained using molecular methods and cell viability. The use of a combination of the above indirect molecular methods provides a better correlation with cell viability than the use of only a single analytical technique (Keer *et al*., 2003).

1.4.2 Flow cytometry

Flow cytometry is an appealing technique for fast analysis of bacterial viability, which measures the physical and physiological characteristic of individual cells as they flow past an electrical or optical sensor (Diaper *et al*., 1992; Bunthof *et al*., 2001). The principle of flow cytometry is that single particles suspended within a stream of liquid are examined individually within a short time period as they pass through a light source. The optical signals generated are mostly spectral bands of lights in the visible spectrum, which represent the detection of various chemicals or biological components, mostly fluorescence (Robinson, 2004). It uses fluorescent labelling and can analyse 5 000- 10 000 cells at a time (Bunthof *et al*., 2001). The basis of the flow is a jet of isotonic sheath fluid, which travels at a speed of 20 km h^{-1}, into which the bacterial samples are introduced at controlled rates. This flow of cells moves in a single file into the cytometer. The light source used is either a mercury vapour lamp or different lasers. Three factors are measured using photomultiplier tubes as the

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particles pass through the light beam. These factors are forward scatter (FS), side scatter (SSC) and fluorescence. FS is related to cell size while SSC measures the internal composition of the cell (Veal *et al*., 2000).

The most widely used dye for determining bacterial or cell viability is the esterase substrate carboxyfluorescein diacetate (cFDA), which measures intracellular esterase activity. This is a non-fluorescent dye that diffuses across undamaged or intact cell membranes and yields a positively charged green fluorescent compound, carboxyfluorecein (cF), upon hydrolysis by non-specific cellular esterases. Another nucleic acid dye used as a probe for mortality studies, is propidium iodide (PI), which enters cells with damaged membranes but not those with intact membranes and forms a red fluorescent DNA-complex. Stained cells correspond to cells having compromised or damaged membranes and are considered dead (Rault *et al*., 2007; Paparella *et al*., 2008).

Multiparametric flow cytometry uses cFDA and PI at the same time and is a very useful and sensitive tool for measuring viability of bacteria. It has been used for this purpose for *Bifidobacterium* spp. that are sensitive to bile salts, as well as *Streptococcus macedonicus* which is sensitive to acid (Rault *et al*., 2007). Flow cytometry has also been used to assess the viability of *Lactococcus*, *Lactobacillus*, *Leuconostoc*, *Enterococcus* and *Pediococcus* spp. among others (Bunthof *et al*., 2001). The LIVE/DEAD *Bac*Light kit is widely used in microbial flow cytometry to measure viability, although to obtain useful results one must ensure appropriate controls are used (Winson and Davey, 2000). It comprises two fluorescent nucleic acid stains: SYTO9 and PI. SYTO9 penetrates both viable and nonviable bacteria, while PI penetrates bacteria with damaged membranes only, as mentioned above. Therefore, bacterial cells with compromised membranes fluoresce red and those with intact membranes fluoresce green (Auty *et al*., 2001).

Subpopulations can also be physically selected or sorted for further studies. Cell sorting is a very useful procedure as it can be used for confirmation of a particular population. A specific population can be isolated for further analysis and growth and it can also be used to isolate a rare event (Veal *et al*., 2000; Winson and Davey, 2000; Bunthof *et al*., 2001). In addition to estimation of cell viability, there are many other applications of flow cytometry and cell sorting. A wide range of fluorescent probes are available for directly estimating cellular parameters such as nucleic acid content, enzyme activity, calcium flux, membrane potential

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and intracellular pH. The use of flow cytometers for cell sorting is also widespread and applications range from separation of large numbers of cells for functional studies or chromosomes for preparing gene libraries (Ormerod, 2000).

The major advantages of flow cytometry are that a large number of bacterial cells can be examined simultaneously and data acquired quickly. Both physical as well as physiological characteristics can be studied. The cells of interest can also be recovered by sorting. Disadvantages are that the data corresponds to the population of the cells, making it difficult if not impossible, to tell which individual cell produced a particular measurement. The flow cytometer is also a very complex instrument, requiring the possession of knowledge of the underlying principles and operation in order to use the machine and for accurate interpretation of results. There are also many different methods and staining procedures for different parameters measured (Ormerod, 2000; Winson and Davey, 2000; Robinson, 2004).

1.4.3 Microscopy

Microscopy is also very useful for assessing bacterial viability, although it is somewhat laborious, and some microscopic techniques require the differentiation of live and dead bacteria by using fluorescent stains or dyes (Auty *et al*., 2001; Alakomi *et al*., 2005).

1.4.3.1 Fluorescent microscopy

These fluorescent stains allow the differentiation of viable, dead and damaged cells in a sample by staining the viable cells with one stain followed by counterstaining of dead or all cells with another stain (Alakomi *et al*., 2005). The direct epifluorescent microscope is used for enumeration of environmental samples and conventional epifluorescence microscopy is used for viability staining of liquid samples such as milk. Fluorescence microscopy is a rapid and direct method for assessing bacterial viability but it has a disadvantage that certain strains cannot be identified. The fluorescent indicators of viability are based on membrane integrity, enzyme activity, membrane potential, respiration and pH gradient, similar to flow cytometry (Auty *et al*., 2001).

Confocal scanning laser microscopy (CSLM) has been used to study the viability of *Escherichia coli* and *Salmonella* among other bacteria (López-Amorós *et al*., 1997). CSLM

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has many advantages such as increased sensitivity and clearer focus, which enables observation of subsurface structures of food *in situ*. The digital attainment of images by this microscope enables quick enumeration of bacteria by image analysis. It has been used for direct *in situ* viability assessment of bacteria in probiotic dairy products such as the human probiotic strains of *Lactobacillus paracasei* and *Bifidobacterium* spp. (Auty *et al*., 2001).

1.4.3.2 Scanning electron microscopy (SEM)

Microscopes that do not need fluorescent dyes can also be used to determine the viability of bacterial samples. SEM is one such microscope used to test bacterial viability by viewing the structural characteristics of the cells outer surface. During the last 40 years, SEM has been a very useful tool for scientists to observe the external surfaces of microorganisms. A beam of electrons is scanned back and forth across the specimen and focused on a very small point on the specimen of interest, resulting in a transfer of energy to that area. These primary electrons will then dislodge electrons from the specimen itself. The resulting secondary electrons will then be attracted and collected by a detector and then translated into a signal image, allowing scientists to see the intricate details of the material's surface. The specimen is coated with a thin film of heavy metal such as gold, which enhances conductivity at low voltage and contrast at high voltage (Kim and Fane, 1993; Williams and Carter, 1996; Muscariello *et al*., 2005; Madigan and Martinko, 2006). The specimen has to be thin in order to transmit sufficient electrons so that enough intensity falls on the screen to produce an interpretable image in a reasonable time (Williams and Carter, 1996).

Advantages of SEM include its ability to analyse intact biological structures regardless of their shape and it has a wide range of magnification, able to go to more than 300 000x. It has a high depth of field, which allows more of the specimen to be in focus at one time. The image produced is a representation of electronic data, allowing for image analysis and quantification. It has also been described as a technique that combines the best aspects of light microscopy and transmission electron microscopy (Müller and Engel, 2001; James, 2009).

There are certain limitations to SEM such as the requirement of coating samples before viewing. Coatings such as gold are very expensive and they also obscure the fine surface detail on some of the specimens, although SEMs equipped with field emission guns have made such samples easier to view (Kim and Fane, 1993; Muscariello *et al*., 2005). Another

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difficulty is when wet or damp samples, such as biological tissue, need to be viewed. The high vacuum requirements in the chamber means that in order to remove or fix the water before imaging, long specimen preparation techniques are required. This also raises the risk of introducing artefacts which can destroy the finer features and leach out the ions that could otherwise be analyzed by microanalysis (Muscariello *et al*., 2005; James, 2009). Drying of the sample can also lead to damage of the sample's structure (Kim and Fane, 1993). These problems can be overcome by the new environmental scanning electron microscope (ESEM) that allows the observation of wet samples without the need of prior sample preparation (Muscariello *et al*., 2005). Despite these disadvantages, SEM is still a very useful tool for estimating cell viability.

1.4.3.3 Infrared (IR) and Raman spectroscopies

Vibrational spectroscopies such as IR and Raman are nowadays extensively used to study the molecular and chemical (lipids, proteins, membranes, nucleic acids, polysaccharides, ect.) composition of cells based on the fact that all organic molecules absorb IR light. Useful information on the chemical composition of cells can therefore be attained (Beekes *et al*., 2007; Zoumpopoulou *et al*., 2010; Lu *et al*., 2011b). There have been many studies on the application of these techniques to identify changes at cellular level after cells have been exposed to certain stress factors which cause internal damage to the cell (Zoumpopoulou *et al*., 2010). Fourier transform infrared (FT-IR) spectroscopy is a physiochemical technique that "fingerprints' the entire cell and is able to detect even the slightest compositional changes that cannot be revealed by other methods (Alvarez-Ordóñez *et al*., 2010).

Advantages of these techniques is that they are non-invasive, inexpensive, simple to use, rapid, no reagents are used and all biological specimens can be distinguished; including bacteria (Davis and Mauer, 2010; Lu *et al*., 2011b). Detection, enumeration, classification and identification can all be carried out using one single instrument when using IR spectroscopes. There is also little or no sample preparation required and therefore bacterial cells remain intact during analysis (Naumann, 2000; Lin *et al*., 2004; Davis and Mauer, 2010). Despite the many advantages of IR spectroscopy, there are a few disadvantages. Only microorganisms that are grown in culture can be analyzed and factors such as cultivation time, temperature and culture medium need to be rigidly controlled in order to obtain reproducible results. Multiple scans of the same sample are therefore required (Naumann, 2000; Davis and Mauer, 2010).

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Both Raman and IR spectroscopies are non-invasive, rapid alternatives to study changes and injury in bacterial cell membranes by various treatments such as heat and cold treatment, antibiotic, bacteriocin and chemical treatment (Lu *et al*., 2011a; Lu *et al*., 2011b). FT-IR spectroscopy has been successfully used in Microbiology (Marcotte *et al*., 2007). The FT-IR spectra of microorganisms represent a unique "fingerprint" which is highly representative for different strains and species. Gram-positive and Gram-negative bacteria can be faultlessly discriminated on their FT-IR spectra (Ngo-Thi *et al*., 2003). Changes in the IR spectra of bacteria can replicate modifications in protein and polysaccharide contents using vibrational methods related with the functional group characteristics of these species (Marcotte *et al*., 2007).

FT-IR has been used in numerous studies to detect bacterial cell damage. Lu and colleagues (2011c) successfully used FTIR together with Raman spectroscopy to detect cell injury caused by garlic and its diallyl constituents on *Listeria monocytogenes* and *Escherichia coli* as well as on *Campylobacter jejuni* in another study. Zoumpopoulou and colleagues (2010) detected changes in the cellular composition of *Salmonella enterica* serovar Typhimurium once exposed to antimicrobial compounds of *Lactobacillus* strains. FT-IR spectroscopy has also been used to differentiate intact and sonication-injured cells of *L. monocytogenes*, heat-induced lethal and sub lethal injury of *Lactococcus lactis* as well as cell damage of *Lactobacillus helveticus* during vacuum drying (Zoumpopoulou *et al*., 2010). The survival and injury of *E. coli* O157:H7, *C. jejuni* and *Pseudomonas aeruginosa* under cold stress was also determined using FT-IR (Lu *et al* 2011a).

1.4.4 Microplate assays

Microplate scale fluorochrome staining assays are also used for assessing the viability of probiotic preparations. This method also makes use of fluorescent staining. A microplate is a flat plate with multiple wells used as test tubes into which the sample to be studied is transferred. The microplate will then be wrapped in parafilm to prevent evaporation and the samples will then be incubated at room temperature for 24 hours for cell attachment. After incubation the plate is then washed three times in a plate washer with a buffer in order to remove loosely adhered cells. Fresh broth is then added to the wells, wrapped with parafilm, incubated and washed. After the final washing, a fluorescent dye such as cFDA, or in other

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cases labels with red or green fluorescent probes is added and the plate is once again incubated and then washed with buffer. The microplate is then read with a fluorescent-plate reader (Snyder and Champness, 2003; Gamble and Muriana, 2007).

A study was carried out to compare this method with traditional culture-based techniques and with epifluorescent microscopy. In this study the cell state of freeze-dried *L. rhamnosus* and *B. animalis* subsp. *lactis* preparations were assessed. It was found that this method is a rapid and strong tool for viability assessment of probiotic preparations and also enabled kinetic measurements (Alakomi *et al*., 2005). Microplate assays are also safe, easy to use cost effective and has greater objectivity in interpreting results (Tansuphasiri *et al*., 2002).

1.5. GARLIC (*Allium sativum*)

Garlic (*Allium sativum*) is a member of the Lillaceae family which includes onions, chives and leeks (Haciseferoğullari *et al*., 2005). It is a rigid, tall, bulbous herb that reaches heights of 30-60 cm and has a very strong smelling odour when crushed. Underground it consists of a multifarious bulb with lots of fibrous roots. Above ground, narrow, grass-like leaves arise from it (Hovana *et al*., 2011). Garlic has been used since ancient times as a food, spice and herbal remedy for curing various diseases and can be consumed fresh, as pills or capsules as well as extracts (Rees *et al*., 1993; Haciseferoğullari *et al*., 2005; Goncagul and Ayaz, 2010).

1.5.1 History

It has been mentioned throughout history, dating back to the ancient Egyptians where slaves building the pyramids were given garlic as part of their staple diet. It was believed that garlic would maintain and increase their strength (Rivlin, 2001; Daka, 2011). It was also placed in tombs of pharaohs as they believed that if they took garlic to their next life, the food there would always be well seasoned. The use of garlic as a food for the Israelites is also mentioned in the bible in Numbers 11:5 (Rivlin, 2001; Amagase *et al*., 2002).

In Ancient Greece there is evidence that during the earliest Olympic Games garlic was fed to the participants in order to give them strength (Deresse, 2010; Daka, 2011). It is also believed that Hippocrates, the father of medicine, used garlic (Rivlin, 2001). The Romans used garlic

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for a wide variety of illnesses and infections such as asthma, animal and insect bites and GIT disorders among others, and was also fed to soldiers and sailors for strength and endurance (Harris *et al*., 2001; Rivlin, 2001). In Ancient China and Japan garlic was used as a food preservative and was part of the daily diet. Garlic tea was recommended for fever, headaches, cholera and dysentery and was believed to have a positive effect on cardiovascular disease (Rivlin, 2001; Corzo-Martínez *et al*., 2007; Deresse, 2010; Daka, 2011).

Throughout the Middle Ages and in World War II it was used to treat the wounds of soldiers. It was also used as a treatment for the Great Plagues (Rivlin, 2001; Amagase *et al*., 2002). In Early America, the Natives added garlic to their tea as they believed it treated flu (Rivlin, 2001). Today, over 3000 studies have confirmed the beneficial health effects of garlic and it is still being employed in folk medicine all over the world for treatment of various diseases (Amagase *et al*., 2002; Corzo-Martínez *et al*., 2007).

1.5.2 Health benefits of garlic

Alliums have been extensively studied for their health benefits and thousands of publications have been written about them (Amagase *et al*., 2002). Garlic and onions have been shown to be effective in cardiovascular disease due to their hypocholesterolemic, hypolipidermic, anti-hypertensive, anti-diabetic, antithrombotic and anti-hyperhomocysteinemia effect. They also have antimicrobial, antioxidant, anticarcinogenic, antimutagenic, antiarthritic, immune-enhancing and prebiotic activities (Matsuura, 1997; Amagase *et al*., 2002; Corzo-Martínez *et al*., 2007).

1.5.2.1 Effects related to cardiovascular disease

Many studies have been done to confirm the beneficial effects of garlic in the alleviation of cardiovascular disease. In studies where garlic essential oil and raw garlic were fed to rats, chicken, swine and rabbits, the results indicated that garlic consumption significantly lowered the total serum cholesterol, low density lipoproteins (LDL), very low density lipoproteins (VLDL) and increased the level of high density lipoproteins (HDL) in the animals. There have also been several clinical reports which revealed the cholesterol-lowering effects of raw garlic and some garlic supplements (Ackermann *et al*., 2001; Amagase *et al*., 2002; Corzo-Martínez *et al*., 2007; Kojuri *et al*., 2007).

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Kojuri and colleagues (2007) tested the effect of garlic on the lipid profile of hyperlipidemic patients. Patients were given enteric-coated garlic powder tablets twice daily, and their lipid profiles were checked after 6 weeks. The results showed that the garlic tablets lowered cholesterol and LDL-cholesterol and increased HDL-cholesterol. These results indicated that garlic may play a very important role in therapy of hypercholesterolemia. Mansell and Reckless (1991) suggested that garlic essential oil had an effect on reducing blood pressure in hypertensive patients (Corzo-Martínez *et al*., 2007). Other researchers reported that the bioactive constituents of garlic and onion had an anti-diabetic effect by stimulating insulin production and secretion by the pancreas, interfering with the absorption of glucose and favouring the saving of insulin. It has also been determined that garlic has an antithrombotic effect by inhibiting platelet aggregation *in vitro* (Corzo-Martínez *et al*., 2007; Tsuchiya and Nagayama, 2008).

1.5.2.2 Anticarcinogenic and antimutagenic activities

The possibility of the use of garlic as an anticancer agent began in 1958 when Weisberger and Pensky demonstrated *in vitro* and *in vivo* that thiosulfinate extracts from garlic inhibited the growth of tumor cells. Since then, there has been ongoing epidemiological and laboratory investigations to prove the chemopreventative or anticarcinogenic effects of garlic and related *Allium* species. Nakata and Fujiwara (1975) identified a carbohydrate in garlic extract that appeared to have antitumor immunity (Corzo-Martínez *et al*., 2007). Studies on their capabilities to inhibit tumor growth and cellular proliferations have shown that ingestion of garlic or onions decreases the risk of sarcoma and carcinoma in the stomach, colon, oesophagus, prostate, bladder, blood, liver, lungs, skin and brain (Sengupta *et al*., 2004; Corzo-Martínez *et al*., 2007; Tsuchiya and Nagayama, 2008). It has also recently been discovered that garlic has an antimutagenic effect. The sulphur compounds in garlic have an effect on DNA repair mechanisms, removing mutations in DNA and therefore preventing the start of carcinogenesis (Corzo-Martínez *et al*., 2007).

1.5.2.3 Immune-enhancing effects

In 2001, Kyo and colleagues found that garlic could be used as a promising candidate as an immune response modifier, being able to maintain the homeostasis of immune function and suppressing unnecessary functions. The components responsible for garlic's immune-enhancing effects are not fully known, but it is known that several low-molecular weight sulphur compounds, such as diallyl sulphide (DAS) and S-allyl cysteine (SAC) have

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immune-stimulating properties. In 1987, Hirao and colleagues isolated a garlic protein fraction with an immune-stimulating effect (Corzo-Martínez *et al*., 2007).

1.5.2.4. Other health benefits

Studies conducted on animals have shown that garlic may improve brain function. This is due to its antioxidant agents that neutralises free radicals accumulating in the body. Garlic could therefore possibly be used to treat patients with Alzheimer's disease. It has also been used to treat asthma, whooping cough, chronic bronchitis, ring worm, ear infections, and tonsillitis and is even used as a mosquito repellent (Kumar *et al*., 2010).

1.5.3 Antimicrobial activity

Garlic has many antimicrobial activities including antibacterial, antifungal, antiviral and antiprotozoal (Uchida *et al*., 1975; Harris *et al*., 2001). Louis Pasteur was the first person to describe the antibacterial activity of garlic juices (Sivam, 2001). It exhibits antibacterial activity against a wide range of Gram-positive, Gram-negative and acid-fast bacteria which include *Bacillus*, *Clostridium*, *Enterococcus faecalis*, *Escherichia coli*, *Hafnia*, *Klebsiella*, *Lactobacillus acidophilus*, *Micrococcus*, *Mycobacterium tuberculosis*, *Neisseria gonorrhoeae*, *Proteus*, *Pseudomonas*, *Salmonella*, *Shigella dysenteriae*, *S. flexneri*, *S. sonnei*, *Staphylococcus* and *Streptococcus* (Uchida *et al*., 1975; Rees *et al*., 1993; Ankri and Mirelman, 1999; Harris *et al*., 2001; Ruddock *et al*., 2005). *Helicobacter pylori*, the causative agent of stomach ulcers and stomach cancer, is also susceptible to garlic (Cellini *et al*., 1996; Sivam, 2001). Garlic extracts also prevent the formation of *Staphylococcus* enterotoxins A, B and C1. On the other hand some bacteria such as the mucoid strains of *Pseudomonas aureginosa*, *Streptococcus β hemolyticus* and *Enterococcus faecium* are resistant to the action of allicin (Ankri and Mirelman, 1999). The antimicrobial effect of garlic on *Bifidobacterium* spp. has not yet been investigated.

Antifungal activity of garlic was discovered in 1936 by Schmidt and Marquardt. *Aspergillus*, *Candida*, *Cryptococcus*, *Epidermophyton*, *Microsporum*, *Rhodotorula*, *Torulopsis*, *Trichophyton* and *Trichosporon* are all susceptible to garlic extracts. Garlic also inhibits the growth of some protozoan species such as *Balantidium entozoon*, *Crithidia fasciculata*,

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Entamoeba histolytica, *Giardia lamblia*, *Leishmania major*, *Leptomonas colosoma*, *Opalina ranarum*, *O. dimidicita* and *Trypanosomes* (Ankri and Mirelman, 1999; Harris *et al*., 2001).

Garlic also exhibits antimicrobial activity against viruses although there have been very little investigations on its antiviral properties. It has been found to have *in vitro* activity against cytomegalovirus, herpes simplex virus 1 and 2, HIV, influenza A and B, parainfluenza virus type 3, rhinovirus, rotavirus, vaccinia virus, vesicular stomatitis virus and viral pneumonia (Rees *et al*., 1993; Ankri and Mirelman, 1999; Harris *et al*., 2001; Goncagul and Ayaz, 2010).

1.5.4 Antimicrobial compounds in garlic

In 1944, Cavallito and colleagues isolated and identified the compound responsible for the antimicrobial activity of crushed garlic cloves. They used different methods of extraction and isolated diallyl disulfide, the main component of garlic oil (Harris *et al*., 2001). This very unstable and rapidly decomposing oxygenated sulphur compound giving garlic its characteristic odour, was called allicin. It is produced when alliin, the stable precursor in garlic, is rapidly converted to allicin by the enzyme allinase once intact garlic tissues are damaged by crushing or cutting (Ankri and Mirelman, 1999; Harris *et al*., 2001; Ruddock *et al*., 2005).

Cross-section studies have shown that alliin and allinase are situated in different compartments in garlic cloves. Fungi and other soil pathogens invade the clove and destroy the membranes enclosing the compartments containing alliin and allinase, thereby causing the reaction between two, producing allicin which in turn inactivates the invading microbes (Ankri and Mirelman, 1999). This suggests that allicin is designed as a potential defence mechanism against soil microbes. Garlic also contains various other compounds such as adjoene, enzymes (peroxidise and miracynase), carbohydrates (glucose and sucrose), amino acids like cysteine, glutamine and methionine; bioflavonoids such as quercetin and cyanidin; vitamin B and C; allistatin I and allistatin II as well as minerals such as iron, calcium, zinc and phosphorous. Some of these compounds may also be responsible for garlic's antimicrobial activity (Goncagul and Ayaz, 2010; Kumar *et al*., 2010).

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1.5.5 Mechanism of action of garlic

Allicin, which is the main active compound in garlic, is readily permeable through phospholipid membranes and is easily taken up by the cell causing damage by altering their membranes which in turn causes autolysis leading to cell death or cessation of growth (Lu *et al*., 2011b). It is known to affect microorganisms by a number of different actions. It acts by immediately and totally inhibiting RNA synthesis and partially inhibiting DNA and protein synthesis (Focke *et al*., 1990; Al-Waili *et al*., 2007; Deresse, 2010). Lipid and fatty acid biosynthesis as well as NO formation are also influenced which has significant changes in the viability of cells (Focke *et al*., 1990; Miron *et al*., 2000). Allicin is able to alter a cells signal transduction and electrochemical ability and even causes cells to undergo apoptosis. In addition, it also reacts with cysteine and thiol containing enzymes in cells (Miron *et al*., 2000; Gruhlke *et al*., 2010).

1.5.6 Adverse effects

The most common side effects from the intake of garlic are bad breath and body odour due to garlic's strong smell. Studies have also shown that excessive intake of garlic can cause GIT problems or upsets such as diarrhoea, flatulence and changes in the intestinal microflora. There have also been instances where skin problems have occurred in individuals where fresh or crushed garlic came into direct contact with the skin and caused blisters, burns and allergic dermatitis. In 1982, Lybarger and colleagues found that workers exposed to garlic powder in garlic-growing or industrial processing developed asthmatic symptoms (Corzo-Martínez *et al*., 2007). There have also been reports that raw garlic and garlic powder decrease serum protein and calcium, cause anemia and inhibit spermatogenesis. However, aged garlic extract (AGE) has no adverse effects and has been studied extensively and its safety has been well established (Amagase *et al*., 2001).

1.5.7 Garlic preparations

Garlic can be consumed fresh, in the form of pills, capsules and extracts (Goncagul and Ayaz, 2010). There are many commercial garlic preparations available and these are classified into four groups, namely, essential oil, garlic oil macerate, garlic powder and garlic extract. All of

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these preparations are produced from the garlic cloves. The manufacturing processes influence the composition of the garlic product and the manufacturers must ensure these products are safe, effective and stable and don't have any adverse effects (Amagase *et al*., 2001).

Garlic essential oil is obtained by heating crushed garlic cloves to 100°C and collecting it as a steam distillate. All water-soluble compounds including allicin are removed from the oil (O' Gara *et al*., 2000; Amagase *et al*., 2001). Garlic oil macerate is a product made of encapsulated mixtures of whole garlic cloves ground into vegetable oil. During the manufacturing process some alliin is converted to allicin (Amagase *et al*., 2001). Garlic powder is mainly used as a spice for condiments and processed foods and is prepared by slicing, drying and pulverizing garlic cloves which then form allicin upon the addition of water. Garlic extract is produced by soaking whole or sliced garlic cloves in an extracting solution such as purified water and diluted alcohol. After separation of the solution the extract is concentrated and is then ready for use (O' Gara *et al*., 2000; Amagase *et al*., 2001).

Aged garlic extract (AGE) is another form of garlic used and is processed differently. The extract is aged for up to 20 months wherein the odorous, harsh and irritating compounds are converted into a safe and natural form (Amagase *et al*., 2001). AGE, in addition to being the safest garlic preparation, has a greater effectiveness than other preparations, even raw garlic and is therefore probably the most suitable preparation used to enrich foods. In addition, it is the only garlic preparation that has been studied to prove its safety for human consumption (Corzo-Martínez *et al*., 2007).

These different garlic preparations have varying effects on the different microorganisms mentioned above as some exert a greater inhibitory effect compared to others. Ross and colleagues (2001) examined the antimicrobial properties of garlic oil and garlic powder against human enteric bacteria. They found that garlic oil had the strongest inhibitory effect on the bacteria and shows that garlic oil contains a much greater concentration of antibacterial substances than garlic powder which needs a liquid medium to activate the allicin. This was also proven in a previous study where the activities of garlic oil and garlic powder were tested against *Helicobacter pylori* (O' Gara *et al*., 2000). Iwalokun and colleagues (2004) showed that AGE has a broad spectrum of antimicrobial activity against many multi-drug resistant Gram-positive and Gram-negative bacteria, as well as *Candida*

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spp. This antimicrobial activity was comparable to that of ciprofloxacin and fluconazole antibiotics. Therefore AGE has a very strong inhibitory effect and is active against many pathogenic microbes.

1.6. CONCLUSION

The viability of probiotic bacteria, in particular bifidobacteria, is essential in order for consumers to get the promised health benefits provided by these probiotic products. Susceptibility of bifidobacteria to numerous antimicrobials has been studied but to our knowledge, to date, there have been no studies on how garlic affects the probiotic strains of bifidobacteria. This question is essential to answer because although garlic has certain beneficial effects, it also kills or inhibits certain bacteria.

Bifidobacteria are used as probiotics due to their numerous health benefits and are also prescribed by doctors to help treat AAD. Therefore, it is essential to know whether probiotics and garlic can be used simultaneously, as it would be a disadvantage and waste of money to the consumer if garlic were to inhibit or decrease the viability of these products. In this study the susceptibility of different probiotic *Bifidobacterium* strains to the antimicrobial effects of garlic will be tested using numerous methods. The disk diffusion method as well as MIC and MBC determination will be performed firstly to see whether garlic inhibits bifidobacteria. Morphological alterations and changes in cell surface properties will be monitored by SEM. FT-IR will be used to assess any biochemical changes taking place inside the cells once exposed to garlic. It will also be used to investigate the mechanism of action of the garlic on bifidobacterial cells. Viability after treatment with garlic will be assessed using flow cytometry as well as determination of possible recovery of garlic-damaged cells.

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CHAPTER 2

Assessment of *Bifidobacterium* susceptibility to inhibitory effects of different garlic (*Allium sativum*) preparations: An *In vitro* study

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2.1 Abstract

Probiotic containing foods and products need to contain high enough numbers of viable probiotic cells in order to be of optimal benefit to the consumer. Therefore it is significant to identify any products or materials that may have a negative influence on probiotic viability. The antimicrobial effect of garlic (*Allium sativum*) on numerous pathogenic microorganisms is well recognized but there is inadequate knowledge on its effects on beneficial probiotic bacteria, in particular bifidobacteria. This study investigated the antibacterial effects of different garlic preparations against selected strains of probiotic bifidobacteria. The disk diffusion assay revealed the antibacterial activity of the different garlic preparations which were characterized by zones of inhibition ranging from 13.0 ± 1.7 to 36.7 ± 1.2 mm. All *Bifidobacterium* strains were inhibited by fresh garlic clove extract and time kill curves established that the inhibitory effect of this extract was apparent within approximately 1 h of incubation. Minimum inhibitory concentration (MIC) values ranged from 75.9 to 303.5 mg/ml garlic (estimated 24.84-99.37 µg/ml allicin) for garlic clove extract. From the selected strains tested it was observed that the *B. bifidum* strain was most susceptible, followed by the *B. longum* strains. The *B. lactis* strains were most resistant to the antibacterial effects of garlic. These results indicate that probiotic bifidobacteria are susceptible to garlic. Therefore probiotic-containing foods and products should not be ingested in conjunction with garlic as this could possibly lower probiotic viability.

Keywords: garlic, *Allium sativum*, allicin, probiotics, antimicrobial, minimum inhibitory concentration values, *Bifidobacterium bifidum*.

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2.2 Introduction

Probiotics are viable microorganisms which when administered in adequate amounts provide significant health benefits to the host by inhibiting the growth of pathogenic organisms in the GIT, thereby sustaining the intestinal microbial balance (Leahy *et al*., 2005; Moubareck *et al*., 2005; Reid, 2006; Wohlgemuth *et al*., 2010). Further health benefits include the treatment of many GIT disorders such as constipation, IBS as well as diarrhoea associated with antibiotics and travel (Schrezenmeir and de Vrese, 2001; Kaur *et al*., 2002). Probiotics are also known to possess antimutagenic and anticarcinogenic properties as well as enhancing immune responses, reducing cholesterol and for their vaccine adjuvant effects (Kaur *et al*., 2002). It is therefore important that consumers get high quality probiotic containing products which possess high enough numbers of viable probiotic cells (Alakomi *et al*., 2005).

The most common strains used as probiotics are those from the LAB which include lactobacilli and bifidobacterial species both belonging to the normal human gut microflora (Sullivan and Nord, 2002). Bifidobacteria have antagonistic activity against many microbes such as *Campylobacter*, *Salmonella*, *Listeria*, *Shigella* and *Vibrio cholera* as well as pathogens such as *Escherichia coli*, *Shigella dysentriae* and *Yersinia enterocolitica* (Biavati *et al*., 2000; Reyed, 2007). Although bifidobacteria do inhibit many microbes, they themselves are susceptible to a wide range of antimicrobials, specifically antibiotics, and this has been confirmed by many studies (Masco *et al*., 2006; Domig *et al*., 2007; Mättő *et al*, 2007). It is therefore important to test the susceptibility of bifidobacteria against various compounds and food products that may have an antimicrobial effect as it would render the specific probiotic ineffective if used in conjunction with the antimicrobial.

Garlic (*Allium sativum*) is a perennial plant which belongs to the Allium family (Liliaceae) (Iwalokun *et al*., 2004; Hovana *et al*., 2010). Throughout history garlic has been used as a food spice and herbal medicine due to its health benefits in preventing and treating a wide variety of diseases (Amagase *et al*., 2001; Haciseferoğullari *et al*., 2005; Belguith *et al*., 2010). There is extensive literature on the health benefits of garlic. Some examples include relief of digestive system disorders, asthma, skin disorders, prevention of cardiovascular disease, kidney and bladder problems, high blood pressure, as well as reduction of pain and inflammation. It has even been proposed to treat malaria, sexual debility, and Alzheimer's

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disease (Kumar *et al*., 2010; Daka, 2011). Garlic is mainly consumed as a condiment in an assortment of dishes (Belguith *et al*., 2010). Allicin is the main active compound in crushed garlic cloves. It is produced by the rapid reaction between the substrate alliin with the enzyme allinase once intact garlic tissue is damaged; by the attack of a microbe, once it is crushed, cut or chewed, or when it is dehydrated, pulverized and then exposed to water (Miron *et al*., 1998; Amagase *et al*., 2001; Miron *et al*., 2002). Antimicrobial agents such as allicin are able to alter bacterial cell membranes which lead to cell leakage as well as autolysis which prevents growth and causes cell death (Lu *et al*., 2011). Allicin concentration in garlic products can be measured using spectrophotometrical analysis by the reaction between 4-mercaptopyridine (4-MP) and allicin (Miron *et al*., 2002). It is this compound, allicin, which is responsible for its antimicrobial activity and acts by immediately and totally inhibiting RNA synthesis as well as partial DNA and protein synthesis (Al-Waili *et al*., 2007; Deresse, 2010).

The therapeutic uses of garlic have been well documented throughout the ages and these include its antimicrobial, antihelmantic, antiseptic and anti-inflammatory properties. The antimicrobial properties of garlic comprise its antibacterial, antifungal, antiviral and antiprotozoal activities (Uchida *et al*., 1975; Rees *et al*., 1993; Deresse, 2010; Daka, 2011). It inhibits both Gram-positive and Gram-negative bacteria which include species such as *Bacillus*, *Candida*, *Enterococcus*, *Escherichia*, *Proteus*, *Pseudomonas*, *Saccharomyces*, *Salmonella*, *Staphylococcus*, *Helicobacter*, as well as *Mycobacterium*, among others (Uchida *et al*., 1975; Rees *et al*., 1993; O' Gara *et al*., 2000; Belguith *et al*., 2010; Deresse, 2010).

Although there have been numerous studies on the effects of garlic on pathogens there are few studies on the susceptibility of beneficial bacteria, particularly probiotic strains of *Lactobacillus* (Rees *et al*., 1993; Naganawa *et al*., 1996; Ross *et al*., 2001) and to our knowledge there is currently no tests that have been done to date on its effects on *Bifiobacterium* species.

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2.3 Materials and Methods

2.3.1 Microrganisms

The following bacterial cultures were tested in this study; *Bifidobacterium lactis* Bb12 which was obtained as freeze-dried sachets from CHR-Hansen, Denmark. *B. bifidum* LMG 11041 and *B. longum* LMG 13197 were purchased from the LMG culture collection, Belgium and revived as per instructions from the supplier. *B. lactis* Bi-07 300B, *B. longum* Bb536 and *Lactobacillus acidophilus* La14 150B were obtained from the Department of Food Science, University of Pretoria, South Africa as subcultures on selective media. The *Lactobacillus* culture was used as a comparison strain.

2.3.2 Garlic preparations

Fresh garlic cloves (GC), garlic paste (GP), garlic powder (Gp) and garlic spice (GS) were bought from local supermarkets in Pretoria. GC, Gp and GS were stored at room temperature, whilst GP was stored at 4°C for no longer than 2 weeks.

2.3.2.1 Preparation of garlic extract

The garlic extract was prepared as described by Bakri and Douglas (2005), with slight modifications. Fresh cloves were separated and peeled and a predetermined amount of 10 g was weighed and crushed using a mortar and pestle. This was then suspended in 5 ml sterile distilled water. The same amounts of GP, Gp and GS were weighed and GS and Gp were suspended in 10 ml of sterile distilled water, whilst GP was suspended in 5 ml. These suspensions were then centrifuged and filtered through a 0.22 µm filter (Minisart).

2.3.2.2 Determination of final concentration of garlic and allicin in solution

The weight of the insoluble material of the GC, GP, Gp and GS was subtracted from the weight of the original material to get the final concentration of GE in solution. The sterile extract was used within 30 min of its preparation for the inhibitory studies.

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2.3.3 Determination of actual allicin concentration

The concentration of allicin in each garlic preparation was determined spectrophotometrically by reaction with thiol, 4- mercaptopyridine (Bakri and Douglas, 2005). A 1:1 dilution of each garlic extract was incubated at room temperature in 1 ml 4- mercaptopyridine (10^{-4}) in 50 mM phosphate buffer, 2 mM EDTA, pH 7.2. The decrease in optical density at 324 nm after 1 h was used to calculate the allicin concentrations in each garlic preparation used (Miron *et al*., 2002; Bakri and Douglas, 2005). This was repeated in duplicate.

2.3.4 Preparation of inoculums

Lactobacillus acidophilus La14 150B was grown in MRS broth while *Bifidobacterium* strains were grown in MRS-cys-HCL broth. Both of the inoculated broths were then incubated at 37°C for 48 h in anaerobic jars with Anaerocult A gaspacks (Merck Ltd, Mofferfontein, SA). For the disk diffusion assay and the broth dilution assay for MIC testing the bacterial cell suspensions were adjusted to a 0.5 McFarland standard (approximately 1 x 10^8 cfu/ml).

2.3.5 Phosphoketolase assay

Verification of the *Bifidobacterium* cultures was performed by using the phosphoketolase assay, according to Orban and Patterson (2000). Bifidobacterial cells were harvested by centrifugation (5000 rpm) for 10 min at 4°C. A nutrient broth culture of *E. coli*, which had been left overnight, was also harvested and the cells were used as a negative control. Overnight MRS-cys-HCL cultures of bifidobacteria were used as positive controls. Cells were washed twice with phosphate buffer (KH_2PO_4, 0.05M, and cys-HCl, 500mg/L, mixed 1:1 (V/V) and adjusted to pH of 6.5 by centrifugation for 15 min (5000 rpm) and resuspended in 1 ml phosphate buffer. 0.4 ml CTAB (450 µg/ml) was then added and incubated for 5 min at 37°C. Following pretreatment with CTAB, 0.25 ml of a solution containing NaF (3 mg/ml) and NaI (5 mg/ml) in dH_2O was added followed by addition of 0.25 ml sodium fructose-6-phosphate (80 mg/ml in dH_2O). It was then vortexed and incubated at 37°C for 30 min. 1.5 ml hydroxylamine-HCl (13 g/100 ml) was added, vortexed and incubated at 37°C for 15 min. following this, 1ml TCA (15% w/v), 1 ml 4N HCl and 1 ml ferric chloride (5% w/v in 0.1 ml HCl) was added after which colour formation was observed.

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2.3.6 Disk diffusion (DD) assay

The antimicrobial activity of the garlic preparations were tested using the disk diffusion method according to Benkeblia (2004), with minor modifications. A lawn of bifidobacteria was prepared by spreading 0.1 ml of each the broth cultures onto MRS-cys-HCl agar plates. Filter disks (1 cm in diameter) were used and impregnated with 0.1 ml garlic extract of fresh GC, GP, Gp and GS of varying concentrations. Sterile dH_2O was used as a negative control and ampicillin (Amp) (100 mg/ml), which was passed through a 0.22 µm filter (Minisart), was also used as a positive control for comparison with the garlic preparations. Filters were impregnated with 0.1 ml ampicillin on separate plates for each culture. *L. acidophilus* was used for comparison with the *Bifidobacterium* cultures. The inoculated plates were then incubated anaerobically at 37°C for 48-72 h, after which the resultant inhibition zones were measured and expressed in millimeters (mm) as its antimicrobial activity. Each combination of the isolate and antimicrobial agent was repeated in triplicate.

2.3.7 Viable plate counts

Each of the *Bifidobacterium* cultures was suspended in sterile ½ strength Ringers solution. The suspensions were then serially diluted up to 10^{-6} using Ringers solution and 0.1 ml of each dilution pour-plated onto triplicate MRS-cys-HCl plates. The plates were then incubated anaerobically at 37°C for 72 h, after which the resultant colonies were counted on plates containing 30-300 colonies. This then gave us a confirmation of the initial amount of bacteria before the garlic preparations were added and enabled us to determine the minimum exposure time.

2.3.8 Determination of minimum exposure times (Time kill curves)

MRS-cys-HCl broth was inoculated with each of the *Bifidobacterium* spp. respectively and incubated overnight (24 h) anaerobically at 37°C. The cultures were then diluted to a concentration equivalent to 0.5 McFarland standards. The different garlic extracts were then added to each of the *Bifidobacterium* broth cultures respectively. The cultures were then incubated at 37°C for 6 h. A 1 ml sample was then taken from each of the broth cultures, containing the garlic extracts, immediately after the addition of garlic extracts, then after 0.5, 1, 2, 3, 4, 5 and 6 h of incubation. Cells were recovered by centrifugation at 13.5 rpm for 15

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min and then rinsed with ½ Ringers solution and once again vortexed for 15 min at 13.5 rpm. These samples were then serially diluted in ½ Ringers solution up to 10^{-6} and 0.1 ml of each dilution was pour-plated onto MRS-cys-HCl plates and incubated anaerobically at 37°C. Ampicillin was used as a positive control while bacterial cell suspensions without garlic extract were used as negative controls. This experiment was repeated in triplicate.

2.3.9 MIC and MBC determinations

The minimum inhibitory concentrations (MICs) and minimum bactericidal concentrations (MBCs) were determined using the broth dilution method according to Bakri and Douglas (2005) with minor modifications. The garlic extracts were serially diluted in MRS-cys-HCl broth in Eppendorf tubes and inoculated with 100 µl of the *Bifidobacterium* cultures. The cultures were all adjusted to a concentration equivalent to 0.5 McFarland's standard. The Eppendorf tubes were then incubated anaerobically overnight at 37°C and the highest dilution where there was no growth was recorded as the MIC. The MBC was then determined by taking 100 µl from the tubes that had no visible growth and plating out onto MRS-cys-HCl agar plates. The plates were then incubated anaerobically overnight at 37°C. The highest dilution where there was no growth at all on the plates was recorded as the MBC. Sterile MRS-cys-HCl broth was used as a control for both of the above mentioned methods. MIC and MBC determinations were performed in triplicate.

2.3.10 Statistical analysis

Statistical analysis of results was performed using STATISTICA version10, which included determination of standard deviations of means. Student's *t* test was performed to analyze the difference between the mean values and was performed at 5% significance level. Values where $p < 0.05$ indicated a marked significant difference, and $p > 0.05$ was non-significant.

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2.4 Results and Discussion

2.4.1 Determination of the antibacterial activity using the disk diffusion (DD) assay

Identity of all *Bifidobacterium* strains was confirmed by performing the phosphoketolase assay. The final concentration of garlic extract in solution was determined as 60.7% (w/v), 10.7% (w/v), 9% (w/v) and 8.9% (w/v) for the GC, GP, Gp and GS, respectively. The allicin concentration of each garlic preparation was determined spectrophotometrically (Table 2.1) (Miron *et al*., 2002). All extracts inhibited most strains except for *B. lactis* Bi-07 300B and *L. acidophilus*.

Table 2.1: Allicin concentration of garlic preparations.

Garlic preparation	Allicin concentration (µg/ml)
Garlic clove	198.74
Garlic paste	124.98
Garlic powder	26.63
Garlic spice	10.24

To determine the sensitivity of the *Bifidobacterium* strains to the garlic preparations the disk diffusion assay was performed. All garlic extracts exhibited different levels of inhibition against the tested strains as shown by results in Table 2.2 and the zones of inhibition in Figure 2.1. The zone of inhibition indicates the effectiveness of the garlic in inhibiting the growth of the bifidobacteria. The larger the zone, the more sensitive the bifidobacteria is to garlic. These reported results are means (mm) ± SD of triplicate measurements. All strains, excluding the control *L. acidophilus* were susceptible to most of the extracts. This is the first time that the sensitivity of bifidobacteria to garlic is being reported. Ampicillin was used as a positive control and all *Bifidobacterium* strains were inhibited with inhibition zones ranging from 31.0 ± 1.7 to 52.0 ± 2.0 mm. These results correlate with those reported by Domig and colleagues (2007) whom obtained zones of inhibition ranging from ±35-±67 mm for ampicillin against *Bifidobacterium* strains. This was expected as previous research shows bifidobacteria are susceptible to this antibiotic (Domig *et al*., 2007; Mättő *et al*., 2007; Reyed, 2007). For the GC extract the susceptibility pattern was as follows: *B. bifidum* LMG 11041 >

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B. longum Bb536 > *B. longum* LMG 13197> *B. lactis* Bb12 > *B. lactis* Bi-07 300B. *L. acidophilus* La14 150B was not inhibited by GC extract. There was no association between the susceptibilities of the strains to any of the extracts. The susceptibility pattern for GP extract was: *B. bifidum* LMG 11041 > *B. longum* Bb536 > *B. lactis* Bb12 ~ *B. lactis* Bb12 ~ *B. longum* LMG 13197. Gp and GS had the following patterns: *B. bifidum* LMG 11041 > *B. longum* Bb536 > *B. longum* LMG 13197 > *B. lactis* Bb12 > *B. lactis* Bi-07 300B and *B. longum* LMG 13197 > *B. bifidum* LMG 11041 > *B. longum* Bb536 > *B .lactis* Bb12 > *B. lactis* Bi-07 300B, respectively.

Table 2.2: Antibacterial activity of different garlic preparations

Strains	Zones of inhibition (mm)					
	Garlic preparations					
	Garlic clove	Garlic paste	Garlic powder	Garlic spice	Ampicillin (+ control)	dH2O (- control)
B. lactis Bb12	22.3 ± 1.5	0.0 ± 0.0	17.3 ± 2.1	19.3 ± 2.1	34.7 ± 2.1	-[a]
B. longum LMG 13197	28.0 ± 1.0	0.0 ± 0.0	19.7 ± 2.1	24.0 ± 2.0	43.3 ± 2.5	-[a]
B.lactis Bi-07 300B	13.0 ± 1.7	-[a]	-[a]	-[a]	31.0 ± 1.7	-[a]
B.longum Bb536	31.3 ± 2.3	20.7 ± 2.1	24.0 ± 3.0	19.7 ± 1.5	44.7 ± 1.5	-[a]
B.bifidum LMG 11041	36.7 ± 1.2	21.3 ± 2.5	28.0 ± 2.0	22.3 ± 0.6	52.0 ± 2.0	-[a]
Lactobacillus acidophilus La14 150B	-[a]	-[a]	-[a]	-[a]	44.0 ± 2.7	-[a]

No inhibition zone
Mean ±SD zones of inhibition are given.

The diameter of the zones of inhibition ranged from 13.0 ± 1.7 to 36.7 ± 1.2 mm, 0.0 ± 0.0 to 21.3 ± 2.5 mm, 17.3 ± 2.1 to 28.0 ± 2.0 mm and 0.0 ± 0.0 to 24.0 ± 2.0 mm for GC, GP, Gp and GS, respectively, as compared with 31.0 ± 1.7 to 52.0 ± 2.0 mm for ampicillin. These results indicate a considerable antibacterial activity of garlic, specifically GC. All of the garlic extracts were most effective against *B. bifidum* LMG 11041 and least effective against *B. lactis* Bi-07 300B. We can compare this to *L. acidophilus* which was used as a comparison and had no inhibition zones. *B. lactis* Bi-07 300B therefore had little or no sensitivity towards the garlic extracts except for GC. Minimal antibacterial activity was observed for GP on all tested *Bifidobacterium* strains.

Our strain of *L acidophilus* had no inhibition zones for GC which did not correspond to a previous study performed by Owhe-Ureghe and colleagues (2010) where they recorded a mean zone of inhibition of 18 mm. This could be because the two strains used were different

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and from different origins, theirs being isolated from extracted carious teeth. A smaller concentration of garlic was also used compared to that of Owhe-Ureghe and colleagues (2010). Another reason, according to Deresse (2010), is that garlic species are different in various countries as well as the means of the processing methods for garlic preparations.

It was anticipated that if there were any antimicrobial effects, it would most likely be observed in the fresh GC extract, as raw garlic contains the active compound allicin which is released upon crushing of the cloves. There have been countless studies proving that this compound exhibits antimicrobial activities against a wide variety of both Gram-positive and Gram-negative bacteria including some fungi and viruses (Harris *et al*., 2001; Benkeblia, 2004; Ruddock *et al*., 2005; Durairaj *et al*., 2009; Goncagul and Ayaz, 2010). GC extract also revealed the highest allicin concentration which therefore coincides with the results obtained. GS and Gp had lower allicin concentrations. This is probably due to their preparation process where they are dehydrated, dried and stored for long periods of time before they are utilized. The active ingredients found in fresh garlic extract, such as allinase, often become inactive; thereby producing insignificant amounts of allicin and thus negligible antimicrobial properties (Yu and Shi-ying, 2007). These preparations should therefore theoretically have less effect on the strains compared to that of GC, which was observed. GS and Gp may possibly contain other compounds, besides and less potent than allicin, which have an antagonistic effect on bifidobacteria. Other compounds with antimicrobial effects such as ajoene, vinyl dithiins and thiosulfanates have been isolated as products of garlic degradation (Harris *et al*., 2001). Another reason for the low allicin concentrations for Gp and GS could be due to volatilization as allicin is a very unstable compound and may undergo various reactions forming other derivatives depending on conditions and processing actions (Hovana *et al*., 2011). GP had little or no visible antibacterial effect on the strains.

There have been very few studies done on the influence that different culture media may have on the antimicrobial activity of garlic components but it is known that cysteine has an effect on allicin (Ross *et al*., 2001). Therefore this may have influenced our disk diffusion assay results on the antimicrobial activity of not only our GP preparation, but for all the other preparations, as bifidobacteria require cysteine in its culture media.

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(A) (B)

(C) (D)

(E) (F)

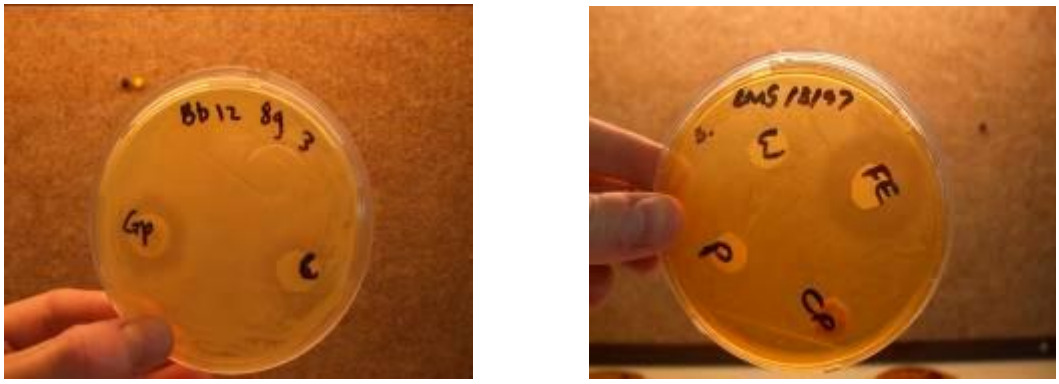

Figure 2.1: Inhibition zones of the *Bifidobacterium* strains to the garlic preparations. *L. acidophilus* La14 150B (A), *B. lactis* Bi-07 300B (B), *B. longum* Bb536 (C), *B. longum* LMG 11041 (D), *B. lactis* Bb12 (E) and *B. longum* LMG 13197 (F) exposed to distilled water (W), garlic clove extract (C and FE), garlic paste (P), garlic powder (Gp) and garlic spice (GS).

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2.4.2 Minimum exposure times

The minimum exposure times established the time required for the garlic preparations to start decreasing the viability of each strain as well as revealing what effects they had on cell growth. The initial concentration of bacteria for all strains was approximately equal to 0.5 McFarland's standard.

2.4.2.1. *B. bifidum* LMG 11041

This strain showed the highest ($p<0.05$) decrease in viability overall to all the garlic preparations after the 6 h period (Fig. 2.2). The initial lag phase for GC lasted approximately 30 min, which was very similar to that of the positive control, ampicillin. According to Ross and colleagues (2001) lag phases may be an indication of the waiting period for the garlic molecules to be taken up and/or the start of the toxic effect on the metabolic processes of the cell. This lag phase was then followed by a gradual drop in viability. After 5 h another steep drop in viability occurred. GP, Gp and GS had longer lag phases of ± 2 h before a slight drop was even observed followed by another drop after the 4th hour. Negative control cell suspensions that contained no garlic showed no drop in viability over the same period, there was actually an increase in viable cells indicating that the garlic preparations, once exposed, obviously do have an effect on the strain's viability. This is evident in the marked difference ($p < 0.05$) between the drop in viability when exposed to the garlic preparations as opposed to the negative control. Total reductions of approximately 1.2log, 0.4log, 0.6log and 0.7log were observed after 6 h to GC, GP, Gp and GS, respectively. Results indicate that this strain was most susceptible to GC which showed the highest drop in viability ($p < 0.05$), followed by GS, Gp and GP.

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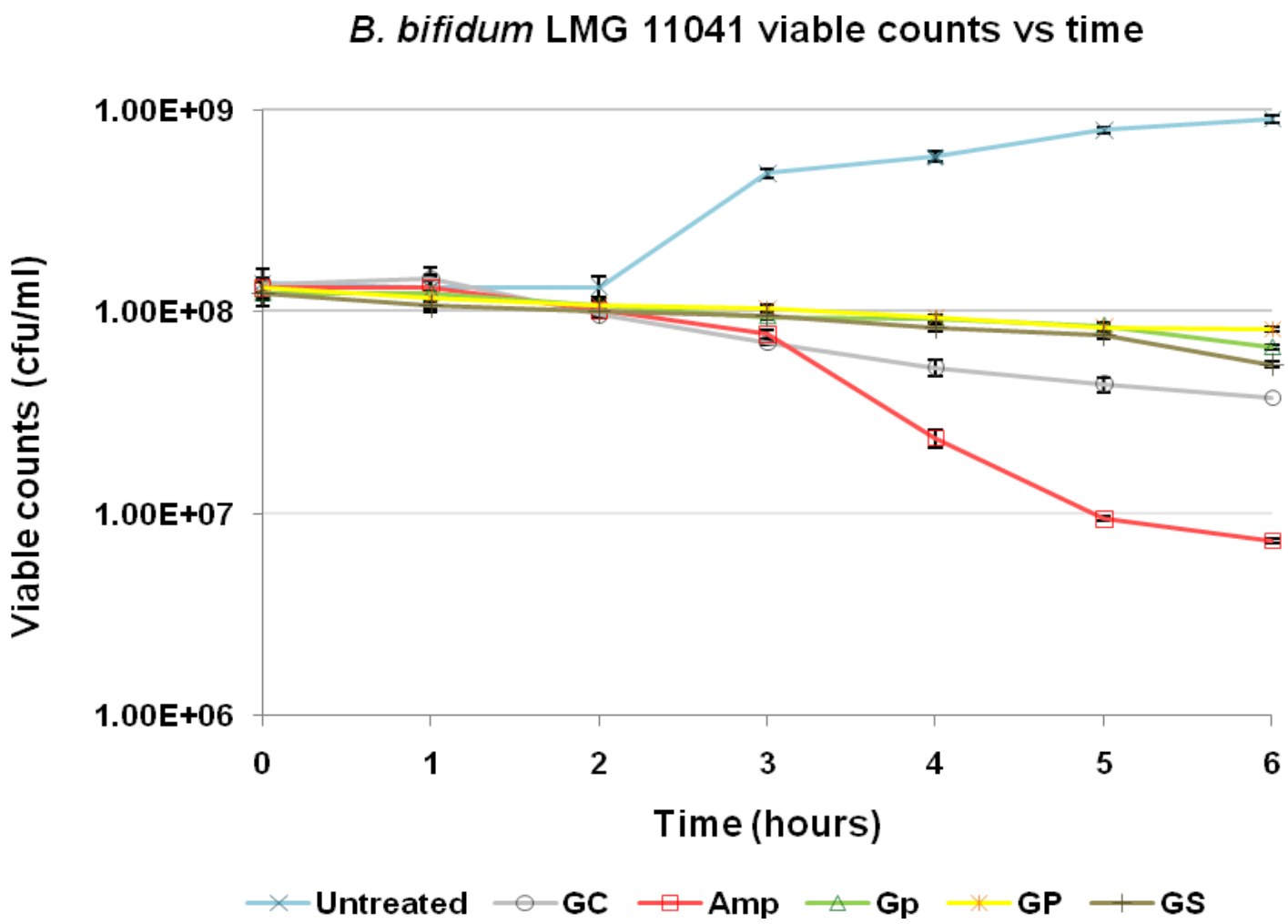

Figure 2. 2: The effect of GC, Gp, GP and GS on *B. bifidum* LMG 11041 viability with time with untreated cell suspensions and ampicillin (Amp) as negative and positive controls, respectively.

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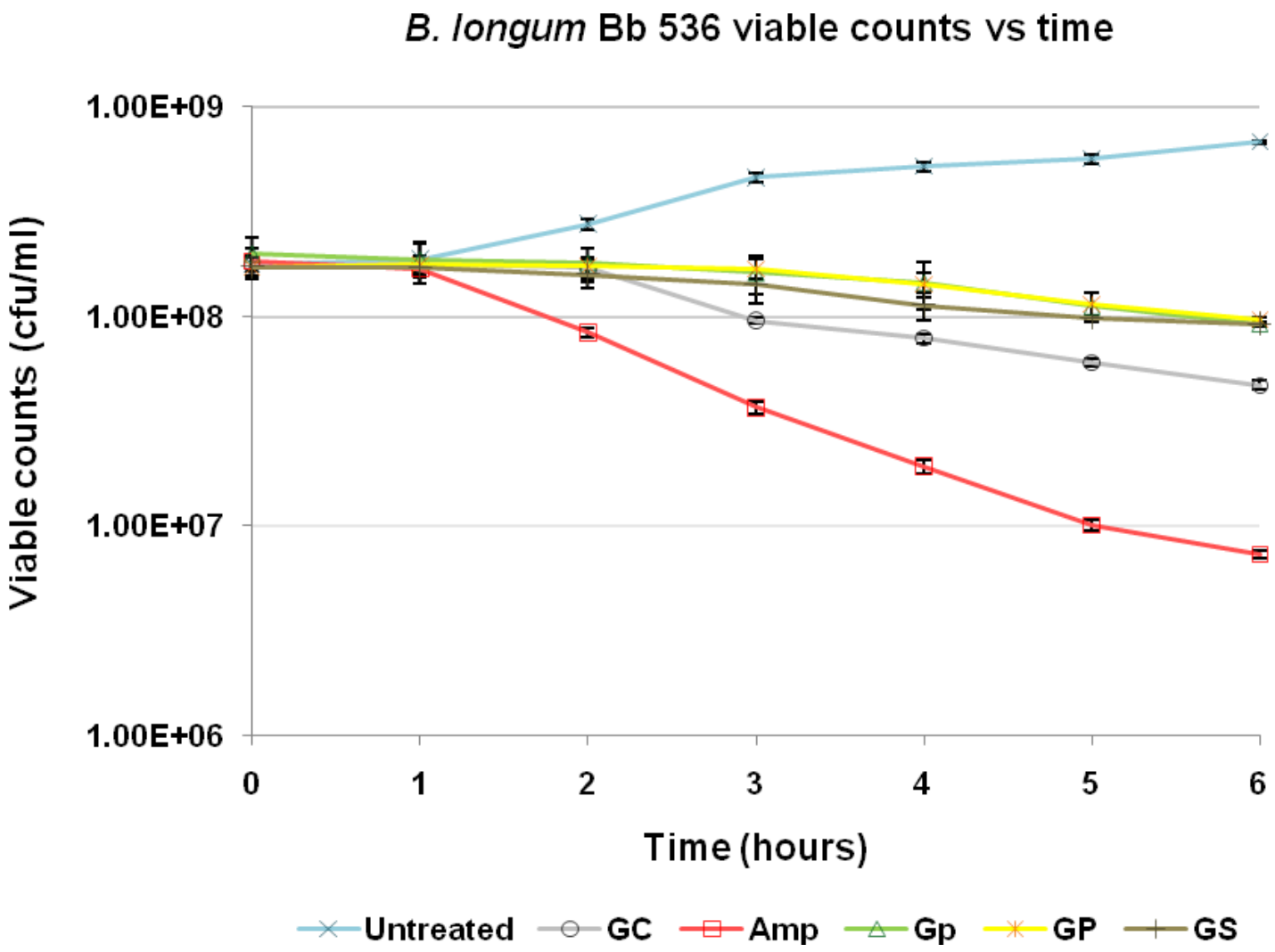

Figure 2. 3: The effect of GC, Gp, GP and GS on *B. longum* Bb 536 viability with time with untreated cell suspensions and ampicillin (Amp) as negative and positive controls, respectively.

2.4.2.2. *B. longum* Bb 536

B. longum Bb 536 was also susceptible to all garlic preparations and therefore showed a significant decrease ($p < 0.05$) in viability for each garlic preparation, compared to the negative control, during the exposure period (Fig. 2.3). With GC, a longer initial lag phase of approximately 1.5 h occurred in comparison with *B. bifidum* LMG 11041's 0.5 h lag phase. Therefore it took longer for the garlic molecules to start reacting with this strain before there was a considerable drop in cell numbers. After 2 h viability dropped at a gradual rate for the remainder of the exposure period. Lag phases of 1 to 2 h were observed for GP, Gp and GS after which there was a slight decrease in viability for 2 h before the numbers seemed to start leveling out. A total reduction of approximately 0.8log was observed after 6 h to GC and 0.3log to GP, Gp as well as to GS. *B. longum* Bb 536 showed the highest drop in viability for GC extract followed by GS. GP and Gp showed a very similar, if not identical, effect on this strains cell growth and viability with a p-value higher than 0.05.

2.4.2.3. *B. longum* LMG 13197

Interestingly, *B. longum* LMG 13197 experienced an even longer lag phase upon exposure to GC than the previous two strains (Fig. 2.4). It had an initial lag phase of 2 h before the GC began to have a decreasing effect on its viability. On the contrary, it took GS only 0.5 h to start lowering the viability of *B. longum* LMG 13197 and ampicillin initiated an immediate decrease in viability. Gp only started having an inhibitory effect after 3 h, whereas viability remained relatively constant throughout the exposure period to GP and actually started to increase slightly after 4 h. This may be an indication that the active compounds in GP, responsible for its antibactertial activity, became diminished and the remaining population of *B. longum* cells started multiplying again. Instability of the active compound or its transformation into stable compounds such as polysulfides and thiosulphonates may be the reason for the diminished antimicrobial activity (Cellini *et al*., 1996; Belguith *et al*., 2010). This has happened previously to bacterial cells exposed to garlic. Regrowth of *Salmonella enterica* cells exposed to garlic powder was observed by Margues and colleagues (2008). Feldberg and colleagues, (1988) suggested that regrowth of *Salmonella typhimurium* could be owed to its capability to titrate allicin or possibly to metabolize it into non-inhibitory compounds (Belguith *et al.,* 2010). At 6 h an approximate reduction of 0.9log, 0.3 log and 0.4log was observed for GC, Gp and GS. For GP there was an increase of approximately 0.1log after the exposure period.

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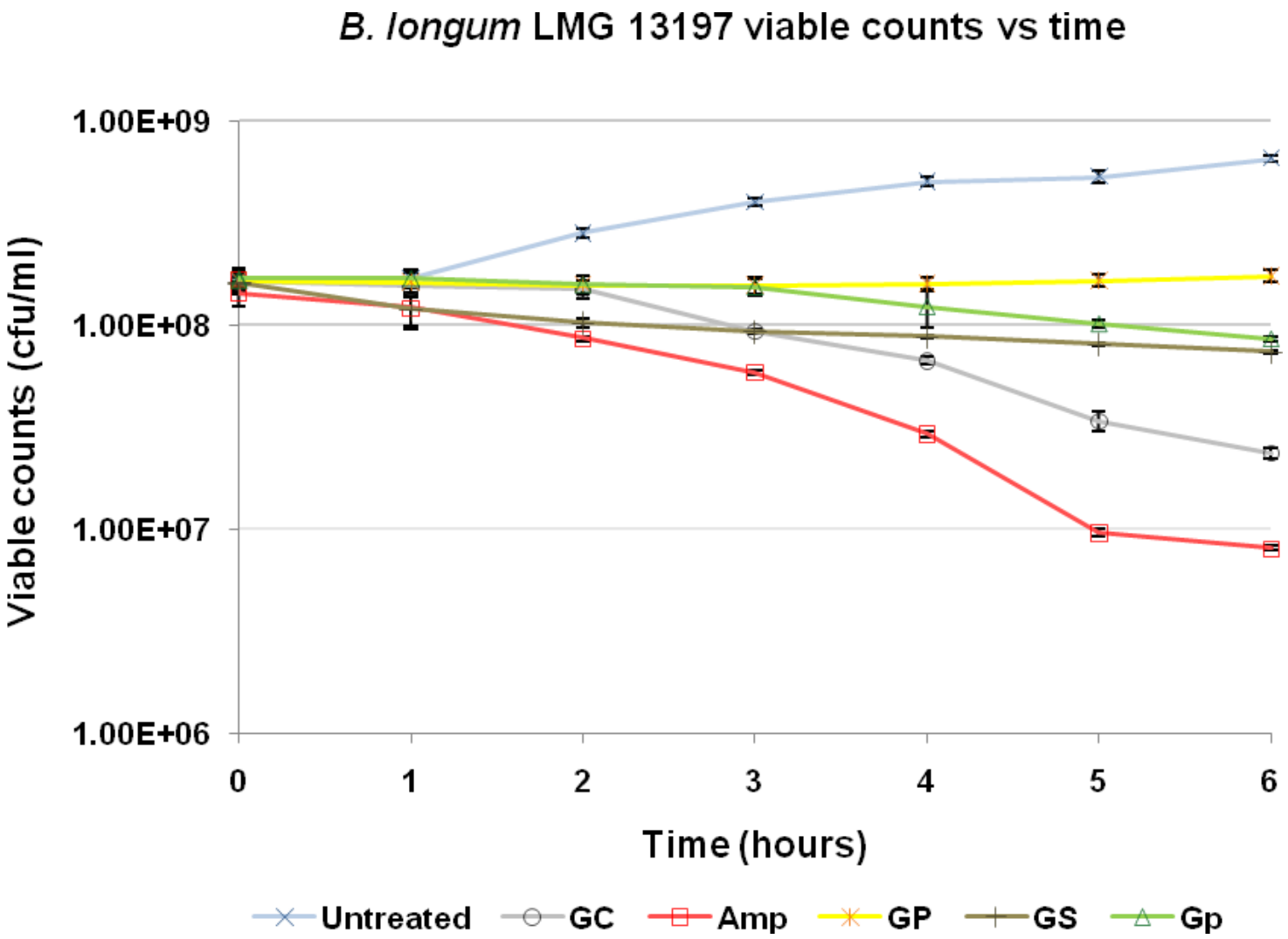

Figure 2.4: The effect of GC, Gp, GP and GS on *B. longum* LMG 13197 viability with time with cell suspensions and ampicillin (Amp) as negative and positive controls, respectively.

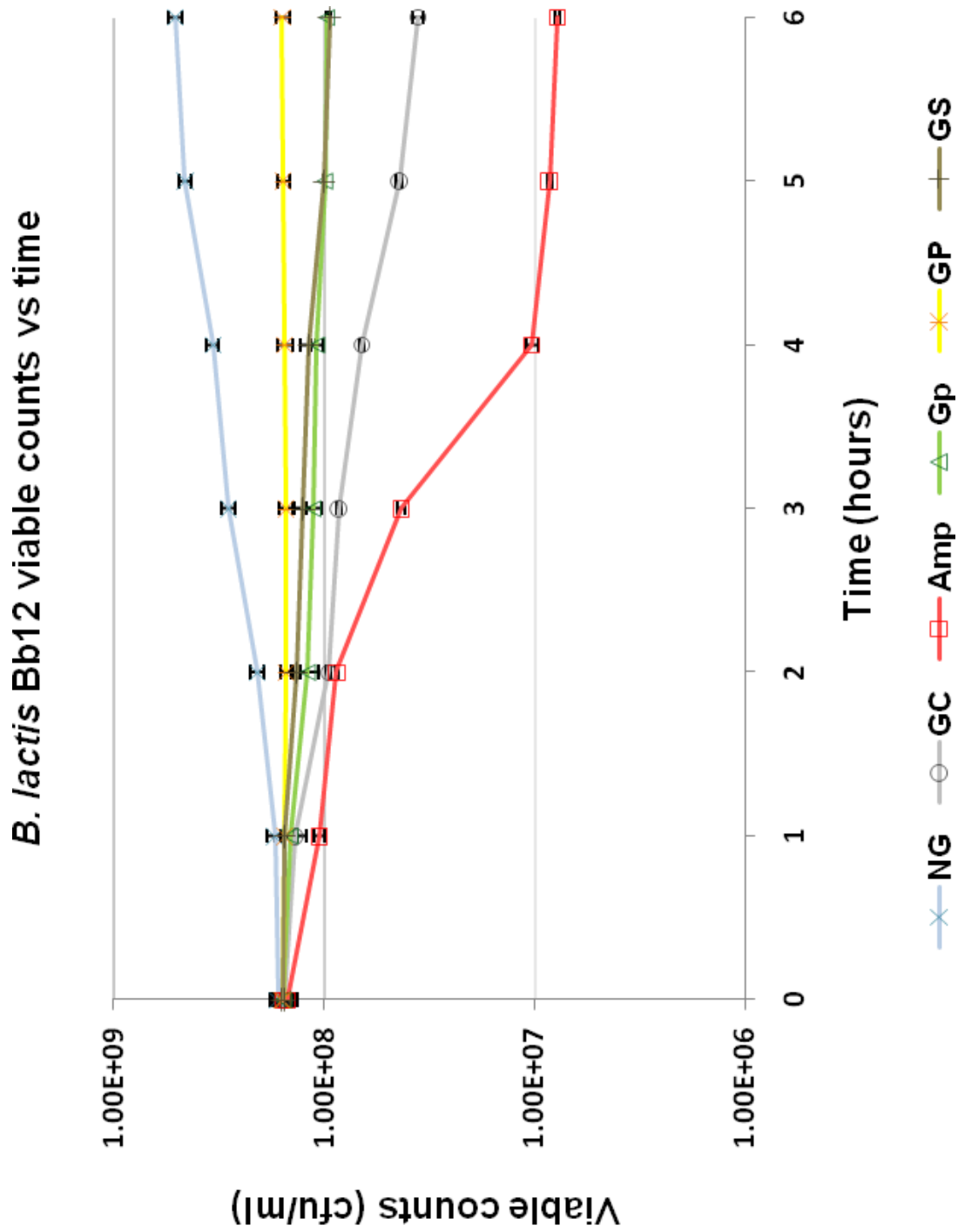

Figure 2. 5: The effect of GC, Gp, GP and GS on *B. lactis* Bb12 viability with time with untreated cell suspensions and ampicillin (Amp) as negative and positive controls, respectively.

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2.4.2.4. *B. lactis* Bb12

The initial lag phase for GC, Gp and GS was 1 h, after which there was only a very slight decrease in viability for both Gp and GS (Fig. 2.5). There was somewhat more of a drop after 1 h exposure to the GC extract. There was a noteworthy difference ($p < 0.05$) between viability loss to GC as opposed to the other preparations. Total reductions in the region of 0.8log and 0.2log were observed for GC and for Gp and GS, respectively. This strain was resistant to GP with no drop in viability after the 6 h of exposure. Viability remained fairly constant with the same number of viable cells observed at the end of exposure to GP compared with the initial amount at 0 h.

2.4.2.5. *B. lactis* Bi-07 300B

This strain was least susceptible to the antimicrobial effects of all the garlic preparations with only a small decrease in viability after exposure to GC and no decrease in viability after exposure to Gp, GP and GS (Fig. 2.6). After an initial lag phase of 1 h for GC there was a drop in viability for the next hour followed by a relatively small and slow decline in cell numbers for the remainder of the exposure period. The viable population decreased by roughly 0.4log after 6 h exposure to GC.

2.4.2.6. *L.acidophilus* La14 150B

This strain was used as a comparison for all the *Bifidobaterium* strains as it is resistant to Gp, GP and GS, as seen with results from disk diffusion assay, and only slightly susceptible to the antimicrobial effects of GC (Fig. 2.7). If we compare the time kill curves of the tested *Bifidobacterium* strains with the *Lactobacillus* strain we can definitely see that *B. bifidum* LMG 11041 showed the highest ($p < 0.05$) drop in viability for all garlic preparations over the 6 h exposure period. *B. lactis* Bi-07 300B, on the other hand, demonstrated a slight decrease in viability for the GC extract and no decrease for the other three preparations. This strain showed the closest trend to that for *L. acidophilus*.

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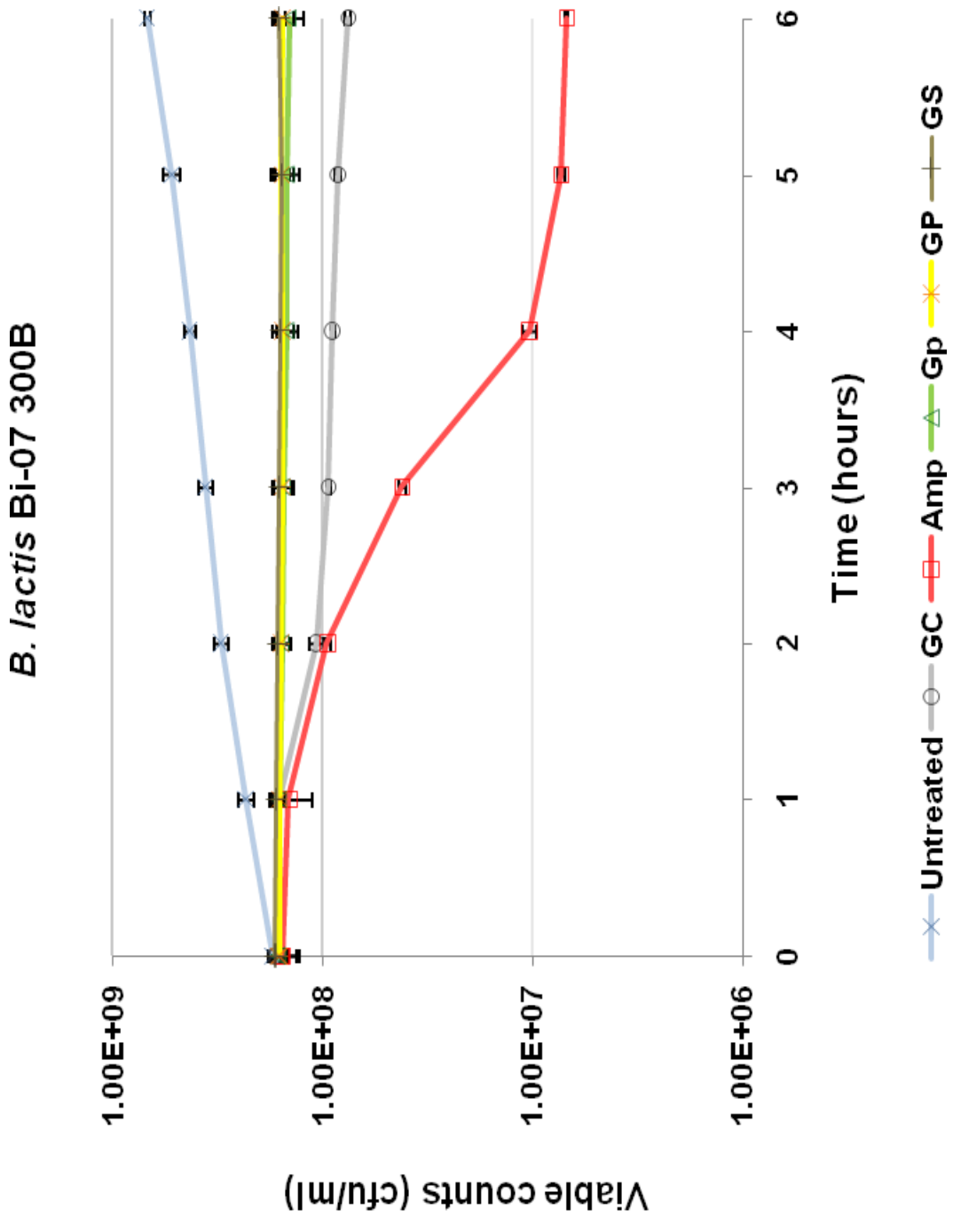

Figure 2.6: The effect of GC, Gp, GP and GS on *B. lactis* Bi-0-7 300B viability with time with untreated cell suspensions and ampicillin (Amp) as negative and positive controls, respectively.

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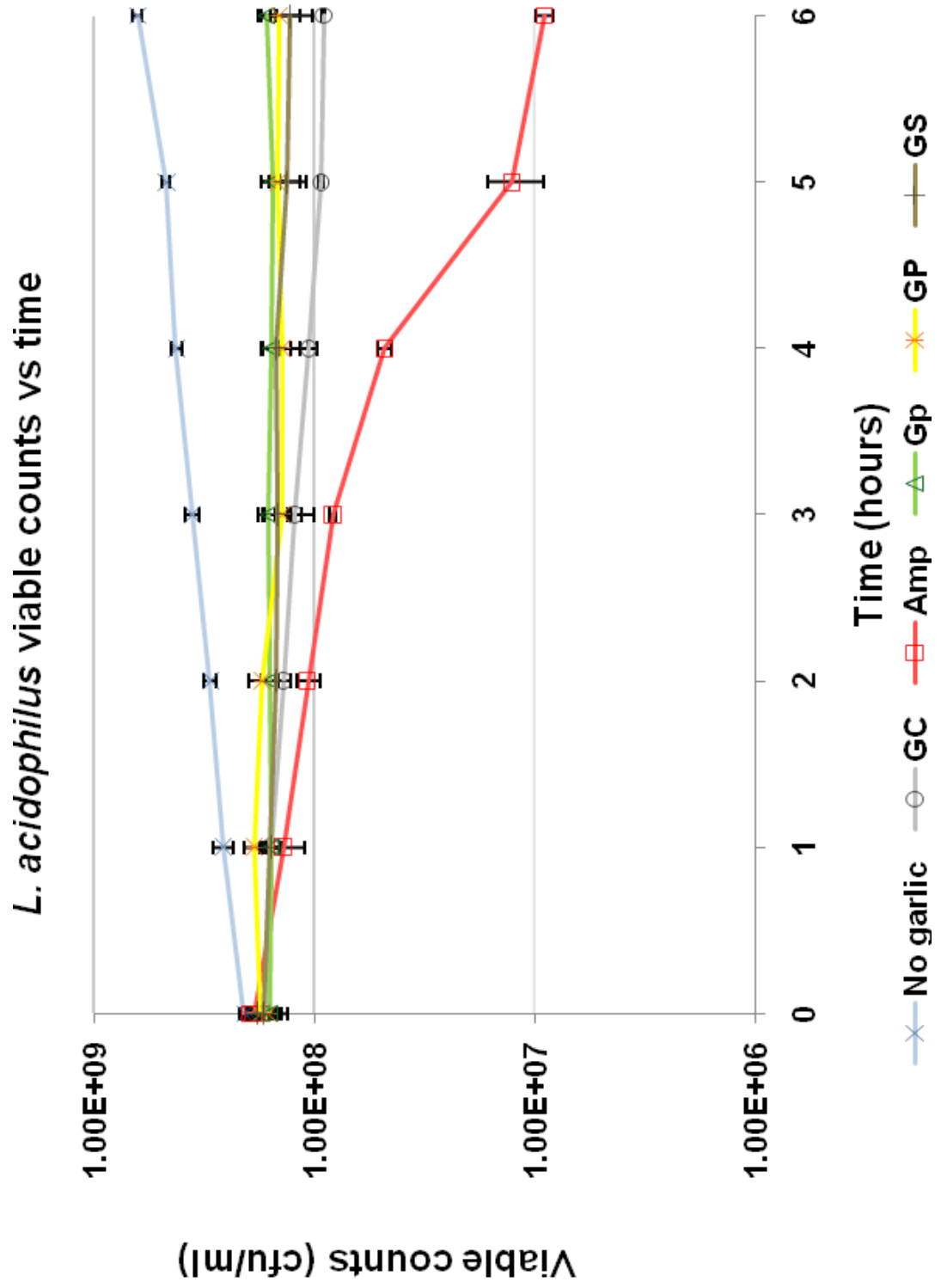

Figure 2.7: The effect of GC, Gp, GP and GS on *L. acidophilus* La14 150B viability with time with untreated cell suspensions and ampicillin (Amp) as negative and positive controls, respectively.

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Overall *B.bifidum* LMG 11041 and *B. longum* Bb 536 were the only two *Bifidobacterium* strains to show a decrease in viability for all garlic preparations over the 6 h exposure period. All strains showed a significant decrease ($p < 0.05$) in viability upon exposure to GC. *B. longum* LMG 13197 experienced a decrease in viability for Gp and GS but not for GP; while *B. lactis* Bb12, upon exposure to Gp and GS, decreased slightly less. *B. lactis* Bi-07 300B showed a very similar pattern to that of the comparison *Lactobacillus* strain which showed practically no decrease in viability upon exposure to GP, Gp and GS and a slight decrease for GC. This could also possibly indicate that *B. lactis* Bi-07 300B requires a longer exposure period of more than 6 h to succumb to the antimicrobial effects of garlic. All bacterial cell suspensions containing ampicillin decreased in viability during the 6 h exposure which was expected with *B. longum* Bb536 experiencing the highest decrease. There was no drop in cell viability in the negative controls after 6 h. All strains, including the *Lactobacillus* strain, if only slightly, showed a drop in viability to that of their initial counts when exposed to the GC extract ($p < 0.05$). The order of strains that demonstrated the highest drop in viability to the lowest was as follows: *B. bifidum* LMG 11041 > *B. longum* Bb 536 > *B. longum* LMG 13197 > *B. lactis* Bb12 > *B. lactis* Bi-07 300B. For most strains, GC had the shortest lag phase when compared to the other garlic preparations and on average took 1 h to start inhibiting the bacterial cells. The most sensitive strain, *B. bifidum* LMG 11041 succumbed to the antibacterial activity of GC within 0.5 h.

Viable cell numbers of all *Bifidobacterium* strains, except *B. bifidum* LMG 11041 and *B. longum* Bb536, were the same; and for others slightly higher, as their initial inoculums for GP. This could possibly be an indication of these strains showing a bacteriostatic response to GP. This could also be the case for both *B. lactis* strains for Gp and GS as their viable cell numbers were also the same as their initial counts, with very slight decreases in viability over the 6 h exposure time to these preparations.

The differences in minimum exposure period observed for these strains signified that sensitivity of *Bifidobacterium* strains to garlic preparations differed between species as well as within strains of the same species. This emphasized that no generalizations should be made for the various strains on their sensitivity to antimicrobial effects of garlic. Inter-strain variations in garlic extract sensitivity was also observed for strains of *Streptococcus mutans* by Chen and colleagues (2009), whom attributed it to be partly due to the different cell surface compositions in different strains.

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2.4.3 MICs and MBCs

As mentioned previously, the garlic extract used for GC, GP, Gp and GS was 60.7% (w/v), 10.7% (w/v), 9% (w/v) and 8.9% (w/v) respectively. The allicin concentration for each preparation can be seen in Table 2.1. Both garlic extract concentration and the determined allicin concentration were used to calculate the MIC and MBC values. MIC and MBC values obtained are shown in Table 2.2.

The MIC values of GC, Gp and GS against the different *Bifidobacterium* strains ranged from 75.9-303.5 mg/ml garlic, 24.84-99.37 µg/ml allicin; 30->90 mg/ml garlic, 8.88->26.63 µg/ml allicin and 44.5>89 mg/ml garlic, 5.12->10.24 µg/ml allicin, respectively. The MIC values for GP against all strains, except for *B. longum* Bb536 and *B. bifidum* LMG 11041, were >107 mg/ml garlic; >124.98 µg/ml allicin. MIC values for ampicillin were significantly lower ($p < 0.05$) against all strains, including *Lactobacillus*, compared to any garlic extract and ranged from 0.00000035-0.5 mg/ml. This was expected as bifidobacteria and lactobacilli are very susceptible to this antibiotic (Zhou *et al*., 2005; D'Aimmo *et al*., 2007).

GC extract was found to inhibit all *Bifidobacterium* strains tested as well as the control *Lactobacillus* culture and had significantly higher ($p < 0.05$) MIC values compared to the other preparations. The maximum effectiveness of GC extract was on *B. bifidum* LMG 11041 (75.9 mg/ml garlic; 24.84 µg/ml allicin) followed by *B. longum* Bb536 (86.7 mg/ml garlic; 28.39 µg/ml allicin), *B. longum* LMG 13197 (151.75 mg/ml garlic; 49.69 µg/ml), *B. lactis* Bb12 (202.3 mg/ml garlic; 66.25 µg/ml allicin) and lastly *B. lactis* Bi-07 300B, with a higher MIC value of 303.5 mg/ml garlic and 99.37 µg/ml allicin for GC. There was no significant difference ($p > 0.05$) between the MIC values obtained for GP for most *Bifidobacterium* strains, besides *B. bifidum* LMG 11041 and *B. longum* Bb536, indicating that the first three were not susceptible to this preparation. GS also showed no negligible differences between MIC values for *B. lactis* strains and *B. longum* LMG 13197, but had lower values for *B. bifidum* LMG 11041 and *B. longum* Bb536 showing that these two strains were more sensitive to the GS preparation. Once again the *B. bifidum* strain showed the lowest MIC for Gp, followed by *B. longum* Bb536 and *B. lactis* Bb12. High MIC values for Gp were obtained for the rest of the strains. Strains that were sensitive to the different garlic preparations had MBC values that were a dilution lower, sometimes more, than those for the MIC values.

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Table 2.3: Inhibitory effect (MIC and MBC) of different garlic extracts on tested *Bifidobacterium* strains.

***Bifidobacterium* species**	**Antimicrobial agent**	**MIC (mg/ml (µg/ml))**	**MBC (µg/ml)**
B. lactis Bb12	**GC**	202.3 (66.25)	1:1 (198.74)
	Gp	45 (13.32)	1:1 (26.63)
	GP	>107 (124.98)	>107 (>124.98)
	GS	89 (10.24)	>89 (>10.24)
	Amp	0.035	1:2
B.bifidum LMG 11041	**GC**	75.9 (24.84)	1:5 (39.75)
	Gp	30 (8.88)	1:2 (13.32)
	GP	107 (124.98)	>107 (>124.98)
	GS	44.5 (5.12)	1:1 (10.24)
	Amp	0.00000035	1:7
B.lactis Bi-07 300B	**GC**	303.5 (99.37)	1:1 (198.74)
	Gp	>90 (>26.63)	>90 (>26.63)
	GP	>107 (>124.98)	>107 (>124.98)
	GS	>89 (>10.24)	>89 (>10.24)
	Amp	0.05	1:2
B.longum Bb536	**GC**	86.7 (28.39)	1:4 (49.69)
	Gp	45 (13.32)	1:1 (26.63)
	GP	107 (124.98)	>107 (>124.98)
	GS	44.5 (10.24)	1:1 (10.24)
	Amp	0.00005	1:4
B.longum LMG 13197	**GC**	151.75 (49.69)	1:2 (99.37)
	Gp	90 (26.63)	>90 (>13.32)
	GP	>107 (124.98)	>107 (>124.98)
	GS	89 (10.24)	>89 (>10.24)
	Amp	0.002	1:3
Lactobacillus acidophilus La14 150B	**GC**	303.5 (99.37)	>607 (>198.74)
	Gp	>90(>26.63)	>90 (>26.63)
	GP	>107 (>124.98)	>107 (>124.98)
	GS	>89 (>10.24)	>89 (>10.24)
	Amp	0.5	1:1

Sterile broth, without garlic, was used for negative control. Ampicillin (Amp) (50 mg/ml) was used as positive control. The allicin concentration was determined spectrophotometrically (Miron *et al*., 2002). Strains not killed by the highest garlic clove concentration are depicted as: >607 mg/ml (The same goes for GP, Gp and GS extracts).

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Bifidobacterium bifidum LMG 11041 had the lowest MIC's and MBC's for all garlic preparations compared to the other bifidobacterial strains, indicating that it was the most sensitive strain overall to all the preparations. These results correspond to the disk diffusion assay as well as the minimum exposure time results. *L. acidophilus*, the strain used as the comparison, had the highest MIC and MBC values and was therefore least susceptible to all the garlic preparations. With these results we can say that *Bifidobacterium* spp. are indeed more sensitive to garlic than the *Lactobacillus* strain. In a study conducted by Rees and colleagues (1993), *Lactobacillus* spp. were also least inhibited by the presence of garlic compared to other microorganisms tested.

For our results GC and Gp have an inhibitory effect against *L. acidophilus* at slightly higher MIC values compared to those recently reported by Ross and colleagues (2001) and Owhe-Ureghe et al (2010). Our estimated allicin concentration for GC extract was also slightly lower than some published allicin MIC results such as Bakri and Douglas, 2005. These differences in results may be due to a number of reasons. One important reason is the origin and type of strains of garlic. According to Deresse (2010) and Hannan and colleagues (2012), garlic spp. tend to vary in different countries as well as the different processing methods in garlic preparation. Therefore there may be varying concentrations of individually and synergistically active bio-substances in different garlic preparations around the world which may explain the differences in antimicrobial potency as well as variations in genetic disparity among bacteria tested (Iwalokun *et al*., 2004; Hannan *et al*., 2012; Gaekwad and Trivedi, 2013).

Our slightly lower allicin concentrations and higher MIC values may also be due to too little precautions taken to prevent loss of garlic components, specifically allicin, by volatilization. Allicin is not only a very volatile compound, but is also very unstable and will undergo numerous reactions forming other derivatives dependent on environmental conditions and the processing reactions. Our garlic preparations were suspended in dH_2O and according to Lawson and Wan (1995), allicin can react with water to form diallyl disulphide, which doesn't show evidence of the same level of antimicrobial activity as allicin does (Hovana *et al*., 2010). Lastly, the type of methodology used for determination of MIC's will also affect results. The depth of agar, lag-time required for diffusion of the garlic extract, pH, size of inoculum and incubation time will all influence the MIC of the extract (Hannan *et al*., 2012). Therefore standardization of garlic extract used as well as methodologies is necessary.

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There has been very little investigation on the antimicrobial activity of GP, Gp and GS. Our results indicate that GP had a slight antimicrobial effect on *Bifidobacterium* spp. and Gp and GS had much less of an antimicrobial effect on the strains compared to GC. According to O' Gara and colleagues (2000), Gp does have a lower potency or inhibitory effect compared to GC due to its high vegetable content. As mentioned before, cysteine in the culture media may have had an impact on the interpretation of our *in vitro* test results on the antimicrobial activity of our GP preparation. Therefore these results may be an underestimate of its full antimicrobial potential, possibly even for GS and Gp. Commercial GP contains acetic acid and benzoic acid and may therefore also have affected our determined allicin concentration and results (Gupta and Ravishankar, 2005).

2.5 Conclusions

These are the first results obtained on the inhibitory effect of GC, GP, Gp and GS on *Bifidobacterium* spp. and there have been no other studies, to our knowledge, on susceptibility tests of garlic on *Bifidobacterium* spp. This study highlighted that garlic displays an antimicrobial activity towards bifidobacteria. Results indicated that fresh GC extract was the most effective with inhibiting the strains, followed by GS, Gp and GP having negligible or no antimicrobial effect. All *Bifidobacterium* strains were inhibited at different extents and it appeared that the *B. bifidum* strain was most susceptible, followed by the *B. longum* and *B. lactis* strains. This therefore shows that susceptibility of bifidobacteria to garlic differs between spp. and among different strains of the same species.

Time kill curves showed that viability of the strains definitely decreased and for most strains began to decrease after 1 h of exposure to GC. According to O' Gara and colleagues (2000) allicin remains stable in an acidic medium and this gives us ample enough reason to believe, that when ingested in conjunction, garlic may inhibit and lower the effectiveness of the probiotic bifidobacteria once they come into contact in the GIT. Much work is put into preparing probiotic products with the optimal number of viable bacterial cells in order to give the consumer the optimal health benefits, and it is therefore important to recognize anything that may hamper the products viability. Therefore this study was significant in identifying garlic as a product that could possibly decrease the viability of probiotic bifidobacteria when ingested simultaneously.

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Future studies will explore the actual antibacterial mechanism of garlic against bifidobacteria as well as the effect that food processing and different food matrices have on the antibifidobacterial effects of garlic. Additional studies can also be done on the physiological changes that occur in bifidobacteria once it is exposed to garlic.

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CHAPTER 3

Morphological properties of garlic (*Allium sativum*) treated bifidobacteria as depicted by scanning electron microscopy (SEM)

Booyens, J, Labuschagne, M.C & Thantsha, M.S 2013, '*In vitro* antibacterial mechanism of action of crude Garlic (*Allium sativum*) clove extract on selected probiotic *Bifidobacterium* species as revealed by SEM, TEM and SDS-PAGE analysis', *Probiotics and Antimicrobial Proteins* (Accepted).

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3.1 Abstract

Bifidobacteria are important beneficial microbes that inhabit the human gastrointestinal tract and are used in probiotic-containing foods and products. Changes in morphology of bifidobacteria occur as a result of stress which may include anything from predation, limitation of nutrients, environmental changes as well as contact with a toxic or antimicrobial compound that is harmful to the cell. Garlic (*Allium sativum*) has been found to have antimicrobial properties and has an inhibitory effect on a number of microorganisms. The morphological alterations as well as behavioural changes of selected *Bifidobacterium* strains induced upon exposure to different garlic extracts were illustrated and discussed in this study using scanning electron microscopy (SEM). All strains were affected by garlic clove extract and SEM micrographs revealed an assortment of changes which included swarming, cell elongation, cell wall and cytoplasm lysis, distortion of the outer surface and shape of the cells, pore-formation as well as swelling of the cells. Initiation of apoptosis was also evident. Exposure to garlic paste extract resulted in morphological alterations in most strains except the *B. lactis* strains. This study provides new insights into the action of garlic on the outer surface and behaviour of bifidobacteria.

Keywords: morphology, bifidobacteria, scanning electron microscopy (SEM), garlic, *Allium sativum*

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3.2 Introduction

The occurrence of bifidobacteria is a significant indication of stability of microflora in the human gastrointestinal tract (GIT) as well as good health. These bacteria have a positive effect against allergies, acute and chronic intestinal infections and immunodeficiency diseases among others (Novic *et al*., 2001). Together with lactobacilli, bifidobacteria are used in probiotic products due to these beneficial health effects (Reyed, 2007). In order for probiotic bacteria to exert their health benefits they must be able to attach to and colonize the intestinal mucosa. Surface properties of these bacteria play an important role in the probiotic effect and colonization capability. Therefore changes to the surface properties may prevent probiotics from colonizing the intestinal mucosa and thereby decrease their effectiveness (Gueimonde *et al*., 2005).

Bifidobacteria are Gram-positive, anaerobic, non-spore-forming bacteria which are pleomorphic, branched rods that may occur as single cells, clumps or in chains (Biavati *et al*., 2000; Giovanna and Dellaglio, 2007). Their rod-shape appearance is inconsistent and can change due to a variety of factors such as stress and nutrient conditions (Boylston *et al*., 2004). Their cell wall is characteristically Gram-positive with a structure that consists of a thick peptidoglycan envelope which contains various proteins, polysaccharides and teichoic acids (Biavati *et al*., 2000). Their inconsistent appearance was already observed in earlier years when they were observed as colon bacteria reproducing by budding and formation of Y-shapes (Biavati *et al*., 2000; Young, 2006).

Morphological changes in bacteria occur due to various environmental demands or basic forces. These may include among other, a limitation of nutrients, predation, cell division, attachment and differentiation. In response to nutrient scarcity, cells may undergo elongation in order to increase total surface area without a noticeable increase in the surface-to-volume ratio. With regards to predation, smaller cells, which are sought out by some predators, will increase their cell length. Individual bacteria adopt a shape that is adaptive and advantageous and they will modify their morphology in order to cope with these changes (Young, 2006; Young, 2007). Exposure to or contact with a harmful compound or agent also induces cell surface changes as the cell surface is the first physical barrier of defence against environmental compounds (Ruiz *et al*., 2007). Bacterial cells also use biofilm formation as an

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alternative survival strategy, where cells cluster together for protection from exposure to harmful chemicals such as antibiotics, or phagocytosis by protozoa (Young, 2006).

Changes in morphology have been observed in *Bifidobacterium* species where the typical rod-shaped cells have become coccoid once there is a nutrient constraint in order to save energy (Young, 2007). The genus *Bifidobacterium* is distinct for its pleomorphic forms within the species and *B. bifidum* has been studied numerous times specifically for its variation in shape (Husain *et al*., 1972; Young, 2006). Research has proven that once bifidobacterial species enter their death phase due to an environmental constraint, they undergo irreversible structural and functional rearrangement. During this phase they can also form cyst-like capsules (Novic *et al*., 2001). In this study we have already established that bifidobacteria are inhibited on exposure to garlic.

Garlic (*Allium sativum*) has been used for centuries due to its numerous medicinal uses and health benefits (Kumar *et al*., 2010; Daka, 2011). Numerous reports have indicated that garlic has a broad antibacterial spectrum against both Gram-positive and Gram-negative bacteria (Ruddock *et al*., 2005; Daka, 2011). Allicin is the compound responsible for garlic's antimicrobial activity and once intact garlic tissue is crushed, cut or damaged; alliin is converted into this compound, allicin (Harris *et al*., 2001; Ruddock *et al*., 2005).

There is much research which indicates that garlic has a morphological effect on bacterial cells. A study done by Belguith and colleagues (2009) proved that outer membrane integrity of *Salmonella hadar* was affected once exposed to aqueous garlic extract. Rod-like projections were also observed on the outer membrane of the cells. O' Gara and colleagues (2000) also observed changes in the morphology and behaviour of *Helicobacter pylori* cells once exposed to garlic oil. Typical rod-shaped cells decreased in numbers and coccoid cells became apparent. There was also an increase in clumping of these cells and cellular debris. Garlic has also been shown to affect the cell surface properties of *Candida albicans* and *Listeria monocytogenes* (Lemar *et al*., 2005; Kim *et al*., 2007). Biofilm formation in *Streptococcus mutans* has also been attributed as a result of exposure to garlic extract (Lee *et al*., 2011). The aim of this study was to investigate the changes in morphology, cell wall structure and behaviour of *Bifidobacterium* species once exposed to different garlic preparations.

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3.3. Material and Methods

3.3.1 Bacterial cultures

Commercial probiotic cultures of *Bifidobacterium lactis* Bb12 (CHR- Hansen), *B. longum* Bb536 (Morinaga Milk company), *B. lactis* Bi-07 300B and *Lactobacillus acidophilus* La14 150B (Danisco) were used. *L. acidophilus* La14 150B (Danisco) was used as a comparison. *B. bifidum* LMG 11041 and *B. longum* LMG 13197 strains were both purchased from the BCCM/LMG culture collection, Belgium and revived as per instructions from the supplier.

3.3.2 Preparation of inoculums

Lactobacillus acidophilus La14 150B was grown in MRS broth while the bifidobacterial cultures were grown in MRS broth supplemented with 0.05% cysteine hydrochloride. Both of the inoculated broths were incubated at 37°C for 48 h in anaerobic jars with Anaerocult A gaspacks (Merck Ltd, Mofferfontein, SA) and Anaerocult C test strips for confirmation of anaerobic conditions inside the jar. Bacterial cell suspensions were adjusted to a 0.5 McFarland standard (approximately 1 x 10^8 cfu/ml).

3.3.3 Garlic preparations

Fresh garlic cloves (GC), garlic paste (GP), garlic powder (Gp) and garlic spice (GS) were bought from a local supermarket in Pretoria. GC, Gp and GS were stored at room temperature, whilst GP was stored at 4°C for no longer than 2 weeks.

3.3.3.1 Preparation of garlic extracts and determination of the final concentration of garlic in solution

The garlic extract was prepared as described by Bakri and Douglas (2005), with minor modifications. Fresh cloves were separated and peeled and 10 g was weighed and crushed using a mortar and pestle. This was then suspended in 5 ml sterile distilled water. The same amounts of GP, Gp and GS were weighed and GS and Gp were suspended in 10 ml of sterile distilled water, whilst GP was suspended in 5 ml. These suspensions were then centrifuged and filtered through a 0.22 µm filter (Minisart). The weight of the insoluble material of the

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GC, GP, Gp and GS was subtracted from the weight of the original material to get the final concentration of garlic extract in solution. The sterile extract was used within 30 min of its preparation for the inhibition studies. One hundred microlitres of each of the different garlic extracts were added to 1 ml of the *Bifidobacterium* and *Lactobacillus* broth cultures. Untreated broth cultures were used as a negative control while ampicillin (Amp) was used as a positive control. The cultures were then incubated at 37°C for 6 h. Samples were taken immediately before exposure to the garlic extracts and then after 6 h of exposure. Eppendorf tubes were then centrifuged for 10 min at 12044.9 g. Cells were resuspended in 1 ml Ringer's solution and centrifuged once again. This was repeated three times after which the cultures were prepared for SEM.

3.3.4 Scanning electron microscopy

To determine the effect that the garlic preparations have on the morphology and membrane of bifidobacteria, a modification of the method by Lai-King et al (1985) was used. The cells, which were suspended in ¼ Ringer's solution, were harvested by filtering through a 0.2 µm filter membrane and then fixed using 2.5% gluteraldehyde for 1 h. This was followed by washing the cells three times with 0.15M phosphate buffer. Following this the cells were dehydrated in a graded alcohol series (25, 50, 75, 90 and 100% ethanol). After critical-point drying for 24 h, the filters were then mounted onto SEM specimen stubs and coated with gold. The specimens were observed under a JEOL JSM-840 and a JEOL JSM-5800LV scanning electron microscope.

3.4 Results and Discussion

SEM micrographs revealed numerous changes to the morphology and outer surface of all *Bifidobacterium* strains exposed to the GC extract for 6 h. The control, *L. acidophilus* strain, also experienced some changes.

3.4.1 *L. acidophilus* La14 150B

It was already revealed in the previous chapter that *L. acidophilus* was not sensitive to the antimicrobial effects of garlic. Contrary to those results, SEM analysis revealed some morphological changes such as bunching or swarming in groups (Fig. 3.1B and C) as well as

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collection of debris on the outer surface (Fig. 3.1B) after exposure to ampicillin and GC extract. Changes in bacterial morphology were expected after *L. acidophilus* cells were treated with ampicillin as it has been proven that antibiotics alter the surface morphology of bacterial cells (Klainer and Perkins, 1970; Nikiyan *et al*., 2010). Ampicillin also inhibits synthesis of the peptidoglycan layer of gram-positive bacterial cell walls such as *Bacillus cereus* and therefore destructs the cell wall (Nikiyan *et al*., 2010). This may be the cause of the cellular debris on the outer surface of the ampicillin-treated cells. Untreated cells retained a uniform rod-shape with a smooth surface (Fig. 3.1A).

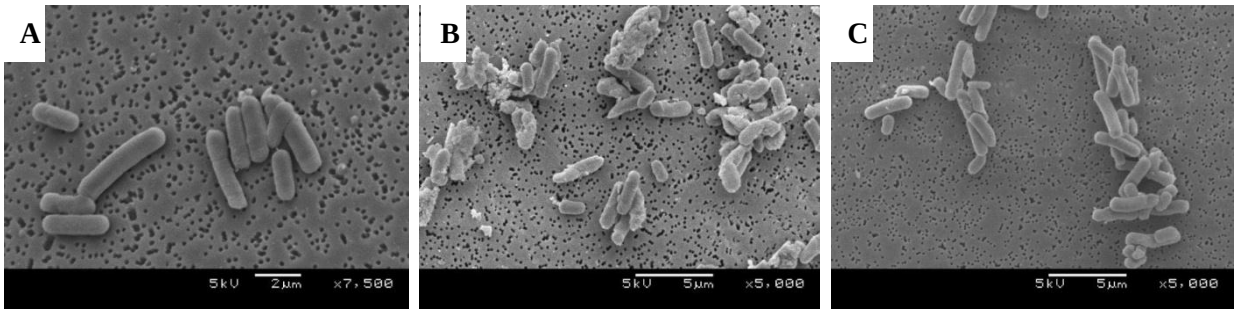

Figure 3.1: Scanning electron micrographs of *L. acidophilus* La14 150B before (A) and after exposure to ampicillin (B) and GC extract (C).

3.4.2 *B. bifidum* LMG 11041

In the disk diffusion test (Chapter 2, section 2.4.1), *B. bifidum* LMG 11041 was most susceptible to the antibacterial effects of all the garlic extracts. SEM analysis revealed a pleomorphic cell culture and untreated *B. bifidum* LMG 11041 cells (Fig. 3.2A) were non-uniform in appearance. These pleomorphic *Bifidobacterium* cells have also been observed by other researchers previously. Gyllenberg (1955) described mycelial cells of bifidobacteria that appeared to swell and form coccoid granules (Poupard *et al*., 1973). In addition, Poupard, and colleagues (1973) documented bifidobacteria that were highly branched, filamentous, with some irregular and so swollen they resembled a bladder with a cross-like structure on the surfaces. Cells with cross-walls are morphology common to some bifidobacterial strains that are in their exponential and early stationary growth phases (Novic *et al*., 2001).

Exposure of this pleomorphic culture to ampicillin resulted in an increase in short, oval rods which resembled cocciod cells. Distorted cells as well as cells with swollen ends were also evident (Fig. 3.2B). Chains of short, oval rods have also been observed when bifidobacteria

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have been exposed to ultrafiltered and skim milk (Ventling and Mistry, 1992). Cells exposed to GP extracts showed a loss in cell wall consistency (Fig. 3.2C). Distortion to the outer surface or cell wall could possibly be a result of lysis of the cell wall. Loss of cell wall integrity has also been observed by Lu and colleagues (2011a) when they exposed *Campylobacter jejuni* cells to garlic. According to Kim and colleagues (2007), cell wall lysis may be due to the weakening of the peptidoglycan layer, possibly in this case as a result of exposure to the GP extract. They observed similar results when they treated *Listeria monocytogenes* with garlic shoot juice. The observed change is not only restricted to bacteria as Ghannoum (1988) observed it in garlic treated *Candida albicans* (Kim *et al*., 2007). Exposure to GP extract also revealed cell debris among the cells (Fig. 3.2C). Debris collection on the outer surface of *C. albicans* cells treated with garlic powder extract was also detected using SEM by Lemar and colleagues (2005) and the debris collection may be a result of the loss in the volume of cellular cytoplasm.

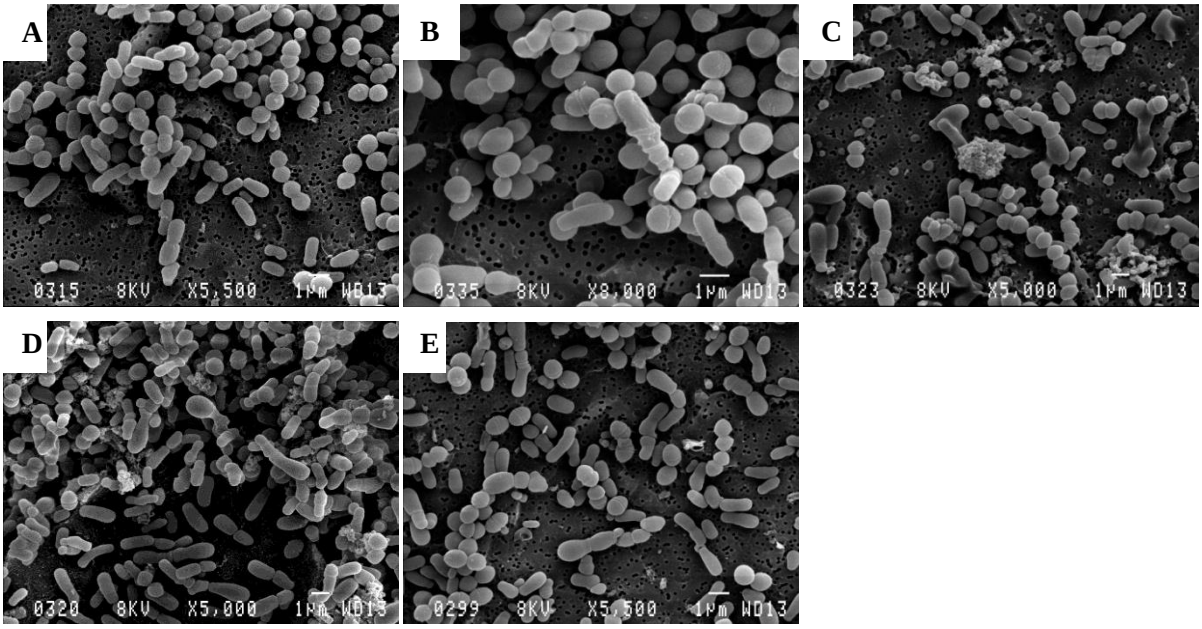

Figure 3.2: Scanning electron images of *B. bifidum* LMG 11041. Morphological characteristics before (A) and after exposure to ampicillin (B), GP (C) and GC (D-E) extract.

B. bifidum cells treated with GC extract (Fig. 3.2D-E) became highly distorted with a club shape appearance and bulbous ends. The cells also appeared to be swollen and appearance of cells with cross walls that looked as if they were budding into cocci-shaped cells was also evident. This has been observed previously in *Bifidobacterium* spp. where typical rod-shaped cells became coccoid once there was a nutrient limitation (Young, 2007). In addition, in a

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study performed by Husain and colleagues (1972), *B. bifidum* cells became pleomorphic and did not resemble rods at all due to a change of nutrients used in the medium. As with exposure to GP extract, debris was also evident in the surrounding medium of GC extract treated cells. In their death phase, bifidobacteria do show various morphologies which include rod-shaped and coccoid cells, budding and branched cells as well as club-shaped cells forms (Novic *et al*., 2001).

3.4.3. *B. longum* LMG 13197

Untreated *B. longum* LMG 13197 cells showed a uniform structure with consistent dispersal (Fig. 3.3A-B). Garlic-treated cells showed morphological surface damage as well as clumping of the cells. Exposure to ampicillin resulted in swarming or bunching of the cells. Possible production of extracellular polysaccharides (EPS) was also observed (Fig. 3.3C). Secretion of EPS probably serves as a survival mechanism protecting the cells from any form of stress. EPS production has been observed in *B. longum* cells which were cultured in skim milk and peptone-yeast lactose media (Biavati *et al*., 2000).

Cells treated with GC extract for 6 h showed various morphological alterations and damage to the outer surface of the cells. Numerous cells appeared longer, varying in sizes between ~5-10 µm compared to 1 µm control cells (Fig. 3.3D-E). Cells become elongated in order to increase their surface-to-volume ratio in response to environmental changes or stress (Koch, 1996), such as in this case, GC extract. Lysis of the cell wall and cytoplasm as well perforation of some cells was also evident (Fig. 3.3F-G). These results correspond to those reported by Kim and colleagues (2007), where they treated *Listeria monocytogenes* with garlic shoot juice. They attributed cell wall lysis to be due to the weakening of the peptidoglycan layer possibly as a result of exposure to the garlic extract. This was also proposed by Ghannoum (1988) where he attributed the change in structure and integrity of the outer membrane of *Candida albicans* to the decrease in lipid content of the membrane due to the presence of garlic (Kim *et al*., 2007).

Exposure to GP extract resulted in bunching or swarming of cells in groups (Fig. 3.3H-I). In a study done Lee et al (2011), it was observed that *Staphylococcus mutans* cells showed an increase in aggregation due to treatment with garlic extract. Aggregation is the first step in biofilm formation (Lee *et al*., 2011). Exposure to GP extract also revealed possible EPS production in the culture as was observed with GC extract.

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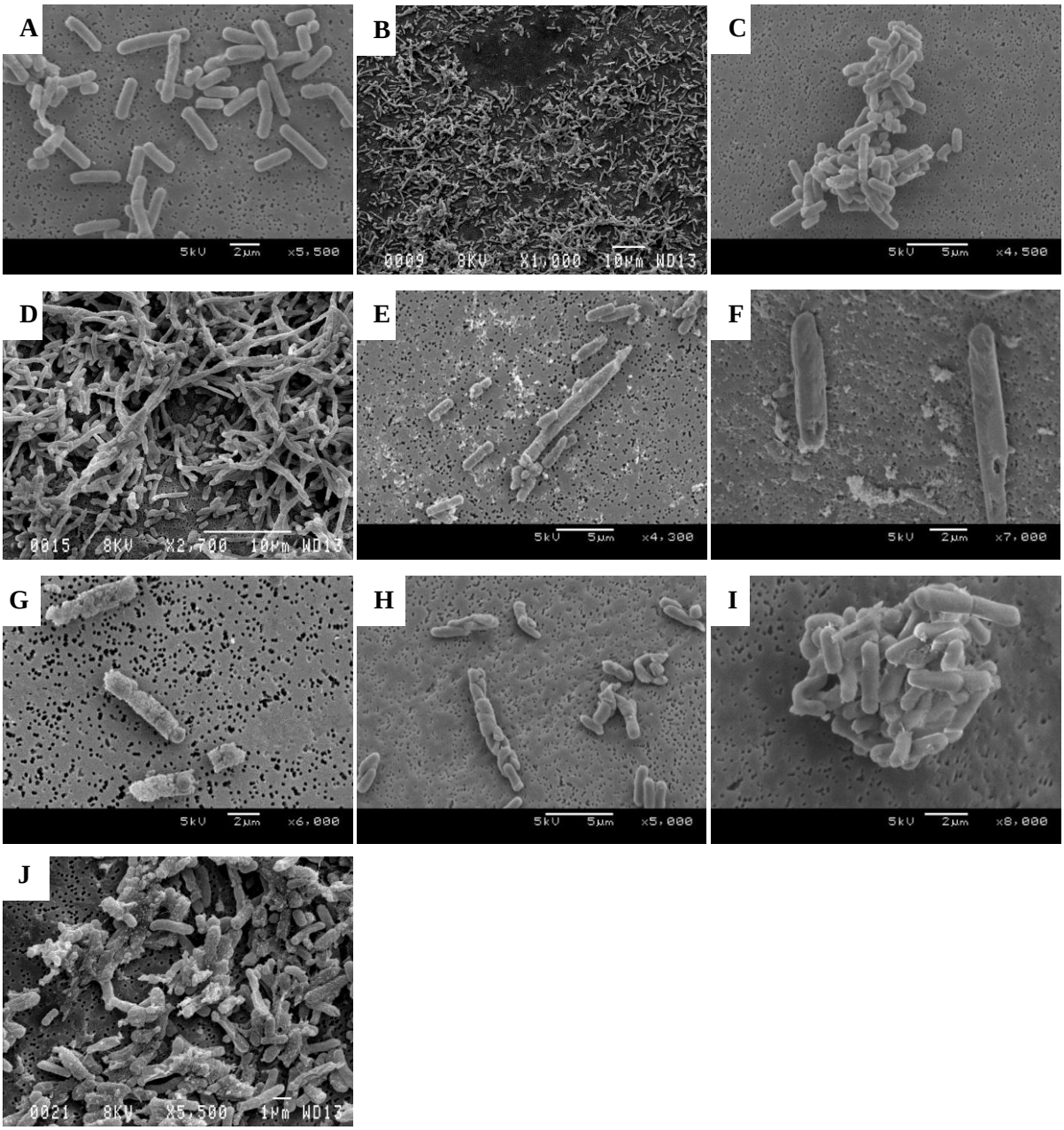

Figure 3.3: SEM images showing morphological changes in *B. longum* LMG 13197 before (A-B) and after exposure to ampicillin (C), GC (D-G) and GP extract (H-J).

3.4.4 *B. longum* Bb536

B. longum Bb536 cells not subjected to garlic showed a smooth, regular surface (Fig. 3.4A) while GC-treated cells showed severe distortion of the cell surface, cell wall lysis and disintegration, EPS production as well as pore formation (Fig. 3.4B-E). Our results correspond with findings performed by Lemar and colleagues (2005) and Kim and colleagues (2007) whom used garlic on different microorganisms. Sinking and distortion of the cell wall may be due to the loss of cytosolic volume possibly as a result of the autolysis

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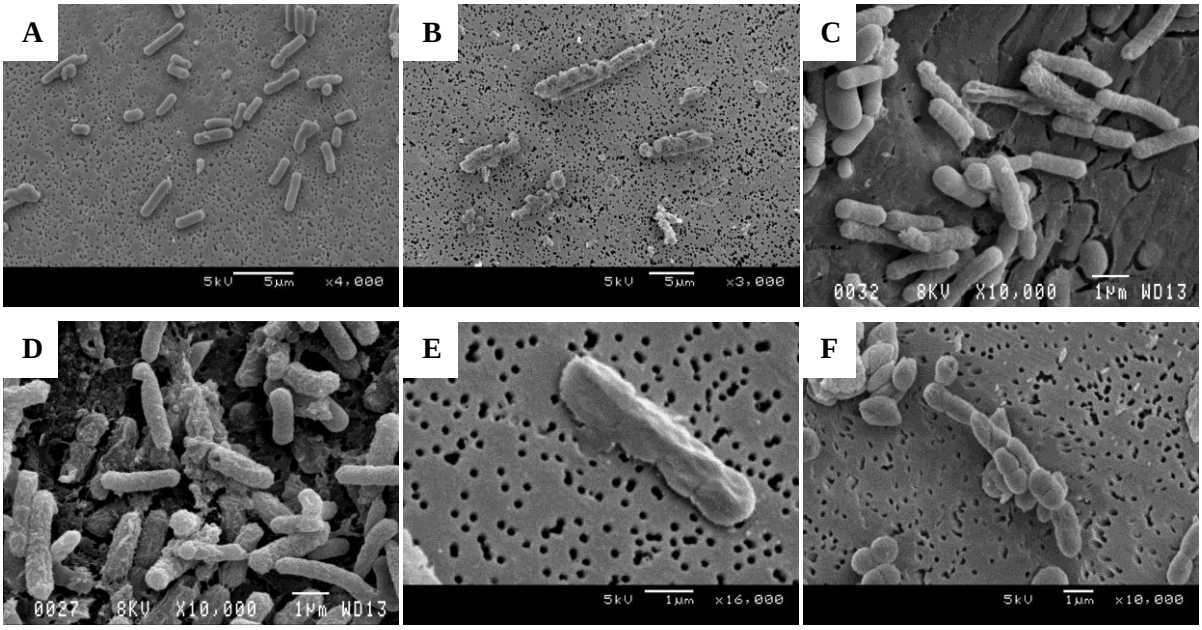

Figure 3.4: SEM images showing changes in morphology to *B. longum* Bb536 before (A) and after exposure to GC (B-E) and GP extract (F).

activity of allicin (Lemar *et al*., 2005; Kim *et al*., 2007). Formation of EPS or possibly a collection of debris was also observed after exposure to GC extract (Fig. 3.4D). EPS is normally produced in biofilms by bacteria in a reaction to stress such as physiological changes and nutrient depletion among others. It may either form a capsule which is made up of high-molecular weight polysaccharide that attaches to the cell surface or it could produce slime which is attached to the cell surface or released into the culture (Wai *et al*., 1998). EPS formation may also indicate that the culture reached its death phase due to exposure to the garlic. Novic and colleagues (2001) showed that once bifidobacterial cells were in their death phase they also began to produce capsular polysaccharides and microfibrils. As was observed with *B. longum* LMG 13197, *B. longum* Bb536 cells also exhibited swarming after treatment with GP extract (Fig. 3.4F).

3.4.5 *B. lactis* Bb12

The morphology of *B. lactis* Bb12 was also pleomorphic as was observed with the *B. bifidum* LMG 11041 strain (Fig. 3.5A). The untreated sample consisted of rod-shaped cells, including very short rods and irregular cocci-shaped cells with cross-walls, in comparison to the uniform cultures of the other strains (Fig. 3.3A and 3.4A). The phosphoketolase assay was performed to confirm that this strain was indeed *Bifidobacterium*. A non-uniform culture has been observed before by other researchers. Poupard and colleagues (1973) documented

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bifidobacteria that were highly branched, filamentous, with some irregular swollen cells that they resembled a bladder with a cross-like structure on the surfaces.

Exposure of *B. lactis* Bb12 to ampicillin (Fig. 3.5B) and GC (Fig. 3.5C-D) resulted in swollen ends or a dumbbell-shaped appearance. Nikiyan and colleagues (2010) found that cells become spherical or coccus in appearance on exposure to ampicillin. It has also been previously suggested that swelling of bacillary forms, which are rod-shaped bacteria, is due to an interference with cell wall synthesis compounds, which in this case could be as a result of allicin (Husain *et al*., 1972). There was an increase in numbers of cocci-shaped cells and cells with distorted shapes and wall surfaces due to exposure to GC extract. As mentioned before when bifidobacteria are entering their death phase, possibly as a result of stress, they have an assortment of morphologies which include coccoid cells (Novic *et al*., 2001).

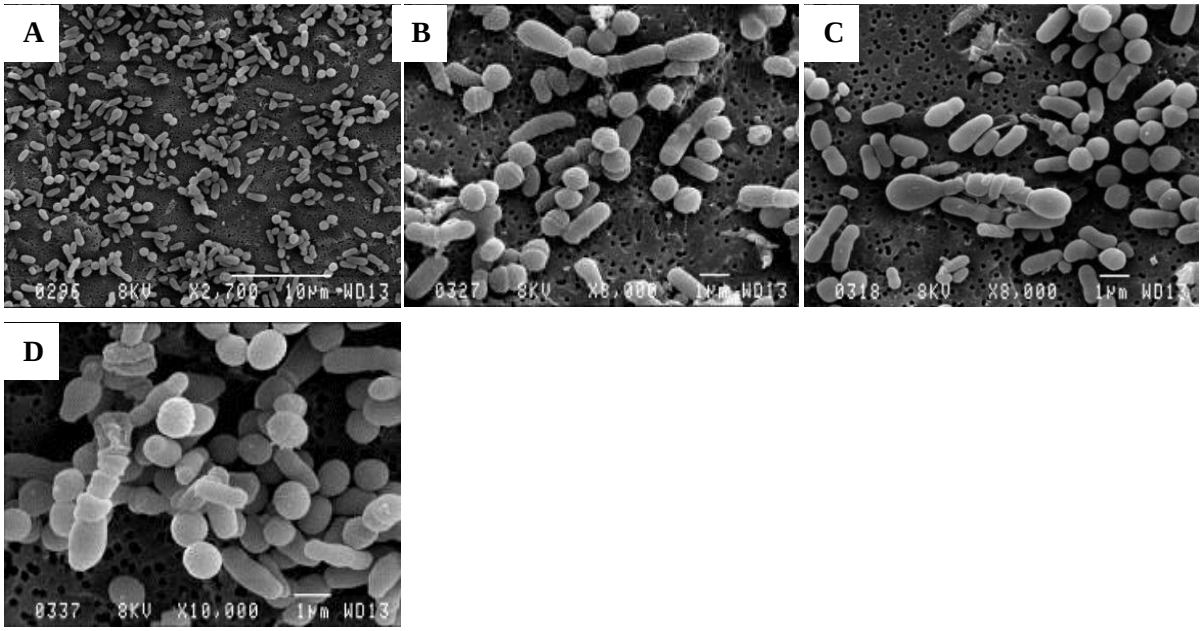

Figure 3.5: SEM images showing *B. lactis* Bb12 before (A) and after treatment with positive control, ampicillin (B) and GC extract (C-D).

3.4.6. *B. lactis* Bi-07 300B

Control cells were smooth and uniform in appearance (Fig. 3.6A) with lengths of 1 µm. On the contrary, cells that were treated with ampicillin increased to lengths of ~5-10 µm. Cell wall lysis and indentations occurring in the middle of the cell wall were also observed (Fig. 3.6B). Sinking or indentation of the cell wall could also be as a result of the autolysis properties of allicin as suggested by Kim and colleagues (2007). The same results were

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observed for cells treated with GC extract (Fig. 3.6C-E). Lysis and disintegration of the cell as a result of exposure to GC extract can clearly be seen in Figure 6E. Elongation of cells was also observed for *B. longum* LMG 13197 cells exposed to GC extract and as mentioned previously. Koch (1996) proposed that cells increase in length in order to increase their surface-to-volume ratio in response to any stress. Allicin may possibly be destroying or altering nutrients or growth conditions for the bifidobacteria therefore they may respond to this by filamentation or elongation of cells in order to increase total surface area without an appreciable increase in the surface-to-volume ratio (Young, 2006).

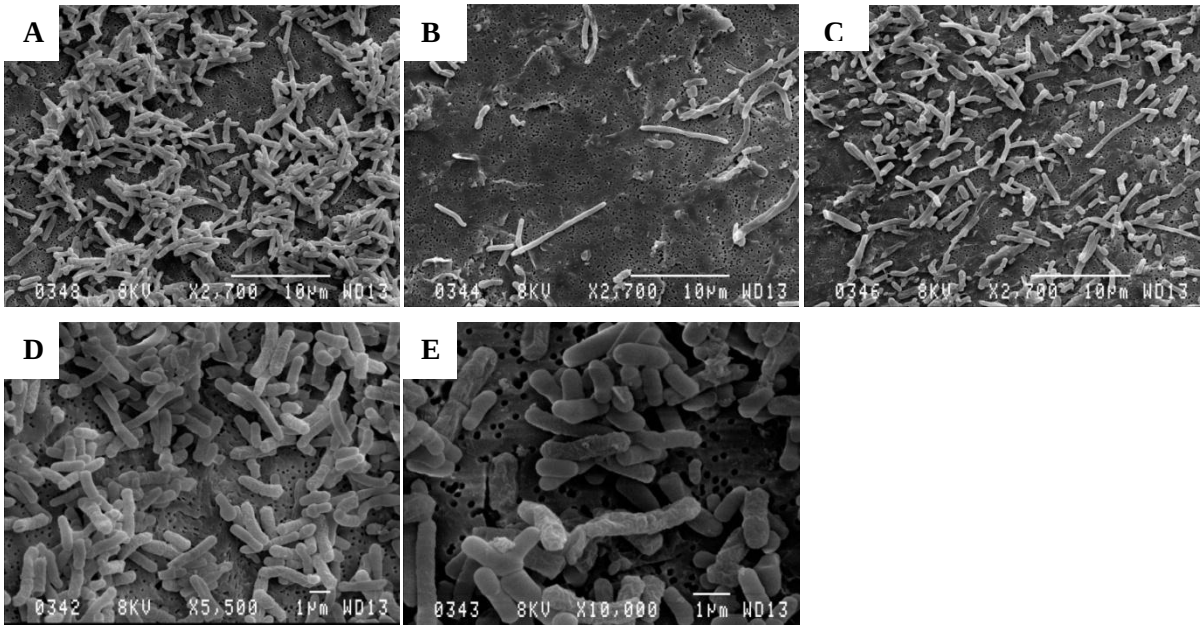

Figure 3.6: SEM images of *B. lactis* Bi-07 300B before (A) and after exposure to ampicillin (B) and GC extract (C-E).

Morphological characteristics for the cultures exposed to Gp and GS extract resembled the untreated cells. This might be an indication of the weaker antimicrobial properties of Gp and GS (as observed in the previous chapter) compared to GC and GP extract, where some of the cells were either not affected or were recovering as was observed with the time kill curves in the previous chapter.

The bacterial cell surface is the first physical target to any harmful compound subjected to the cell therefore if the cell were sensitive to an antimicrobial compound; morphological changes to the cell surface would theoretically be seen. Allicin, the compound responsible for garlic's antimicrobial activity, has been found to react with thiol-containing proteins and therefore

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this could possibly mean that it is able to permeate both biological and artificial membranes (Kim *et al*., 2007). Most antimicrobial agents, such as allicin, are able to modify cell membranes which may cause leakage and autolysis therefore preventing growth and causing cell death (Lu *et al*., 2011b). Therefore this could be a strong indication why the tested *Bifidobacterium* strains undergo the above mentioned changes in morphology. Allicin has been found to have a bacteriocidal effect on many bacteria and is effective against most Gram-positive and Gram-negative bacteria which include methicillin-resistant *Staphylococcus aureus*. Most bacteria cannot develop resistance against allicin as it directly modifies thiol enzymes or proteins involved in cell division. Allicin is known to inhibit cell growth and apoptosis and inhibits RNA synthesis more strongly than DNA and protein synthesis (Fujisawa *et al*., 2008; Lui *et al*., 2010).

It has also been found that garlic alters the electrochemical ability of cells and induces apoptosis (Gruhlke *et al*., 2010). Apoptosis or programmed cell death result in morphological changes such as shrinkage of the cell and cytoplasm condensation (Saraste and Pulkki, 2000). When *Escherichia coli* undergoes apoptosis, cell elongation and lysis also take place (Lewis, 2000). Some of these changes were also observed for *Bifidobacterium* strains tested in this study after exposure to the GC extract. Initiation of apoptosis may therefore have been occurring in the *Bifidobacterium* strains exposed to allicin.

Morphological changes have been previously reported for many different bacteria treated with garlic. Belguith and colleagues (2009) observed changes in morphology of *Salmonella hadar* cells when exposed to aqueous garlic extract. *Campylobacter jejuni* endured morphological damage as well as cell wall changes once in contact with garlic (Lu *et al*., 2011a). Changes in morphology of the structure of an organism exposed to garlic have also been studied by Awny and colleagues (2005). The formation of various projections on the outer membrane of the cell envelope of *Salmonella hadar* has also been observed after exposure to garlic. Garlic aqueous extract has also altered the morphology and ultrastructure integrity of *Salmonella typhimurium, Escherichia coli, Bacillus subtilis* and *Bacillus pumilus* (Belguith *et al*., 2009).

This study provides new insights into the action of garlic on the morphology and cell surface properties of bifidobacteria. All of the *Bifidobacterium* strains tested showed considerable and various alterations in their morphology, cell surface properties as well some changes in

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the actual behaviour of the cells once exposed to the different garlic preparations. There were no significant changes observed for the control strain, *L. acidophilus*. This is probably due to the fact that the composition of the cell wall of lactobacilli is significantly different from bifidobacteria (Exterkate *et al*., 1971; He *et al*., 2006). Therefore, garlic would naturally affect these two species differently. Results showed garlic stress-associated morphological changes for all *Bifidobacterium* strains. The strains were affected differently; some having a number of different changes to their morphology while others had one or two of the same or different modifications. These differences in morphology between the different strains may be due to variations in the composition of fatty acids in *Bifidobacterium* spp. A study done by Veerkamp (1971) showed that there were slight differences in fatty acid composition for different *Bifidobacterium* strains.

These variations in morphology included cell elongation, a decrease in rod-shaped cells and increase in coccoid cells, formation of EPS or biofilm, bulbous and distorted ends of the cells, cell shrinkage or disintegration. Behavioural aspects such as swarming in groups or bunching were also observed. The beginning stages of cell apoptosis were also evident. We couldn't deduce which *Bifidobacterium* strain was most affected, in terms of morphology but *B. bifidum* and *B. longum* strains both underwent morphological changes after exposure to GC and GP extract whereas *B. lactis* strains were only morphologically affected by the GC extract.

3.5 Conclusions

In conclusion, it can be seen from these results that garlic promotes various morphological changes, external structural changes as well behavioural changes for all the *Bifidobacterium* strains tested. All strains experienced changes in morphology after exposure to GC extract, while *B. bifidum* LMG 11041, *B. longum* LMG 13197 and *B. longum* Bb536 were the only strains to show any changes after being treated with GP extract. Gp and GS extract did not affect the morphology of any of the strains which could possibly indicate that, although it inhibited some strains as seen with the disk diffusion assay, its antimicrobial effects were not as strong as GC and GP extract to morphologically alter the cells. It could also be because of these preparations' lower allicin concentration as allicin seems to be the main compound which affects the cells. Changes in the cell surface properties of these *Bifidobacterium* strains

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may also alter their ability to colonize the intestinal mucosa and therefore prevent them from conferring their health benefits.

These changes in morphology on the external structure of treated cells may be surface reflections of biochemical reactions occurring within the cell wall and further biochemical studies as well as TEM analysis should be considered. Future studies also include the determination of the physiological changes occurring within the cells as well as changes in lipid composition.

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CHAPTER 4

Fourier-transform infrared (FT-IR) spectroscopy analysis of selected *Bifidobacterium* spp. treated with garlic (*Allium sativum*) clove extract

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4.1 Abstract

Fourier transform infrared (FT-IR) spectroscopy (4000-850 cm^{-1}) was used to detect damage of probiotic *B. bifidum* LMG 11041, *B. longum* LMG 13197 and *B. lactis* Bb 12 after exposure to garlic clove extract. Probiotic *Bifidobacterium* species are used in pharmaceutical products as well as certain foods and they need to be present in high viable numbers. It is therefore important to test these strains against compounds that may decrease their viability. FT-IR spectroscopy confirmed damage to the cells as indicated by changes in spectral features of lipids and fatty acids in cell membranes, proteins and polysaccharides as well as nucleic acids. Spectral data was analyzed by principle component analysis (PCA). It revealed segregation of garlic-treated and untreated cells for all bifidobacterial strains tested, with *B. bifidum* LMG 11041 showing the most variation. Allicin appeared to be the main compound responsible for garlic's antibifidobacterial activity. Therefore, FT-IR could detect biochemical differences between intact and injured bifidobacterial strains once exposed to garlic clove extract, indicating that garlic has a negative effect on these probiotic strains.

Keywords: Fourier-transform infrared (FT-IR) spectroscopy, *Bifidobacterium*, probiotics, garlic, allicin, biochemical changes, principle component analysis (PCA)

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4.2 Introduction

Bifidobacteria are used as probiotics and are added to a variety of different food products and pharmaceutical preparations due to their numerous health benefits (Perez *et al*., 1998; Mattarelli *et al*., 1999; Gueimonde *et al*., 2005). In order for probiotic bacteria to exert their health benefits they must have high enough viable counts as well as be able to attach to and colonize the intestinal mucosa. Surface properties of these bacteria play an important role in their probiotic effect and colonization capability. The most important cell wall components involved in colonization include polysaccharides, teichoic and lipoteichoic acids and proteins (Mattarelli *et al*., 1999; Gueimonde *et al*., 2005). Therefore changes to the surface properties may prevent probiotics from colonizing the intestinal mucosa and thereby decrease the effect of probiotics (Gueimonde *et al*., 2005). It is therefore important to test these probiotics against compounds that may render them ineffective or decrease their capacity to perform.

Fourier transform infrared (FT-IR) spectroscopy is a fairly new technique used to study the entire molecular composition of microbial cells. This is possible because all functional groups of organic molecules are able to absorb IR light (Zoumpopoulou *et al*., 2010). Not only is FT-IR spectroscopy a rapid and non-invasive alternative to study changes or injury that takes place in bacterial cells, sample preparation is also very minimal (Lu *et al*., 2011b). The infrared spectra of bacterial cells is able to reveal the biochemical composition of their cellular constituents which include the cell wall and membrane (composed of phospholipid bilayer, peptidoglycan and lipopolysaccharides), as well as the cytoplasm (fatty acids, water, nucleic acids, proteins and polysaccharides) (Al-Qadiri *et al*., 2008; Zoumpopoulou *et al*., 2010).

Due to the fact that FT-IR spectroscopy reveals the biochemical composition of bacterial cellular components, it has recently been used to investigate the mode of action of bactericidal compounds as well as to determine changes in membrane fluidity and membrane phase behavior in response to environmental stresses (Alvarez-Ordóñez *et al*., 2010). When bacteria are exposed to or grow under stressful conditions, changes of cell structures and molecules may take place which causes different FT-IR spectra. Certain bacteria, such as *Salmonella typhimurium* and *S. enteritidis*, develop adaptive responses to changes in their environment or under stressful conditions, such as changes in salt concentration, pH,

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temperature and oxygen tension (Alvarez-Ordóñez *et al*., 2010). FT-IR spectroscopy has been used in numerous studies to obtain information on compositional changes of internal molecules in bacterial cells as a result of injury or treatment with some antimicrobial compound. Zoumpopoulou and colleagues (2010) successfully used FT-IR spectroscopy to detect internal cellular changes in *Salmonella enterica* serovar typhimurium due to antimicrobial compounds. Sub lethal thermal injury in *S. enterica* and *Listeria monocytogenes*, sonication injury in *L. monocytogenes* as well as injury caused by cold stress in *Campylobacter jejuni* and *Pseudomonas aeruginosa* have also been identified by means of FT-IR spectroscopy (Lin *et al*., 2004; Al-Qadiri *et al*., 2008; Lu *et al*., 2011a). FT-IR methods have also been used to study chlorine-injured *P. aeruginosa* and *Escherichia coli* in water, radical induced damage of *Micrococcus luteus* and heat-killed *E. coli* O157:H7 in ground beef (Davis and Mauer, 2010).

In order to understand IR spectra of microbial cells, one should have a good knowledge of the composition of cells, chemical structures present and be able to differentiate between cells and tissues. Bifidobacteria are Gram-positive and therefore have a cytoplasmic membrane and cell wall, unlike Gram-negative cells which also have an outer membrane. Cell walls are firm high-molecular complexes which consist of peptidoglycan (30-70%), which gives the cell shape and protects it against osmotic disturbances. It consists of disaccharide-pentapeptide subunits with alternating D- and L- amino acids and a γ-bonded D-glutamic acid residue. Many Gram-positive bacteria have an additional polymer, covalently bonded to the peptidoglycan, the teichoic and teichuronic acids (Schleifer and Kandler, 1972; Naumann, 2000).

Garlic (*Allium sativum*) possesses antibacterial, antioxidant and anti-inflammatory activities and contains allicin, ajoene and the diallyl sulfides which are responsible for its bioactive properties. Antimicrobial agents, such as garlic, are easily taken up by the cell and damage bacterial cells by altering their membranes causing autolysis thereby inhibiting growth or causing cell death (Lu *et al*., 2011b). Allicin, which is the main active compound in garlic and responsible for its antimicrobial activity has been known to totally inhibit RNA synthesis as well as partial DNA and protein synthesis and is also known alter the electrochemical ability and induce apoptosis in cells (Ross *et al*., 1988; Al-Waili *et al*., 2007; Deresse, 2010; Gruhlke *et al*., 2010). Allicin is also known to affect lipid biosynthesis in microbes, signal transduction, Nitric oxide (NO) formation as well as reacting with thiol-containing proteins

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(Focke *et al*., 1990; Rabinkov *et al*., 1998; Miron *et al*., 2000; Lu *et al*., 2011b; Lu *et al*., 2011c). FT-IR spectroscopy has also been able to detect sub lethal damage in foodborne pathogens, such as *Campylobacter jejuni*, *Escherichia coli* O157:H7 and *Listeria monocytogenes*, as a result to garlic exposure (Lu *et al*., 2011b; Lu *et al*., 2011c).

We have already shown sensitivity of bifidobacteria to antibacterial effects of garlic and also investigated the effect of garlic on morphology of the treated bifidobacteria using SEM. Here we aim to analyze, using FT-IR spectroscopy, the biochemical changes that take place in *Bifidobacterium* spp. after cells are treated and incubated with garlic clove extract in order to better understand the mechanism of bacterial injury from exposure to garlic.

4.3 Materials and Methods

4.3.1 Culture preparation

B. bifidum LMG 11041, *B. longum* LMG 13197 both purchased from the LMG culture collection, Belgium and revived as per instructions. *B. lactis* Bb12 was obtained as freeze-dried sachets from CHR-Hansen (Denmark). These strains were grown in MRS-cys-HCl broth and incubated at 37°C for 48 h in anaerobic jars containing Anaerocult A gaspacks (Merck Ltd, Modderfontein, SA). Cultures were diluted to a concentration equivalent to 0.5 McFarland standards. Garlic clove extract (100 µl), prepared as previously mentioned (Chapter 2, section 2.3.2.1), was then added to 1 ml broth cultures and further incubated for 6 h. Cells were recovered from 1 ml broth cultures by centrifugation at 13.4 rpm for 15 min (Eppendorf minispin centrifuge), supernatant discarded and the pellet resuspended in ½ Ringers solution. This was repeated twice as to remove media components. The pellet was then resuspended in 1 ml distilled water (dH_20) and ready for FT-IR sample preparation and measurement. The average cell concentration of the bacterial samples was kept constant at 1 x 10^9 cfu/ml in order to generate consistent FT-IR signals.

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4.3.2 Preparation of bacteria for FT-IR

All of the bacterial samples were prepared according to Marcotte and colleagues (2007) with slight modifications. Five microlitres of each sample was deposited onto a CaF_2 slide and air-dried at room temperature under low vacuum for 15 min. A further 2 µl of bacterial sample was deposited on top of the already dried sample to form a homogenous dried film of cells for FT-IR analysis. Samples were dried before analysis as absorption of water greatly affects FT-IR spectra. Samples were analyzed directly after preparation to prevent any changes associated with aging of the samples.

4.3.3 FT-IR spectroscopy and spectral measurements

A Bruker Vertex 70V FT-IR spectrometer, equipped with a Hyperion microscope attachment was used to view the samples and record the bacterial IR spectra. The CaF_2 slides, coated with the bacteria, were placed under the microscope objective and IR spectra were recorded in transmission mode from 4000 to 850 cm^{-1} at a spectral resolution of 4 cm^{-1}. Fifteen spectra for each sample were acquired at room temperature to get a total of 45 spectra for each treatment. Experiments were done in triplicate.

4.3.4 Data analysis

OPUS software (version 6, Bruker) was used to perform data analysis. Spectra were smoothed with a Savitsky-Goly function algorithim with 25 smoothing points, base-line corrected and normalized. PCA was used on the raw data to separate and group control and garlic-treated spectra in order to show a clear difference between the two data sets

4.4 Results and Discussion

FT-IR spectroscopy was used in addition to SEM (Chapter 3) as sample preparation is minimal for this technique. SEM samples had to undergo a lot of preparation which may possibly have damaged the cell surface giving inaccurate results. To confirm that the morphological changes were due to the garlic and not due to damage during SEM sample preparation, it was decided to utilize FT-IR spectroscopy. FT-IR spectroscopy was also able

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to reveal even the slightest biochemical changes that may not have been picked up by other techniques employed.

Spectra obtained for all *Bifidobacterium* strains were comparable to spectra previously reported for other bacteria (Al-Qadiri *et al*., 2008; Vodnar *et al*., 2010; Zoumpopoulou *et al*., 2010; Lu *et al*., 2011a; Lu *et al*., 2011b; Lu *et al*., 2011c). Strong absorptions were obtained in all four spectral regions (4000-850 cm^{-1}), which distinguish the main cellular components. Table 4.1 shows the most common spectral band assignments found in microorganisms. Variation in spectra between the controls and garlic-treated bacterial samples was evident once the spectra were converted by smoothing, normalization and base-line correction.

Various aspects affect FT-IR spectra and these may include the incubation time of the samples and temperature as well as the optical properties of the filter membranes used for sample preparation (Lin *et al*., 2004). For this study, preparation of samples was consistent to reduce these aspects and to generate dependable spectral patterns in order to obtain reproducible data. The average cell concentration of the samples was always 1 x 10^9 cfu/ml with the aim to provide strong FT-IR signals.

Mean FT-IR spectral features of *B. bifidum* LMG 11041 are shown in Figure 4.1, illustrating all four distinguishable regions. Region I (3000-2800 cm^{-1}) depicts the fatty acids of the bacterial cell membrane, with 3 peaks (2960, 2925, and 2860 cm^{-1}) depicting the alkyl group of lipids (Davis and Mauer, 2010; Zoumpopoulou *et al*., 2010; Lu *et al*., 2011a). Region II (1700-1500 cm^{-1}) contains the amide I (1650 cm^{-1}) and amide II (1550 cm^{-1}) bands of proteins and peptides (Davis and Mauer, 2010). According to Davis and Mauer (2010) region I and II are the most useful regions when identifying bacteria while the other regions are useful in identifying structural and compositional changes. Region III (1500-1200 cm^{-3}) represents fatty acids and ester bending vibrations as well as proteins and phosphate-carrying compounds. The three major peaks include the peak at 1455 cm^{-1} which represents the CH_2 bending vibrations of lipids and proteins, 1400 cm^{-1} the $C(CH_3)_2$ bending vibrations of proteins and carbohydrates.

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Table 4.1: Assignment of bands frequently found in FT-IR spectra of microorganisms (4000-400 cm^{-1})

Wavelength (cm^{-1})	**Assignment of bands**
~3500	O-H stretch of hydroxyl groups (Beekes *et al*., 2007)
~3200	N-H stretching of Amide A in proteins (Beekes *et al*., 2007; Davis and Mauer, 2010)
~2959	C-H asymmetric stretching of CH_3 (Beekes *et al*., 2007)
~2930	C-H asymmetric stretching of $>CH_2$ (Beekes *et al*., 2007)
~2921	C-H asymmetric stretching of $>CH_2$ in fatty acids (Beekes *et al*., 2007)
~2898	C-H stretching of →C-H methane (Beekes *et al*., 2007; Davis and Mauer, 2010)
~1740	>C=O stretching of lipid esters (Davis and Mauer, 2010; Lu *et al*., 2011)
~1715	>C=O stretching of esters, nucleic acids and carbonic acids (Davis and Mauer, 2010; Lu *et al*., 2011)
1695-1675	Amide I components of proteins resulting from antiparallel pleated sheets and β-turns of proteins (Beekes *et al*., 2007, Davis and Mauer, 2010)
~1655	Amide I of α-helical structures of proteins (Beekes *et al*., 2007; Lu *et al*., 2011)
~1637	Amide I of β-pleated sheet structures of proteins (Davis and Mauer, 2010; Lu *et al*., 2011)
~1620	Stretch of base carbonyl and ring breathing mode of nucleic acid (Lu *et al*., 2011)
1550-1520	Amide II band of proteins (Davis and Mauer, 2010)
1515	"Tyrosine"band (Beekes *et al*., 2007; Davis and Mauer, 2010)
~1469	CH_2 bending of acyl chains (phospholipids) (Lu *et al*., 2011)
~1458	C-H deformation of $>CH_2$ of proteins (Lu *et al*., 2011)
~1415	C-O-H in-plane bending in carbohydrates, DNA/RNA backbone, proteins (Davis and Mauer, 2010)
~1400	C=O symmetric stretching of COO^- group in amino acids and fatty acids (proteins) (Davis and Mauer, 2010; Lu *et al*., 2011)
1310-1240	Amide III band components of proteins (Beekes *et al*., 2007; Davis and Mauer, 2010)
1250-1220	P=O asymmetric stretching of phosphodiesters in phospholipids (Beekes *et al*., 2007)
1200-900	C-O-C, C-O dominated by ring vibrations in various carbohydrates C-O-P, P-O-P (associated with cell wall glycopeptides and lipopolysaccharides (Beekes *et al*., 2007; Vodnar *et al*., 2010)
~1161	Stretch of C-OH of serine, threonine and tyrosine residues of cellular proteins (Lu *et al*., 2011)
~1150	C-O stretch of carbohydrates (Lu *et al*., 2011)
~1085	P=O symmetric stretching in DNA, RNA and phospholipids (Davis and Mauer, 2010; Lu *et al*., 2011)
~1078	C-OH stretch of oligosaccharide (Lu *et al*., 2011)
~1028	$-CH_2OH$ and C-O stretch coupled with C-O bending of carbohydrates (Lu *et al*., 2011)
900-600	"Fingerprint region" (Beekes *et al*., 2007; Davis and Mauer, 2010)
720	C-H rocking of $>CH_2$ in fatty acids, proteins (Beekes *et al*., 2007; Davis and Mauer, 2010)

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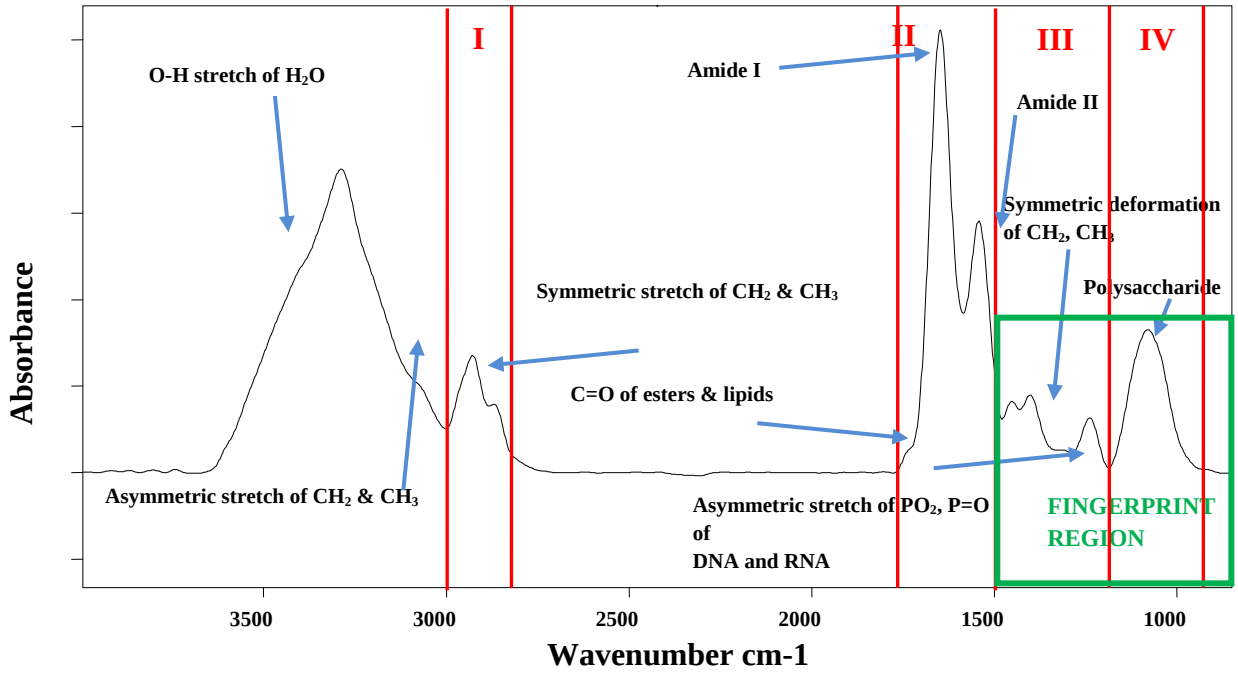

Figure 4.1: Mean FT-IR spectra of untreated treated *B. bifidum* LMG 11041 showing the major bands and regions (I-IV) (4000 to 850 cm^{-1}) of most bacterial specimens.

Absorption at 1240 cm^{-1} is due to the asymmetric stretching mode of phosphate groups in nucleic acids or phospholipids. Lastly, region IV (1200-900 cm^{-3}) contains absorption bands typical of polysaccharides or carbohydrates of microbial cell walls with absorption peak occurring at 1100-950 cm^{-1} (Davis and Mauer, 2010; Zoumpopoulou *et al*., 2010). The band at 1080 cm^{-1} is related to nucleic acids (Lu *et al*., 2011a). All spectra shown are means of the averages of 45 spectra of the 3 independent experiments performed.

Due to the fact that bacterial spectra usually show fairly small compositional differences in cellular constituents, spectra were modified to amplify the spectral changes. Owing to the fact that the spectrometer produces high frequency instrumental noise, smoothing of spectra (25 points) was performed to eliminate noise as well as to enhance the information content of the spectra (Al-Qadiri *et al*., 2008; Davis and Mauer, 2010). Normalization was done to balance differences in path length strength as well as base-line correction (Al-Qadiri *et al*., 2008). The change in spectra of the different bifidobacteria, due to exposure to garlic, is discussed next.

Three strains with differing susceptibility to garlic were chosen, namely, *B. bifidum* LMG 11041 (most sensitive), *B. lactis* Bb12 (most resistant) and *B. longum* LMG 13197 (moderately sensitive).

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4.4.1 FT-IR spectral measurements

4.4.1.1 *B. bifidum* LMG 11041

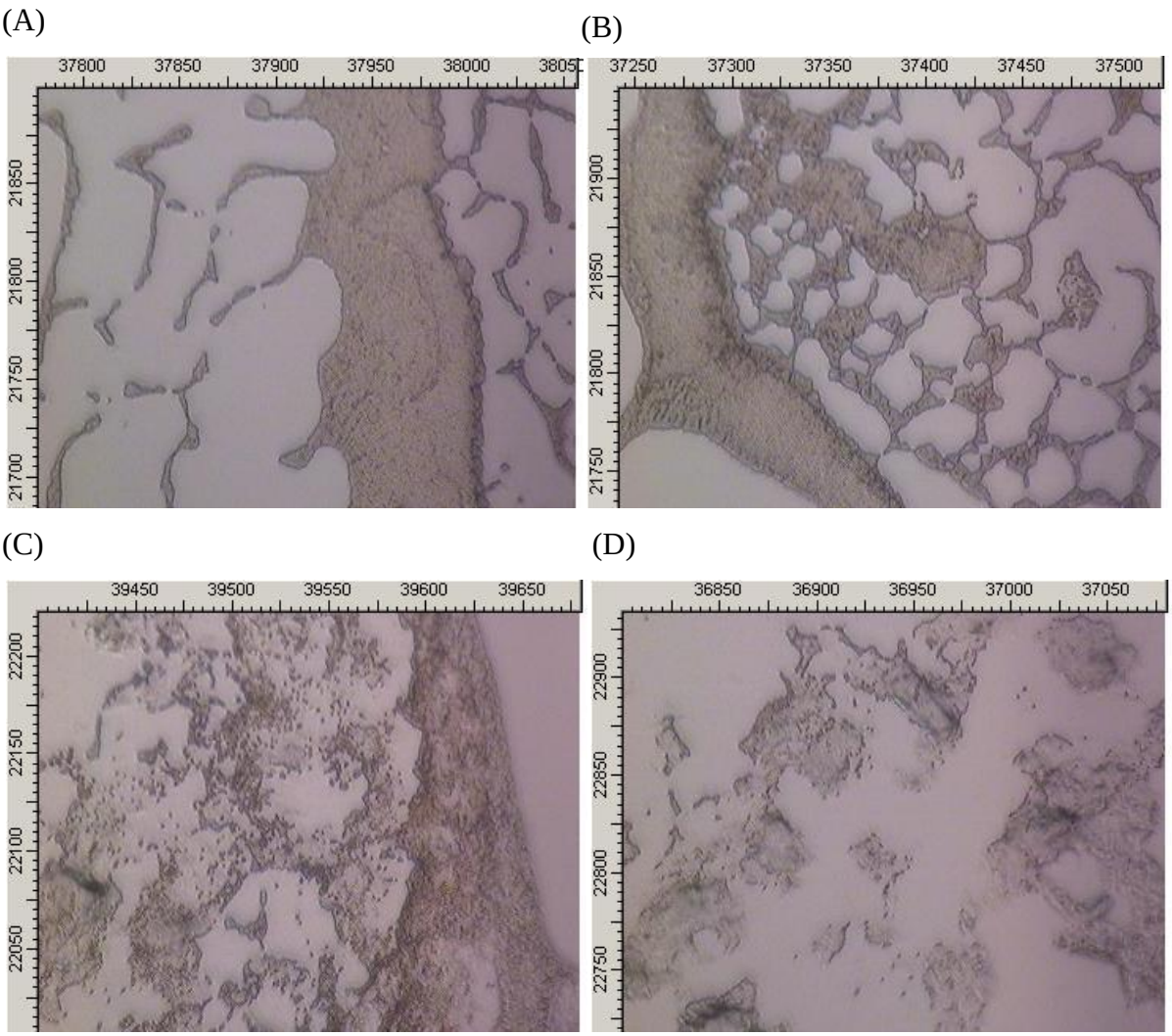

Figure 4.2: Micrographs of untreated (A, B) and garlic-treated (C, D) *B. bifidum* LMG 11041 samples.

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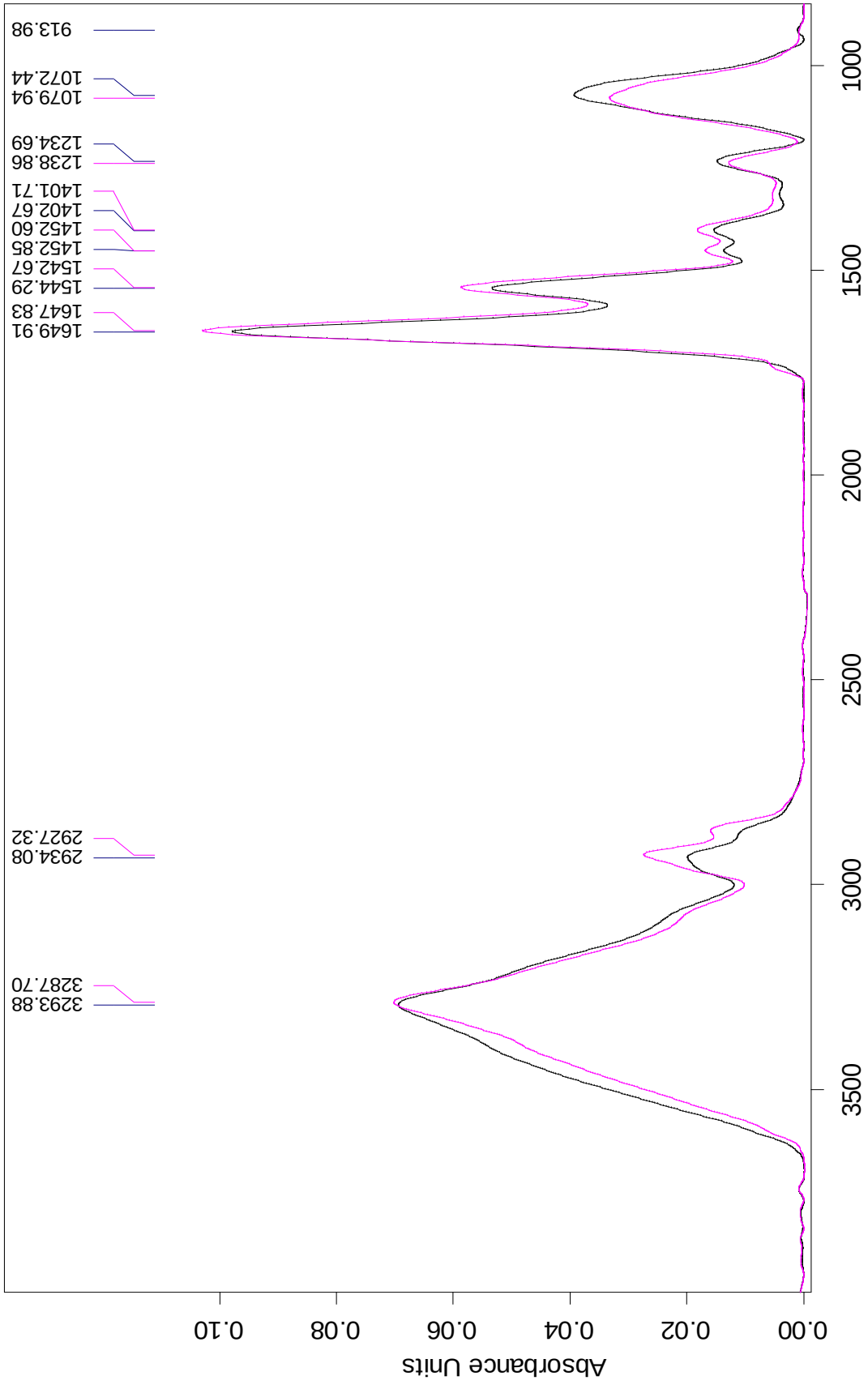

Figure 4.3: Average FT-IR spectra of *B. bifidum* LMG 11041 (Untreated): black; Garlic-treated: pink)

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Results obtained from SEM (Chapter 3, section 3.4.2) clearly showed a change in morphology once *B. bifidum* LMG11041 was exposed to garlic. Micrographs (Fig. 4.2) taken with the Hyperion microscope attachment showed a clear difference between untreated (A, B) and treated (C, D) samples. Garlic-treated cells appeared to have condensed and disintegrated.

Changes in spectral patterns between the control and garlic-treated samples for *B. bifidum* LMG 11041 are illustrated in Figure 4.3. There were shifts at all major peaks (3285, 2930, 1655, 1550, 1452, 1400, 1238, 1078, and 913 cm^{-1}) once this strain was exposed to garlic clove extract for 6 h indicating changes in biochemical composition in the cells.

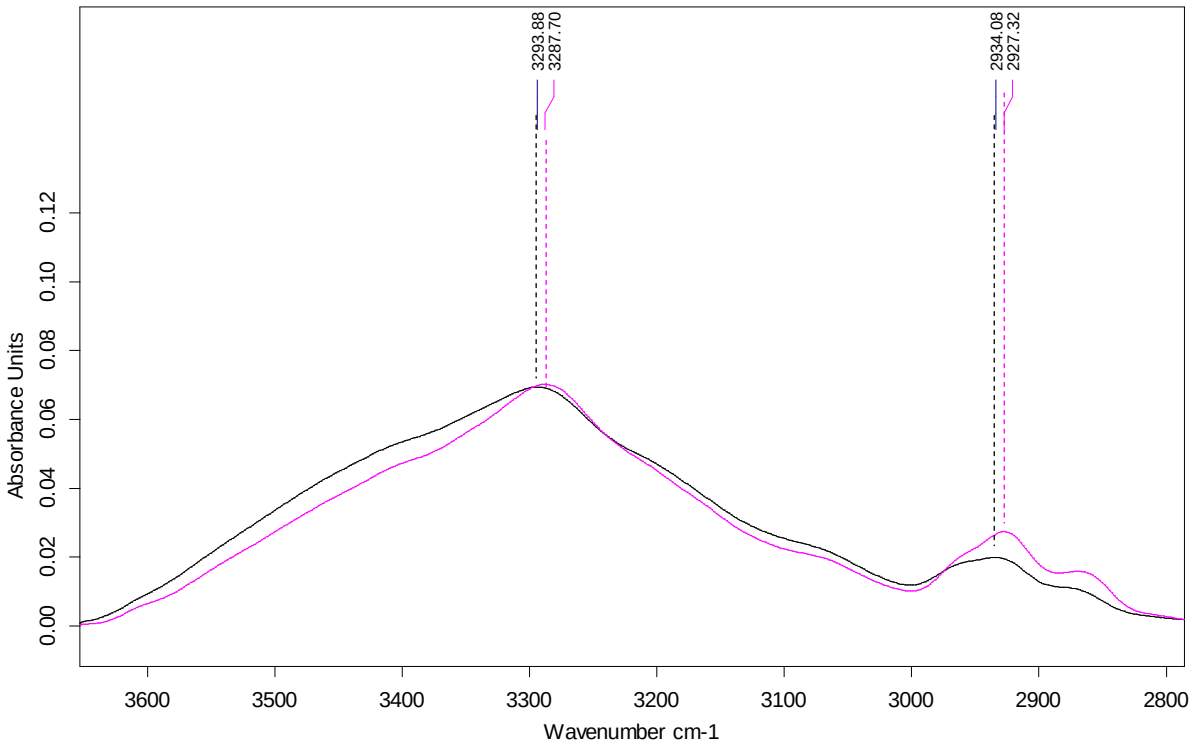

Figure 4.4: Close up of the shift in peaks at 3290 cm^{-1} and 2930 cm^{-1} for *B. bifidum* LMG 11041. Untreated (black), garlic-treated (pink).

There were definite shifts in peaks (Fig. 4.4) which correspond to the N-H stretch of proteins and the O-H stretch of polysaccharides and water (~3290 cm^{-1}) as well as at the peak representing lipids or the asymmetric stretching of $>CH_2$ in fatty acids (~2930 cm^{-1}). Therefore there were changes in these areas once the cells were treated with garlic. In addition there was also an increase in spectral frequency/intensity of the peak at 2934 cm^{-1}.

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An increase in spectral frequency around this peak denotes an increase in membrane fluidity as well as conformation disorder of the acyl chains of cell membrane phospholipids (Fang *et al*., 2007; Alvarez-Ordonez *et al*., 2010) therefore possibly changing cell membrane morphology. It is known that allicin is membrane-permeable and affects lipid and fatty acid biosynthesis in microbes thereby causing changes in the viability of cells (Focke *et al*., 1990; Gruhlke *et al*., 2010).

Slight shifts were also observed at the peaks in the region of Amide I (~1649 cm^{-1}) and Amide II (1544 cm^{-1}) of proteins (Fig. 4.5). In addition, the intensity of spectral features associated with these two peaks increased compared to the control. According to Lu et al (2011a) this may mean that there was an increase in the amount of polysaccharides and oligosaccharides. This could serve as a survival mechanism for bacteria to down regulate most functions in order to save energy and protect itself from a stress, against which in this case could possibly be the garlic.

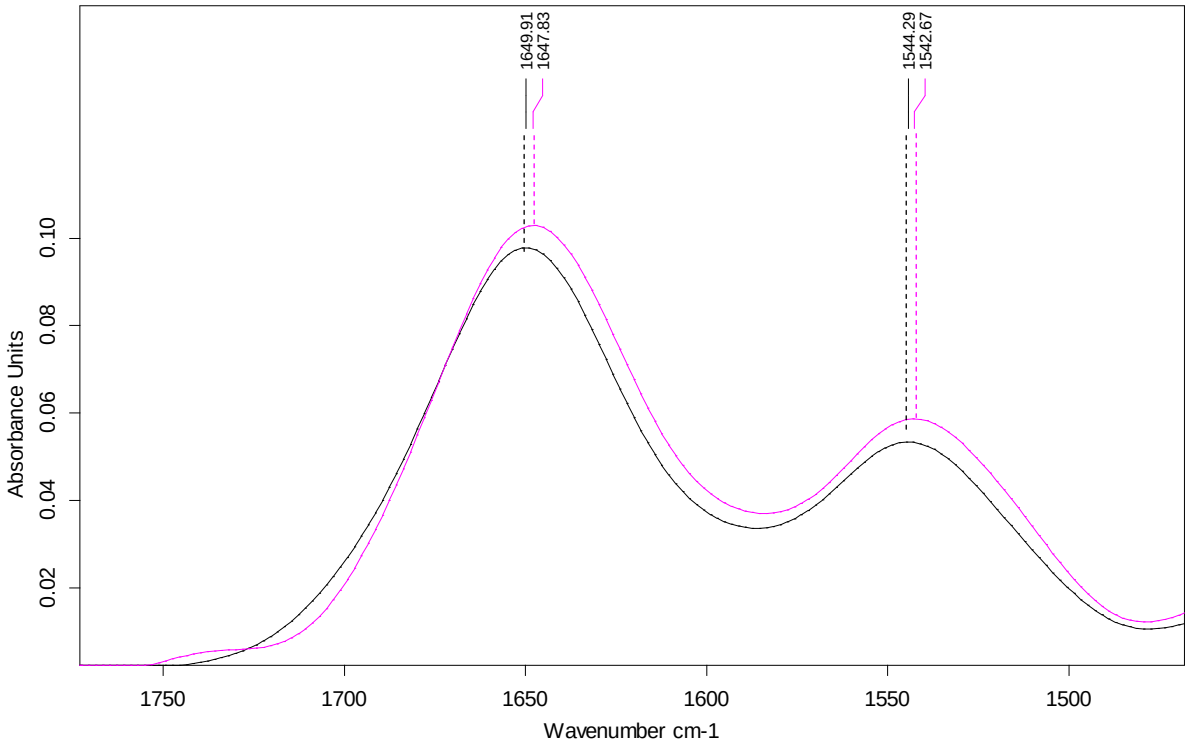

Figure 4.5: FT-IR spectra of *B. bifidum* LMG 11041 denoting shifts in the Amide I and Amide II peaks as well as an increase in band intensities (Black: untreated; Pink: garlic-treated).

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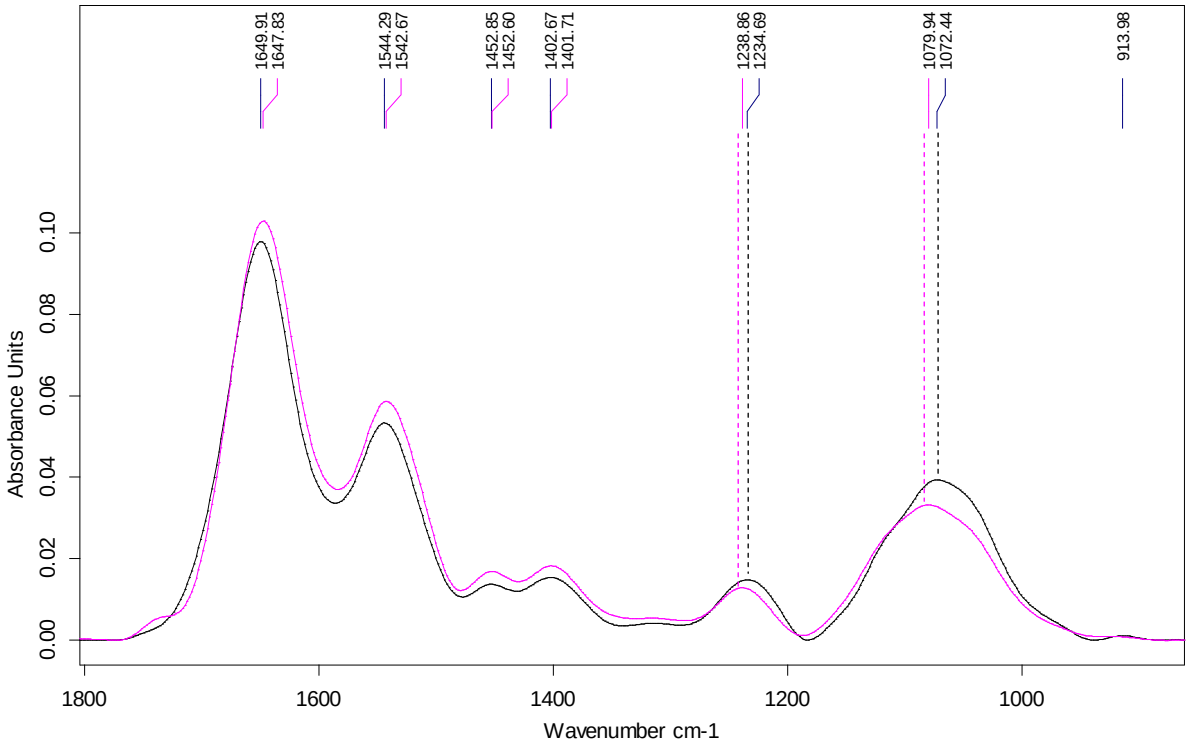

Figure 4.6: Spectra of *B. bifidum* LMG 11041 showing a shift at peaks 1234 and 1072 cm^{-1} as well as drop in intensity between 1280 and 1000 cm^{-1}.

The biggest shift in peaks occurred at 1238 cm^{-1} and 1079 cm^{-1} which represent phosphodiesters, and nucleic acids and carbohydrate regions in the cell wall respectively. There was also a decrease in spectral intensities in these two areas (Fig. 4.6). This was also found in a previous study by Lu and colleagues (2011a) and they attributed this decrease to a reduction in viable counts, prevention of cell growth or cell death.

The difference between treatments at these two peaks may possibly indicate nucleic acid denaturation associated with the P=O antisymmetric stretching mode of the phosphodiester backbone of nucleic acids (1238 cm^{-1}) as well as effects on nucleic acid ribose and deoxyribose structure observed in the difference between treatments at 1080 cm^{-1} (Al-Qadiri *et al*., 2008). It is expected to find changes in the nucleic acid region as previous studies have proved that one of allicin's main mechanisms of action is to completely inhibit RNA synthesis and partially inhibit protein and DNA synthesis (Ross *et al*., 1988).

Lastly, there was a clear peak at 913 cm^{-1} for the control sample (indicated by arrow in box in Fig. 4.3), whereas the peak was lost once *B. bifidum* LMG 11041 was exposed to garlic clove. This can be seen clearly in Figure 4.3 and 4.6. This peak represents phosphodiesters

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(Lu *et al*., 2011b). This may therefore indicate damage to the phospholipids in the cell wall and therefore a change in the structure of the bacterial envelope polysaccharides. The same differences were observed in *Escherichia coli* and *Listeria monocytogenes* once they were exposed to garlic in a study performed by Lu and colleagues (2011b) as well as in *Pseudomonnas auruginosa* once exposed to chlorine (Al-Qadiri *et al*., 2008). There were minor peak shifts at 1452 and 1402 cm^{-1} which correspond to the asymmetrical CH_3 and symmetrical CH_3 bending vibrations.

4.4.1.2 *B. longum* LMG 13197

Micrographs taken of *B. longum* LMG 13197 also showed clear variations between non-treated and garlic-treated samples (Fig. 4.7). Differences in spectral features between the control and the garlic-treated bacterial samples for this strain can be seen in Figure 4.8.

There were a number of shifts in all the main peaks; 3291, 2928, 1649, 1544, 1453, 1403, 1236 and 1073 cm^{-1}. As with *B. bifidum* LMG 11041, a big shift occurred at peak 3291 cm^{-1} (Fig. 4.9) which represents the N-H stretch of proteins and O-H stretch of polysaccharides and water. There was also a slight shift at peak 2928 cm^{-1} associated with lipids in the cell membrane (C-H asymmetric stretch of CH_2 in fatty acids). Another prominent change that could be seen in the region 3600-2800 cm^{-1} was the drop in intensity as well as a significant decrease in the area of bands 3291 cm^{-1} and 2928 cm^{-1} compared to the control. The drop in the area associated with lipids might be related with a drop in viability or cessation of growth. The fall in intensity indicates cell death according to Lu and colleagues (2011a), who also observed similar results for *Escherichia coli* O157:H7 once this organism was placed under cold stress and in low nutrient media. Another possible reason for the drop in intensity of the CH_2 vibration may be as a result of a decrease in membrane fluidity, which results in an increase in saturated fatty acids and a decrease in unsaturated fatty acids (Fang *et al*., 2007).

This change in intensity of the CH_2 stretching may suggest changes in the conformational order of the membrane acyl chains (Fang *et al*., 2007). Many studies on bacteria have shown that variations in the cell membrane lipid composition results from environmental stresses (Scherber *et al*., 2009) and that allicin affects lipid and fatty acid formation in microbes which causes large changes in viability of cells (Focke *et al*., 1990).

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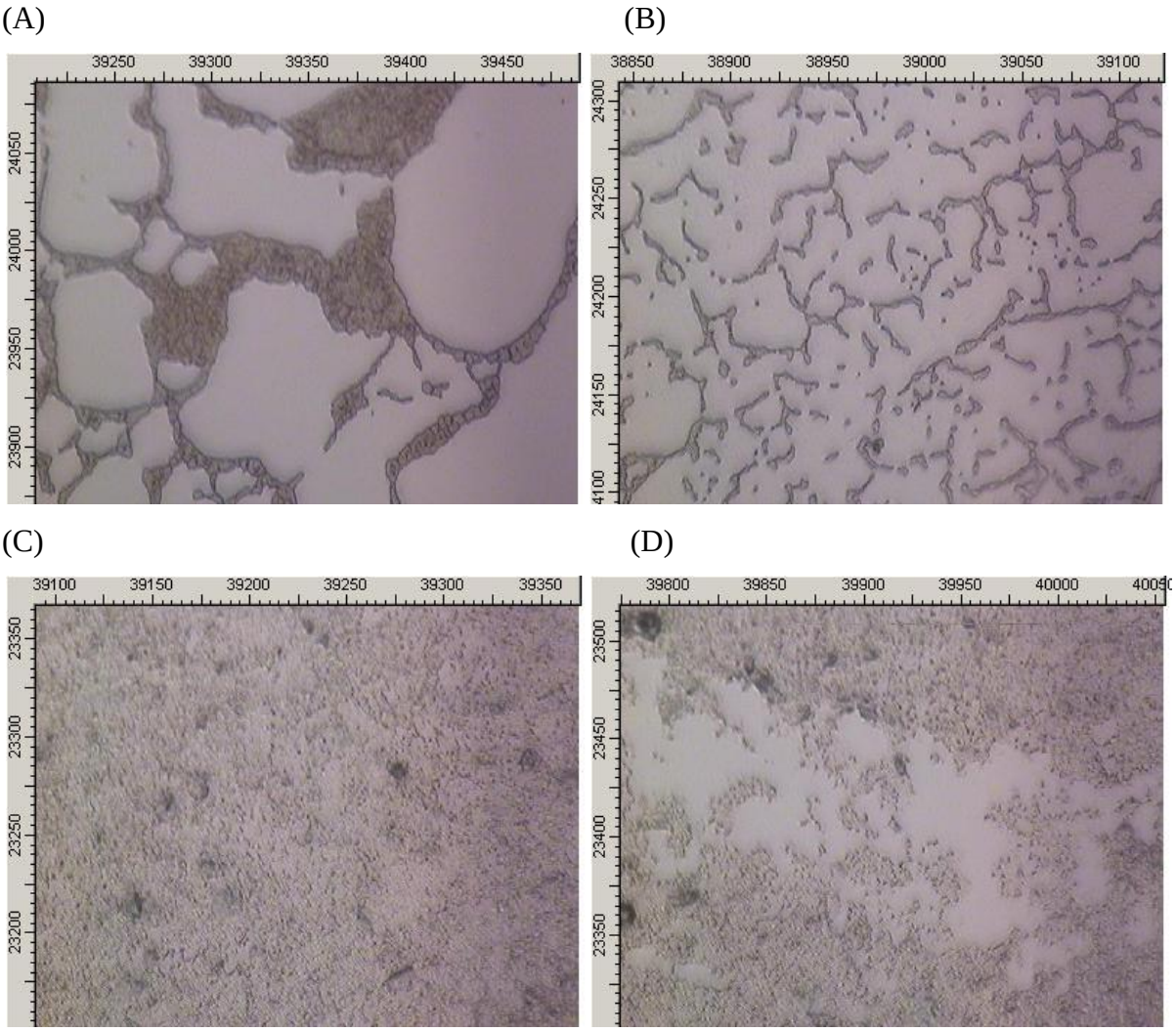

Figure 4.7: Micrographs of untreated (A, B) and garlic-treated (C, D) *B. longum* LMG 13197 samples.

Definite shifts in peaks were observed at bands assigned to Amide I (1655 cm^{-1}) and Amide II (1545 cm^{-1}) as well at the peak representing the C=O symmetric of COO^- of proteins (1399 cm^{-1}), when compared to control samples. Therefore biochemical changes occurred within these molecules after exposure to garlic. According to Lu and colleagues (2011c) it is the phenolic and organosulfur compounds in garlic that contribute to protein damage as observed at 1399 cm^{-1}. As with the *B. bifidum* strain, when comparing the control and treated strain, there was an increase in intensities which once again may mean that there was an increase in the amount of polysaccharides produced. This is probably the way the bacteria acts in response to stress and uses the increase in polysaccharides as a survival mechanism in order to save energy and protect itself (Lu *et al*., 2011a). EPS and biofilm formation was also observed using SEM.

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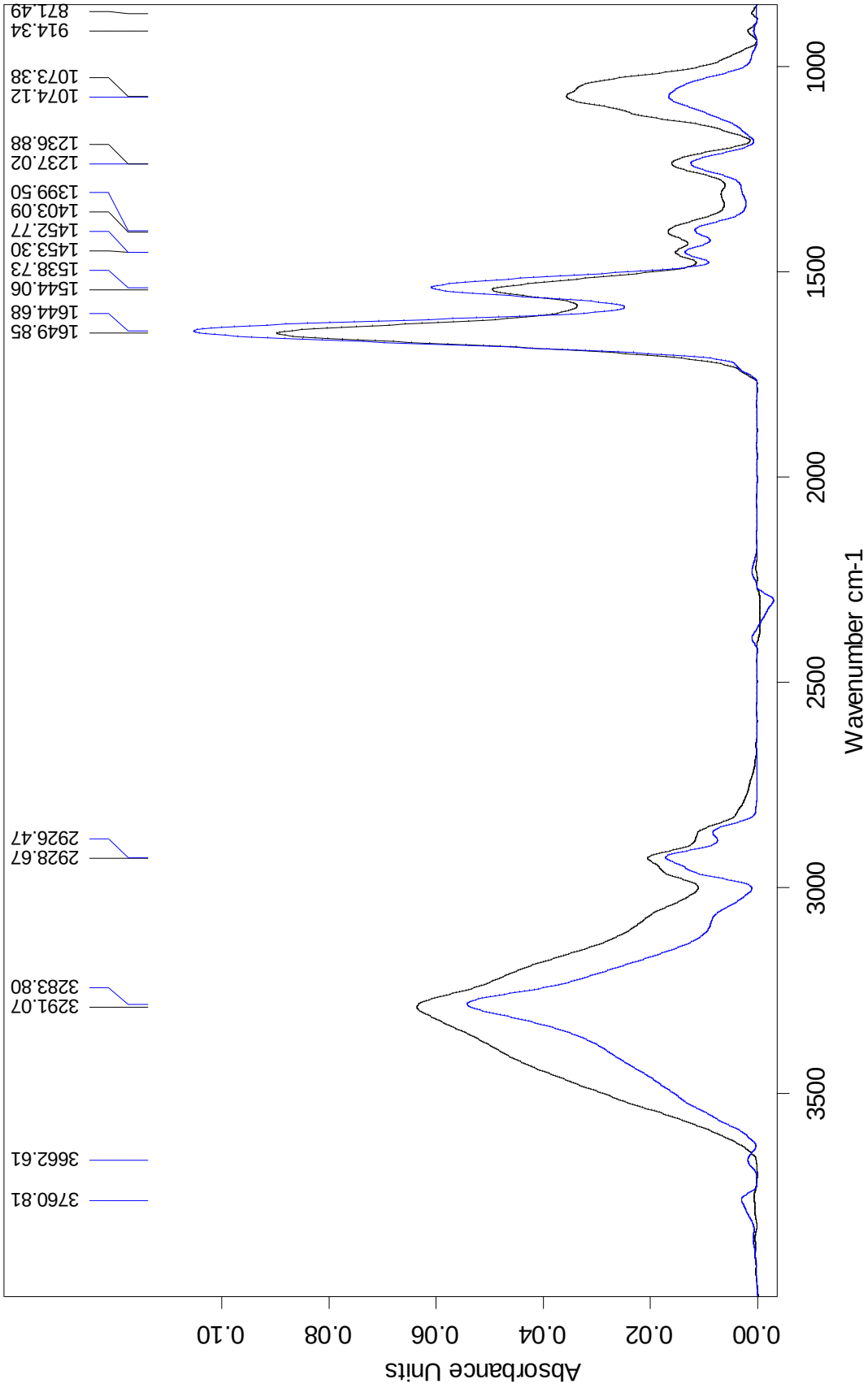

Figure 4.8: FT-IR spectra of untreated and garlic-treated *B. longum* LMG 13197 samples (Non-treated: black; garlic-treated; blue).

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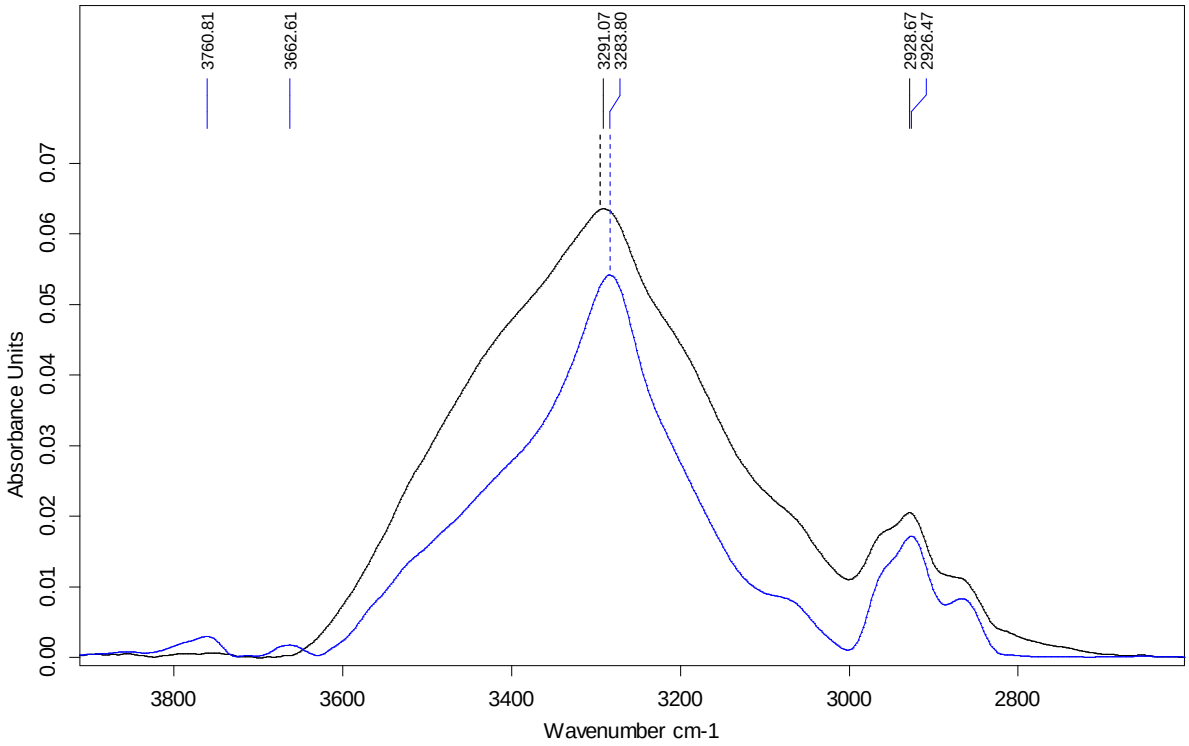

Figure 4.9: FT-IR spectra of *B. longum* LMG 13197 untreated (black) and garlic-treated (blue) samples showing peak shifts, drop in intensity as well as decrease in area in the lipid region.

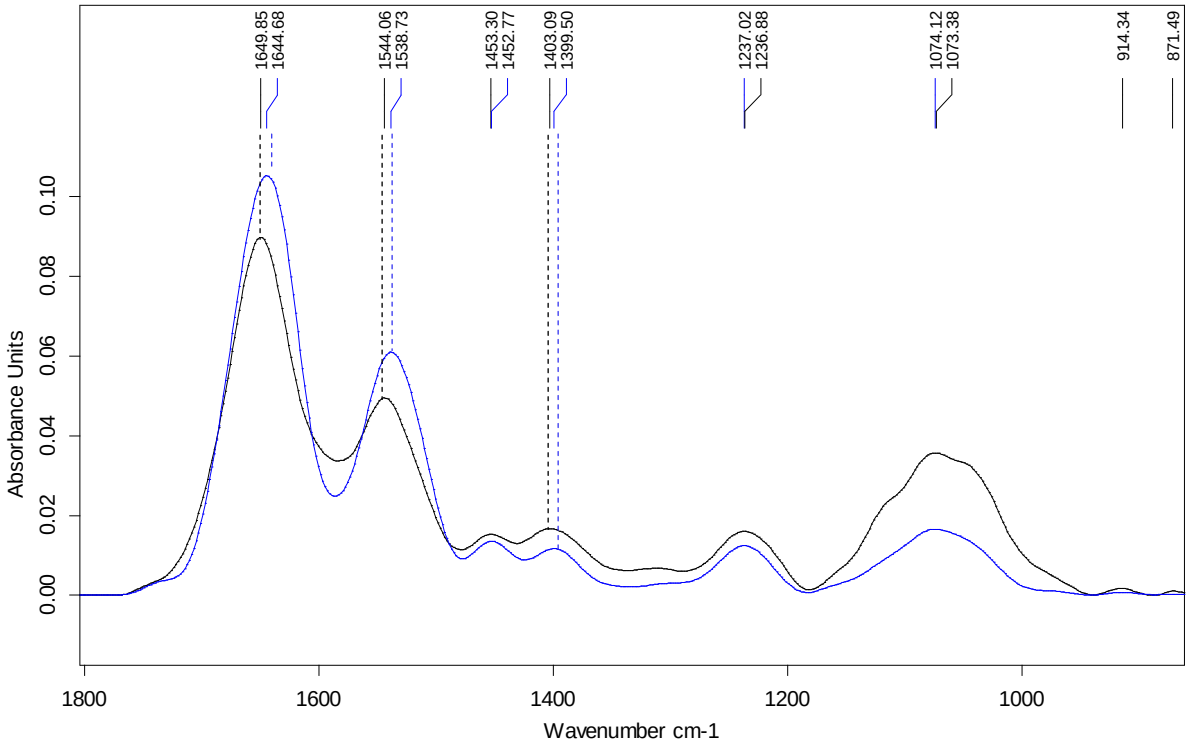

Figure 4.10: Shift in peaks at Amide I (1650 cm^{-1}), Amide II (1545 cm^{-1}) and 1395 cm^{-1}.

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When compared with the spectra of *B. bifidum* LMG 11041, *B. longum* LMG 13197 showed a decrease in intensity in the region of 1490 cm^{-1} – 1260 cm^{-1} whereas the *B. bifidum* strain showed a slight increase. Drops in spectral intensities usually indicate cell death, a decrease in viable cells or cessation of growth, vice versa (Lu *et al*., 2011a). Compared to the *B. bifidum* strain, there were slight shift in peaks at 1236 cm^{-1} and 1074 cm^{-1} representing nucleic acid denaturation of the phosphodiester backbone as well as damage to DNA and RNA, although not as significant as the *B. bifidum* strain. In addition there was a significant drop in intensity and band area at peak 1074 cm^{-1} (nucleic acids (DNA and RNA)), which according to Lu and colleagues (2011a) indicates microbial death.

Disappearance of peaks 914 cm^{-1} and 871 cm^{-1} may possibly mean there were changes or damages to glycopeptides and lipopolysaccharides associated with the cell wall, therefore changing the structure of the bacterial envelope polysaccharides (914 cm^{-1}) as well as possible changes in aromatic ring vibrations of various nucleotides (871cm^{-1}) (Naumann, 2000; Vodnar *et al*., 2010). Disappearance of peak 914 cm^{-1} was also seen with the *B. bifidum* strain.

4.4.1.3 *B. lactis* Bb12

Micrographs taken of *B. lactis* Bb12 showed slight differences between garlic-treated and untreated samples.

FT-IR spectra of untreated compared to garlic-treated *B. lactis* Bb12 samples showed slight differences in spectral features (Fig. 4.12). There were slight shifts in spectral peaks at 2930 cm^{-1} (lipid), 1648 cm^{-1} (Amide I), 1542 cm^{-1} (Amide II), 1453 cm^{-1} (C-H deformation of CH_2 proteins) and 1235 cm^{-1} (P=O asymmetric stretch of PO_2 phosphodiesters). A notable peak shift occurred at 3292 cm^{-1} (N-H stretch of proteins and O-H stretch of polysaccharides and water) as well as 1395 cm^{-1} (C=O symmetric stretch of COO^- of proteins).

As with *B. bifidum* LMG 11041, there was a slight increase in spectral intensity round the lipid regions (2929 cm^{-1}) for this strain when comparing treated and untreated spectra. Once again this could mean an increase in membrane fluidity as well as conformation disorder of the acyl chains of membrane phospholipids (Fang *et al*., 2007; Alvarez-Ordonez *et al*., 2010).

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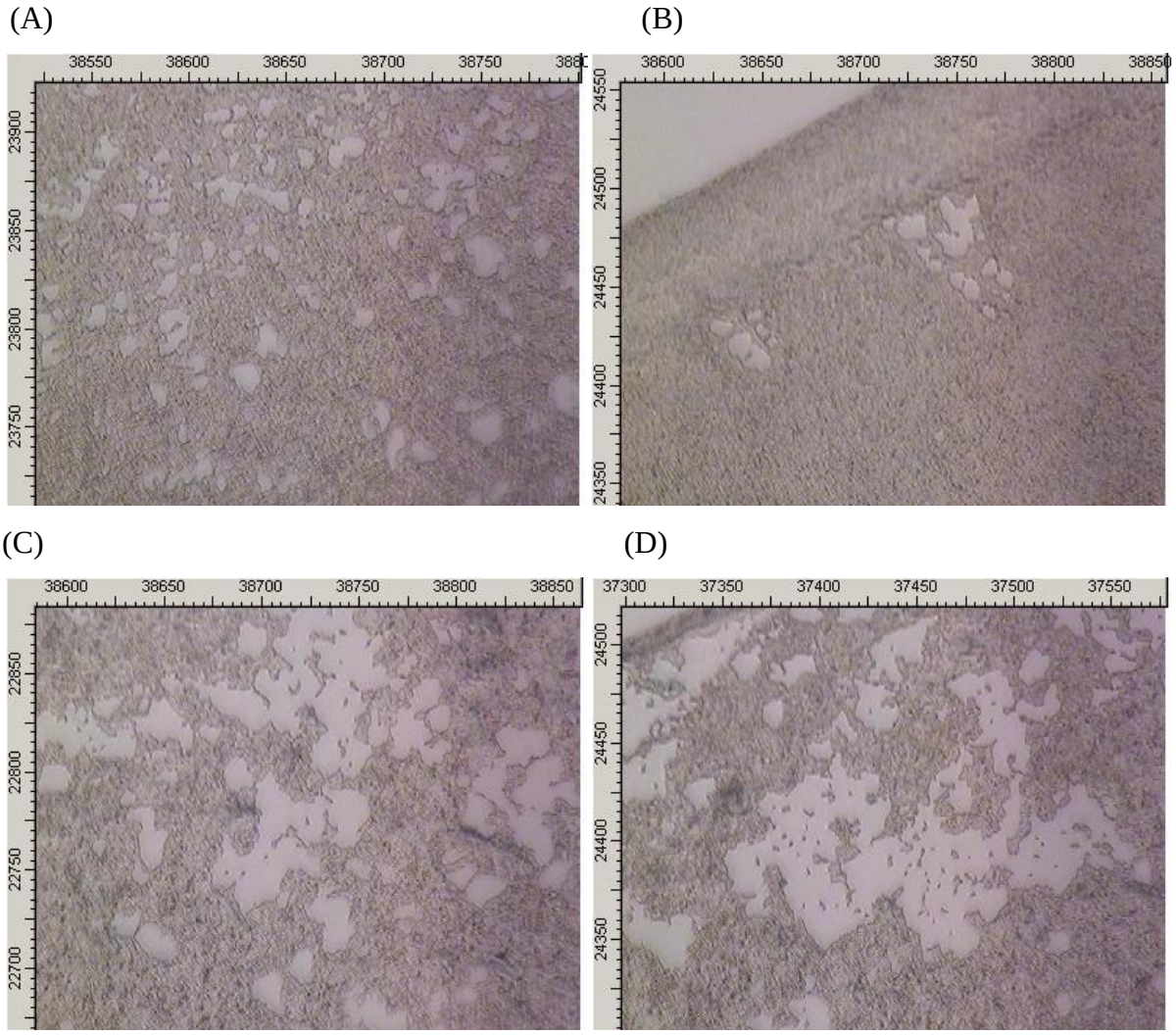

Figure 4.11: Micrographs of untreated (A, B) and garlic-treated (C, D) *B. lactis* Bb12 samples.

There were no significant changes in spectral intensities at peaks depicting Amide I and II. There was however a slight decrease in spectral intensity at peak 1235 cm^{-1} (phosphodiesters) compared to control samples which may possibly indicate a decrease in viable cell counts or cell death (Lu *et al*., 2011a) as was seen with the previous strains. According to Al- Qadiri and colleagues (2008) a change at this peak indicates nucleic acid denaturation associated with the P=O anti-symmetric stretching mode of the phosphodiester backbone of nucleic acids.

In addition, there was also quite a big loss in spectral intensity at peak 1073 cm^{-1} which corresponds to nucleic acids. Therefore garlic clove extract could possibly be altering or affecting RNA and DNA which may indivertibly lead to microbial death or cessation of growth as was seen with previous strains.

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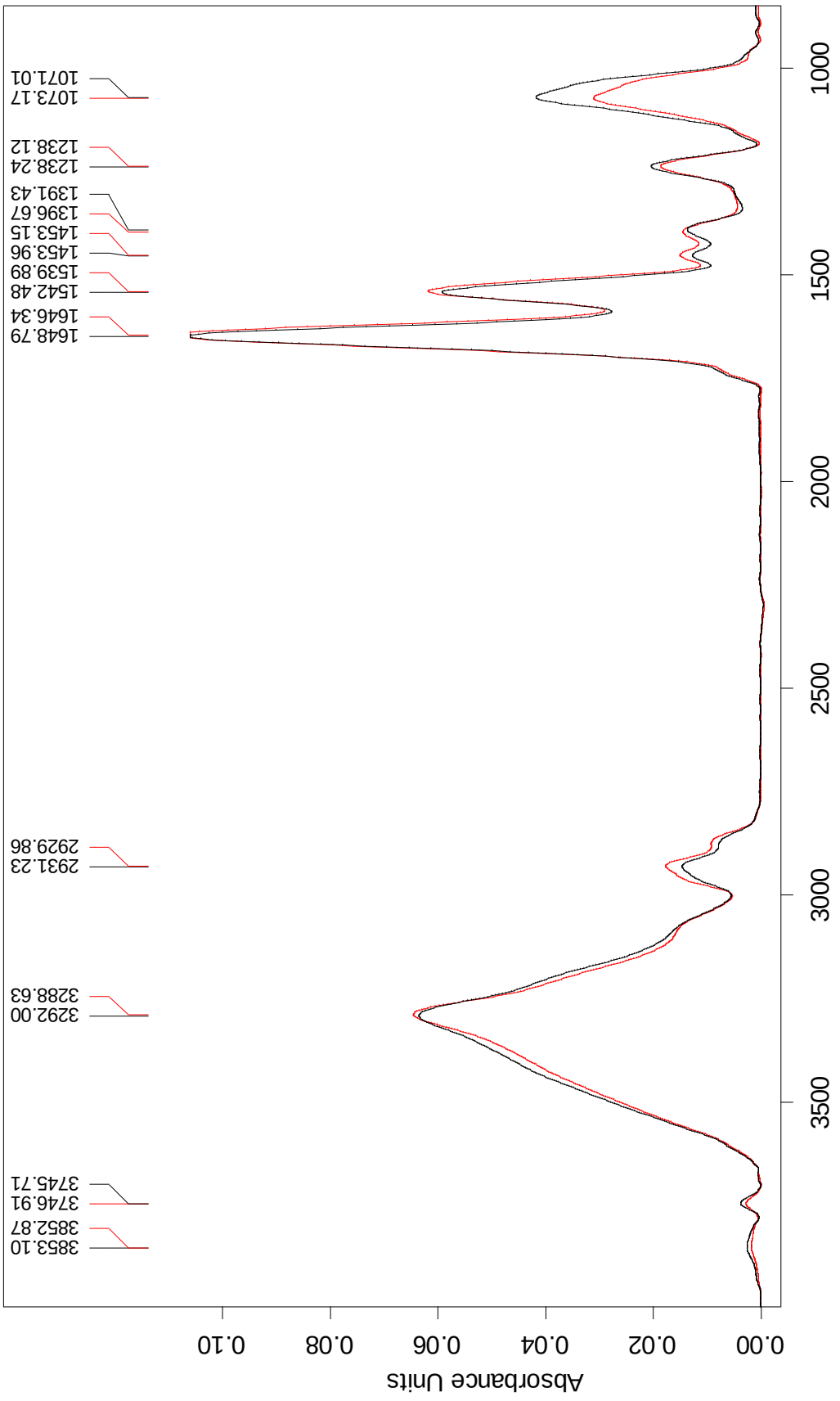

Figure 4. 12: FT-IR spectra of garlic-treated (red) and untreated (Black) samples of *B. lactis* Bb12

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Significant spectral features related to bacterial cell injury included changes in nucleic acid and lipid features of the cell membrane for the three strains tested. Differences were also observed between control and garlic-treated samples in spectral regions associated with proteins for *B. bifidum* LMG 11041 and *B. longum* LMG 13197 only. *B. longum* LMG 13197 also showed changes or damage to the cell wall structure. According to Lu and colleagues (2011b), proteins and phospholipids in bacterial cell membranes are the main targets affected by plant antioxidants. Once these antimicrobials alter the cell membrane cell leakage and lysis occur resulting in cessation of growth or microbial death and this was seen in the spectral features for *B. bifidum* LMG 11041 and *B. longum* LMG 13197. It is known that allicin acts by immediately and totally inhibiting RNA synthesis as well as partial DNA and protein synthesis (Al-Waili *et al*., 2007; Deresse, 2010). Allicin is also known to affect lipid biosynthesis in microbes which causes changes in cell viability (Focke *et al*., 1990). Nucleic acid changes were seen in the FT-IR spectra in all three strains treated with garlic.

4.4.2 PCA data

PCA has been extensively used in interpreting infrared spectra in microbiology, medicine agricultural and food sciences. It reduces a multidimensional data set to its most dominant features, removes random variation, and maintains the relevant variation between the data points (Lin *et al*., 2004; Davis and Mauer, 2010). PCA shows whether there are natural clusters in the data and describes similarities or differences from multivariate data sets (Lin *et al*., 2004). In this study PCA confirmed a clear segregation between control and garlic-treated samples. Clear segregations with distinct sample clusters were observed between control and garlic-treated samples. PCA was performed for all four regions as well as for the separate regions including region I the fatty acids (lipids) of the cell membrane (3000-2800 cm^{-1}), region II representing structural proteins (1800-1500 cm^{-1}), region III associated with proteins, fatty acids and phosphate carrying compounds (1500-1200 cm^{-1}) and region IV typical for polysaccharides in the cell wall (1200-900 cm^{-1}). Groupings of the spectra represent differences or similarities among the regions and can be compared with variations in the molecular composition of the bifidobacterial strains.

Figure 4.13 shows the PCA of the first derivative and multiplicative scatter correction (MSC) of *B. bifidum* LMG 11041 for all the different regions. When comparing all the regions together between the control and garlic-treated samples segregation between the two sample

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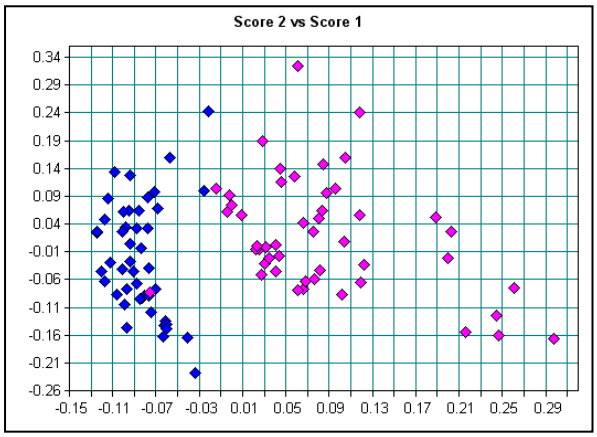
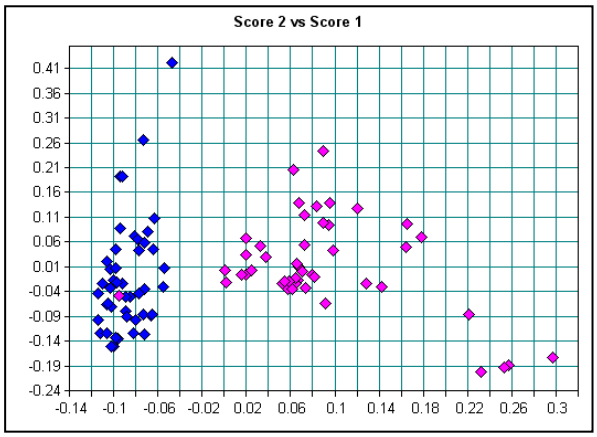
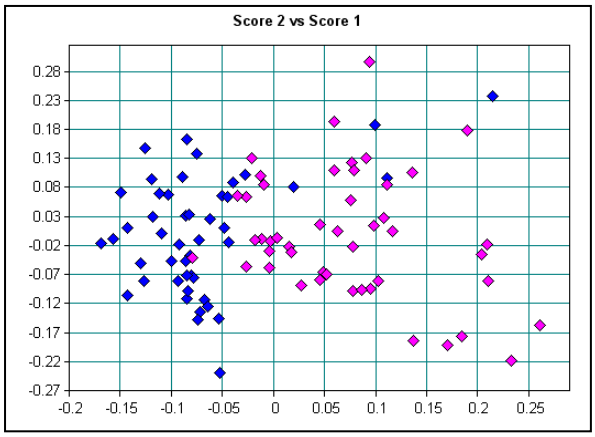
(A)



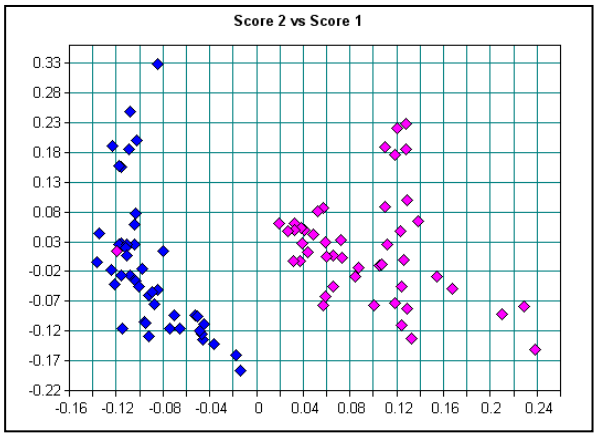
(B)



(C)



(D)



(E)

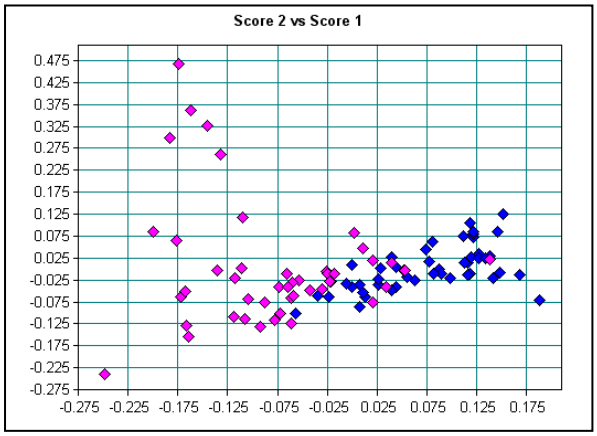


Figure 4.13: PCA of the first derivative and multiplicative scatter correction (MSC) of *B. bifidum* LMG 11041 for all spectral regions (4000-850 cm^{-1}) (A), region I (3000-2400 cm^{-1}) (B), region II (1800-1500 cm^{-1}) (C), region III (1500-1200 cm^{-1}) (D) and region IV (1200-900 cm^{-1}(E). Blue=untreated samples; pink=garlic treated samples.

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clusters for most regions can be observed (Fig. 4.13A). On closer inspection when comparing the separate regions, one can observe that there was a distinct separation between clusters in region I (lipids/fatty acids) and III (mixed region) (Fig. 4.13B and D). This means that there were more significant changes between control and treated samples in these two regions than all the other regions for *B. bifidum* LMG 11041. In spectral region III, control and garlic-treated samples were distinguished even better than in region I (Fig. 4.13D). There was no distinct clustering achieved related to spectral region II and IV. Therefore, these findings show that the most significant changes induced by the garlic clove extract occurred in the region representing cell structure proteins and phosphodiesters associated with phospholipid bilayers, while other cellular constituents were less affected. This could be a reason why *B. bifidum* LMG 11041 showed unusual morphological changes as indicated by SEM results (Chapter 3, section 3.4.2).

When comparing PCA of *B. longum* LMG 13197 to *B. bifidum* LMG 11041 it can be observed that there is a significant separation between the control and garlic-treated samples over the all regions (Fig. 4.14). There were major spectral differences in region I, II and III (Fig. 4.14B-D). Nucleic acids and polysaccharides of the cell wall (Region IV) were less affected than the other cellular components as was seen with less distinct clustering in Figure 4.14 (E). Therefore garlic clove extract caused more biochemical changes in this strain compared to *B. bifidum* LMG 13197.

Clear separations with distinct sample clusters were seen among non-treated and garlic-treated *B. lactis* Bb12 in the entire spectral region (Fig. 4.15). There were fewer differences between control and treated samples in region III and IV as these clusters were closer together (Fig. 4.15D-E). Most differences were seen within the lipid and protein regions of the cell for *B. lactis* Bb12 as can be seen with the more isolated clusters (Fig. 4.15B-C). Damage was confined to the cell wall for this strain whereas for the more sensitive strains (*B. bifidum* LMG 11041 and *B. longum* LMG 13197), damage extended to the nucleic acid region.

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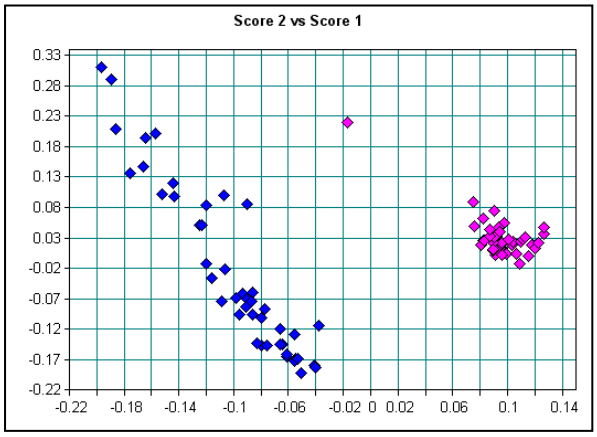
(A)



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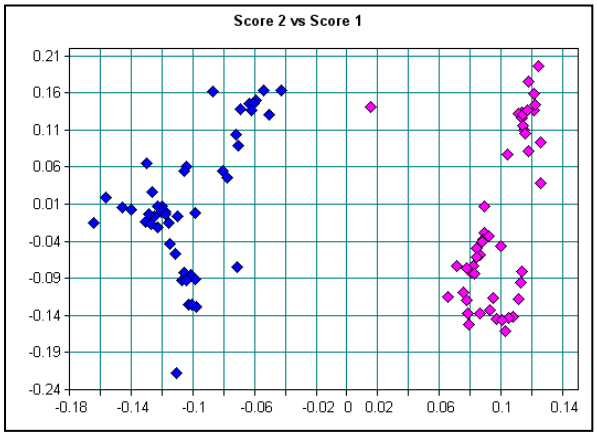
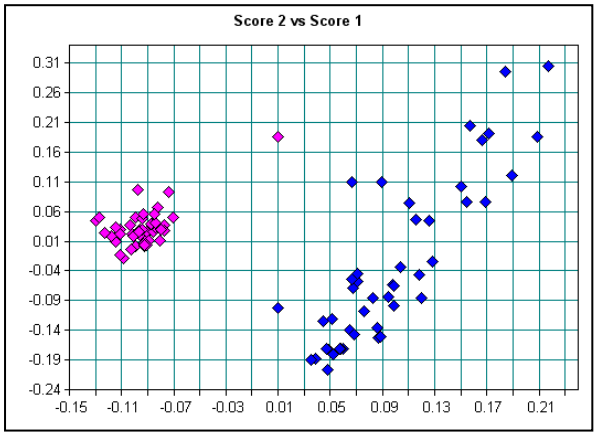


(C)



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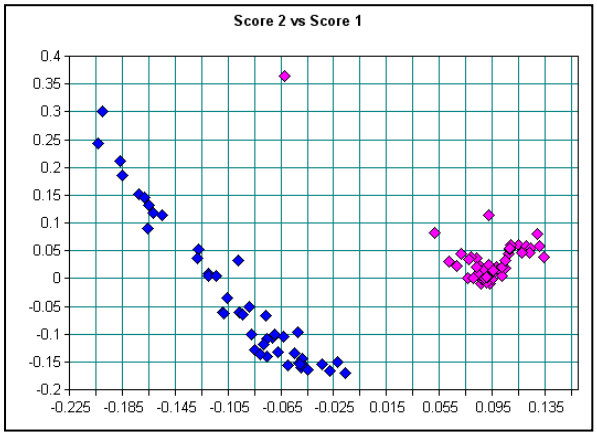

(E)

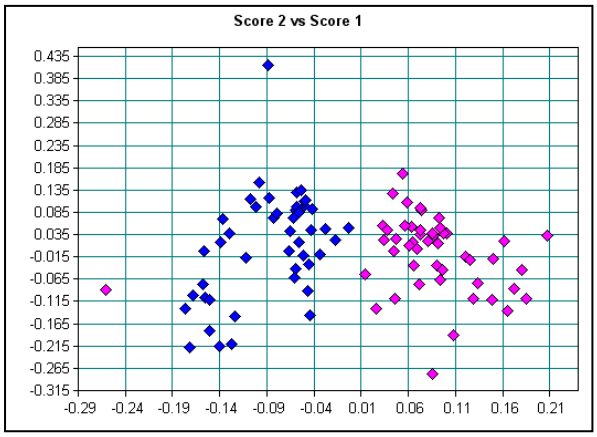

Figure 4.14: PCA of the first derivative and multiplicative scatter correction (MSC) of *B. longum* LMG 13197 for all spectral regions (4000-850 cm^{-1}) (A), region I (3000-2400 cm^{-1}) (B), region II (1800-1500 cm^{-1}) (C), region III (1500-1200 cm^{-1}) (D) and region IV (1200-900 cm^{-1}(E). Blue=untreated samples; pink=garlic treated samples.

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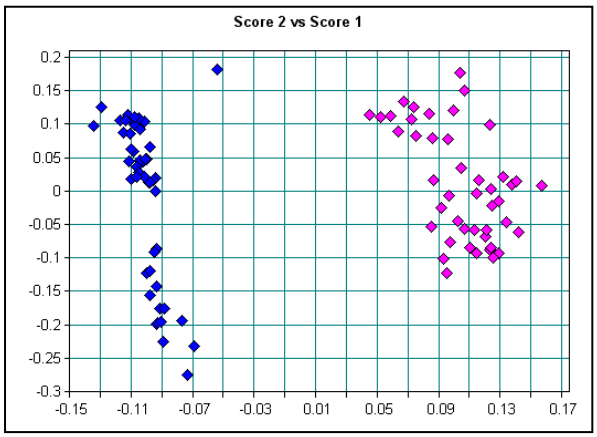
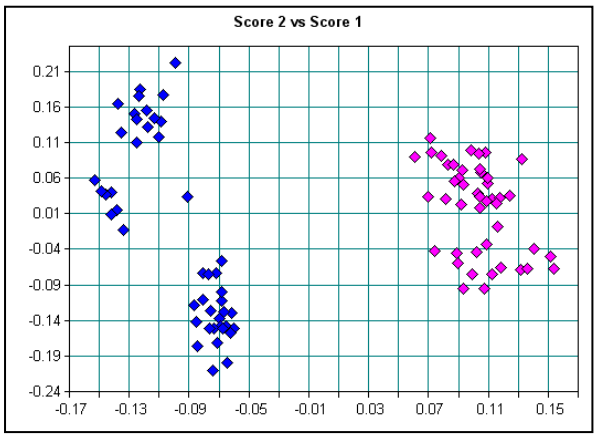
(A)



(B)



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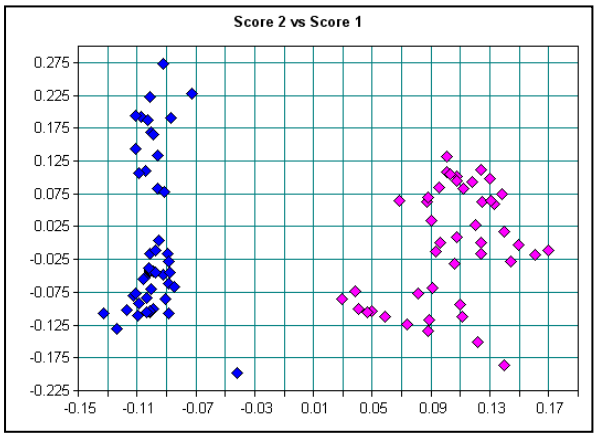

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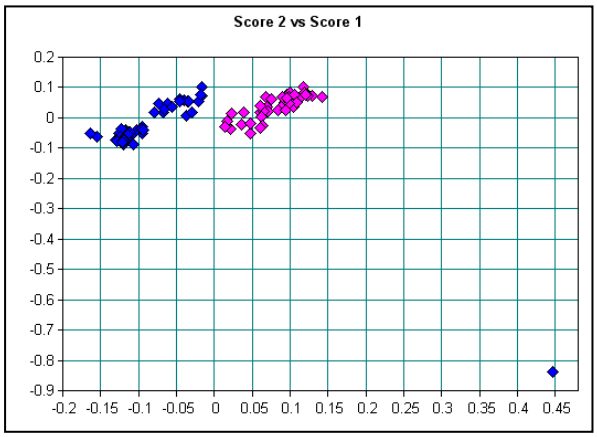

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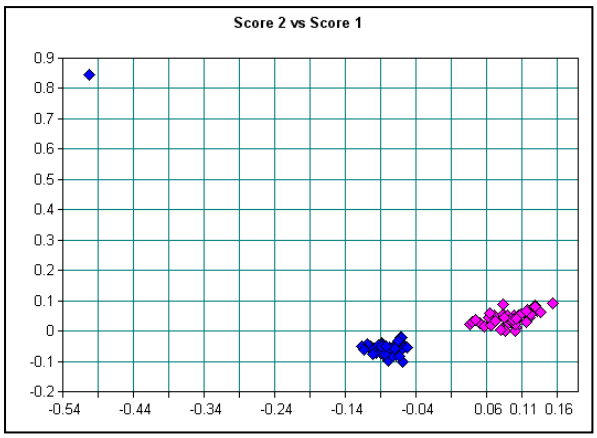

Figure 4.15: PCA of the first derivative and multiplicative scatter correction (MSC) of *B. lactis* Bb12 for all spectral regions (4000-850 cm^{-1}) (A), region I (3000-2400 cm^{-1}) (B), region II (1800-1500 cm^{-1}) (C), region III (1500-1200 cm^{-1}) (D) and region IV (1200-900 cm^{-1}(E). Blue=untreated samples; pink=garlic treated samples.

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Taken as a whole, PCA revealed distinctive features of the FT-IR spectra among *Bifidobacterium* cells. It showed that all bifidobacterial strains tested showed definite differences in most if not all of their regions and for some strains fewer differences once treated with garlic clove extract. When the strains were exposed to garlic clove extract, significant changes were seen in lipids or fatty acids in the cell membrane as well as cell structure proteins and phosphodiesters associated with phospholipid bilayer in all three strains. For *B. longum* LMG 13197 and *B. lactis* Bb12 noteworthy differences were observed in the amide groups of proteins as well as in the nucleic acids and polysaccharides of the cell wall.

It was observed that *B. bifidum* LMG 11041 showed more variation between control and treated samples therefore representing less distinct changes in spectral features. A reason for this could be the difficulty of obtaining a condensed sample during sample preparation as can be seen in the micrographs in Figure 4.2. Therefore this may have influenced results making it difficult to obtain reproducibility of control and treated samples of this strain therefore showing more variation in the clusters in PCA.

4.5 Conclusions

This study showed that FT-IR could differentiate between non-treated and garlic-treated *Bifidobacterium* species, confirming that garlic clove extract does indeed have a damaging effect on the tested *Bifidobacterium* spp.

Upon studying the spectral data, the bactericidal effect of garlic on *Bifidobacterium* cells causes apparent variations in the spectral properties associated with secondary structure proteins, fatty acids and phospholipids in the cell membrane as well as nucleic acids (damage to the DNA and RNA). *B. bifidum* LMG 11041 and *B. longum* LMG 13197 also showed variations in spectral features associated with carbohydrates or polysaccharides of the cell wall, whereas *B. lactis* Bb12 showed no apparent difference in spectral features for this region. *B. lactis* Bb12 appeared to have been least affected by the garlic clove extract than the other two strains as was seen with the smaller amount of biochemical changes occurring within the cell. This study therefore supports previous studies that protein and membrane damage are the main factors in inactivation of gram-positive bacteria by garlic.

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PCA clearly showed detection and separation of both garlic-treated (injured) and control cells and showed a somewhat slightly different but more reliable picture of the variations in the spectra. It was much more representative and removes any random variations encountered. It showed changes in all spectral features for *B. lactis* Bb12 including changes in region III which was not observed by just interpreting the spectra.

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Results from this study prove that garlic clove extract does indeed induce biochemical changes within *Bifidobacterium* cells and therefore also confirms that garlic does damage and inhibit these cells. The main targets for garlic clove extract for all the strains appear to be the nucleic acids (DNA and RNA) and the fatty acids (lipids) in the cell membrane. Changes to the surface properties may therefore prevent these probiotic *Bifidobacterium* strains from colonizing the intestinal mucosa and thereby decrease their positive effect as well as their viability.

Further studies could be done using Raman spectroscopy to study spectral changes in the sulfur region (500-700 cm^{-1}) which indicates protein structural changes, as previous studies (Lu *et al*., 2011b) have suggested that garlic reacts with thiol-containing enzymes, causing cell injury or death.

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CHAPTER 5

Flow cytometric viability assessment of selected *Bifidobacterium* spp. exposed to garlic (*Allium sativum*) clove extract

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5.1 Abstract

The objective of this work was to use flow cytometry as a tool to assess viability of *Bifidobacterium bifidum* LMG 11041, *B. longum* LMG 13197, *B. lactis* Bb12 and *Lactobacillus acidophilus* La14 150B after exposure to garlic clove (GC) extract. Furthermore, we aimed to determine the extent of damage induced by garlic on the cells. The LIVE/DEAD *Bac*Light viability kit was used to stain the cells. Results revealed a decrease in percentage of viable cells with an associated increase in damaged cells and, possibly lysed cells, after treatment with GC extract for all three *Bifidobacterium* spp. tested. Subsequent inoculation of the stained treated cells into fresh growth medium in the absence of GC extract resulted in an increase of the percentage of live cells. This suggested that injured cells were able to recover and revert to the physiologically active state, as well as assume their original shapes and sizes. Thus, cells exposed to sub-lethal concentrations of garlic can repair their damage and start multiplying again. This work showed that flow cytometry in combination with fluorescent techniques is useful to improve understanding of the effects of GC extract on *Bifidobacterium* species.

Keywords: Probiotics, *Bifidobacterium* spp., garlic, viability, flow cytometry, membrane integrity.

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5.2 Introduction

Allicin, the main active compound of garlic responsible for its antimicrobial activity, can readily pass through phospholipid membranes and be taken up by the cells. This causes damage to their membranes, leading to cessation of growth, autolysis and eventually cell death (Bakri and Douglas, 2005; Lu *et al*., 2011). It is known to affect microorganisms by a number of different mechanisms of action. It mainly acts by immediately and totally inhibiting RNA synthesis and partially inhibiting DNA and protein synthesis (Focke *et al*., 1990; Al-Waili*et al*., 2007; Deresse, 2010).

We have shown that garlic has antibacterial activity and decreases viability of selected *Bifidobacterium* spp. using the disk diffusion assay and conventional plate count methods. Traditional plate count methods only take into account cells that can replicate under conditions provided for growth and therefore some cells which are viable but nonculturable are not counted and are considered as dead, dormant, damaged, injured or inactive cells (Rault *et al*., 2007). These traditional culture methods therefore examine bacterial death only in retrospect and intermediate states such as cell injury and stress are very difficult to distinguish (Hewitt *et al*., 1999; Berney *et al*., 2007).

It has been reported in the past that cells in injured populations can possibly enter a brief noncultivable state, presumably due to sub-lethal injury mechanisms, which include damage of the cell membrane or reversible and temporary membrane permeabilization (Rault *et al*., 2007). This is known as the "viable but not culturable" (VBNC) state. These nonculturable bacteria may remain in this state for more than a year (Paparella *et al*., 2008). This has been observed for starved *Micrococcus luteus* or bile salt-stressed *Bifidobacterium* cells (Ben Amor *et al*., 2002; Rault *et al*., 2007).

Therefore more rapid and sensitive techniques have been developed for quicker assessment of the viability of samples. Such techniques use a variety of stain-based methods such as fluorescent probes or dyes (Rault *et al*., 2007). Flow cytometry is one such technique. Flow cytometric analysis allows rapid enumeration of individual cells and can simultaneously measure the morphological and functional cellular functions of cells. Analysis of different physiological parameters such as membrane integrity, cellular viability, membrane potential,

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intracellular pH and intracellular enzyme activity among others, has been performed using this technique (Bunthof *et al*., 2001a; Ananta *et al*., 2004; Rault *et al*., 2007).

The use of flow cytometric methods in bacterial assessment of viability in probiotic products and dairy starter cultures has become a very popular and successful method (Auty *et al*., 2001; Bunthof and Abee, 2002). To name but a few, it has been used to assess the viability of *Bifidobacterium* exposed to bile salt as well to study the effects of different stress conditions on the viability of lactic acid bacteria (Ben Amor *et al*., 2002; Rault *et al*., 2007; Paparella *et al*., 2008). Bunthof and colleagues (2001a) used it to assess the survival of different probiotic strains when exposed to bile salt and acid. Viability changes in different *Bifidobacterium* spp. was also performed using flow cytometry by Lahtinen and colleagues (2006).

The LIVE/DEAD *Bac*Light kit (Molecular Probes Inc.) is commercially available and popular in assessing bacterial viability. It consists of two stains: red fluorescing propidium iodide (PI) and green fluorescing SYTO9, which both stain nucleic acids. Propidium iodide enters only cells with damaged cytoplasmic membranes and SYTO9 enters and stains all cells. It displaces SYTO9 from the DNA in damaged cells due to its higher affinity or attraction for DNA (Bunthof *et al*., 2001a). The LIVE/DEAD *Bac*Light kit allows differentiation between intact and damaged cytoplasmic membranes as well as active and dead cells (Berney *et al*., 2007). Membrane integrity is a good indicator for cell death, as cells with damaged membranes cannot provide stable conditions for their molecular structures and may therefore eventually decompose and die. Membrane potential and membrane integrity are therefore good factors to discriminate living-active, dormant and dead cells (David *et al*., 2011). The aim of this study was to apply multiparametric flow cytometry of the LIVE/DEAD *Bac*Light kit stained cells in order to assess and compare the viability of selected strains of *Bifidobacterium* exposed to garlic clove (GC) extract and to determine the extent of damage thereof.

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5.3 Materials and methods

5.3.1 Bacterial cultures

Commercial probiotic cultures of *Bifidobacterium lactis* Bb12 (CHR- Hansen), *B. bifidum* LMG 11041 and *B. longum* LMG 13197 (BCCM/LMG culture collection, Belgium) and *Lactobacillus acidophilus* La14 150B (Danisco) were used. *L. acidophilus* La14 150B was used as a comparison.

5.3.2 Preparation of inocula and exposure to garlic clove extract

Bifidobacterium strains were grown in MRS broth supplemented with 0.05% cysteine hydrochloride and incubated at 37°C for 16 h in anaerobic jars containing Anaerocult A gaspacks (Merck Ltd. Modderfontein, SA). *Lactobacillus acidophilus* La14 150B was cultured in MRS broth without cysteine hydrochloride. Overnight cultures were then diluted 10-fold in fresh MRS broth at 37°C and subcultured cells allowed to grow anaerobically to reach the mid-exponential phase (0.5 McFarland standard), which corresponds to a concentration of approximately 10^8 cell/ml. One hundred microlitres of garlic clove (GC) extract, prepared as previously mentioned (Chapter 2, section 2.3.2.1) and ampicillin (Amp) were added to 1 ml of the bacterial suspensions and further incubated for 6 h at 37°C. Bacterial cells were then harvested by centrifugation at 13400 rpm for 15 min (Eppendorf minispin centrifuge) and the pellet washed twice with ½ Ringers solution by centrifugation. Washed cells were resuspended in phosphate buffered saline (PBS) and the concentration adjusted to 10^8 cells/ml. Untreated and heat-killed (incubated at 70°C for 30 min) cells were used as control samples.

5.3.3 Staining of bacteria for flow cytometric analysis

Live cells that were in their mid-exponential phase were used as positive controls while heat killed cells (70°C heat-killed for 30 min) were used as negative controls. Samples were stained with the Live/Dead *Bac*Light bacterial viability kit (Molecular Probes, L7012). Three replicates of positive and negative control cultures were individually stained with 2 µl PI, 1.5 µl SYTO9 and 1.5 µl of mixed SYTO9 and PI, respectively. The same amount of stains were

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added to the untreated, heat killed samples, the three GC extract treated and to the ampicillin-treated samples. All cultures (live, heat-killed, GC extract treated and ampicillin treated cells) were stained separately with 1.5 µl (Diluted 1:20) of SYTO 9, PI or a mixture of two stains (dual staining). Dual staining was performed for one sample of each of the untreated live, untreated dead and GC treated sample sets in order to differentiate live, dead and compromised cells. After addition of the stains to the culture, the tubes were incubated in a dark cupboard for 15 min. Then the samples were centrifuged at 13 400 rpm for 10 min. The cell pellets were resuspended in PBS and placed on ice before flow cytometric analysis.

5.3.4 Flow cytometry analyses

Flow cytometric analysis was performed with a BD FACSAria, equipped with fourargon lasers (488-nm, 633-nm, 405-nm laser and 375-nm near UV laser) for excitation. BD FACSflow (Becton Dickinson) was used as sheath fluid and the flow cytometer was adjusted to count 10 000 events per sample. The green fluorescence from SYTO9 was detected through a 530-nm, 30-nm-bandwidth band-pass filter which amounts to a range of 675-715-nm (FITC). Red fluorescence was detected through a 695-nm, 40-nm band-pass filter (Per-C-P), which amounts to a range of 515-545-nm. The forward scatter (FSC), sideward scatter (SSC), green and red fluorescence of each single cell were measured. All signals were collected using exponential amplifications. A combination of FSC and SSC was used to discriminate bacteria from background. All experiments were performed in triplicate.

5.3.5 Data analysis

Data compensation and analysis was performed using FlowJoVersion 7.6.1.

5.3.6 Revival of stained treated cells

The dual stained GC extract-treated sample was centrifuged at 13400 rpm for 15 min after the initial flow cytometric analysis. The supernatant was discarded and the pellet resuspended in 1 ml fresh MRS-cys broth, and incubated for 24 h at 37°C. After incubation, bacteria were harvested from the broth by centrifugation at 13 400 rpm for 15 min and washed twice with ½ Ringers solution. The cells were then resuspended in PBS and the concentration adjusted to

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10^8 cells/ml. The cells were stained as described above and analysed using flow cytometry, with live untreated and heat-killed cells used as controls.

5.3.7 Statistical analysis

Microsoft Excel 2007 was used to determine means and standard deviations of the samples.

5.4 Results and Discussion

5.4.1 Flow cytometric data interpretation

Flow cytometric data allowed us to estimate the impact of GC extract on microbial viability. Dual-parameter dot plots of the green fluorescence (SYTO9) (*y*-axis) and the red fluorescence (PI) (*x*-axis) were used to differentiate bacterial populations based on their fluorescence properties. Each dot represents one detected event, which is identified as a single cell which is plotted as a co-ordinate of their green (SYTO9) and red (PI) fluorescence value. PI and SYTO9 were simultaneously used for viability assessment of the strains before and after addition of GC extract.

Dot plots with results closest to the average means of triplicate experiments (Appendix) were used to illustrate our results. The different quadrants were set to represent the following population of cells: Quadrant 1 (Q1), upper left quadrant, SYTO9-stained ($SYTO9^+PI^-$) cells, quadrant 2 (Q2) upper left quadrant, double-stained ($SYTO9^+PI^+$) cells, quadrant 3 (Q3) lower right quadrant, PI-stained (PI^+SYTO9^-) and quadrant 4 (Q4), unstained ($SYTO9^-PI^-$) cells. Thus events appearing in Q1 ($SYTO9^+PI^-$) correspond to the live/viable cells in the population, Q2 ($SYTO9^+PI^+$) represents cells which suffered membrane damage (compromised cells), Q3 (PI^+SYTO9^-) shows dead cells while unstained viable, or lysed cells with depolarized cytoplasmic membranes are located in Q4 ($SYTO9^-PI^-$). Figures 5.1 and 5.2 show the flow cytometric detectors and compensation settings as determined using control samples. Results from these two figures show that a good discrimination was achieved between live and dead/compromised cells by the dual staining method.

Double staining of the GC extract treated *Bifidobacterium* cells with PI and SYTO9 was used to determine the effect of garlic on the membrane integrity of the cells, thereby giving an

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indication of whether the cell is viable, dead or damaged. If the cell membrane is intact and not damaged, the cells exclude the nucleic acid dye PI, which binds to RNA and DNA. Damage to the cell membrane makes it permeable to PI, which upon entry into the cells stains nucleic acids resulting in red fluorescence. PI is therefore used as a marker for dead cells and indicates that cells have lost their membrane integrity and tend to reduce culturability (Volkert *et al*., 2008). SYTO9 stains all cells in a population, whether they are dead or alive. Fluorescent stains, such as PI and SYTO9, have been used in many studies on lactic acid bacteria to differentiate between intact and permeable (damaged) cells (Bunthof *et al*., 2001a; Bunthof *et al*., 2001b; Bunthof and Abee, 2001; Ananta *et al*., 2005). The results obtained for each of the tested strains in the current study are discussed separately below.

5.4.1.1 *L. acidophilus* La14 150B

In Chapter 2, it was documented that *L. acidophilus* La14 150B was not susceptible to GC extract, whereas it was very susceptible to ampicillin. Flow cytometric data confirmed these findings. Compensation controls represented by dot plots (Fig. 5.1A-D) worked well and it can be seen that once *L. acidophilus* La14 150B cells were treated with GC extract (Fig. 5.2E) there was not much difference when compared to the untreated control (Fig. 5.2A). After GC extract treatment approximately 99.2% (Fig. 5.2E, Q1) did not take up the PI stain, indicating that cells remained healthy with intact polarized cytoplasmic membranes and were able to withstand GC extract treatment.

Only 0.56% (Fig. 5.2E, Q2 and Q3) of the cell population took up PI and showed cytoplasmic membrane damage as a result of the GC extract treatment. Ampicillin was used as a positive control and it was expected that there would be a decline in viability as well as considerable cell damage or death once *L. acidophilus* La14 150B was treated with this antibiotic. Upon treatment of *L. acidophilus* with ampicillin, the percentage of healthy cells with intact polarized cytoplasmic membranes decreased from 99.6% to 48.6% (Fig. 5.2D, Q1). There was an increase of 44% for cells with damaged or depolarized membranes (Fig. 5.2D, Q2) and 6.99% of the dead cells (Fig. 5.2D, Q3).

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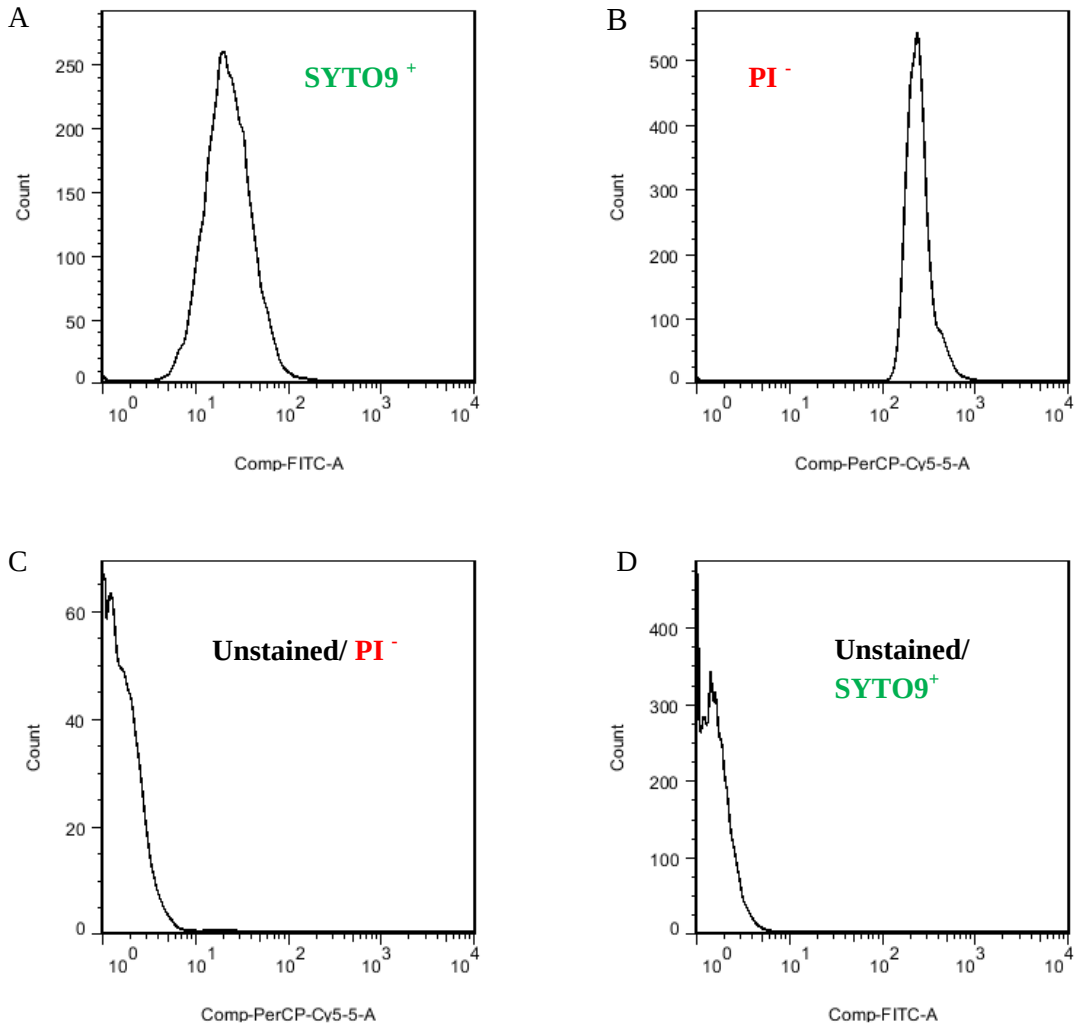

Figure 5.1: Fluorescence histograms showing compensation controls for *L. acidophilus* La14 150B: A: SYTO9 stained/SYTO9^{+}; B: PI stained/PI $^{+}$; C: Unstained control/ PI^{-}; D: Unstained control/SYTO9^{-}.

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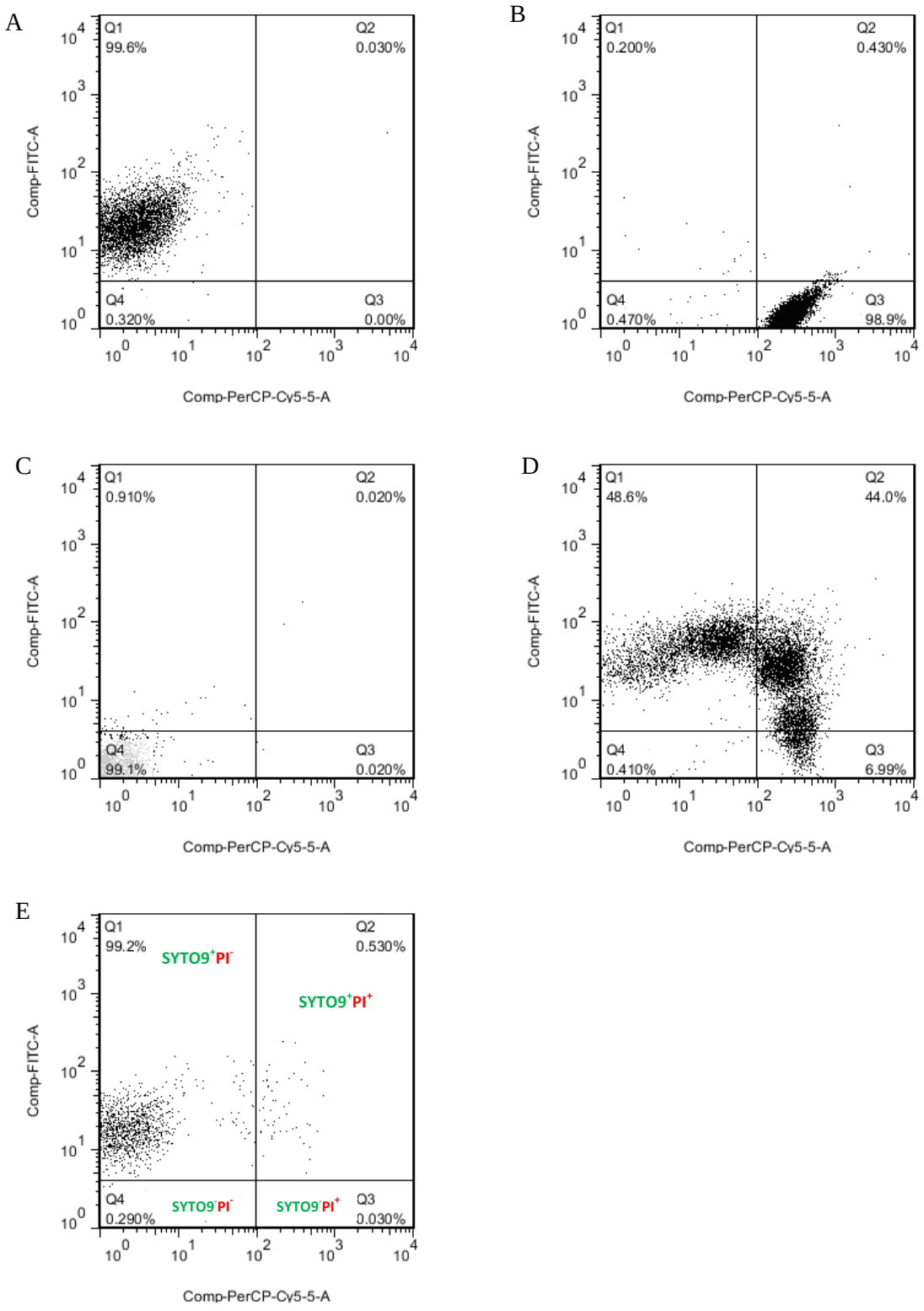

Figure 5.2: Flow cytometry dot plots of viable untreated (A), dead (B), unstained (C), ampicillin-treated (D) and GC extract treated (E) *L. acidophilus* La14 150B cells.

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According to Nikiyan and colleagues (2010), ampicillin inhibits the synthesis of the peptidoglycan layer of Gram-positive bacteria cell walls and therefore destructs the cell wall. These results once again correlated with the results that were obtained in the previous Chapters for effect of ampicillin on this bacterium. This therefore confirms that *L. acidophilus* La14 150B is relatively resistant to GC extract as has been observed in the previous Chapters.

5.4.1.2 *B. bifidum* LMG 11041

Figure 5.3 shows successful controls for *B. bifidum* LMG 11041. The live cell sample however did not have 100% viable cells, but 98.7% viable and 0.34% of damaged cells (Fig. 5.4A). This indicated that even in a sample which is presumed to be totally healthy by classical microbiology, there are already some cells that are damaged or injured. This has also been observed by Ben Amor and colleagues (2002) and Hayouni et al (2008). The heat-killed *B. bifidum* LMG 11041 sample contained 98.4% cells that were permeable to PI, with a corresponding fluorescence intensity of 10^3 (Fig. 5.4B). Dot plots obtained for this strain showed significant differences between untreated and GC extract treated cells (Fig. 5.4A and E). Not only were there cells visible in three of the four quadrants after exposure to GC extract, but the entire population also changed shape and density (Fig. 5.4E). The relative percentage of freshly harvested viable cells in the treated sample decreased from 98.7% to 82.2% (Fig. 5.4A, Q1), whereas there were increases from 0.34% to 8.66% and 1% to 9.08% in Q2 and Q4, respectively. The increase of cells located in Q2 ($SYTO9^+PI^+$ stained population) of Figure 5.4E is evident of progressive cell damage and membrane deterioration or permeabilization. Cell depolarization indicates a decline in cell functionality, due to energy depletion, but it does not imply cell death (Cánovas *et al*., 2007).

It is known that allicin, the main active compound found in garlic, can easily pass through phospholipid membranes and be taken up by the cell, which in turn causes cell membrane damage and eventually cell lysis and termination of growth (Al-Waili *et al*., 2007; Deresse, 2010). Similar flow cytometric data for bifidobacteria exposed to bile salt stress was obtained by Ben Amor et al (2002). Ananta and colleagues (2005) also found cell membrane damage using flow cytometry when *Lactobacillus rhamnosus* underwent spray drying.

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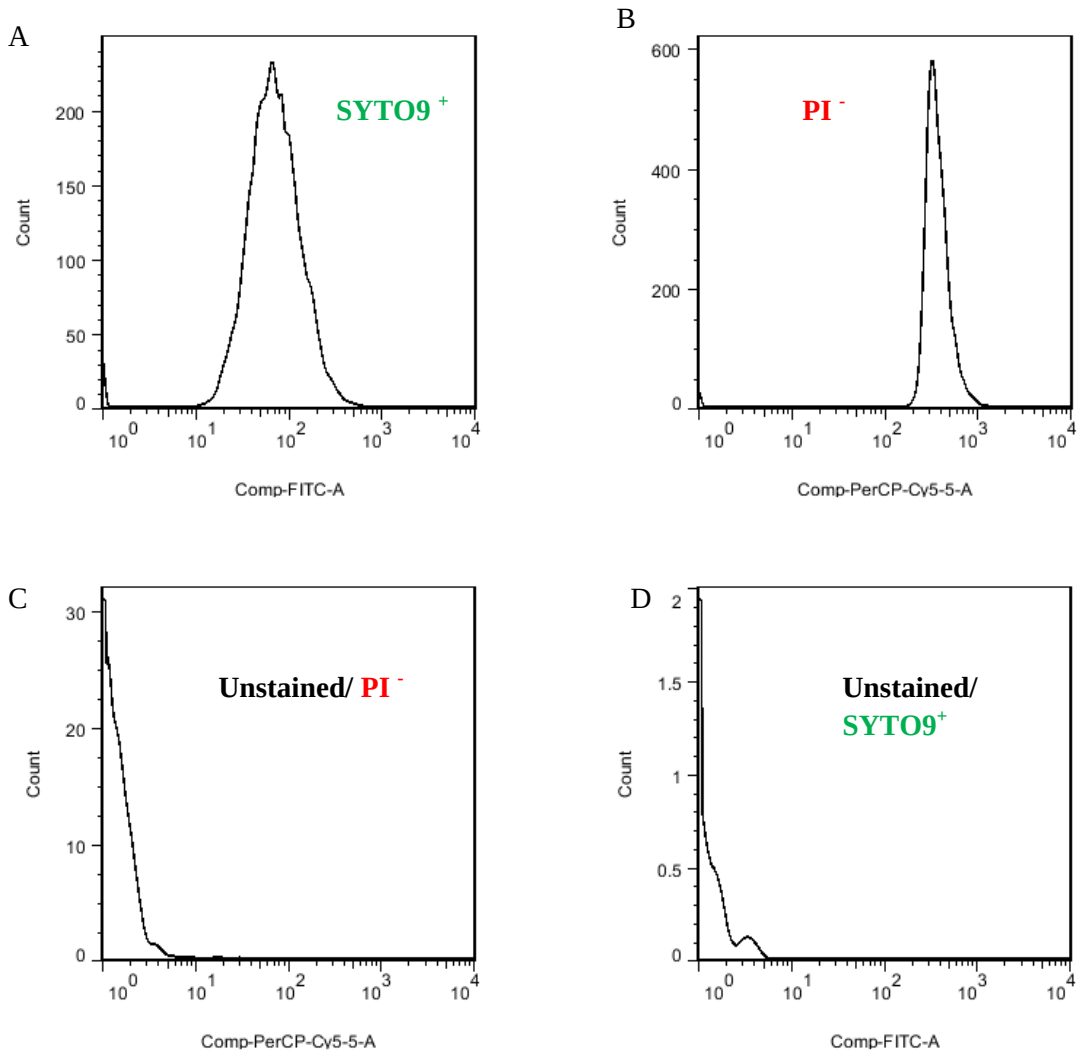

Figure 5.3: Fluorescence histograms showing compensation controls for *B bifidum* LMG 11041: A: SYTO9 stained/SYTO9+; B: PI stained/PI+; C: Unstained control/ PI$^-$; D: Unstained control/ SYTO9$^-$.

Interestingly, after GC extract treatment the percentage of the cell population in Q4 (SYTO9$^-$ PI$^-$) increased to 9.08% (Fig. 5.4E). An increase in numbers of unstained cells could be possibly due to a number of reasons. Firstly, these cells could correspond to cells that have undergone lysis, as a result have lost their nucleic acids, thereby rendering them unstainable (Martínez-Abad *et al*., 2012). Secondly, they may also be cells that were unstained possibly due to the fact that bacterial species may exist or are able to clump together or form interlaced chains which, according to Hayouni et al (2008), may provide a single increasing signal resulting from the input of each cell to it, therefore decreasing staining accuracy and producing misleading results. These 'ghost cells' have also been described by other researchers elsewhere (da Silva *et al*., 2005; Shena *et al*., 2009).

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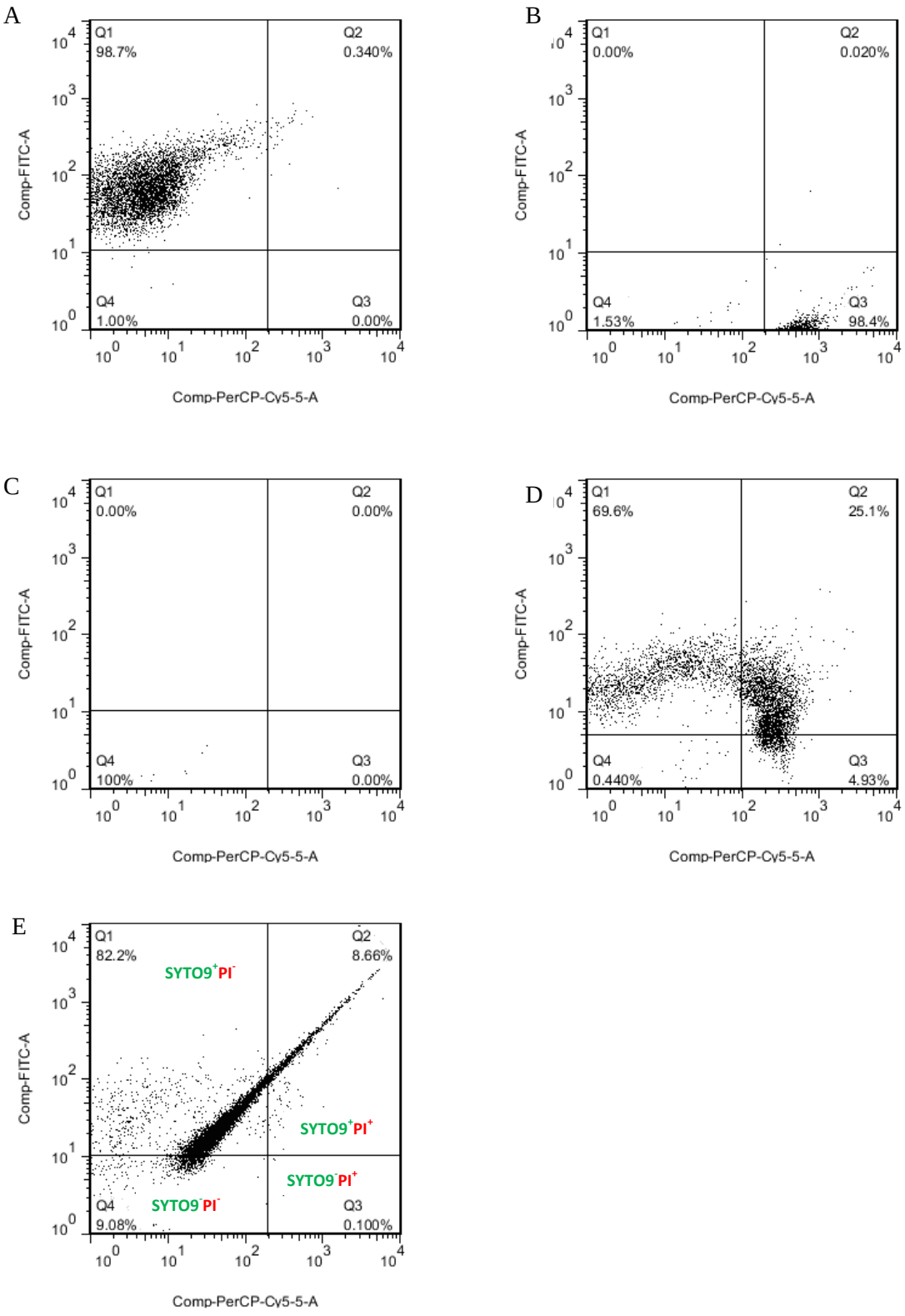

Figure 5.4: Flow cytometry dot plots of viable untreated (A), dead (B), unstained (C), ampicillin-treated (D) and GC extract treated (E) *B. bifidum* LMG 11041cells.

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It can also clearly be observed that there was a significant difference in the shape and density of the populations' light scatter pattern before and after exposure to GC extract (Fig. 5.4A and E). Flow cytometric analysis is able to measure each cell's individual light dispersion pattern, which supplies information on cell size and granularity (Schenk *et al*., 2011). There was a definite change in light scatter of the bacterial cell population after treatment, which became less diffuse and much more concentrated. This could possibly be due to a change in cellular structure and external morphology as a result of the GC extract treatment. It could also indicate that the size of the cells decreased after GC extract treatment possibly changing form a rod-shape to coccoid in shape, as was evident with scanning electron microscopy (Chapter 3, section 3.4.2). Similar results were obtained by Schenk and colleagues (2011) after *Escherichia coli* and *Listeria innocua* cells were exposed to UV-C light. *Bifidobacterium bifidum* LMG 11041 therefore showed a considerable drop in viability and cell damage when exposed to GC extract. Comparing the results for GC extract treatment with those obtained for cells treated with ampicillin, a significant change in cell population due to treatment was also observed. The percentage of viable *B. bifidum* LMG 11041 cells decreased from 98.7% to 69.6%, (Fig. 5.4D, Q1) while there was an increase to 25.1% (Fig. 5.4D, Q2) and 4.93% (Fig. 5.4D, Q3) for that of stressed cells with damaged membranes and dead cells, respectively. This confirms results shown in Chapter 3 (Section 3.4.2) that *B. bifidum* LMG 11041 was very susceptible to ampicillin.

5.4.1.3 *B. longum* LMG 13197

As with *B. bifidum* LMG 11041, this strain also gave good results for the controls (Fig. 5.5). Good fluorescent peaks for both SYTO9 and PI were obtained and for the unstained sample both stains were absent. When comparing untreated (Fig. 5.6A) and GC extract treated (Fig. 5.6E) cells, dot plots revealed that *B. longum* LMG 13197 followed a similar pattern to that of *B. bifidum* LMG 11041 (Fig. 5.4). The viable cell population decreased from 99.8% to 88.8% after GC extract treatment, whereas cell numbers increased in Q2 to 9.4% (Fig. 5.6E), indicating damaged cells with depolarized membranes. The population shape and density also changed after exposure to GC extract (Fig. 5.6E) compared to the untreated bacterial population (Fig. 5.6A), with the light scatter pattern becoming more concentrated indicating a change in size and molecular content of the cells.

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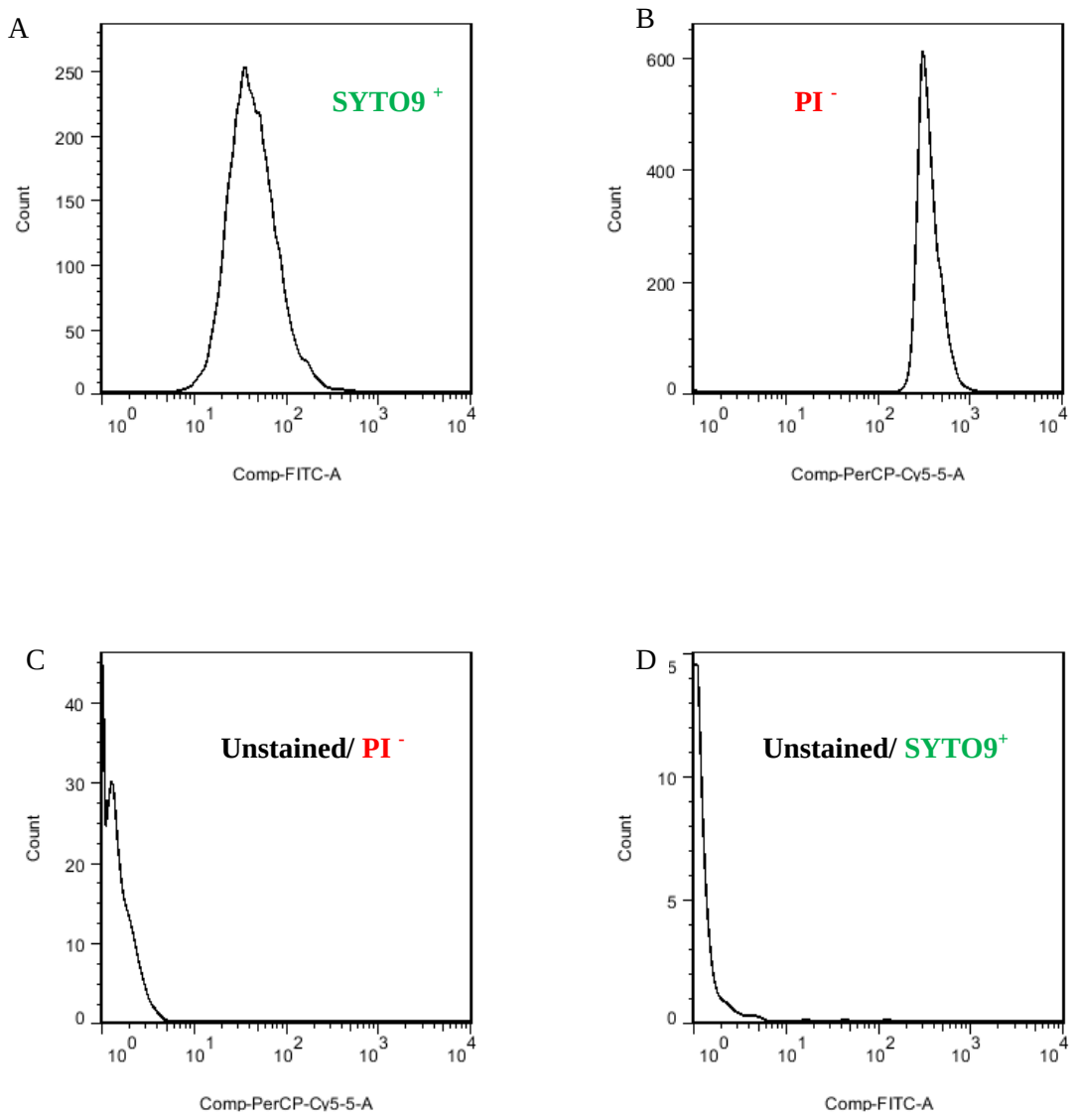

Figure 5.5: Fluorescence histograms showing compensation controls for *B. longum* LMG 13197: A: SYTO9 stained/SYTO9+; B: PI stained/PI+; C: Unstained control/ PI$^-$; D: Unstained control/ SYTO9$^-$.

A concentrated light pattern indicates smaller cells whereas a more diffuse light scatter pattern indicates bigger cells (Schenk *et al*., 2011). Therefore the cells may have changed from their normal rod-shape appearance to more coccoid-shape after GC extract treatment. According to Novik et al (2001), bifidobacteria entering their death phase have an assortment of morphologies which include coccoid-shaped cells as a result of stress. This clearly shows that GC extract affects both the viability of the cells and their shape and morphology. A study by Young et al (2007) showed that typical rod-shaped *Bifidobacterium* spp. became coccoid under stress, such as a nutrient limitation.

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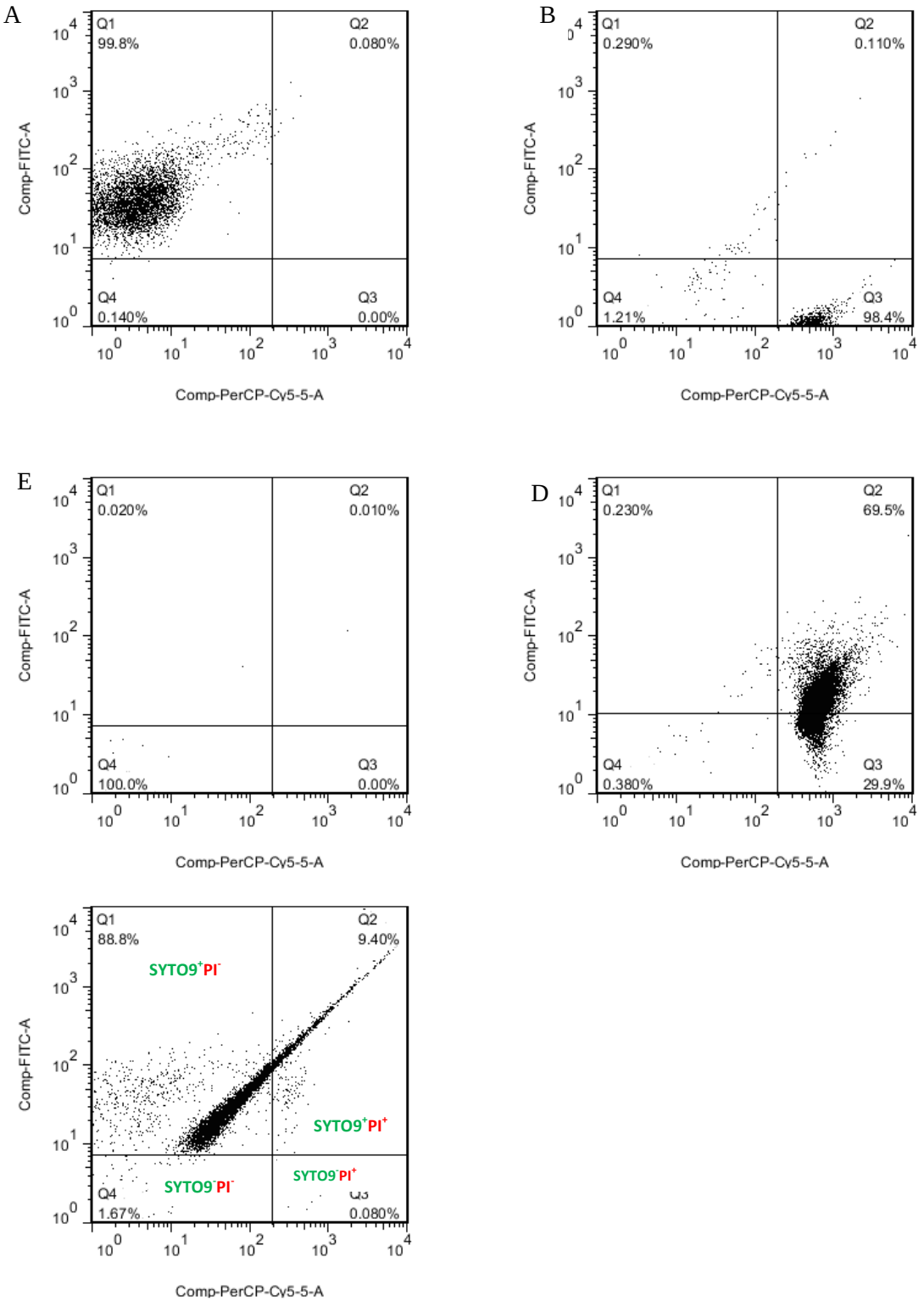

Figure 5.6: Flow cytometry dot plots of viable untreated (A), dead (B), unstained (C), ampicillin-treated (D) and GC extract treated (E) *B. longum* LMG 13197cells.

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Therefore GC extract negatively affected *B. longum* LMG 13197, causing cell membrane damage and a drop in viable cells. The only difference between *B. longum* LMG 13197 and *B. bifidum* LMG 11041 is that the percentage of unstained cells did not increase as much as was observed with the later strain, with only 1.68% of cells recorded (Fig. 5.4D, Q4). This suggests that this strain was probably less susceptible to the effects of GC extract.

Ampicillin had a much more detrimental effect on *B. longum* LMG 13197 (Fig. 5.6D) than it had on *B. bifidum* LMG 11041 (Fig. 5.4D). There were barely any viable cells remaining after treatment (0.23%) as compared with 99.8% before treatment. A percentage of 69.5% of cells were membrane compromised (Fig. 5.6D, Q2) and 29.9% appeared dead (Fig. 5.6D, Q3). The light scatter pattern was also much more concentrated and less diffuse when compared to untreated cells (Fig. 5.6A, Q1) indicating a change in the size and contents (granularity) of the cells as mentioned previously.

5.4.1.4 *B. lactis* Bb12

Compensation controls for *B. lactis* Bb12 were successful with good fluorescent peaks for both stains (Fig. 5.7). Results for GC extract treated sample showed that 8.09% of the cell population had membrane damage (Fig. 5.8E, Q2) and 24.8% of the population was unstained (Fig. 5.8E, Q4) due to severe cell lysis. Previous studies have confirmed that garlic damages cell membranes and causes cell lysis. Kim et al (2007) reported cell wall lysis in *Listeria monocytogenes* cells treated with garlic shoot juice. Similar results were also observed by Ghannoum (1988), where he attributed the change in structure and integrity of the outer membrane of *Candida albicans* to the decrease in lipid content of the membrane due to the presence of garlic (Kim *et al*., 2007).

Only 67% of the cells remained viable and healthy after treatment (Fig 5.8E, Q1) as opposed to 99.7% before treatment (Fig. 5.8A). As with the *B. bifidum* LMG 11041 (Fig. 5.4) and *B. longum* LMG 13197 (Fig. 5.6), the entire cellular population changed in terms of the size of each cell as well as the granular content of the cells, meaning GC extract had an effect on the intracellular contents of the cells. These results confirmed that *B. lactis* Bb12 is susceptible to GC extract and there is a significant drop in viable cells after treatment. Dot plots for ampicillin treated *B. lactis* Bb12 cells revealed that this strain was very also very susceptible to ampicillin.

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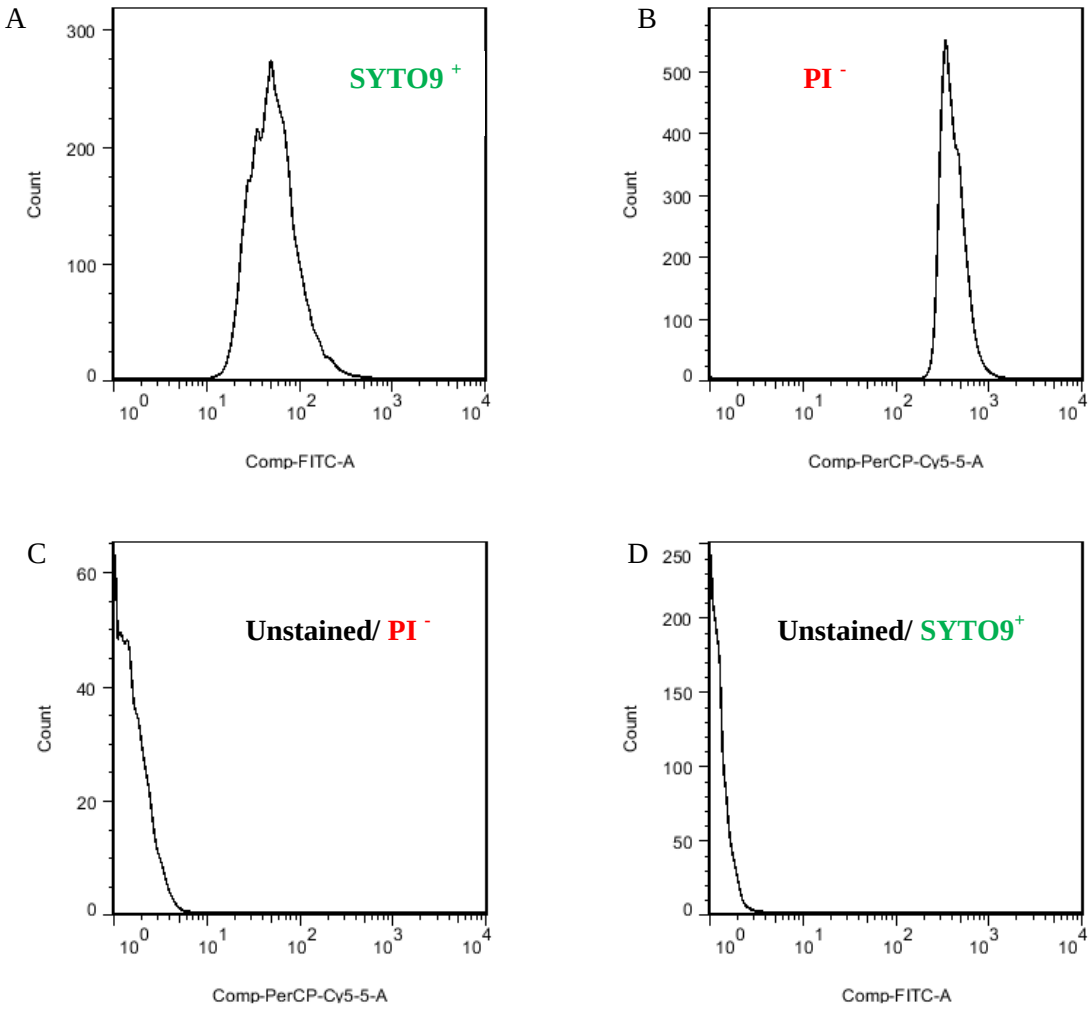

Figure 5.7: Fluorescence histograms showing compensation controls for *B. lactis* Bb12: A: SYTO9 stained/SYTO9+; B: PI stained/PI+; C: Unstained control/ PI$^-$; D: Unstained control/ SYTO9$^-$.

Ampicillin treatment reduced the population of viable cells from 99.7% to only 44.7% (Fig. 5.8D, Q1). The rest of the cells were membrane damaged (42.7%), dead (11.2%) or lysed (1.49%), (Fig. 5.8D, Q2, Q3 and Q4, respectively). The population shape of *B. lactis* Bb12 was similar to that of *B. bifidum* LMG 11041 after ampicillin treatment.

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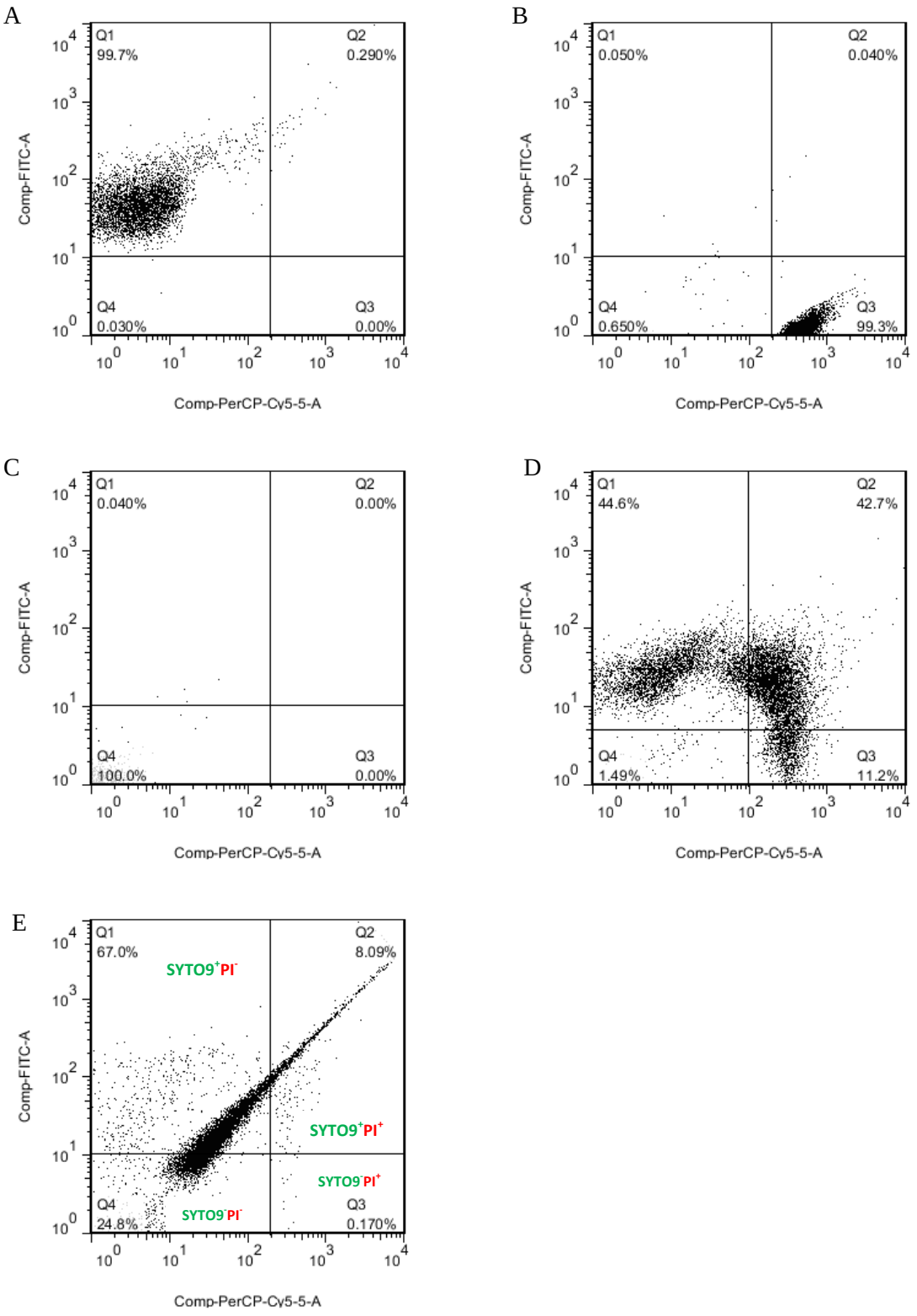

Figure 5.8: Flow cytometry dot plots of viable untreated (A), dead (B), unstained (C), ampicillin-treated (D) and GC extract treated (E) *B. lactis* Bb12 cells.

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Untreated cells for all *Bifidobacterium* strains and *L. acidophilus* stained with SYTO9 (Fig. 5.2A, 5.4A, 5.6A and 5.8A) showed a homogeneous behavior in their fluorescent labeling properties. All cells yielded high green fluorescence and were situated in Q1. For all three *Bifidobacterium* strains, exposure to GC extract resulted in a sub-population increase of 9.32%, 12.87% and 7.76% (means of triplicate experiments, Appendix) for *B. bifidum* LMG 11041, *B. longum* LMG 13197 and *B. lactis* Bb12, respectively in the number of events encountered in Q2 ($SYTO^{+}PI^{-}$), indicating damage to the cell and a drop in viability. We know that allicin, the active agent of garlic, is able to penetrate and enter cell membranes causing damage to the cell (Bakri and Douglas, 2005). Additionally, sub-population increases of 11.01%, 5.04% and 22.83% for *B. bifidum*, *B. longum* and *B. lactis* spp., respectively, appeared in Q4 (Fig. 5.4E, 5.6E and 5.8E) after GC extract exposure. This indicated either severe cell lysis occurred, thereby probably inhibiting the cell to take up any stains, especially since control samples indicated good staining.

In order for a cell to be healthy and fully functional, it needs both an intact polarized cytoplasmic membrane as well as an active transport system (Hewitt *et al*., 1999; da Silva *et al*., 2005). Therefore with the use of PI, a DNA stain that cannot enter a cell with an intact cytoplasmic membrane, we established that GC extract does indeed damage the cell and renders it non-functional and unhealthy. When a cell is excessively stressed, the cytoplasmic membrane depolarizes and ultimately permeabilzes which indicates cell death and cell lysis (da Silva *et al*., 2005; Hewitt *et al*., 1999). Garlic causes cell membrane damage which can result in cell lysis which ultimately leads to death (Lu *et al*., 2011). Lipid and fatty acid biosynthesis as well as NO^{-} formation are also influenced by garlic, which has significant changes in the viability of cells (Focke *et al*., 1990; Miron *et al*., 2000). This could support our results where cells were found in Q2 and Q4, indicating cell membrane damage and cell lysis, respectively. Dot plots for *L. acidophilus* La14 150B showed no real susceptibility to GC extract, which substantiates results obtained in previous chapters.

5.4.2 Determination of recovery of damaged cells

Cells that have undergone stress such as damaged membrane, proteins and/or DNA, possibly from sub-lethal injury, can generally assume a dormant or nonculturable condition in which they do not grow, but they are able to recover by repairing or replacing damaged molecules, and come back to their physiologically active condition (Ben Amor *et al*., 2002). In order to

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determine the ability of compromised or injured bifidobacteria cells to recover from sub-lethal damage due to GC extract exposure, we harvested the stained cells after flow cytometry analysis, re-inoculated them into fresh medium and incubated the suspension under appropriated growth conditions. If the DNA or damaged molecules are damaged beyond repair or if the damage/stress caused by culture conditions requires more energy than the cell can safely generate for its repair; no growth will occur (Nebe-von-Caron *et al*., 2000). Table 5.1 shows percentages of live, dead, lysed or unstained cells and damaged (compromised) cells of the three *Bifidobacterium* strains treated with GC extract and after they were re-inoculated into fresh broth in the absence of GC extract. The results show that generally, the numbers of viable cells in all the samples increased after re-inoculation, indicating growth of the cells. The differences in the extent of increase in viability for the *Bifidobacterium* strains are discussed briefly below.

Table 5.1: Percentages of live, dead, lysed/unstained cells and compromised/damaged cell of *B. bifidum* LMG 11041, *B. longum* LMG 13197 and *B. lactis* Bb12 treated with GC extract and after re-inoculation in the absence of GC extract

Bifidobacterium strains	Live (%)		Damaged (%)		Dead (%)		Lysed/Unstained (%)	
	Treated	After reinoculation	Treated	After reinoculation	Treated	After reinoculation	Treated	After reinoculation
B. bifidum LMG 11041	82.2	98.7	8.66	0.73	0.1	0.01	9.08	0.57
B. longum LMG 13197	88.8	97.4	9.4	2.35	0.08	0.02	1.67	0.19
B. lactis Bb12	67	97.4	8.09	1.83	0.17	0.08	24.8	0.7

5.4.2.1 *B. bifidum* LMG 11041

After treatment with GC extract, it was observed that 8.66% of the cells were damaged and had compromised membranes (Fig. 5.9A, Q2). After cells were re-inoculated into fresh broth in the absence of GC extract, it was observed that the percentage of previously damaged cells decreased from 8.66% (Fig. 5.9A, Q2) to 0.73% (Fig. 5.9B, Q2). The percentage of 'lysed' cells decreased from 9.08% (Fig. 5.9A, Q4) to 0.57% (Fig. 5.9B, Q4). This possibly suggests

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that these cells were able to recover in the absence of GC extract and return to their physiologically active and growing state. Therefore, the damaged cells exposed to GC extract entered a dormant state in which they could not grow. Once the source of stress, in this case GC extract, was removed and the cells received a fresh medium with adequate nutrients, they were able to recover and repair the damage induced by the GC extract. The reduction in the percentage of lysed cells is an indication that these numbers were fewer than other cells in the population, as it is highly unlikely that cells are able to recover after cell lysis. The light scatter pattern of the re-inoculated cells also showed that they returned to their original size and shape (Fig. 5.9B) as they were before GC extract treatment (Fig. 5.9A), as the pattern became more diffuse in appearance.

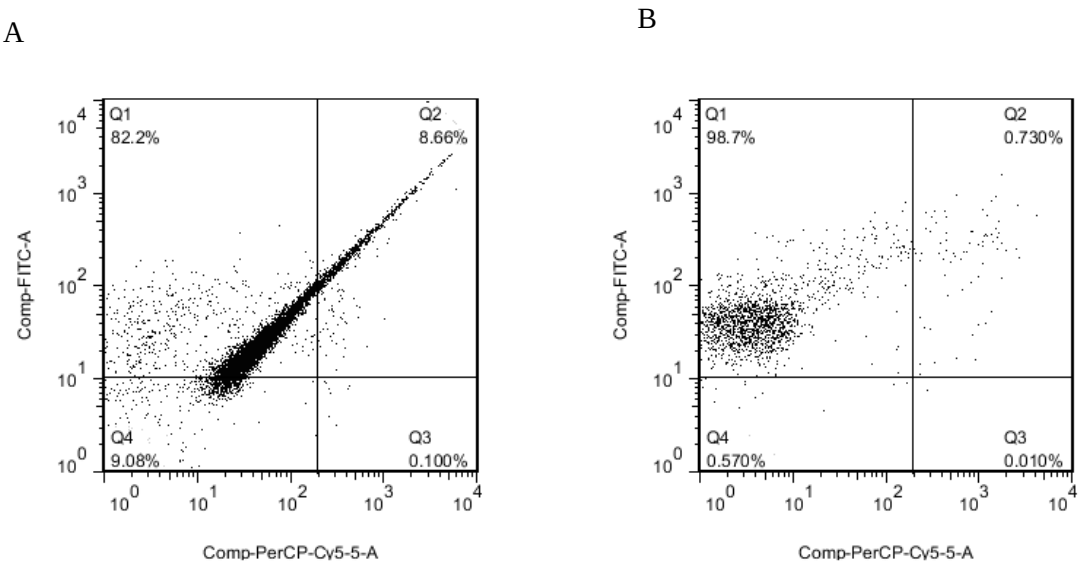

Figure 5.9: Flow cytometry dot plots of GC extract treated *B. bifidum* LMG 11041 cells after initial staining (A) and after re-inoculation into fresh broth (B).

5.4.2.2 *B. longum* LMG 13197

For *B. longum* LMG 13197, slightly fewer cells recovered after removal of GC extract and re-inoculation into fresh medium (Fig. 5.10B). The percentage of live cells increased from 88.8% (Fig. 5.10A, Q1) to 97.4% (Fig. 5.10B, Q1). A decrease from 9.4% (Fig. 5.10A, Q2) to 2.35% (Fig. 5.10B, Q2) in the percentage of damaged cells was also observed. A possible reason for fewer cells recovering could be that GC extract had a more permanent damaging effect on this strain or the recovery time may be longer than 24 h in order for the cells to revert to the physiologically active state and start multiplying again. Once again the

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concentrated light scatter pattern became more diffuse indicating that the cells assumed their original size and shape.

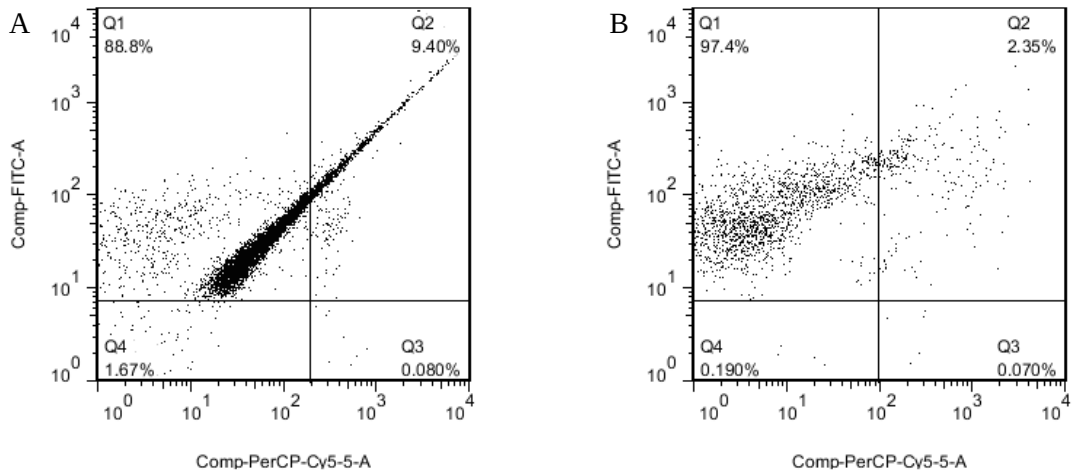

Figure 5.10: Flow cytometry dot plots of GC extract treated *B. longum* LMG 13197 cells after initial staining (A) and after re-inoculation into fresh broth (B).

5.4.2.3 *B. lactis* Bb12

The results obtained for *B. lactis* Bb12 after re-inoculation were similar to those of *B. bifidum* LMG 11041. Most of the compromised cells for *B. lactis* Bb12 recovered after resuscitation in fresh broth without GC extract (Table 5.1 and Fig. 5.11B). This once again suggests that these cells assumed a dormant (non-culturable) state after GC extract treatment, but were able to recover by repairing the damaged molecules, and return back to their physiologically active condition once the source of stress was removed and fresh nutrients became available.

In summary, re-inoculation of the damaged GC extract treated cells into fresh broth, resulted in apparent re-growth of the injured cells for all the three *Bifidobacterium* spp. All three *Bifidobacterium* spp. showed a decrease in the amount of events occurring in Q2 after the cells were inoculated into fresh broth without GC extract as compared to cells that were exposed to GC extract (Table 5.1). This could mean that a fraction of the stressed cells adopted a latent state in which they couldn't reproduce but were induced to a physiologically active state after recovery.

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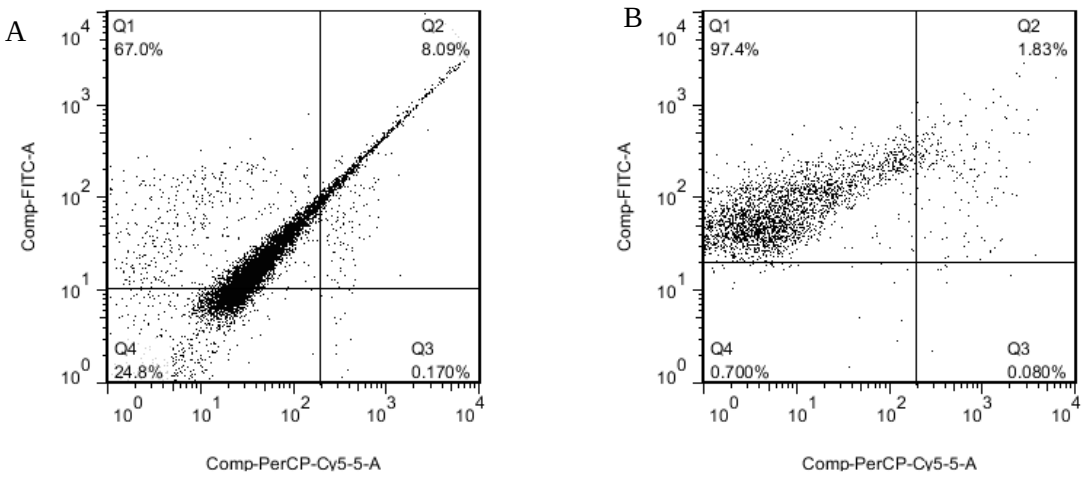

Figure 5.11: Flow cytometry dot plots of GC extract treated *B. lactis* Bb12 cells after initial staining (A) and after re-inoculation into fresh broth (B).

Garlic clove may have induced sub-lethal injury within the *Bifidobacterium* populations through a reversible and transient membrane permeabilization which resulted in loss of viability, but these cells could then regain growth after being resuscitated in fresh broth containing fresh sources of growth. According to Hewitt and colleagues (1999), cells are able to exist without a membrane potential for a short period of time after which they either quickly progress to permeabilisation and death or re-polarise in the presence of a fresh energy source, as was the case with our results. This was also observed by Ben Amor et al (2002), for *Bifidobacterium* cells exposed to bile salt.

5.5 Conclusion

Multiparameter flow cytometric analysis and simultaneous use of LIVE/DEAD *Bac*Light viability kit provided a good discrimination between viable, damaged and dead cells in the mixtures of untreated and GC extract treated *Bifidobacterium* cells. The decrease in the populations of SYTO9-stained *Bifidobacterium* cells with concomitant increase in PI stained cells for all three species indicated membrane deterioration after GC treatment. The bacterial membrane was identified as one of the main sites of injury for the *Bifidobacterium* spp. tested after GC treatment. These findings showed that for all three *Bifidobacterium* cells, GC extract treatment destroyed membrane integrity as PI was able to enter the cells.

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B. bifidum LMG 11041 and *B. lactis* Bb12 were the most vulnerable to GC extract, having higher increases in events occurring in Q4 possibly indicating lysed cells. This signifies that these two strains had significant cytoplasmic membrane damage and lysis eventually occurred. Once lysis occurrs the cells cellular contents are exposed to the external environment and the cell ultimately dies. The treated cells showed changes in light scatter patterns after exposure to GC extract, which indicates a change in cell shape and granularity. All three *Bifidobacterium* spp. were able to recover after damaged cells when re-inoculated into fresh broth. Cell sorting would be a much better representation of whether damaged cells were really able to recover as one would easily be able to gate the cells and sort them in order to obtain only the damaged cells to plate them out. Dead cells and "lysed" cells could then also be obtained and plated to confirm their physiological state as indicated by flow cytometry.

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CHAPTER 6

General conclusions and recommendations

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General conclusions

- Garlic has an antibacterial activity on bifidobacteria, with garlic clove extract having the highest activity, followed by garlic powder, garlic spice and garlic paste. Garlic paste only inhibited *B. bifidum* LMG 11041 and *B. longum* Bb 536.

- All *Bifidobacterium* strains tested were inhibited at different extents with the *B. bifidum* strain being most susceptible, followed by *B. longum* and *B. lactis* strains. Susceptibility to garlic differed between *Bifidobacterium* spp. and among strains of the same spp. All the *Bifidobacterium* spp. tested were more susceptible to garlic than *L. acidophilus* La14 150B.

- The probiotic bifidobacteria succumb to antibacterial effects of garlic within a relatively short period. It takes only 30 min of exposure to garlic clove extract to decrease viability of *B. bifidum* LMG 11041 and at least 1 h for viability of all other tested *Bifidobacterium* strains to start decreasing.

- Minimum inhibitory concentration (MIC) values for garlic were lower for most bifidobacterial strains compared to the control *Lactobacillus* strain, indicating that the bifidobacterial strains were more sensitive to the garlic preparations than *L. acidophilus*.

- Garlic promotes unusual morphological as well as behavioural changes in probiotic *Bifidobacterium* spp. with garlic clove and garlic paste showing the most evident changes.

- Exposure to garlic causes an increase in highly distorted cells, elongated and swollen cells with bulbous ends and cocci-shaped cells as well as pore formation, cell lysis and an increase in the presence of debris in the area surrounding the treated *Bifidobacterium* cells.

- Fourier-transform infrared (FT-IR) spectroscopy showed a much clearer picture for the damaging effects of garlic happening inside the celland it clearly differentiated between non-treated and garlic-treated *Bifidobacterium* cells. Biochemical changes

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took place inside garlic-treated cells and spectral variations were evident in regions associated with fatty acids and phospholipids in the cell membrane, secondary structure proteins and nucleic acids.

- The main targets for garlic clove extract appeared to be the nucleic acids (DNA and RNA) and the fatty acids (lipids) in the cell membrane. Therefore, these changes to the surface properties may possibly prevent probiotic *Bifidobacterium* strains from colonizing the intestinal mucosa and thereby decrease their viability as well as beneficial effect, which would disadvantage both the consumers as well as jeopardize the probiotic industry.

- Flow cytometric results revealed a decrease in viable cells and an increase of 8-10% in damaged cells after exposure to garlic for all three *Bifidobacterium* spp. Fluorescent dyes (PI) confirmed that the membrane integrity of the cells were compromised in the presence of garlic proving that the cell membrane of bifidobacteria is indeed a main target site for garlic. Flow cytometry also confirmed that garlic-damaged cells changed in terms of size and shape.

- Garlic-damaged cells were able to recover and return to their physiologically active state and were also able to assume their original shape and size, indicating that garlic damage is temporary once cells areexposed to a fresh medium in the absence of garlic.

Recommendations and future work

- It would be necessary to determine the effect that food processing and different food matrices could have on the antibidobacterial activity of garlic.

- Another important study would be to simulate gastrointestinal tract conditions when exposing bifidobacteria to garlic as this would give a much more representative depiction of how the two will interact. It will also be used to determine how long the active compounds in garlic remain stable under these conditions.

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- Physiological changes taking place inside bifidobacteria once exposed to garlic can also be determined.

- Transmission electron microscopy should be considered to confirm possible cell lysis as well as loss of nucleic acid and other cytoplasmic contents resulting from garlic exposure.

- Further studies could be done using Raman spectroscopy to study spectral changes in the sulfur region (500-700cm^{-1}) which would indicate effect on protein structures.

- Cell sorting, which will allow selective harvesting and culturing of only damaged cells from flow cytometry, is necessary to confirm the actual recovery and resumption of growth by garlic-damaged bifidobacteria. Re-inoculation results reported in this study were obtained from culturing of all cells including those that were still viable and therefore are not a very good indication of this phenomenon.

- Microencapsulation is currently the main strategy used to improve or protect viability of probiotic cultures. It would be worthwhile to investigate the ability of the coating matrices to limit or prevent contact between encapsulated probiotics and garlic.

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APPENDIX

Mean values and standard derivative values for flow cytometric analysis data of triplicate experiments.

Fluorescent dye	Mean (%) (Standard derivative) of triplicate experiments															
	B. bifidum LMG 11041				*B. longum* LMG 13197				*B. lactis* Bb12				*L. acidophilus* La14 150B			
	Q1	Q2	Q3	Q4	Q1	Q2	Q3	Q4	Q1	Q2	Q3	Q4	Q1	Q2	Q3	Q4
Syto9	98.7 (0.4)	0.74 (0.64)	0 (0)	0.57 (0.31)	99.2 (0.22)	0.37 (0.37)	0 (0)	0.19 (0.09)	99.63 (0.09)	0.27 (0.04)	0 (0)	0.1 (0.05)	99.75 (0.15)	0.7 (0.04)	0 (0)	0.17 (0.15)
PI	0.06 (0.04)	0.02 (0)	99.03 (0.46)	0.86 (0.86)	0.16 (0.12)	0.11 (0.07)	99.17 (0.54)	0.61 (0.42)	0.04 (0.03)	0.06 (0.03)	98.37 (0.78)	1.55 (0.8)	0.11 (0.09)	5.42 (5)	94.2 (4.7)	0.27 (0.21)
Unstained	0 (0)	0 (0)	0 (0)	100 (0)	0.04 (0.05)	0.01 (0)	0 (0)	99.99 (0.05)	0.11 (0.13)	0 (0)	0 (0)	99.9 (0.14)	8.26 (7.35)	0.04 (0.02)	0.01 (0.01)	91.85 (7.25)
Amp mix (Syto9 + PI)	31.62 (28.21)	50.53 (20.9)	17.51 (21.22)	0.38 (0.05)	20.21 (19.47)	51.59 (32.05)	14.6 (10.82)	13.64 (18.85)	22.49 (22.12)	34.65 (8.05)	41.5 (30.3)	1.34 (0.16)	24.43 (24.18)	24.51 (19.5)	50.45 (43.46)	0.61 (0.2)
GC mix (Syto9 + PI)	79.37 (5.26)	9.32 (2.45)	0.34 (0.37)	11.01 (7.09)	81.8 (7.31)	12.87 (8.13)	0.27 (0.25)	5.04 (3.49)	68.97 (3.74)	7.76 (0.4)	0.47 (0.26)	22.83 (3.89)	98 (1.2)	1.86 (1.33)	0.02 (0.01)	0.15 (0.14)

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