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Characterization of Commercial Probiotics: Antibiotic Resistance, Acid and Bile Resistance, and Prebiotic Utilization

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by

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Probiotics, live microorganisms that beneficially affect the health of their host, must undergo extensive research to ensure they are safe for consumption and possess certain functional properties. Antibiotic resistance in probiotics has raised concern due to the possibility of its transfer to pathogens. Acid and bile tolerance ensures that organisms will survive passage into the intestines. Prebiotic utilization indicates ability to ferment specific carbohydrates for enhanced growth. The objective of this study was to characterize a group of commercial probiotics for their suitability as probiotics.

Nine commercial probiotic strains (7 *Lactobacillus*, 1 *Lactococcus lactis*, and 1 *Bifidobacterium longum*) were evaluated. Two methods, disk diffusion and broth microdilution, were utilized to determine susceptibility to 9 antibiotics (ampicillin, chloramphenicol, clindamycin, erythromycin, gentamycin, kanamycin, oxytetracycline, streptomycin, and vancomycin). Most strains were susceptible to 4 or more antibiotics. Only one strain, *Lactobacillus salivarius*, showed resistance to three antibiotics. The two methods tested were in agreement for 76.8% (63/82) of the bacteria-antibiotic combinations tested.

The cultures were assessed for their ability to utilize four prebiotics: galactooligosaccharides (GOS), two fructooligosaccharide (FOS), and inulin. Glucose was used as a positive control for growth. Galactooligosaccharides were fermented by seven strains and fructooligosaccharides derived from chicory, by two strains. Inulin did not promote significant growth of any of the strains.

Additionally, *Bacillus coagulans* ProDura is a sporeforming bacterium that has recently been identified and marketed as a novel probiotic with a greater ability to survive the low pH of the stomach when in spore form. This organism showed high tolerance to acid and bile conditions, with only a two log reduction after four hours at pH 2.0, and only a one log reduction in bile salts. It was susceptible to all antibiotics tested and was able to utilize GOS and FOS, but not inulin. In conclusion, the strains evaluated comply with the functional and safety characteristics of probiotics, except for the strain of *Lactobacillus salivarius* which demonstrated an unacceptable level of antibiotic resistance.

DEDICATION

To my family; you are my wings and my roots.

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1.1. Lactic acid bacteria

Lactic acid bacteria (LAB) are a group of Gram-positive bacteria that excrete lactic acid as their main fermentation product (1). Typical LAB members are Gram-positive, facultative anaerobic, catalase-negative organisms with low G+C content, of the genera *Lactobacillus*, *Lactococcus*, *Leuconostoc* and *Pediococcus*. Though the genera *Propionobacterium* and *Bifidobacterium* belong to the high G+C branch, they have been grouped with the LAB for practical and ecological reasons (2).

Most LAB have a long history of being consumed as part of traditional fermented foods and have been awarded the status of "Generally Regarded As Safe" (GRAS) by the Food and Drug Administration (FDA) (2). Lactobacilli are naturally present or deliberately added as starter cultures in unpasteurized milk and dairy products such as cheeses, yogurts and fermented milks (3). *Leuconostoc*, *Lactobacillus* and *Pediococcus* species are commonly established in plant material and are essential for the manufacture of fermented vegetable products (e.g. miso, soy sauce, pickled vegetables and kimchi) (4). LAB starters are also used in dry sausage, where they affect the texture, flavor, shelf-life and safety of the product (5).

1.2. Probiotics

Probiotics are defined as cultures of living microorganisms which beneficially affect the health of the host when administered in adequate amounts (2). These microorganisms survive passage through the gastrointestinal tract and eventually establish in the colon.

However, they must be taken regularly and at sufficiently high levels to avoid washout and to ensure sustained benefits (4). Their benefits are related to the prevention of growth of harmful bacteria by competitive exclusion and by the production of organic compounds (5). Associated effects of probiotics include prevention and treatment of diarrhea, alleviation of lactose intolerance, immunomodulation and prevention or alleviation of allergies in children (4, 5).

Global sales of probiotic products reached US$21.6 billion in 2010 and US$24.23 billion in 2011 (6). The global probiotic market is expected to reach US$44.9 billion in 2018 (6). Asia-Pacific is currently the largest probiotic market and is likely to remain the market leader; however, European and North American markets continue to grow (6). In the United States, probiotics are available primarily as capsules or sachet preparations, although food formats are increasing. Unlike Canada and some European countries, the United States has no governmental standards for probiotics, which leads to variable products that do not contain the bacteria or the number of bacteria stated in the product's label (7). Consumer education and clearer regulations are needed for the probiotic market to reach its full potential in the United States.

1.3. Bacterial species used as probiotics

Lactic acid bacteria are normal residents of the human gastrointestinal tract, especially in the colon, where their numbers can be up to 9 log CFU/g (5). The two genera most

commonly used as probiotics are *Lactobacillus* and *Bifidobacterium*. Neither genera includes any significant pathogenic species (8).

In healthy humans, lactobacilli are normally present in the oral cavity (10^3-10^4 CFU/g), the ileum (10^3-10^7 CFU/g), and the colon (10^4-10^8 CFU/g) (9). They are also widely found in raw milk and fermented dairy products, which continue to be the preferred way to market probiotic strains in food products in the United States (7). Beneficial effects of lactobacilli include control of intestinal inflammation, treatment of infections during pregnancy, management of allergic diseases, control of antibiotic-related diarrhea and prevention of urinary tract infections, amongst others (9).

Bifidobacterium are not considered true LAB, given their high G+C content and their production of a combination of lactic and acetic acid. However, they are normal inhabitants of the gastrointestinal tract of humans, making up to 25% of the cultivable fecal bacteria in adults and 80% in infants (8). They are commonly used as probiotics, with a long history of safe use in fermented dairy products. Their positive effects on human health include prevention of infection by pathogenic bacteria, immunostimulatory and anti-carcinogenic capabilities, protection against infectious diarrhea, lowering of serum cholesterol and alleviation of lactose intolerance (10).

Spore forming bacteria, such as *Bacillus coagulans*, *Bacillus racemilacticus* and *Bacillus laevolacticus*, have only recently received attention as potential probiotics. However, many factors make them good candidates for probiotic use, such as the ease of culturing them in bulk, their production of organic acids and their capacity to sporulate (11). Spores are hardy dormant life forms. One of their most important traits is that they are heat stable, so they can be included in products stored at room temperature without losing viability (12). Both *in vitro* and mouse model experiments have shown that *Bacillus coagulans* spores are capable of germinating in the small intestine, where they could arguably have a beneficial effect (13, 14). Certain strains of *Bacillus coagulans* are capable of producing a bacteriocin-like inhibitory substance, coagulin, which has shown *in vitro* activity against *Enterococcus* and *Listeria* (15). Mice studies have shown *Bacillus coagulans* consumption improves some indices of *Clostridium difficile*-induced colitis (16). Human trials suggest a certain strain of this species may have positive effects on functional intestinal gas symptoms and rheumatoid arthritis symptoms (17, 18). However, there are still very few human studies on the effects of *Bacillus* probiotics for them to achieve the same level of acceptance as lactobacilli and *Bifidobacterium* probiotics.

1.4. Properties of probiotics

The Food and Agriculture Organization (FAO) and the World Health Organization (WHO) released a joint report in 2002 for the evaluation of probiotics. To be classified as probiotics, strains must be identified by phenotypic and genotypic methods, since many

probiotic effects are strain-specific. Then, they should go through a functional characterization and a safety assessment, both *in vitro* and with animal studies, before being tested for efficacy in a double blind, randomized, placebo-controlled human trial (19). A second independent trial is preferred before the strain can be considered a probiotic and be added to food products.

1.4.1. Functional properties of probiotics

The beneficial effects of probiotics depend on their colonization of the gut and their effect on harmful bacteria, for which certain functional properties are necessary. Probiotics must survive gastric and bile acids in order to reach the intestinal tract (11). Once there, they must be capable of adhering to human epithelial cells. Lastly, they should prevent colonization by pathogenic bacteria, either by immune exclusion, competitive adhesion or synthesis of antimicrobial substances (13, 19).

1.4.1.1. Acid and bile resistance

Probiotic strain selection can start with screening for acid and bile resistance. The pH of excreted hydrochloric acid in the stomach is 0.9. However, the presence of food raises the pH value to around pH 3 (5). The ability to survive and grow in a low pH environment is characteristic of LAB, although their tolerance mechanism is not clarified yet. (20) Bile salts, on the other hand, are released into the small intestine after ingestion of fatty foods. They have a detergent-like function, which may disrupt the lipids and fatty acids of

bacterial cell membranes (21). Certain microorganisms, including several species of *Lactobacillus*, can reduce this detergent effect by hydrolyzing bile salts with the bile salt hydrolase (BSH) enzyme (5, 20).

In vivo methods are available for investigating the survival of probiotic bacteria in the human gut. As these methods are expensive, laborious and pose ethical constraints, *in vitro* methods are preferred for the first selection of strains (14). In these *in vitro* methods, cultures are usually exposed to acid conditions (pH 2 to 3) or bile presence (0.3% w/v) in broth for up to four hours. Growth or survival is then monitored (21).

Bacillus species generate dormant spores resistant to heat, desiccation, enzymatic degradation and acidic conditions (22). This increases their potential as probiotics for human or animal use. *In vitro* experiments have shown that spores from different strains of *Bacillus coagulans*, *Bacillus laevolacticus* and *Sporolactobacillus* are able to tolerate pH conditions as low as 2.0 to a varying degree, according to strain and environment conditions (11, 14, 22–24). Similar tests have been performed for bile resistance, with only a slight loss of viability for most strains of *Bacillus* tested (11, 14, 22). Germination of *Bacillus subtilis* spores in the intestine of mice has been reported (25), which is necessary as only the vegetative cells are able to have a probiotic effect.

1.4.1.2. Prebiotic utilization

Prebiotics are defined as non-digestible food ingredients that beneficially affect the host by selectively stimulating the growth and/or the activity of one or a limited number of bacteria in the colon that have the potential to improve host health (8, 10). This is based on the knowledge that the availability of carbohydrates that escape metabolism and adsorption in the small intestine has a major influence on the microflora that becomes established in the colon (26). The main prebiotics used are carbohydrates such as resistant starch, wheat bran, inulin or oligosaccharides (27).

Inulin is a natural component of several fruits and vegetables. It is a mixture of fructooligosaccharides and fructopolysaccharides (28). Oligosaccharides are short polymers of glycosidic residues such as fructose in fructooligosaccharides (FOS) or galactose in galactooligosaccharides (GOS) (27). FOS is the most commonly used commercial prebiotic, which is a mixture of oligosaccharides containing a varying number of fructose moieties connected by β(2→1) glycosidic bonds. GOS originates from enzymatic transgalactosylation of lactose (10). These soluble and fermentable fibers cannot be digested by α-amylase or other hydrolases in the upper section of the intestinal tract and they also resist digestion by gastric acid and pancreatic enzymes (29).

To exert a prebiotic effect, fibers have to be present in foods in amounts of 30-60 mg/g in solid foods and 15 mg/g in liquid foods (30). The effect will also depend on the actual

number of beneficial bacteria, such as bifidobacteria, in the host, which has led to development of products that combine both a probiotic and a prebiotic (28). Prebiotics by themselves as well as their symbiotic combination with probiotic bacteria have been shown to increase bifidobacteria and lactobacilli populations and to inhibit various human and animal pathogenic bacteria *in vitro*, or in mice, piglets or humans (28). The addition of prebiotics to products containing probiotics can also protect and stimulate growth of lactobacilli and bifidobacteria during the product's shelf life (31, 32). However, it is important to determine whether the probiotic strain is capable of metabolizing the prebiotic.

1.4.2. Safety properties of probiotics

There has always been a level of concern about the safe use of lactic acid bacteria as probiotics. The following safety criteria have been proposed: strains intended for human use should have a human origin and be isolated from healthy human gastrointestinal tract and they need to have a non-pathogenic history, not associated with diseases (33). Now, it is considered necessary to also establish the absence of transmissible antibiotic resistance genes.

1.4.2.1. Antibiotic resistance

According to the WHO (34), antimicrobial resistance (AMR) is "the resistance of a microorganism to an antimicrobial medicine to which it was originally sensitive". The

genes that code for resistance are components of natural microbial populations and the exposure to antibiotics exerts a selective pressure, favoring the microorganisms capable of surviving (35). Humans encourage the spread of resistant strains by the misuse of microbial medicines and by poor infection control practices (34).

Awareness of this fact has encouraged major official bodies, such as the European Union (EU), the Centers for Disease Control and Prevention (CDC) and the WHO, to address the need of controlling the increase in resistance (36). Most agencies agree that a global strategy is needed and that it should include comprehensive surveillance of antibiotic resistant organisms and their associated infections; improved control and monitoring of antibiotic use in animals and humans; and increased research in the area of resistance mechanism identification and antibacterial drug product development (34, 37, 38).

Antimicrobial susceptibility testing allows the detection of possible drug resistance in common pathogens and commensals and to identify the most appropriate drug treatment for patients (39). Conventional phenotypic methods are based on the assessment of the growth of bacteria when exposed to the antimicrobial of interest. The results are then compared to interpretative criteria provided by responsible organizations, such as the Clinical and Laboratory Standards Institute (CLSI; formerly the National Committee for Clinical Laboratory Standards, or NCCLS) in the United States and the European Union Committee for Antimicrobial Susceptibility Testing (EUCAST) in Europe (40).The most

commonly used methods are broth dilution tests, antimicrobial gradient methods and the disk diffusion test (39).

The known mechanisms of antibiotic resistance can be broadly classified into four classes: 1) lack or decrease of cell wall permeability, which limits or prevents the intake of the drug; 2) efflux mechanisms, which allow the bacteria to pump out the antibiotic from the cell before it reaches an effective concentration; 3) enzymatic deactivation mechanisms, in which inactivating enzymes alter the antibiotic's chemical structure to render it useless; and 4) modification of target mechanisms, in which the target site of the antibiotic has been altered so it retains its function in the cell but the antibiotic can no longer bind to it (41). Certain mechanisms, such as lack of cell wall permeability or the total absence of the target site, are more likely to be inherent to a bacterial species or genus; therefore this type of resistance is classified as intrinsic or natural (Figure 1.1). However, strains belonging to a group naturally susceptible to an antibiotic can acquire resistance through gain of exogenous DNA or by mutation of indigenous genes (42).

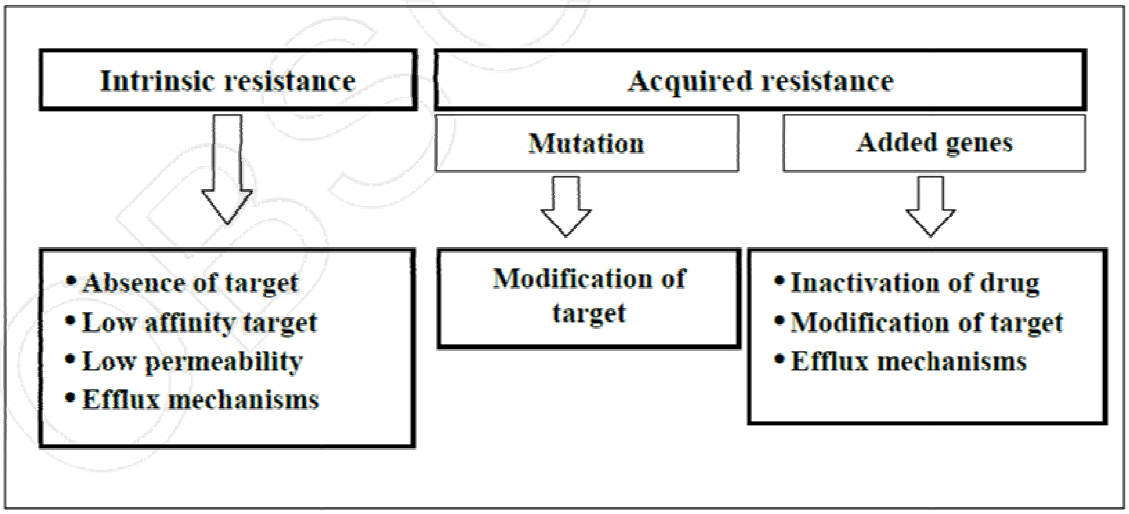

Figure 1.1 Major mechanisms of intrinsic and acquired resistance (42).

Both intrinsic resistance and acquired resistance by chromosomal mutation have a low risk of horizontal dissemination between different bacterial species. In contrast, acquired resistance has a higher potential for transference when the genes are present on mobile genetic elements, such as plasmids and transposons (43). Determinants on these mobile elements move between diverse bacteria and disseminate resistance genes into a variety of microbial communities (35)

1.4.2.2. Antibiotic resistance in LAB

Researchers in the last decade have discovered the presence of antibiotic resistance genes in LAB from different sources. Herreros *et al*. (44) found multiple drug resistance in LAB isolated from Armada cheese, a Spanish goat milk cheese. Masco *et al*. (45)

discovered tet(W) genes responsible for tetracycline resistance in 15 *Bifidobacterium* strains, including 7 probiotic isolates. Surveys of antibiotic resistance phenotypes and determinants have been conducted with lactic acid bacteria strains of European, African and Asian origin and resistance genes have been found in all of them (46–49). There is very little published information about American strains.

However, it is important to distinguish between intrinsic and acquired resistance. For example, enterococci are intrinsically resistant to cephalosporins and low levels of aminoglycosides; while lactobacilli, pediococci and *Leuconostoc* spp. have a high natural resistance to vancomycin (1). There are both intergenus and interspecies differences. Danielsen and Wind (50) surveyed 62 strains of *Lactobacillus* and found that susceptibility varied several folds between species for drugs such as vancomycin, tetracycline and clindamycin. These results emphasize the importance of surveillance and publication of resistance profiles of LAB and other commensal bacteria of industry interest for the development of safety guidelines for their commercial use.

The European Food Safety Authority (EFSA), for example, took published data about resistance profiles into account for the development of its safety assessment scheme (Figure 1.2). This assessment must be done for all strains aiming to be used as additives in the European Union and represents a complete framework for the characterization of resistance of any microorganism (42).

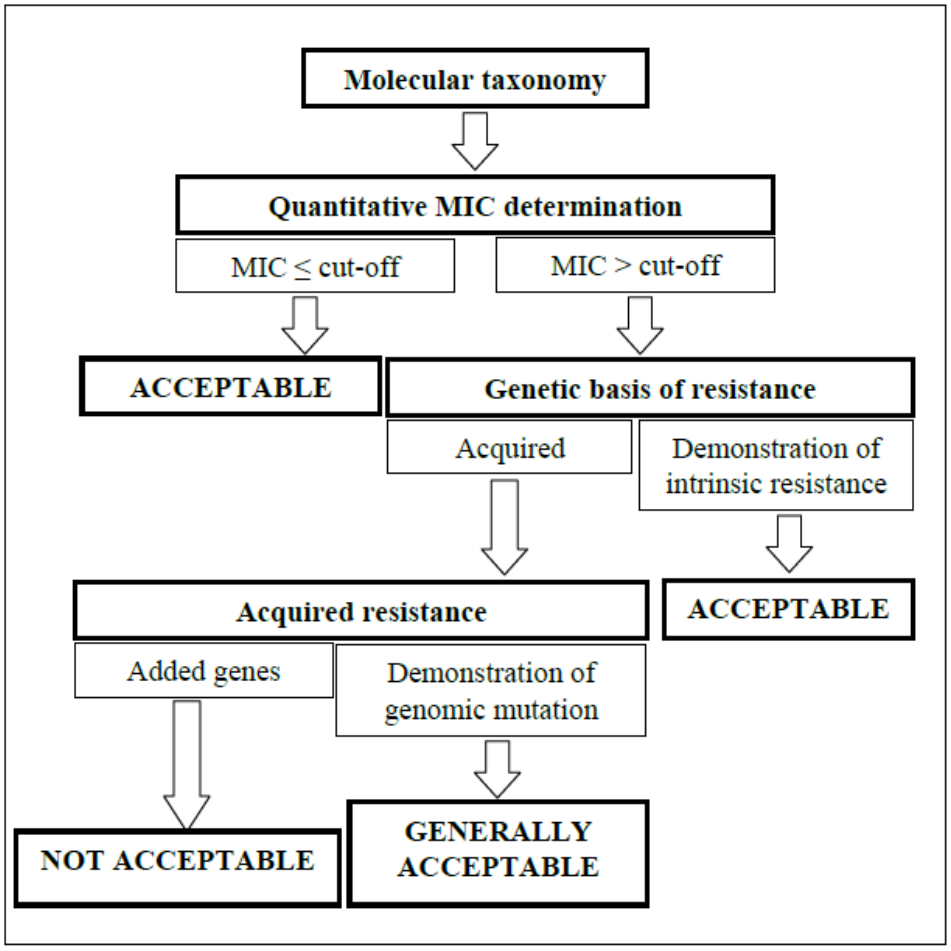

Figure 1.2 EFSA proposed scheme for antimicrobial resistance assessment of a bacterial strain used as feed or food additive (42).

In this proposed scheme, cut-off values are set by studying the distribution of minimum inhibitory concentrations (MICs) of the antimicrobials in bacterial populations of the same species or genus (42). Data about the distribution of MICs is derived from the published body of research and monitoring programs and may be updated as more information becomes available (51). Therefore, resistant strains are those for which MICs

clearly deviate from those of normal susceptible populations. EFSA recognizes nine antibiotics as antimicrobials of human and veterinary importance; susceptibility to these substances is considered a basic requirement for bacterial products intended for use as food and feed additives (Table 1.1) (51).

Table 1.1 Antibiotics recognized as having human and veterinary importance by EFSA (51).

Mechanism of action	Antibiotic Family	Antibiotics of Importance
Inhibition of cell wall synthesis	Penicillins	Ampicillin
	Glycopeptides	Vancomycin
Inhibition of protein synthesis	Aminoglycosides	Gentamycin
		Kanamycin
		Streptomycin
	Macrolides	Erythromycin
	Lincosamides	Clindamycin
	Tetracyclines	Tetracycline
	Single antibiotics	Chloramphenicol

The characterization of potentially probiotic strains is a comprehensive process. Beyond health properties, safety and functionality are also important. The bacteria should be able to survive processing, storage and digestion conditions. Acid and bile resistance is essential for its delivery to the small intestine, where it can exert its positive effect. Prebiotic utilization allows for the formulation of synbiotic products that can protect bacteria during the product's shelf life and increase their activity in the intestinal tract. Most importantly, probiotic strains should be safe for human consumption and inclusion

in the food chain. The definition of a safe strain has expanded to include the absence of antibiotic resistance genes, given the alarming increase of resistance in pathogenic and commensal bacteria worldwide.

Both safety and functional characteristics can be specific to a certain species or a certain strain of LAB. This makes it necessary to characterize each new strain intended for probiotic use, especially emerging species like *Bacillus coagulans*. Therefore, the current research focused on characterizing the functional and safety properties of ten probiotic strains, aiming to determine their ability to utilize prebiotics and their antibiotic resistance. This will contribute to their proper use in the food industry and to the general knowledge about these species.

- ObjectivesTo characterize the prebiotic utilization profile of ten probiotic strains in order to suggest potential synbiotic combinations.
- To evaluate the antibiotic susceptibility of ten probiotic strains, by both the broth microdilution and the disk diffusion method, to determine the safety of the strains and compare the performance of both methods.
- To determine the *in vitro* acid and bile tolerance of *Bacillus coagulans* to predict its survival in the gastrointestinal tract.

CHAPTER 2 : MATERIALS AND METHODS

2.1. Probiotic strains

Ten bacterial strains were obtained from a commercial probiotics supplier (Nebraska Cultures, Walnut Creek, CA) (Table 2.1). Control strains were purchased from the American Type Culture Collection (ATCC, Manassas, VA). The freeze-dried cultures were diluted 1:99 in Phosphate Buffered Saline (PBS; Sigma, St. Louis, MO) and 5 µl were streaked in appropriate media. Tryptic Soy Agar (TSA, Acumedia, Neogen Corporation, Lansing, MI) was used for *Escherichia coli* ATCC 25299 and *Streptococcus pneumoniae* ATCC 49619. De Man Rogosa Sharpe agar (MRS, Acumedia, Neogen Corporation, Lansing, MI) was used for all *Lactobacillus*, *Lactococcus*, *Bifidobacterium* and *Bacillus*. Plates were then incubated for 24 hours at 37°C. Plates were incubated anaerobically with the exception of *E. coli* ATCC 25922 and *S. pneumoniae* ATCC 49619. Single colonies were picked and a Gram stain was performed. Colonies with the expected morphology were grown overnight at 37°C, either in Tryptic Soy Broth (TSB, Acumedia, Neogen Corporation, Lansing, MI) or MRS broth (Acumedia, Neogen Corporation, Lansing, MI). Sterile glycerol at 7% (v/v) was added to the cultured broth and 1-ml aliquots were stored at -80°C. Isolates were then sent for identification by 16s sequencing (Midi Labs, Newark, DE).

Table 2.1 Bacterial strains used.

Probiotic strains	Control strains
Lactobacillus acidophilus DDS 1-10	*Escherichia coli* ATCC 25922
Lactobacillus brevis	*Streptococcus pneumoniae* ATCC 49619
Lactobacillus delbrueckii subsp. *bulgaricus*	
Lactobacillus casei	
Lactobacillus plantarum	
Lactobacillus rhamnosus	
Lactobacillus salivarius	
Lactococcus lactis	
Bifidobacterium longum	
Bacillus coagulans ProDURA	

2.2. Prebiotic utilization by lactic acid bacteria

Probiotic bacterial cultures were prepared by streaking 10 µL of stock culture into MRS plates and incubating overnight at 37°C. A single colony was then picked and transferred to 12 mL of MRS broth and incubated overnight at 37°C. For this assay, only Bifidobacteria were incubated anaerobically, in both plates and tubes.

MRS broth without any sugar (basal MRS) was prepared and autoclaved. Sugar solutions were prepared, sterilized through a syringe filter (mixed cellulose ester membrane, 0.22 µm pore size, Fisherbrand, Fisher Scientific, Waltham, MA) and added to the sterile medium to achieve the desired concentration (Table 2.2). Prebiotics used included two fructooligosaccharide mixtures from different suppliers (FOS, Nutraflora, GTC Nutrition

Company, Bridgewater, NJ and Orafti P95, Beneo, Manheim, Germany), one galactooligosaccharide mixture (GOS, Purimune, GTC Nutrition Company, Bridgewater, NJ), and one inulin mixture (Orafti Synergy 1, Beneo, Manheim, Germany). Since all of these prebiotics contain residual simple sugars, a background sugar (bg) control was prepared for each one of them. Glucose was used as a positive control. Glucose and the prebiotic sugars were added to the media for a final concentration of 1% (w/v).

MRS solutions were dispensed into sterile tubes and inoculated with the bacterial cultures at a 5% (v/v). Absorbance at 620 nm was read every two hours using a Biomate 3 spectrophotometer (Thermo Scientific, Thermo Fisher Scientific, Waltham, MA). The corresponding sterile MRS solutions were used as blanks for absorbance measurements. The aliquot removed for absorbance measurements was diluted with the corresponding sterile MRS solution when absorbance was higher than 0.500. Growth curves were prepared from the absorbance measurements. Doubling time during the exponential phase was determined, as well as the maximal variation of absorbance (Figure 2.1). Doubling time was defined as the time needed for absorbance to double during the exponential phase of growth, as observed in the growth curves. Maximal variation of absorbance was calculated by subtracting the initial absorbance from the highest observed absorbance. The experiments were performed in triplicate.

Table 2.2 Sugar and prebiotic concentrations tested.

Treatment	Sugars	Final concentration (%, w/v)
Positive control	Glucose	1.0%
Orafti P95 Fructooligosaccharide (FOS)	Mixture of FOS (93%), glucose, fructose and sucrose	0.93% FOS 0.0175% glucose 0.0175% fructose 0.035% sucrose
Orafti FOS background sugars (bg)	Mixture of glucose, fructose and sucrose	0.0175% glucose 0.0175% fructose 0.035% sucrose
Nutraflora FOS	Mixture of FOS (95%), glucose, fructose and sucrose	0.95% FOS 0.0125% glucose 0.0125% fructose 0.025% sucrose
Nutraflora FOS bg	Mixture of glucose, fructose and sucrose	0.0125% glucose 0.0125% fructose 0.025% sucrose
Purimune Galactooligosaccharide (GOS)	Mixture of GOS (93%) and lactose	0.93% GOS 0.07% lactose
GOS bg	Lactose	0.07% lactose
Orafti Sinergy 1 Inulin	Mixture of inulin (92%), glucose, fructose and sucrose	0.92% inulin 0.02% glucose 0.02% fructose 0.04% sucrose
Inulin bg	Mixture of glucose, fructose and sucrose	0.02% glucose 0.02% fructose 0.04% sucrose

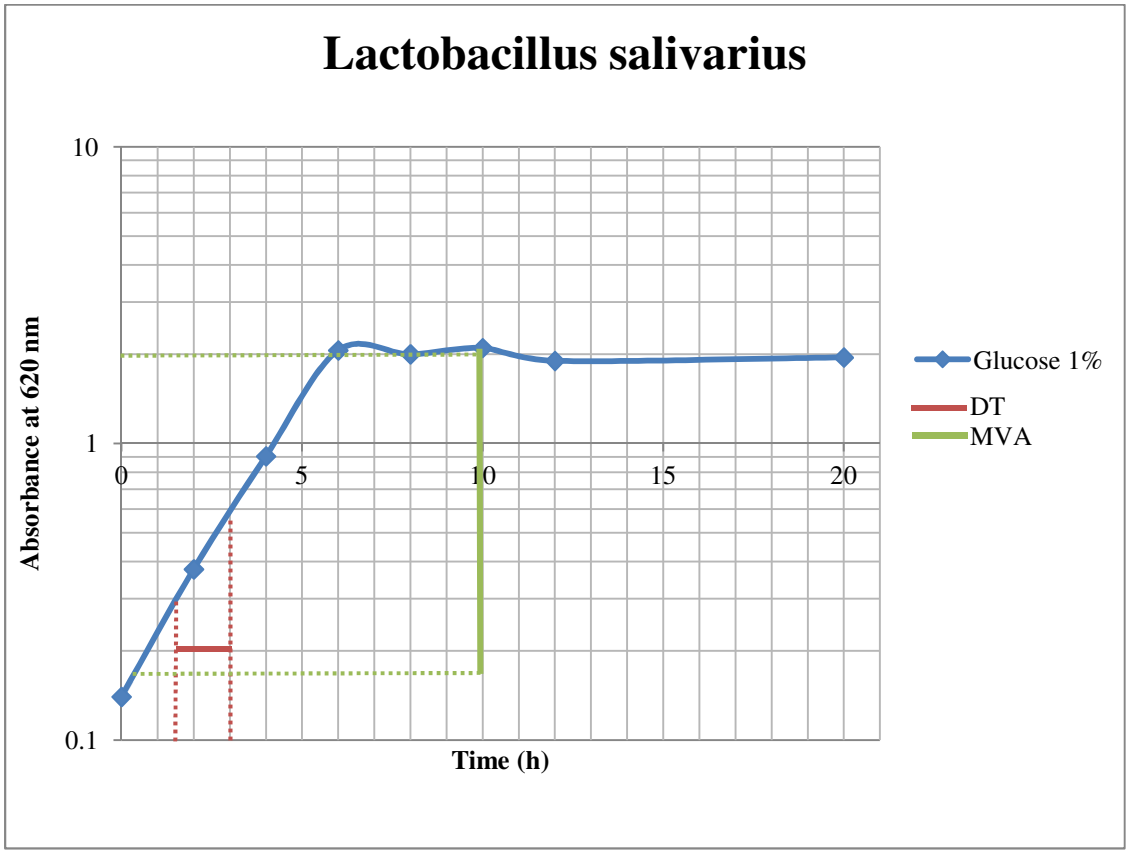

Figure 2.1 Determination of doubling time (DT) and maximal variation of absorbance (MVA) from probiotic growth curves.

2.3. Antibiotic resistance

2.3.1. Broth microdilution method for antibiotic resistance

The procedure for broth microdilution was adapted from the CLSI protocol for antimicrobial susceptibility testing (52). Antibiotic solutions were prepared in LAB susceptibility media (LSM, 9:1 Isosensitest broth: MRS broth, Oxoid, Acumedia). The following antibiotics were tested: ampicillin, chloramphenicol, clindamycin, erythromycin, gentamycin, kanamycin (Sigma Aldrich, St. Louis, MO), oxytetracycline (Acros Organics, Geel, Belgium), streptomycin (MP Biomedicals, Santa Ana, CA) and

vancomycin (Fisher Scientific, Waltham, MA). The range of concentrations tested varied for each antibiotic according to the microbiological breakpoints (bp) defined by EFSA (51).

Cultures for inoculation were grown overnight by mixing 100 µL of stock culture into 9 mL of MRS broth and incubating anaerobically at 37°C overnight. Control strains were grown in TSB broth and incubated aerobically. The absorbance of culture tubes was then adjusted to 0.125-0.132 at 620 nm, the equivalent to a 0.5 McFarland standard (5×10^5 CFU/ml). Absorbance was measured with a Spectronic 20D+ spectrophotometer (Spectronic Instruments, Thermo Fisher Scientific, Waltham, MA), using MRS broth as a blank. The antibiotic solutions were used to prepare a 1:200 dilution of bacteria inoculum.

The mixture of antibiotic solution and inoculum was dispensed into a 96-well plate, with 300 µL per well. Absorbance at 600 nm was measured with iMark Micro Plate Reader (Bio-Rad, Hercules, CA) at 0, 24 and 48 hours of incubation at 37°C. *E. coli* and *S. pneumoniae* controls were incubated aerobically at 37°C. Growth curves were prepared so that growth at the breakpoint could be compared with growth of the positive control to examine susceptibility. For this experiment, it was considered that absorbance of 0.200 or greater corresponds with growth visible to the naked eye. The experiments were performed in triplicate.

2.3.2. Disk diffusion method

The disk diffusion method was a modification of the agar overlay method documented by Charteris *et al*. (53). Plates with 15 ml of MRS agar were overlaid with 4 ml of soft agar containing 200 µL of active culture, prepared as described for the broth microdilution assays. Antibiotic disks (Becton Dickinson, BD, Franklin Lakes, NJ) with the following amounts of the active compound were placed into the dry plates: ampicillin (10 µg), chloramphenicol (30 µg), clindamycin (2 µg), erythromycin (15 µg), gentamycin (10 µg), kanamycin (30 µg), oxytetracycline (30 µg), streptomycin (10 µg), and vancomycin (30 µg). Plates were then incubated anaerobically at 37°C for 24 h. *E. coli* and *S. pneumoniae* controls were incubated aerobically at 37°C. Inhibition zone diameters were measured with a colony counter (Flash and Go, IUL Instruments, Neutec Group Inc, Farmingdale, NY) and interpreted according to the guidelines provided by Charteris *et al*. (53). Strains were classified as susceptible or resistant. The experiments were performed in triplicate.

2.4. Acid and bile tolerance of *Bacillus coagulans*

Freeze-dried spores of *Bacillus coagulans* (about 2.00 x 10^{11} CFU/g) were diluted in PBS to obtain a suspension with 1 x 10^{8} spores/mL. To ensure only spores were present, spore suspensions were heat treated at 80°C for 12 minutes and then chilled in ice. The method for acid and bile resistance was modified from Hyronimus *et al.* (11). Tubes with 10 mL of MRS broth were adjusted to pH 2.0, 2.5 and 3.0 using 3.0 M hydrochloric acid (Sigma Aldrich, St. Louis, MO). MRS with 0.3% bile (Sigma Aldrich, St. Louis, MO) was also

prepared and 10 ml of the broth was dispensed into tubes. MRS broth without addition of acid or bile salts was used as a control. A 10 µL aliquot of the spore suspension was then added to each tube. Counts were performed by plating on MRS agar at times 0, 0.5, 1, 2, and 4h. Plates were incubated aerobically overnight at 37°C. Survival curves were prepared for each treatment and the area under the curve was compared to the control. Survival rates were also calculated, as the percent of the initial population that could be recovered after four hours (log CFU/ml at 4 h divided log CFU/ml at 0 h * 100%). The experiment was performed in triplicate.

2.5. Statistical analysis

Analysis of variance was used to evaluate the effect of different treatments in the acid and bile assay and in the prebiotic utilization assay. The analysis was conducted on the mean doubling time and maximal absorbance variation for the prebiotic growth curves and for the area under the curve for the acid and bile survival curves, with treatment as the independent variable. Statistical analysis was carried out using SAS 9.3 (SAS, Inc, Cary, NC). All tests were conducted at the 5% level of significance.

CHAPTER 3 : RESULTS AND DISCUSSION

3.1 Probiotic strain identification

The identity of the probiotic strains was confirmed by 16s sequencing (MidiLabs, Newark, DE). The reports can be found in Appendix A.

3.2 Prebiotic utilization by lactic acid bacteria

The capacity of nine strains to use prebiotics as a carbon source was evaluated during growth for 24 hours. Glucose was used as a positive control for growth. The background sugars in each prebiotic were also evaluated, to establish their effect on growth and rule out their interference. The mean doubling time during the exponential phase of growth and the maximal absorbance variation were calculated for each strain and prebiotic. The results for lactobacilli (*Lb.*), *Lactococcus lactis* and *Bifidobacterium longum* (*Bb. longum*) are presented in Table 3.1 and Table 3.2. Figures 3.1 to 3.9 show the average of three replicates of the experiment for each strain. Results for *Bacillus coagulans* are included in its particular section.

Table 3.1 Mean doubling time of probiotic strains on selected carbohydrates.

	Mean Doubling Time (h, average ± standard deviation)[1]								
Treatment	*Lb. acidophilus*			*Lb. brevis*			*Lb. casei*		
Glucose	1.5	± 0.4	a	1.4	± 0.3	a	1.1	± 0.2	a
Purimune GOS	1.8	± 0.7	a	1.7	± 0.4	a	1.6	± 0.7	a
Purimune GOS bg[2]	2.2	± 0.9	a	2.0	± 0.3	a	1.6	± 0.6	a
FOS Orafti	1.9	± 0.6	a	1.6	± 0.4	a	1.7	± 0.6	a
FOS Orafti bg	1.7	± 0.5	a	1.7	± 0.4	a	1.9	± 0.9	a
FOS Nutraflora	1.9	± 0.5	a	1.9	± 0.4	a	1.7	± 0.7	a
FOS Nutraflora bg	2.0	± 0.3	a	1.9	± 0.4	a	2.0	± 1.1	a
Inulin	1.9	± 0.4	a	1.8	± 0.3	a	1.8	± 0.8	a
Inulin bg	1.9	± 0.4	a	1.8	± 0.3	a	1.8	± 0.8	a
Treatment	*Lb. delbrueckii*			*Lb. plantarum*			*Lb. rhamnosus*		
Glucose	1.6	± 0.6	a	1.5	± 0.5	a	1.1	± 0.2	a
Purimune GOS	1.5	± 1.0	a	1.5	± 0.4	a	1.6	± 0.4	a,b
Purimune GOS bg	1.8	± 1.4	a	2.4	± 0.6	a,b	2.9	± 0.5	a,b,c
FOS Orafti	1.8	± 1.5	a	3.1	± 0.8	a,b	3.1	± 0.4	b,c
FOS Orafti bg	1.7	± 1.0	a	3.7	± 0.8	b	4.4	± 1.0	c
FOS Nutraflora	1.5	± 0.8	a	1.8	± 0.5	a,b	3.4	± 0.7	b,c
FOS Nutraflora bg	1.6	± 1.1	a	3.5	± 0.9	a,b	3.2	± 0.8	b,c
Inulin	1.6	± 1.1	a	2.8	± 0.7	a,b	2.4	± 0.4	a,b
Inulin bg	1.7	± 1.1	a	2.9	± 0.6	a,b	3.1	± 0.2	a,b,c
Treatment	*Lb. salivarius*			*L. lactis*			*Bb. longum*		
Glucose	2.2	± 0.5	a	2.5	± 0.7	a	1.9	± 0.3	a
Purimune GOS	2.4	± 0.5	a	2.6	± 0.9	a	3.7	± 0.6	a,b,c
Purimune GOS bg	4.4	± 0.7	a,b,c	3.1	± 1.0	a	3.3	± 0.8	a,b,c
FOS Orafti	5.6	± 0.8	c	3.5	± 0.9	a	2.9	± 0.6	a,b,c
FOS Orafti bg	5.0	± 0.9	c	4.4	± 1.0	a	4.4	± 0.7	b,c
FOS Nutraflora	2.8	± 0.6	a,b	4.0	± 1.1	a	4.5	± 0.5	c
FOS Nutraflora bg	5.7	± 0.8	b,c	3.6	± 1.0	a	2.9	± 1.0	a,b,c
Inulin	3.8	± 0.8	a,b,c	3.3	± 1.0	a	2.0	± 0.6	a,b
Inulin bg	4.6	± 1.0	a,b,c	3.4	± 0.6	a	4.0	± 0.8	a,b,c

[1] Mean doubling times with a different letter are significantly different ($p<0.05$) from doubling times for the same strain with other carbohydrates.

[2] bg: Background sugars.

Table 3.2 Maximal absorbance variation of probiotic strains on selected carbohydrates.

	Maximal absorbance variation (average ± standard deviation)[1]								
Treatment	*Lb. acidophilus*			*Lb. brevis*			*Lb. casei*		
Glucose	0.28	±0.12	a	1.97	±0.30	a	1.81	±0.03	a
Purimune GOS	0.29	±0.15	a	1.39	±0.22	a,b	0.67	±0.02	b
Purimune GOS bg[2]	0.28	±0.13	a	0.94	±0.09	b	0.43	±0.06	c
FOS Orafti	0.21	±0.03	a	1.06	±0.13	b	0.34	±0.09	c
FOS Orafti bg	0.27	±0.16	a	1.02	±0.08	b	0.33	±0.08	c
FOS Nutraflora	0.28	±0.12	a	0.98	±0.21	b	0.30	±0.08	c
FOS Nutraflora bg	0.21	±0.07	a	0.96	±0.07	b	0.23	±0.05	c
Inulin	0.31	±0.18	a	1.17	±0.16	b	0.31	±0.07	c
Inulin bg	0.28	±0.17	a	1.16	±0.05	b	0.33	±0.09	c
Treatment	*Lb. delbrueckii*			*Lb. plantarum*			*Lb. rhamnosus*		
Glucose	2.77	±0.72	a	1.75	±0.03	a	1.63	±0.23	a
Purimune GOS	2.23	±0.51	a,b	1.49	±0.03	b	1.14	±0.17	b
Purimune GOS bg	1.56	±0.26	b	0.56	±0.09	d	0.51	±0.06	c
FOS Orafti	1.28	±0.19	b	0.45	±0.02	d	0.40	±0.03	c
FOS Orafti bg	1.31	±0.22	b	0.51	±0.08	d	0.38	±0.02	c
FOS Nutraflora	1.69	±0.37	a,b	0.94	±0.07	c	0.38	±0.06	c
FOS Nutraflora bg	1.22	±0.19	b	0.45	±0.04	d	0.39	±0.05	c
Inulin	1.42	±0.20	b	0.55	±0.05	d	0.39	±0.04	c
Inulin bg	1.42	±0.13	b	0.56	±0.08	d	0.41	±0.05	c
Treatment	*Lb. salivarius*			*L. lactis*			*Bb. longum*		
Glucose	2.15	±0.25	a	0.59	±0.12	a	2.19	±0.00	a
Purimune GOS	1.41	±0.07	b	0.56	±0.09	a,b	1.62	±0.13	b
Purimune GOS bg	0.58	±0.07	d	0.29	±0.04	c	0.38	±0.06	c
FOS Orafti	0.45	±0.02	d	0.29	±0.08	c	0.44	±0.29	c
FOS Orafti bg	0.46	±0.06	d	0.30	±0.07	b,c	0.40	±0.07	c
FOS Nutraflora	1.04	±0.06	c	0.28	±0.06	c	0.39	±0.16	c
FOS Nutraflora bg	0.53	±0.07	d	0.29	±0.08	b,c	0.28	±0.11	c
Inulin	0.65	±0.09	d	0.30	±0.08	b,c	0.46	±0.08	c
Inulin bg	0.54	±0.02	d	0.31	±0.07	b,c	0.49	±0.13	c

[1] Maximal absorbance variations with a different letter are significantly different ($p<0.05$) from maximal absorbance variations for the same bacteria with other carbohydrates.

[2] bg: Background sugars.

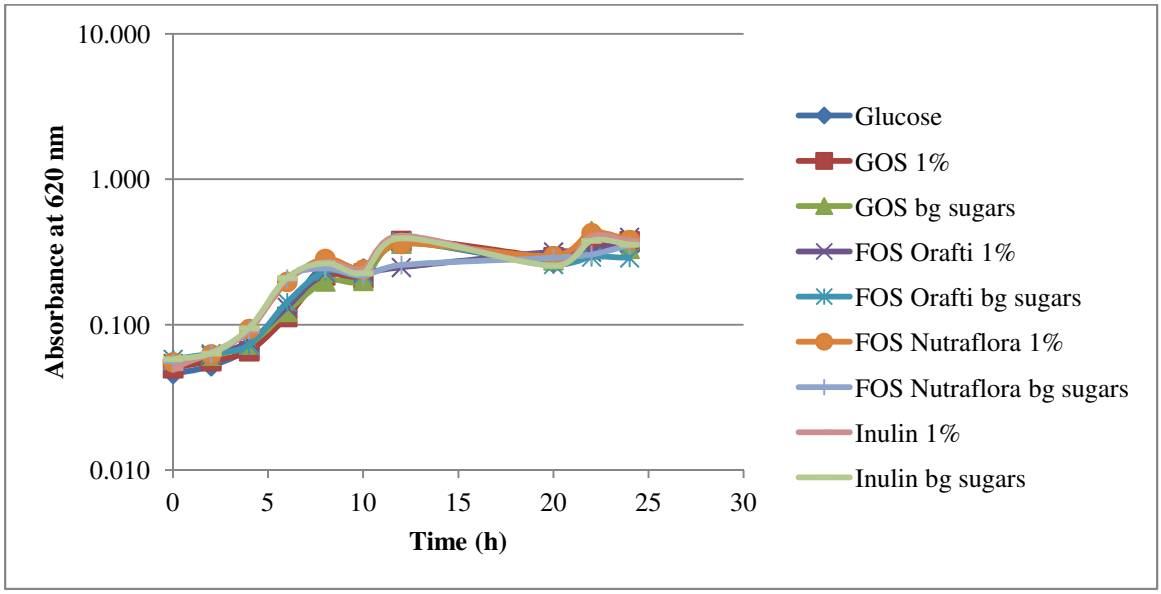

Figure 3.1 Growth of Lactobacillus acidophilus DDS 1-10 on selected carbohydrates.

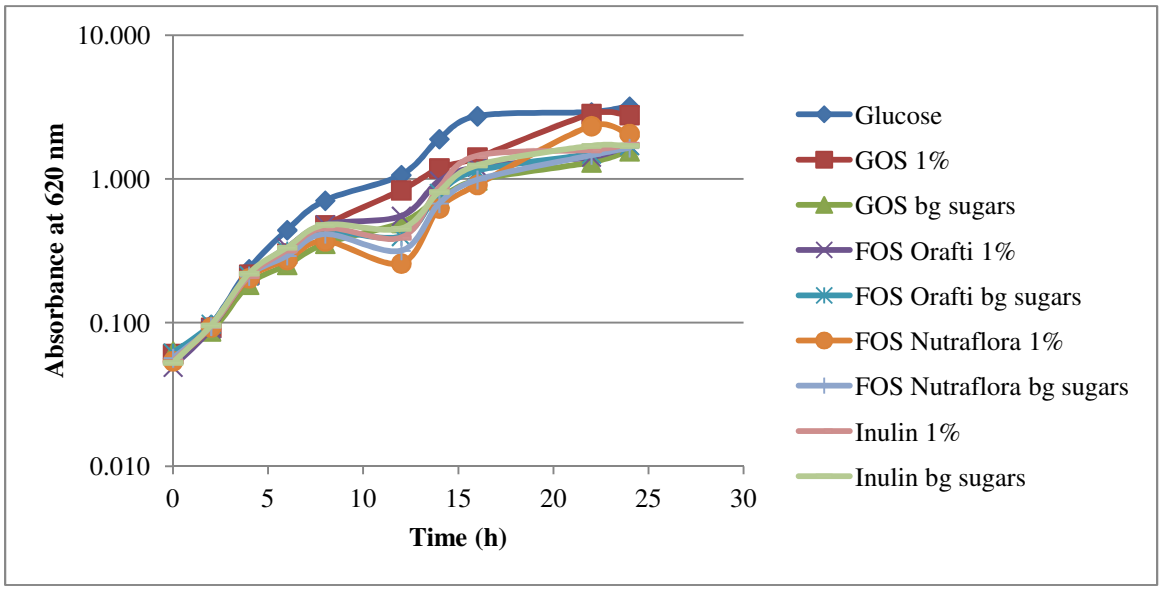

Figure 3.2 Growth of Lactobacillus brevis on selected carbohydrates.

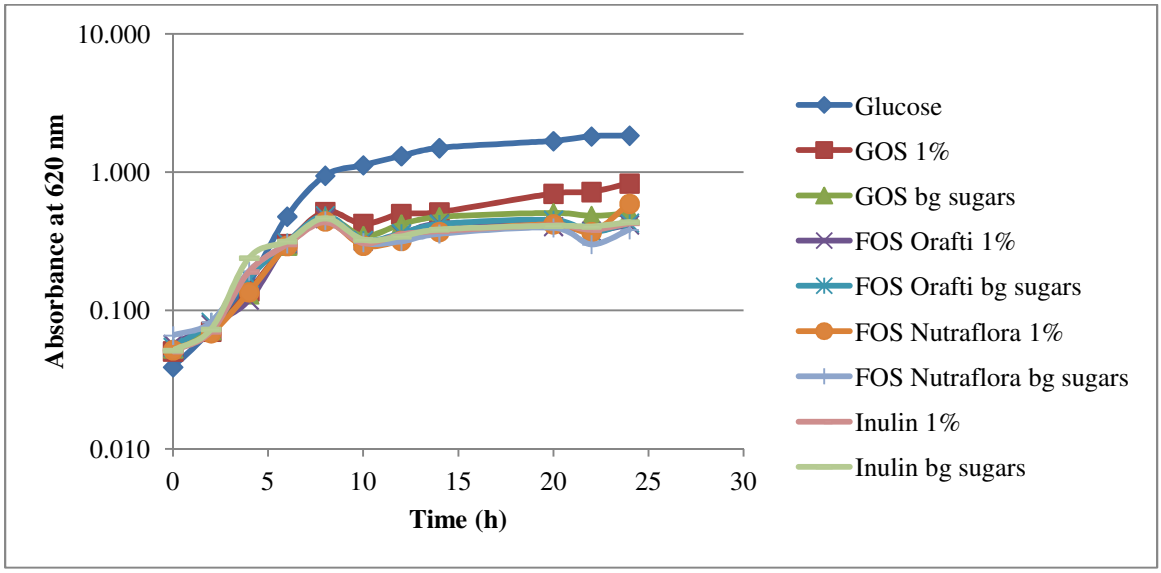

Figure 3.3 Growth of Lactobacillus casei on selected carbohydrates.

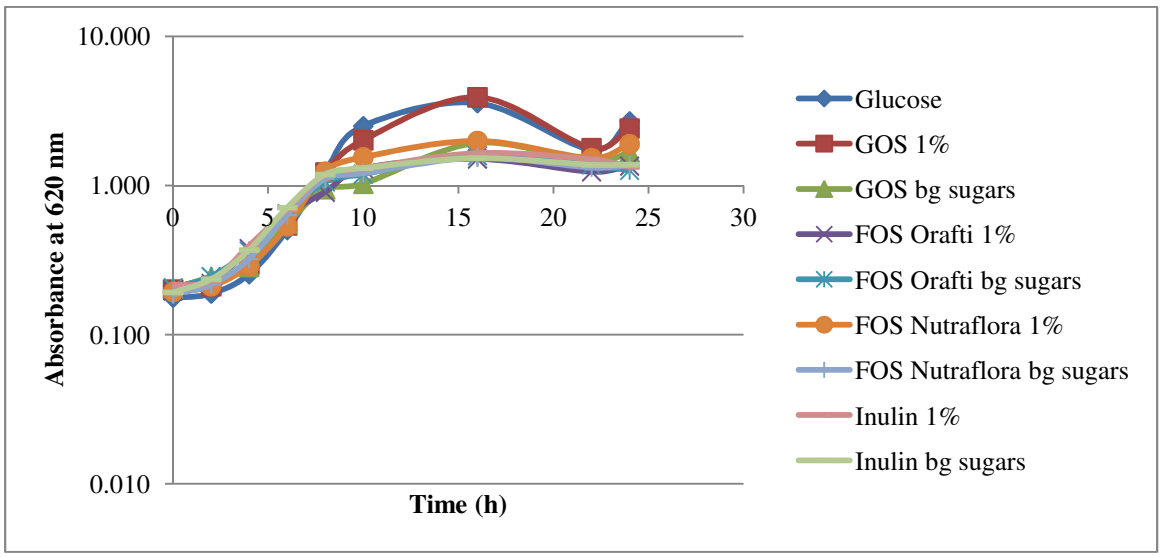

Figure 3.4 Growth of Lactobacillus delbrueckii on selected carbohydrates.

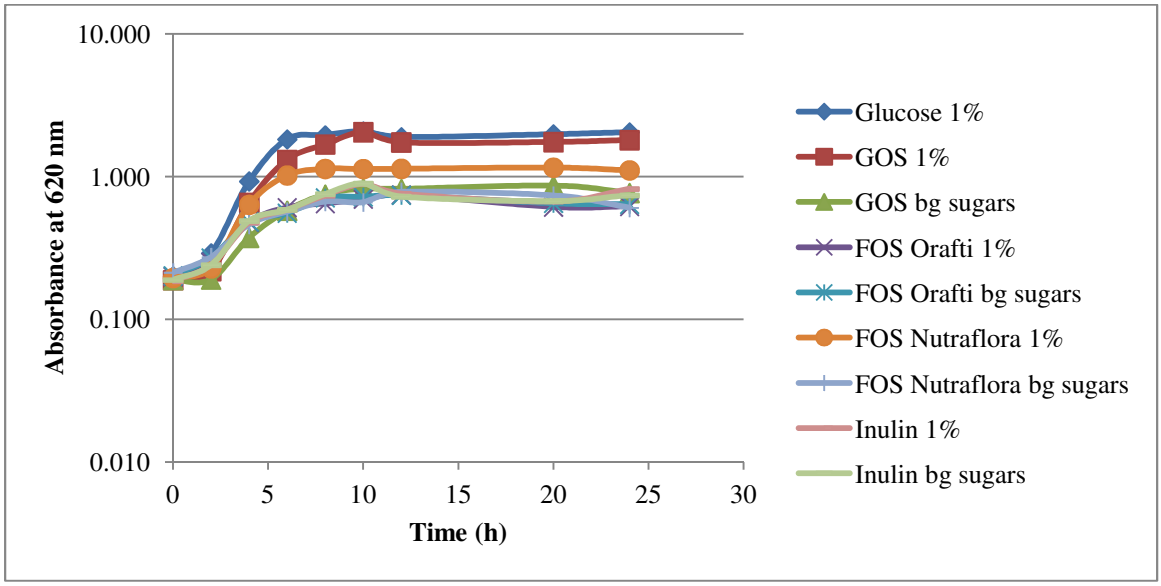

Figure 3.5 Growth of Lactobacillus plantarum on selected carbohydrates.

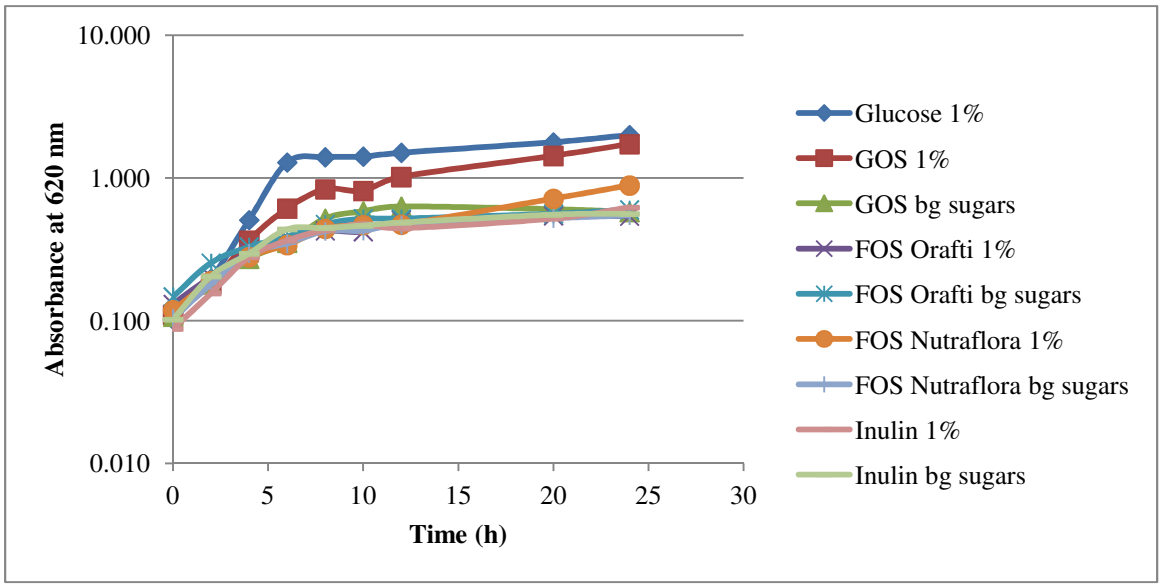

Figure 3.6 Growth of Lactobacillus rhamnosus on selected carbohydrates.

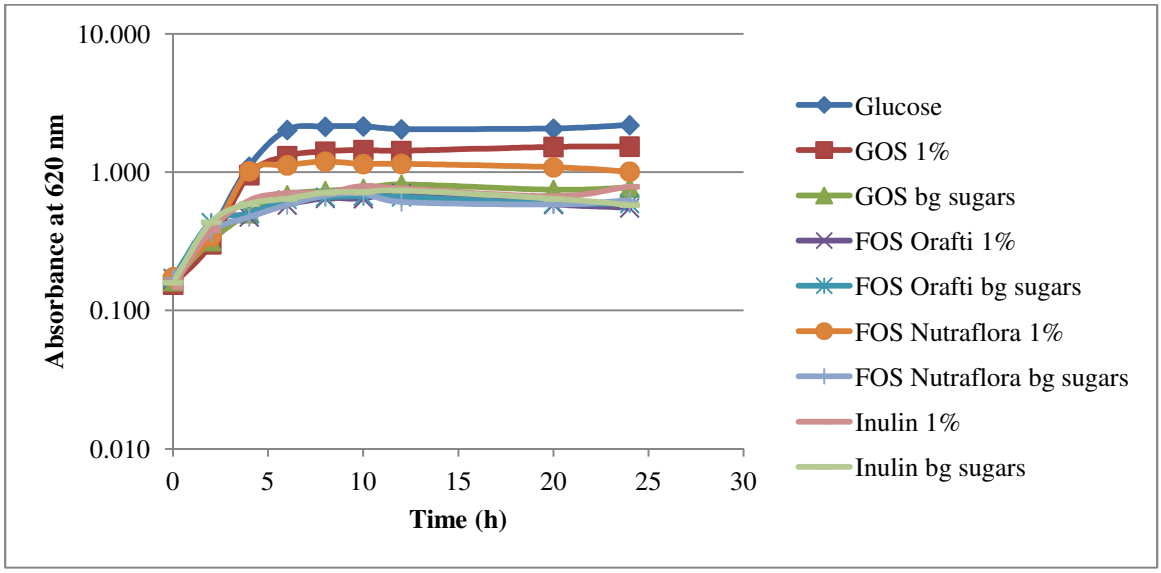

Figure 3.7 Growth of Lactobacillus salivarius on selected carbohydrates.

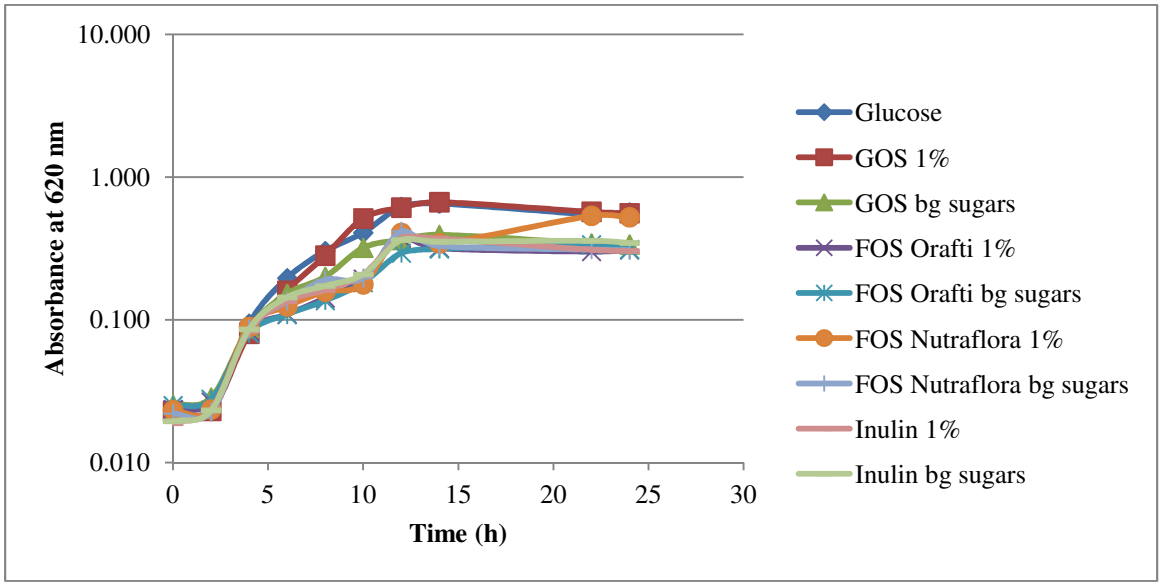

Figure 3.8 Growth of Lactococcus lactis on selected carbohydrates.

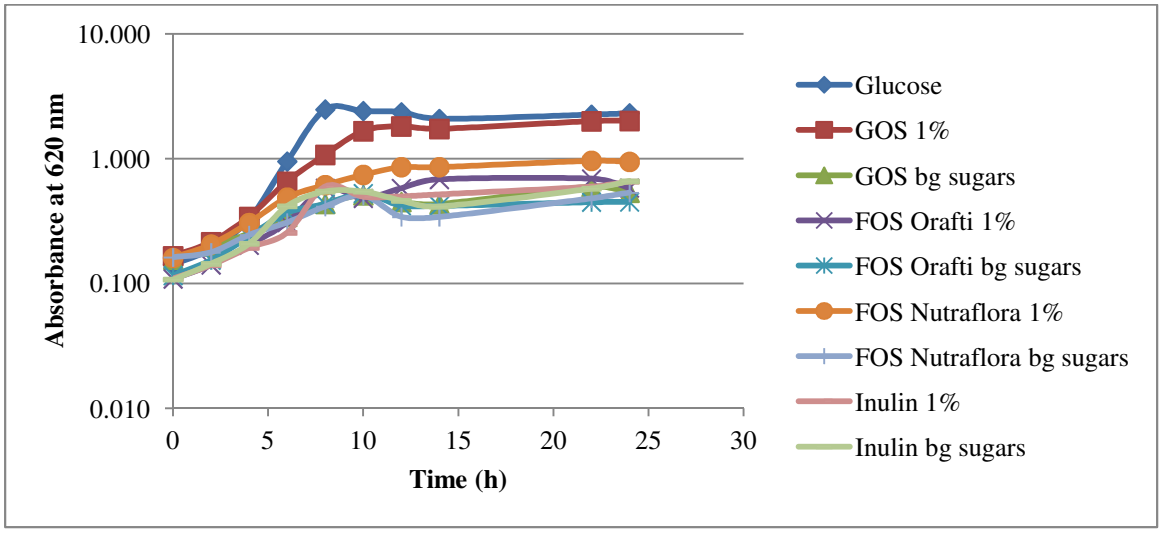

Figure 3.9 Growth of Bifidobacterium longum on selected carbohydrates.

Mean doubling times with glucose ranged from 1.1 ± 0.2 hours for *Lb. casei* and *Lb. rhamnosus* to 2.5 ± 0.7 h for *L. lactis*. Mean doubling times were generally higher for glucose than for any other treatment for all bacteria; however, the difference between glucose and the rest of the treatments was not significant in most of the cases ($p>0.05$). This may be due to the high variability between replicates, as shown by the standard deviation.

There was also no significant difference in doubling time between each prebiotic and its corresponding background sugars. This suggests that the strains utilized the available background sugars and residual glucose from inoculum during the beginning of the exponential phase, which allowed for mean doubling times similar to those of glucose.

Only after these sugars were exhausted would the strains be expected to begin utilizing the prebiotics.

With the exception of *Lb. acidophilus*, all strains showed a higher absorbance increase for glucose than for the majority of background sugars, as shown in Figures 3.1 through 3.9. This was expected, as glucose is the preferred carbon source for most microorganisms and similar results have been reported (54, 55). The lack of differences for *Lb. acidophilus* may be due to the low levels of growth achieved during the experiment. It is possible that different results could be obtained when incubating anaerobically. GOS use by this species has been reported under anaerobic conditions (54, 56).

Based on the maximal absorbance variation (Table 3.2), Purimune GOS was significantly utilized by *Lb. casei*, *Lb. plantarum*, *Lb. rhamnosus*, *Lb. salivarius*, *L. lactis* and *Bb. longum,* as the maximal absorbance increase was higher than the one for background sugars. However, it was not as high as the growth with glucose. *Lb. casei* showed a slight growth with GOS, enough to be different from the background sugars. This agrees with the results of Cardelle-Cobas *et al.* (56), who reported maximum optical densities of up to 0.6 for *Lb. casei* with different galactooligosaccharides as the sole carbon source. In their study, the optical densities obtained with the GOS were similar to those achieved with lactose, which served as their positive control.

Lb. plantarum, *Lb. rhamnosus*, and *Lb. salivarius* were able to achieve significantly higher absorbance variations in GOS than in its background sugars. Cardelle-Cobas *et al.* (56) reported growth of *Lb. plantarum* and *Lb. salivarius* on GOS comparable to that achieved on lactose, their control sugar. *L. lactis* showed similar growth on glucose and GOS, which contrasts with the findings of Gopal *et al.* (57), who reported that none of 20 *Lactococci* strains were able to grow on GOS.

Lb. brevis and *Lb. delbrueckii* were not able to significantly utilize GOS, as there was no difference between GOS and its background sugars. Several studies have shown that the ability to ferment prebiotics is strain and substrate specific, due to differences in metabolic capacity of related strains (56). β-galactosidases are required for degradation of GOS into glucose and galactose (55). Lactobacilli express only intracellular β-galactosidases and lack transport systems for GOS, which explains their usual preference for GOS with a low degree of polymerization so they can be internalized by lactose permeases such as LacS (55, 58). Therefore, utilization of prebiotics requires the presence of specific hydrolysis and transport systems for each prebiotic and the genes coding for these systems may not be present in the different strains (54). Genes encoding for LacS are highly conserved in intestinal lactobacilli, due to their importance for survival in the gastrointestinal tract (58). It is possible that the *Lb. brevis* and *Lb. delbrueckii* strains used in this study are non-intestinal in origin and therefore lack these genes.

Bb. longum was the only bacteria capable of achieving the same level of growth with GOS as with glucose. The ability of GOS to generate a beneficial shift in the colonic microbiota towards Bifidobacteria, known as a "bifidogenic effect", has been widely reported in the literature (10, 59, 60). GOS was the only prebiotic to support growth of *Bb. longum*, as the rest were not significantly different from their corresponding background sugars. This agrees with previous studies using mixed flora samples, where the prebiotic index scores for GOS were higher than those for FOS and inulin (54).

Orafti FOS did not promote significant growth of any of the tested strains. This contrasts with several reports in the literature of satisfactory growth of lactobacilli and bifidobacteria on this prebiotic (61, 62). However, Nutraflora FOS caused a significant increase in absorbance for *Lb. plantarum* and *Lb. salivarius*. Successful utilization of Nutraflora FOS by *Lb. plantarum* strains has been previously reported by Huebner *et al.* (54). They also reported differences in the increase in cell densities between lactobacilli when grown on either Nutraflora P95 FOS or Orafti Raftilose P95 FOS. The difference in utilization could be due to the higher number of fructose chains with a glucose end unit in cane sugar-derived Nutraflora FOS than in chicory root-derived Orafti FOS (63).

Inulin did not promote significant growth of any of the strains tested. Inulin is a fructan of longer chain length than FOS, and non-digestible oligosaccharides of long chain length are less easily fermented by intestinal microbiota (64). Rossi *et al*. (65) reported that only

eight strains from a group of 55 *Bifidobacterium* strains were able to ferment inulin. Their strain set included two *Bb. longum*, from calf and human origin, which were not able to ferment inulin. The capacity to ferment inulin was linked to the presence of extracellular β-fructofuranosidase, the enzyme that hydrolyzes FOS and inulin (65). Van de Wiele *et al*. (64) also reported it took more time to observe significant effects from inulin than from FOS in the Simulator of Human Intestinal Microbial Ecosystem (SHIME). Therefore, it is possible that the strains studied lack the extracellular enzymes needed to hydrolyze FOS and inulin, or that assay time was not enough to observe the prebiotic effect of inulin.

3.3 Antibiotic resistance

The susceptibility profiles of ten probiotic strains were evaluated by both the broth microdilution method and the agar diffusion method. Results for the broth microdilution assay are presented in Table 3.3. Profiles from the disk diffusion method can be found in Table 3.4. Table 3.5 summarizes and compares the results for both methods. Of the 82 bacteria-antibiotic combinations tested, 60 were classified as susceptible by both methods, while the other 12 were classified as resistant by either one or both methods.

Table 3.3 Antibiotic susceptibility profiles of strains by the broth microdilution method using EFSA susceptibility breakpoints.

Antibiotic	Bacteria	MIC (mg/L)	Breakpoint (mg/L)	Classification
Ampicillin	*Lb. acidophilus*	1 - 2	1	Variable
	Lb. brevis	2	2	Susceptible
	Lb. casei	1	4	Susceptible
	Lb. delbrueckii	1	1	Susceptible
	Lb. plantarum	1	2	Susceptible
	Lb. rhamnosus	1	4	Susceptible
	Lb. salivarius	2	4	Susceptible
	Bb. longum	1	2	Susceptible
	L. lactis	1	2	Susceptible
	B. coagulans	n.r.[1]	n.r.	n.r.
Chloramphenicol	*Lb. acidophilus*	4	4	Susceptible
	Lb. brevis	4	4	Susceptible
	Lb. casei	8	4	**Resistant**
	Lb. delbrueckii	4	4	Susceptible
	Lb. plantarum	8	8	Susceptible
	Lb. rhamnosus	4	4	Susceptible
	Lb. salivarius	4	4	Susceptible
	Bb. longum	2	4	Susceptible
	L. lactis	4	8	Susceptible
	B. coagulans	2	8	Susceptible
Clindamycin	*Lb. acidophilus*	0.5	1	Susceptible
	Lb. brevis	>2	1	**Resistant**
	Lb. casei	>2	1	**Resistant**
	Lb. delbrueckii	1	1	Susceptible
	Lb. plantarum	1	2	Susceptible
	Lb. rhamnosus	1	1	Susceptible
	Lb. salivarius	0.5	1	Susceptible
	Bb. longum	>1	1	**Resistant**
	L. lactis	4	1	**Resistant**
	B. coagulans	4	4	Susceptible

[1] n.r. not required by EFSA

Table 3. 3. Antibiotic susceptibility profiles of strains by the broth microdilution method using EFSA susceptibility breakpoints (continued).

Antibiotic	Bacteria	MIC (mg/L)	Breakpoint (mg/L)	Classification
Erythromycin	*Lb. acidophilus*	0.5	1	Susceptible
	Lb. brevis	1	1	Susceptible
	Lb. casei	1	1	Susceptible
	Lb. delbrueckii	2	1	**Resistant**
	Lb. plantarum	0.5	1	Susceptible
	Lb. rhamnosus	0.5	1	Susceptible
	Lb. salivarius	0.5	1	Susceptible
	Bb. longum	0.5 - >2	1	Variable
	L. lactis	0.5	1	Susceptible
	B. coagulans	1	4	Susceptible
Gentamycin	*Lb. acidophilus*	16	16	Susceptible
	Lb. brevis	16	16	Susceptible
	Lb. casei	16	32	Susceptible
	Lb. delbrueckii	16	16	Susceptible
	Lb. plantarum	16	16	Susceptible
	Lb. rhamnosus	16	16	Susceptible
	Lb. salivarius	32	16	**Resistant**
	Bb. longum	16	64	Susceptible
	L. lactis	16	32	Susceptible
	B. coagulans	4	4	Susceptible
Kanamycin	*Lb. acidophilus*	64	64	Susceptible
	Lb. brevis	32	32	Susceptible
	Lb. casei	64	64	Susceptible
	Lb. delbrueckii	16	16	Susceptible
	Lb. plantarum	64	64	Susceptible
	Lb. rhamnosus	64	64	Susceptible
	Lb. salivarius	>64	64	**Resistant**
	Bb. longum	n.r.[1]	n.r.	n.r.
	L. lactis	16	64	Susceptible
	B. coagulans	8	8	Susceptible
Oxytetracycline	*Lb. acidophilus*	4	4	Susceptible
	Lb. brevis	8 – 16	8	Variable
	Lb. casei	4	4	Susceptible
	Lb. delbrueckii	4	4	Susceptible
	Lb. plantarum	32	32	Susceptible
	Lb. rhamnosus	8	8	Susceptible
	Lb. salivarius	4	8	Susceptible
	Bb. longum	4	8	Susceptible

Table 3. 3. Antibiotic susceptibility profiles of probiotic strains by the broth microdilution method using EFSA susceptibility breakpoints (continued).

Antibiotic	Bacteria	MIC (mg/L)	Breakpoint (mg/L)	Classification
Oxytetracycline	*L. lactis*	8	4	**Resistant**
	B. coagulans	4	8	Susceptible
Streptomycin	*Lb. acidophilus*	16	16	Susceptible
	Lb. brevis	16	64	Susceptible
	Lb. casei	16	64	Susceptible
	Lb. delbrueckii	16	16	Susceptible
	Lb. plantarum	n.r.[1]	n.r.	n.r.
	Lb. rhamnosus	32	32	Susceptible
	Lb. salivarius	>64	64	**Resistant**
	Bb. longum	128	128	Susceptible
	L. lactis	16	32	Susceptible
	B. coagulans	8	8	Susceptible
Vancomycin	*Lb. acidophilus*	2	2	Susceptible
	Lb. brevis	n.r.	n.r.	n.r.
	Lb. casei	n.r.	n.r.	n.r.
	Lb. delbrueckii	1	2	Susceptible
	Lb. plantarum	n.r.	n.r.	n.r.
	Lb. rhamnosus	n.r.	n.r.	n.r.
	Lb. salivarius	n.r.	n.r.	n.r.
	Bb. longum	2	2	Susceptible
	L. lactis	4	4	Susceptible
	B. coagulans	1	4	Susceptible

[1] n.r. not required by EFSA

Table 3.4 Antibiotic susceptibility profiles of strains by the disk diffusion method using susceptibility criteria established by Charteris et al.(53)

Antibiotic	Bacteria	Inhibition diameter (mm, average ± standard deviation)	Classification
Ampicillin	*Lb. acidophilus*	38 ± 0.2	Susceptible
	Lb. brevis	29 ± 1.4	Susceptible
Resistant ≤ 12 mm	*Lb. casei*	36 ± 1.3	Susceptible
Moderate 13-15 mm	*Lb. delbrueckii*	38 ± 1.2	Susceptible
Susceptible ≥ 16 mm	*Lb. plantarum*	45 ± 0.7	Susceptible
	Lb. rhamnosus	38 ± 0.6	Susceptible
	Lb. salivarius	32 ± 0.7	Susceptible
	Bb. longum	39 ± 0.4	Susceptible
	L. lactis	34 ± 2.4	Susceptible
	B. coagulans	n.r.[1]	n.r.
Chloramphenicol	*Lb. acidophilus*	36 ± 0.0	Susceptible
	Lb. brevis	35 ± 1.8	Susceptible
Resistant ≤ 13 mm	*Lb. casei*	33 ± 0.7	Susceptible
Moderate 14-17 mm	*Lb. delbrueckii*	36 ± 1.3	Susceptible
Susceptible ≥ 18 mm	*Lb. plantarum*	29 ± 0.5	Susceptible
	Lb. rhamnosus	31 ± 4.0	Susceptible
	Lb. salivarius	31 ± 0.2	Susceptible
	Bb. longum	25 ± 0.4	Susceptible
	L. lactis	30 ± 0.4	Susceptible
	B. coagulans	39 ± 4.1	Susceptible
Clindamycin	*Lb. acidophilus*	18 ± 7.0	Susceptible
	Lb. brevis	15 ± 1.5	Susceptible
Resistant ≤ 8 mm	*Lb. casei*	14 ± 0.8	Susceptible
Moderate 9-11 mm	*Lb. delbrueckii*	39 ± 2.0	Susceptible
Susceptible ≥ 12 mm	*Lb. plantarum*	12 ± 2.4	Susceptible
	Lb. rhamnosus	20 ± 0.0	Susceptible
	Lb. salivarius	26 ± 3.6	Susceptible
	Bb. longum	14 ± 2.1	Susceptible
	L. lactis	22 ± 15.1	Susceptible
	B. coagulans	43 ± 3.5	Susceptible

[1] n.r. not required by EFSA

Table 3.4. Antibiotic susceptibility profiles of strains by the disk diffusion method using susceptibility criteria established by Charteris et al. (53) (continued).

Antibiotic	Bacteria	Inhibition diameter (mm)	Classification
Erythromycin	*Lb. acidophilus*	40 ± 1.2	Susceptible
	Lb. brevis	35 ± 1.3	Susceptible
Resistant ≤ 13 mm	*Lb. casei*	40 ± 0.4	Susceptible
Moderate 14-17 mm	*Lb. delbrueckii*	39 ± 1.7	Susceptible
Susceptible ≥ 18 mm	*Lb. plantarum*	29 ± 1.2	Susceptible
	Lb. rhamnosus	40 ± 0.2	Susceptible
	Lb. salivarius	30 ± 0.2	Susceptible
	Bb. longum	39 ± 0.0	Susceptible
	L. lactis	32 ± 1.1	Susceptible
	B. coagulans	44 ± 4.1	Susceptible
Gentamycin	*Lb. acidophilus*	14 ± 0.3	Susceptible
	Lb. brevis	16 ± 0.0	Susceptible
Resistant ≤ 12 mm	*Lb. casei*	15 ± 0.7	Susceptible
Moderate - mm	*Lb. delbrueckii*	19 ± 1.4	Susceptible
Susceptible ≥ 13 mm	*Lb. plantarum*	16 ± 0.6	Susceptible
	Lb. rhamnosus	16 ± 0.9	Susceptible
	Lb. salivarius	9 ± 0.6	**Resistant**
	Bb. longum	16 ± 0.1	Susceptible
	L. lactis	20 ± 0.3	Susceptible
	B. coagulans	31 ± 0.0	Susceptible
Kanamycin	*Lb. acidophilus*	7 ± 0.0	**Resistant**
	Lb. brevis	7 ± 0.0	**Resistant**
Resistant ≤ 13 mm	*Lb. casei*	7 ± 0.0	**Resistant**
Moderate 14-17 mm	*Lb. delbrueckii*	22 ± 0.7	Susceptible
Susceptible ≥ 18 mm	*Lb. plantarum*	7 ± 0.0	**Resistant**
	Lb. rhamnosus	10 ± 0.6	**Resistant**
	Lb. salivarius	7 ± 0.0	**Resistant**
	Bb. longum	n.r.[1]	n.r.
	L. lactis	7 ± 0.0	**Resistant**
	B. coagulans	30 ± 0.4	Susceptible

[1] n.r. not required by EFSA

Table 3.4. Antibiotic susceptibility profiles of strains by the disk diffusion method using susceptibility criteria established by Charteris et al. (53) (continued).

Antibiotic	Bacteria	Inhibition diameter (mm)	Classification
Tetracycline	*Lb. acidophilus*	43 ± 0.9	Susceptible
	Lb. brevis	28 ± 0.1	Susceptible
Resistant ≤ 14 mm	*Lb. casei*	39 ± 2.3	Susceptible
Moderate 15-18 mm	*Lb. delbrueckii*	37 ± 0.6	Susceptible
Susceptible ≥ 19 mm	*Lb. plantarum*	23 ± 0.2	Susceptible
	Lb. rhamnosus	28 ± 1.9	Susceptible
	Lb. salivarius	24 ± 0.1	Susceptible
	Bb. longum	30 ± 0.2	Susceptible
	L. lactis	33 ± 0.2	Susceptible
	B. coagulans	54 ± 4.8	Susceptible
Streptomycin	*Lb. acidophilus*	18 ± 0.3	Susceptible
	Lb. brevis	7 ± 0.1	**Resistant**
Resistant ≤ 11 mm	*Lb. casei*	10 ± 0.1	**Resistant**
Moderate 12-14 mm	*Lb. delbrueckii*	22 ± 0.3	Susceptible
Susceptible ≥ 15 mm	*Lb. plantarum*	n.r.	n.r.
	Lb. rhamnosus	12 ± 0.5	Moderate
	Lb. salivarius	7 ± 0.0	**Resistant**
	Bb. longum	13 ± 0.8	Moderate
	L. lactis	19 ± 1.0	Susceptible
	B. coagulans	29 ± 1.2	Susceptible
Vancomycin	*Lb. acidophilus*	27 ± 0.4	Susceptible
	Lb. brevis	n.r.[1]	n.r.
Resistant ≤ 14 mm	*Lb. casei*	n.r.	n.r.
Moderate 15-16 mm	*Lb. delbrueckii*	28 ± 1.0	Susceptible
Susceptible ≥ 17 mm	*Lb. plantarum*	n.r.	n.r.
	Lb. rhamnosus	n.r.	n.r.
	Lb. salivarius	n.r.	n.r.
	Bb. longum	7 ± 0.0	**Resistant**
	L. lactis	23 ± 0.2	Susceptible
	B. coagulans	42 ± 4.6	Susceptible

[1] n.r. not required by EFSA

Table 3.5 Comparison of antibiotic susceptibility profiles of probiotic strains by the broth microdilution method and disk diffusion method.

Bacteria	Susceptibility (Broth microdilution / Disk diffusion)								
	Ampicillin	Chloramphenicol	Clindamycin	Erythromycin	Gentamycin	Kanamycin	Oxytetracycline	Streptomycin	Vancomycin
Lb. acidophilus	V / S	S / S	S / S	S / S	S / S	S / **R**	S / S	S / S	S / S
Lb. brevis	S / S	S / S	**R** / S	S / S	S / S	S / **R**	V / S	S / **R**	n.r.
Lb. casei	S / S	**R** / S	**R** / S	S / S	S / S	S / **R**	S / S	S / **R**	n.r.
Lb. delbrueckii	S / S	S / S	S / S	**R** / S	S / S	S / S	S / S	S / S	S / S
Lb. plantarum	S / S	S / S	S / S	S / S	S / S	S / **R**	S / S	n.r.	n.r.
Lb. rhamnosus	S / S	S / S	S / S	S / S	S / S	S / **R**	S / S	S/MS	n.r.
Lb. salivarius	S / S	S / S	S / S	S / S	**R** / **R**	**R** / **R**	S / S	**R** / **R**	n.r.
Bb. longum	S / S	S / S	**R** / S	V / S	S / S	n.r.	S / S	S/MS	S / **R**
L. lactis	S / S	S / S	**R** / S	S / S	S / S	S / **R**	**R** / S	S / S	S / S
B. coagulans	n.r.	S / S	S / S	S / S	S / S	S / S	S / S	S / S	S / S

R: Resistant, S: Susceptible, MS: Moderately Susceptible, V: Variable, N.R.: Not required.

3.2.1 Lactobacilli strains

Lactobacillus acidophilus was susceptible to all antibiotics by the broth microdilution method. However, it was classified as resistant to kanamycin by the disk diffusion method. Several studies have found that intrinsic resistance to kanamycin in the *Lactobacillus acidophilus* group is higher than initially described (50, 66). For example, Danielsen and Wind (50) suggested a breakpoint of >256 mg/L for kanamycin for all *Lactobacillus* species after testing 37 strains. The new distribution data prompted the European Food Safety Authority (EFSA) to change their regulatory limits accordingly, increasing the resistance breakpoint for this species from 16 mg/L in 2008 to 64 mg/L in 2012 (42, 51). The MICs found for all antibiotics were comparable to those reported by

Mayrhofer *et al.* (66), who characterized the susceptibility of 101 strains belonging to the *Lb. acidophilus* group, including 10 strains of *Lb. acidophilus*.

Lactobacillus plantarum, *Lactobacillus rhamnosus, Lactobacillus brevis* and *Lactobacillus casei* were also classified as resistant to kanamycin solely by the disk diffusion assay. The MIC for kanamycin for all strains coincided with the EFSA breakpoint. This suggests that the limits for the disk diffusion test might need to be reviewed for this antibiotic when testing *Lactobacillus* species. The cut-off values for the disk diffusion method were proposed by Charteris *et al.* in 1998 (53) and have been used in several studies since then (67–69). Mayrhofer *et al.* (70) recently compared the broth microdilution and agar disk diffusion methods for susceptibility testing of *Lb. acidophilus* group members, finding a strong correlation between MICs and inhibition zone diameters for several antibiotics. However, they did not establish guidelines for classification and their study did not include kanamycin.

Lb. brevis and *Lb. casei* both had a similar susceptibility profile, as they were classified as resistant to streptomycin only by disk diffusion and as resistant to clindamycin only by broth microdilution. *Lb. casei* also exhibited resistance to chloramphenicol in the broth microdilution assay, while *Lb. brevis* showed variable susceptibility to tetracycline. Both organisms showed no inhibition zones in the disk diffusion assay for streptomycin; yet, their MIC was much lower than the EFSA breakpoint (16 mg/L vs 64 mg/L).

Streptomycin belongs to the aminoglycoside category of antibiotics, which also includes kanamycin. Increased MICs for aminoglycosides have been reported when using MRS agar for testing. MRS agar has a lower pH (6.2 ± 0.2) than the pH optimum for this group of antibiotics (pH 7.8) (71).

Lb. casei or *Lb. brevis* resistance to clindamycin is rare in the available literature, only reported for one *Lb. casei* strain by Charteris *et al.* (53) and two *Lb. brevis* strains by Delgado *et al.* (72) and Herreros *et al.* (44). For chloramphenicol, D'Aimmo *et al.* (33) reported a MIC of 16 mg/L for 6 strains of *Lb. casei*, which is higher than the MIC found in this study (8 mg/L) and the EFSA breakpoint (4 mg/L). Resistance has also been reported for 10 out of 29 *Lb. casei* isolates from European probiotic products examined by the disk diffusion assay (73). Resistance to chloramphenicol, clindamycin or tetracycline is not intrinsic to lactobacilli; therefore, it would be important to study for the presence of known genes providing such resistance in these strains.

Lactobacillus delbrueckii subsp. *bulgaricus* only showed resistance to erythromycin in the broth microdilution assay. It was classified as susceptible to all other antibiotics. The erythromycin resistance determinant *erm*(B) has been reported previously in different lactobacilli from different sources (46, 74–76). As an acquired resistance determinant, it can be potentially be transferred to other bacterial species, as shown by Nawaz *et al.* (76).

This raises a concern regarding the safety of this strain, making it necessary to screen for the presence of this resistance determinant.

In contrast, *Lactobacillus salivarius* was the only strain to show clear resistance to more than one antibiotic in both assays. It was not susceptible to the three aminoglycosides: gentamycin, kanamycin and streptomycin. The MIC for kanamycin was >64 mg/L, the highest concentration tested. However, as discussed with *Lb. acidophilus*, Danielsen and Wind (50) have suggested a breakpoint of >256 mg/L for kanamycin for all *Lactobacillus* species after testing 37 strains. The MIC for streptomycin for *L. salivarius* was also >64 mg/L, while the MIC for gentamycin was 32 mg/L. This is in agreement with previous reports of a lower MIC for gentamycin than for the rest of the aminoglycosides (50). The three aminoglycoside antibiotics act as inhibitors of protein synthesis (53). Lactobacilli show intrinsic resistance to this group of antibiotics due to cell wall structure and membrane impermeability, which may be complemented by efflux mechanisms (49). However, the presence of resistance determinants such as *aac*(6')-*aph*(2'') for gentamicin and kanamycin resistance and *ant*(6) and *aph*E for streptomycin resistance, has been reported in *Lb. plantarum* (49) and *Bifidobacterium longum* (75). Multidrug resistance is not common in lactobacilli, so presence of acquired resistance genes cannot be ruled out.

3.2.2 Other probiotic strains

Lactococcus lactis was resistant to kanamycin (inhibition zone diameter = 7 mm) in the disk diffusion assay. This could be due to media interference, as previously discussed. Lactococci also have a high natural resistance to gentamicin and kanamycin (2). Additionally, it was classified as resistant to clindamycin (MIC = 4 mg/L) and oxytetracycline (MIC = 8 mg/L) by the broth microdilution method. Ammor *et al.* (75) characterized the MICs of 50 *L. lactis* strains for 6 antibiotics, including clindamycin and tetracycline. The MICs ranged from <0.032 to 0.5 mg/L for clindamycin and from 0.125 to 0.5 mg/L for tetracycline. The incidence of resistance to antibiotics in lactococci is low in the literature; however, Rodriguez-Alonso *et al.* (77) did find lactococci strains resistant to tetracycline (n = 2) and clindamycin (n =1) during their survey of 46 lactococci isolates from artisanal raw milk cheeses. As broth microdilution is considered the standard method for determining antibiotic resistance, it is recommended to look for genetic determinants for the observed resistance in this strain.

The only *Bifidobacterium* in the group, *Bb. longum*, exhibited a variable profile, with many differences among methods. It showed certain resistance to clindamycin and erythromycin in the broth microdilution assay and resistance to streptomycin and vancomycin in the disk diffusion method. Erythromycin is a member of the macrolide family of antibiotics, while clindamycin is part of the lincosamides. Resistance to both antibiotics in the same strain suggests a common resistance mechanism, which leads to the phenotype known as macrolide-lincosamide-streptogramin (MLS) phenotype (75).

This phenotype is due to the presence of *erm* genes encoding a 23S rRNA methylase that modifies the antibiotic's target (78). Several classes of *erm* genes have been reported in a number of clinically important organisms, as well as in several lactic acid bacteria (73).

Some bifidobacteria species are considered naturally resistant to aminoglycosides (2), which explains the lack of a MIC for kanamycin amongst the EFSA guidelines. Vancomycin resistant bifidobacteria were found by Charteris *et al.* (79) when using the disk diffusion method. However, most reports classify bifidobacteria species as susceptible to this antibiotic (2, 72). The discrepancy may be due to differences in assay methodology, since it has been suggested that vancomycin diffuses poorly in agar (79). Lastly, the variability observed for *Bb. longum* could be explained by the use of methods optimized for lactobacilli, which leads to occasional poor growth and low repeatability of results.

3.2.3 Comparison of broth microdilution and disk diffusion methods

The broth microdilution and disk diffusion methods were in agreement for 76.8% (63/82) of the bacteria-antibiotic combinations tested, as shown in Table 3.6. Both methods found similar numbers of resistant and susceptible bacteria. However, each method seems to have a slight bias. The broth microdilution classified four more bacteria as resistant to clindamycin than the disk diffusion method. The disk diffusion method classified six more bacteria as resistant to kanamycin than the broth microdilution method (Table 3.5).

Table 3.6 Comparison of broth microdilution and disk diffusion methods for bacteria-antibiotic combinations tested.

Method	Resistant	Susceptible	Variable or Moderate	Total
Broth microdilution	10	69	3	82
Disk diffusion	12	68	2	82
Both methods	3	60	19	82

As for precision of the methods, Mayrhofer *et al.* (70) suggests an acceptable repeatability of ±1 log base 2 for MICs and ± 3 to 4 mm for inhibition zone diameters for disk diffusion. All MICs determined were within this suggested range, while 5 inhibition zone diameters had a standard deviation higher than 4 millimeters. Interestingly, 3 of those diameters were related to *Bacillus coagulans*. This may be explained by the optimization of the disk diffusion method for lactobacilli species. The antibiotics with larger standard deviations for inhibition zone diameters were clindamycin (average standard deviation: 2.5 mm), erythromycin (average standard deviation 1.5 mm) and chloramphenicol (average standard deviation: 1.4 mm). This confirms the acceptable repeatability of the disk diffusion method, as well as the broth microdilution method.

3.3 Properties of the potential probiotic strain *Bacillus coagulans* ProDura

3.3.1 Acid and bile tolerance of *Bacillus coagulans*

The *in vitro* tolerance of *Bacillus coagulans* to either acid or bile was assessed during a period of four hours. The average counts (log CFU/g) for each treatment are presented in Figure 3.10. Survival was compared against the control using the area under the curve

and survival rates (Table 3.7). *Bacillus coagulans* spores were able to survive all conditions tested, including pH 2.0. Survival ranged from 72.5 ± 3.3% in pH 2.0 to 91.9 ± 3.6% in 0.3% bile salts. Acidic conditions had a significant effect over survival, decreasing counts by at least 1 log cycle for all the pH values tested. As expected, reductions in counts were higher at the lower pH values.

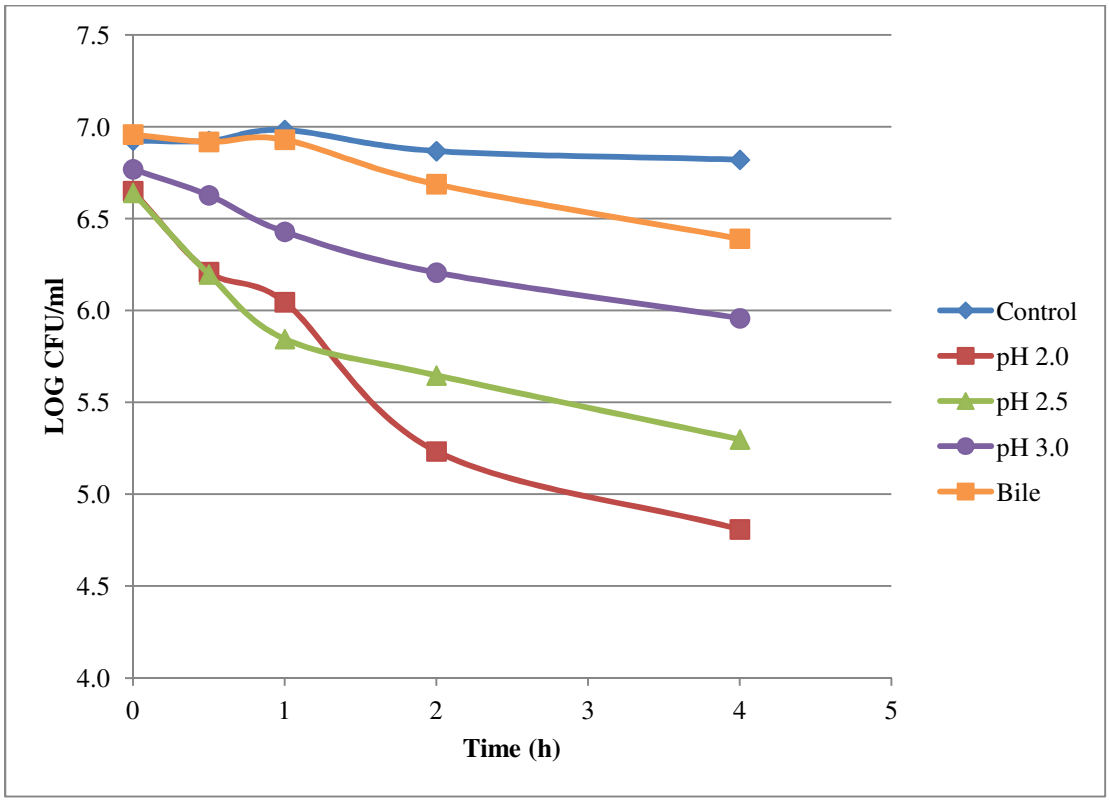

Figure 3.10 Bacillus coagulans cell survival in MRS broth with either acid or bile salts.

Table 3.7 Bacillus coagulans tolerance to acid and bile salts.

Treatment	Average area under the curve (log CFU/ml * h, ± SD)[1]	Average survival (% , ± SD)
Control	27.42 ± 0.30 [a]	99.5 ± 3.0 [a]
pH 2.0	21.34 ± 0.90 [c]	72.5 ± 3.3 [d]
pH 2.5	22.59 ± 0.56 [b,c]	79.4 ± 1.5 [c,d]
pH 3.0	24.60 ± 1.31 [b]	86.6 ± 5.3 [b,c]
Bile salts (0.3%)	26.72 ± 0.09 [a]	91.9 ± 3.6 [a,b]

[1]Treatments with different letters within the same column are significantly different ($p<0.05$).

Bacillus coagulans survival in low pH has been documented before. Sudha *et al.* (80) found a 2 log cycle reduction in *B. coagulans* Unique IS-2 spore counts after exposing the strain to pH 1.5 for 3 hours and a 1 log cycle reduction at pH 2.0 and 3.0. The strain studied here exhibited greater susceptibility to pH 2.0, with a predicted 2 log cycle reduction after 3 hours. Tolerance seems to vary amongst strains of *B. coagulans*, as Hyronimus *et al*. (11) tested 3 different strains (BCI_4 LMAB, CIP5264 and CIP6625) and none of them showed detectable survival after 3 hours at pH 2.5. Those three strains were also weakly tolerant to 0.3% bile. *B. coagulans* Unique IS-2, on the other hand, had only a 1 log cycle reduction after 3 hours in 1.0% bile (80). Maathuis *et al.* (14) tested survival of *B. coagulans* GanedenBC30 during passage through a dynamic model of the stomach and small intestine (TIM-1) and found high survival (70%).

In vitro tolerance to acid and bile has been positively correlated to survival in both human and pig gastrointestinal tracts (81, 82). Guo *et al.* (81) screened *Bacillus* strains for probiotic characteristics, including acid and bile tolerance. *B. subtilis* MA 139 had constant counts during 3 hours at pH 2.0 and was capable of growing in mixed nutrient broth with 0.3% bile. It was also able to exert a probiotic effect in pigs. Succi *et al*. (83) found that the popular probiotic strain*, Lactobacillus rhamnosus* GG, showed reductions of 2 log cycles and 6 log cycles when exposed for 2 hours to pH 3.0 and pH 2.0, respectively. Survival in the gut is supposed to be higher thanks to the buffering effect of food products consumed at the same time as the probiotic. *Bacillus coagulans* strains show higher tolerance to acid than *L. rhamnosus* GG, so that their delivery to the lower intestinal tract is very promising. Their tolerance to acid would also make them suitable for addition to acidified foods, where they could arguably survive and remain viable.

3.3.2 Prebiotic utilization by *Bacillus coagulans*

The capacity of *B. coagulans* to use four commercial prebiotics as a carbon source was evaluated during growth for 24 hours. Glucose was used as a positive control for growth. The background sugars in each prebiotic were also evaluated, to establish their effect on growth and rule out their interference. The mean doubling time during the exponential phase of growth and the maximal absorbance variation were calculated for each prebiotic. Results are presented in Table 3.8 and Figure 3.11.

Mean doubling times ranged from 1.2 to 2.0 hours, but were not significantly different amongst all treatments. Based on maximal absorbance variation, *B. coagulans* grew well on media supplemented with glucose, as expected. It showed similar growth on media supplemented with GOS. Maximal absorbance variation was significantly higher for glucose and GOS, doubling the absorbance obtained with other treatments. It must be noted that the maximal absorbance variation (0.57 ± 0.02) is much lower than those observed with the other probiotic strains tested. This suggests that, while *B. coagulans* is capable of growing in the test conditions, these are not optimal for growth.

Table 3.8 Prebiotic utilization by Bacillus coagulans.

Treatment	Mean doubling time (h, average ± standard deviation)[1]			Maximal absorbance variation (average ± standard deviation)[2]		
Glucose	1.2	± 0.0	a	0.57	± 0.02	a
Purimune GOS	1.4	± 0.1	a	0.50	± 0.07	a
Purimune GOS bg[3]	1.7	± 0.3	a	0.23	± 0.05	b
FOS Orafti	1.8	± 0.3	a	0.14	± 0.03	b
FOS Orafti bg	1.8	± 0.6	a	0.17	± 0.06	b
FOS Nutraflora	2.0	± 0.6	a	0.16	± 0.03	b
FOS Nutraflora bg	1.7	± 0.4	a	0.19	± 0.04	b
Inulin	1.4	± 0.4	a	0.19	± 0.03	b
Inulin bg	1.9	± 0.5	a	0.19	± 0.07	b

[1] Mean doubling times with a different letter are significantly different ($p<0.05$) from doubling times with other carbohydrates.

[2] Maximal absorbance variations with a different letter are significantly different ($p<0.05$) from maximal absorbance variations with other carbohydrates.

[3] bg: Background sugars.

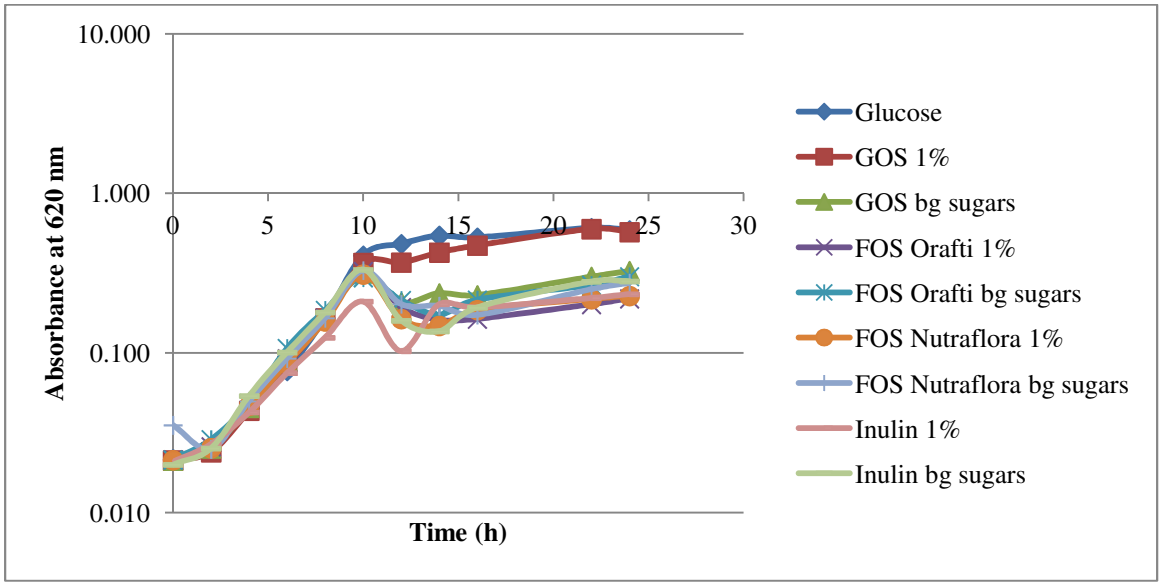

Figure 3.11 Growth of Bacillus coagulans on selected carbohydrates.

There is little available information in the literature about *in vitro* use of prebiotics by *B. coagulans* or other *Bacillus* species. β-galactosidases from *Bacillus circulans*, *B. coagulans* and *B. stearothermophilus* have been widely studied for the production of GOS, since β-galactosidases are capable of catalyzing both the hydrolysis of β-galactoside linkages of lactose to glucose and the transgalactosidation reaction to produce galactooligosaccharides (84–86). It has been reported that *Bifidobacterium* show greater growth on GOS produced by their own β-galactosidase than on commercial products (87). A similar behavior could be expected from *Bacillus* species and GOS.

Based on the results, no difference in growth between FOS and its background sugars was found. However, there are several *in vivo* studies in different species of fish where feed supplementation with both *Bacillus* spp. and FOS has resulted in better growth

performance, survival and non-specific immune response when compared to each supplement by itself or to a control diet (88–90). This suggests a synbiotic relationship between the bacteria and the carbohydrate.

B. coagulans was not able to ferment inulin as a carbohydrate source. This agrees with the results of De Clerck *et al*. (91) who performed the biochemical characterizations of 31 strains of *B. coagulans* using the API tests, and found that none of them was capable of producing acid from inulin.

3.3.3 Antibiotic susceptibility of *Bacillus coagulans*

Antibiotic susceptibility of *B. coagulans* was determined by the broth microdilution method. Results are summarized in Table 3.9.

Table 3.9 Antibiotic susceptibility profile of Bacillus coagulans by broth microdilution method using EFSA susceptibility criteria.

Antibiotic	MIC (mg/L)	Breakpoint (mg/L)	Classification
Ampicillin	n.r.[1]	n.r.	n.r.
Chloramphenicol	2	8	Susceptible
Clindamycin	4	4	Susceptible
Erythromycin	1	4	Susceptible
Gentamycin	4	4	Susceptible
Kanamycin	8	8	Susceptible
Oxytetracycline	4	8	Susceptible
Streptomycin	8	8	Susceptible
Vancomycin	1	4	Susceptible

1 n.r. not required

The strain was susceptible to all antibiotics tested, as the minimum inhibitory concentrations were lower or the same as the EFSA breakpoint. Since interest in *B. coagulans* and other *Bacillus* species as probiotics is fairly recent, there is little available published information on antibiotic susceptibility profiles. Sorokulova *et al.* (92) studied a *B. subtilis* and a *B. licheniformis* strain for antibiotic resistance; while the former was sensitive to all antibiotics listed by EFSA the latter was resistant to chloramphenicol and clindamycin. Hong *et al*. (93) found no antibiotic resistance in a *B. subtilis* strain and clindamycin resistance in a *B. indicus* strain. Clindamycin resistance appears to be a concern in probiotic *Bacillus* strains. The strain studied here, however, shows no resistance and can be classified as safe for use as a probiotic.

CHAPTER 4 : CONCLUSIONS AND FUTURE RESEARCH

4.1 Conclusions

- The prebiotic utilization profile of ten probiotic strains was determined.
    - *Lactobacillus acidophilus*, *Lb. brevis* and *Lb. delbrueckii* subsp. *bulgaricus* were unable to ferment any of the four prebiotics tested.
    - Orafti P95 FOS and Orafti Synergy 1 inulin did not support growth of any of the strains tested.
    - Purimune galactooligosaccharide (GOS) was fermented by *Lb. casei*, *Lb. plantarum*, *Lb. rhamnosus*, *Lb. salivarius*, *Lactococcus lactis*, *Bifidobacterium longum* and *Bacillus coagulans*.
    - Nutraflora P95 fructooligosaccharide (FOS) was fermented by *Lb. plantarum* and *Lb. salivarius*. These prebiotic-probiotic combinations could be used for a synbiotic formulation.
- The susceptibility of ten probiotic strains to nine antibiotics was evaluated by two methods.
    - Only *Lb. salivarius* showed resistance to three antibiotics: gentamycin, kanamycin and streptomycin; and should not be considered safe for probiotic use.
    - The antibiotic susceptibility profiles obtained by the broth microdilution method and the disk diffusion method were in agreement for 76.8% (63/82) of the possible strain-antibiotic combinations, which is considered satisfactory. They also had acceptable repeatability.

- *B. coagulans* was able to survive acid and bile conditions in vitro for up to 4 hours with a 1.0 log reduction in bile salts and a 2.0 log reduction in pH 2.0. This predicts survival of the passage through the intestinal tract and success as a probiotic.

4.2 Future Research

Areas of interest to continue with this research include:

- *In vivo* evaluation of prebiotic utilization by the probiotic strains and possible synbiotic effects.
- Anaerobic evaluation of prebiotic utilization by *Lactobacillus acidophilus*.
- Determination of resistance determinants in *Lactobacillus salivarius* and strains found to be resistant in just one of the two methods tested.
- Examination of a larger set of strains by both microdilution and disk diffusion methods in order to establish updated evaluation criteria for the disk diffusion method.
- Determination of acid and bile resistance of *B. coagulans in vivo* or *in vitro* by a more complex model.

CHAPTER 5 : REFERENCES

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APPENDIX A. ALIGNMENT REPORTS FOR PROBIOTIC STRAINS

Alignment Report - 500BP Identification

Customer: Stratton

125 SANDY DRIVE•NEWARK, DE 19713•PH 302-737-4297•FX 302-737-7781•WWW.MIDILABS.COM

D16M2 Library Revision: 2.22

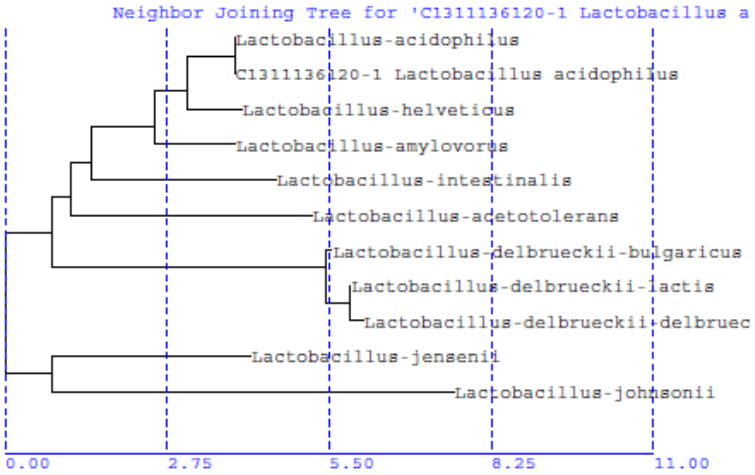
Created: 11/18/2013 1:43:33 PM
Sample ID: C1311136120-1 Lactobacillus acidophilus

16S DNA: 550 base pairs

D16M2 DNA Match Report

Match	%Diff	Length	Library Entry Name
1	0.00	550	Lactobacillus-acidophilus
2	1.73	550	Lactobacillus-helveticus
3	2.91	550	Lactobacillus-amylovorus
4	5.44	550	Lactobacillus-intestinalis
5	7.04	548	Lactobacillus-delbrueckii-bulgaricus
6	7.25	550	Lactobacillus-acetotolerans
7	7.40	548	Lactobacillus-delbrueckii-lactis
8	7.67	548	Lactobacillus-delbrueckii-delbrueckii
9	8.47	550	Lactobacillus-jensenii
10	11.76	557	Lactobacillus-johnsonii

Exact match with Lactobacillus-acidophilus



Closest Match: Lactobacillus-acidophilus
Confidence level: Species

Reviewer's Signature: 11-18-13

Cliff Poindexter, Laboratory Manager

Alignment Report - 500BP Identification

Customer: Stratton

125 SANDY DRIVE•NEWARK, DE 19713•PH 302-737-4297•FX 302-737-7781•WWW.MIDILABS.COM

D16M2 Library Revision: 2.22

Created: 2/21/2014 10:36:22 AM
Sample ID: C1402180158-1. Lactobacillus brevis

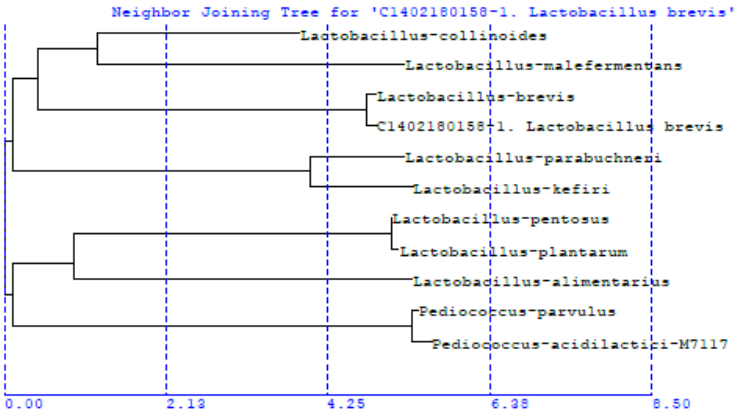
16S DNA: 552 base pairs

D16M2 DNA Match Report

Match	%Diff	Length	Library Entry Name
1	0.27	552	Lactobacillus-brevis
2	6.88	550	Lactobacillus-collinoides
3	9.35	552	Lactobacillus-pentosus
4	9.44	552	Lactobacillus-plantarum
5	10.16	551	Lactobacillus-alimentarius
6	10.27	562	Pediococcus-parvulus
7	10.34	560	Lactobacillus-kefiri
8	10.38	552	Lactobacillus-malefermentans
9	10.41	561	Lactobacillus-parabuchneri
10	10.44	562	Pediococcus-acidilactici-M7117

Concise Alignment with Lactobacillus-brevis

```
Sample:      (139) G (229) C
             ..... .....
LibEnt 1:    (139) A (229) Y
```



Closest Match: Lactobacillus-brevis
Confidence level: Species

Reviewer's Signature: 02-21-14

Cliff Poindexter, Laboratory Manager

Alignment Report - 500BP Identification

Customer: Stratton

125 SANDY DRIVE•NEWARK, DE 19713•PH 302-737-4297•FX 302-737-7781•WWW.MIDILABS.COM

D16M2 Library Revision: 2.22

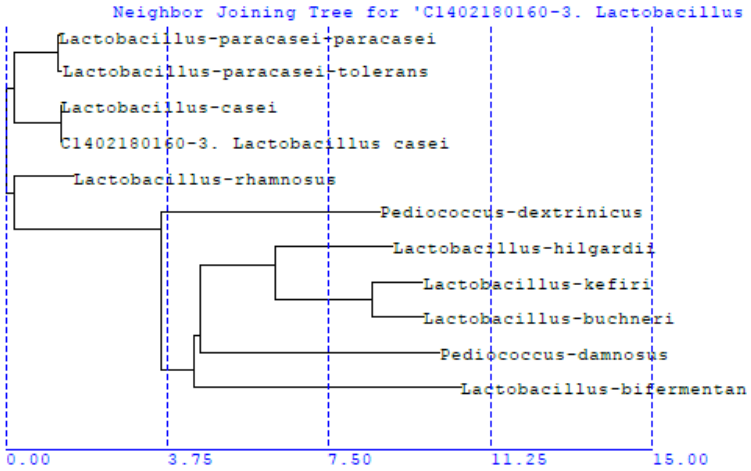
Created: 2/21/2014 10:36:28 AM
Sample ID: C1402180160-3. Lactobacillus casei

16S DNA: 553 base pairs

D16M2 DNA Match Report

Match	%Diff	Length	Library Entry Name
1	0.00	553	Lactobacillus-casei
2	2.08	553	Lactobacillus-paracasei-paracasei
3	2.17	553	Lactobacillus-paracasei-tolerans
4	2.89	553	Lactobacillus-rhamnosus
5	9.82	559	Lactobacillus-hilgardii
6	10.09	558	Pediococcus-dextrinicus
7	11.17	561	Lactobacillus-buchneri
8	11.37	560	Lactobacillus-kefiri
9	11.45	553	Lactobacillus-bifermentans
10	11.57	564	Pediococcus-damnosus

Exact match with Lactobacillus-casei



Closest Match: Lactobacillus-casei
Confidence level: Species

Reviewer's Signature: 02-21-14

Cliff Poindexter, Laboratory Manager

Alignment Report - 500BP Identification

Customer: Stratton

125 SANDY DRIVE•NEWARK, DE 19713•PH 302-737-4297•FX 302-737-7781•WWW.MIDILABS.COM

D16M2 Library Revision: 2.22

Created: 2/21/2014 10:36:25 AM
Sample ID: C1402180159-2. Lactobacillus bulgaricus

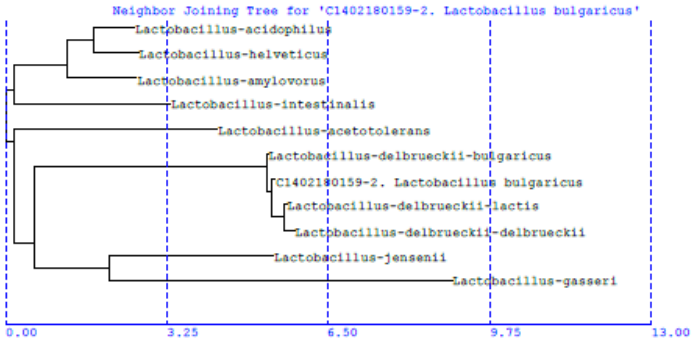
16S DNA: 548 base pairs

D16M2 DNA Match Report

Match	%Diff	Length	Library Entry Name
1	0.18	548	Lactobacillus-delbrueckii-bulgaricus
2	0.46	548	Lactobacillus-delbrueckii-delbrueckii
3	0.55	548	Lactobacillus-delbrueckii-lactis
4	7.22	550	Lactobacillus-acidophilus
5	8.03	550	Lactobacillus-helveticus
6	8.57	550	Lactobacillus-amylovorus
7	8.98	550	Lactobacillus-acetotolerans
8	9.06	550	Lactobacillus-intestinalis
9	9.69	550	Lactobacillus-jensenii
10	13.17	557	Lactobacillus-gasseri

Concise Alignment (maximum difference 0.91):

```
                  81|   90|   103|   227|   435|
Sample:             Y     R      Y      Y      T
Lib Match 1:        T     R      C      Y      T
Lib Match 2:        C     G      Y      C      C
Lib Match 3:        C     A      C      C      C
                    *     *      *      *      *
```



Closest Match: Lactobacillus-delbrueckii
Confidence level: Species

Reviewer's Signature: 02-21-14

Cliff Poindexter, Laboratory Manager

Alignment Report - 500BP Identification

Customer: Stratton

125 SANDY DRIVE•NEWARK, DE 19713•PH 302-737-4297•FX 302-737-7781•WWW.MIDILABS.COM

D16M2 Library Revision: 2.22

Created: 11/18/2013 1:43:39 PM
Sample ID: C1311136124-5 Lactobacillus plantarum

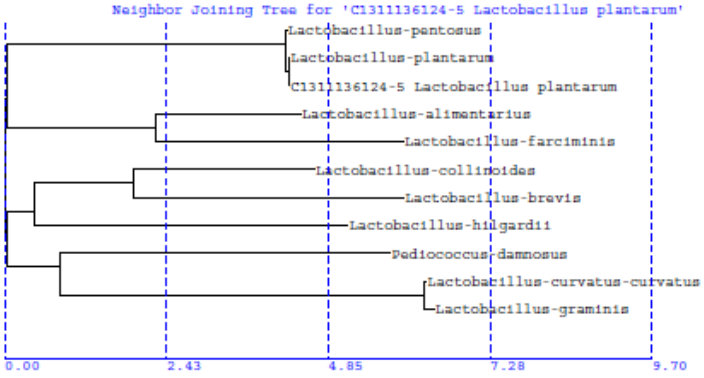
16S DNA: 552 base pairs

D16M2 DNA Match Report

Match	%Diff	Length	Library Entry Name
1	0.00	552	Lactobacillus-plantarum
2	0.09	552	Lactobacillus-pentosus
3	8.70	551	Lactobacillus-alimentarius
4	9.10	550	Lactobacillus-collinoides
5	9.35	552	Lactobacillus-brevis
6	9.89	559	Lactobacillus-hilgardii
7	10.04	556	Lactobacillus-curvatus-curvatus
8	10.04	551	Lactobacillus-farciminis
9	10.22	556	Lactobacillus-graminis
10	10.55	564	Pediococcus-damnosus

Concise Alignment (maximum difference 0.18):

```
                217|
Sample:            Y
Lib Match 1:       Y
Lib Match 2:       T
                   *
```



Closest Match: Lactobacillus-plantarum
Confidence level: Species, Closely Related

Reviewer's Signature: 11-18-13

Cliff Poindexter, Laboratory Manager

Alignment Report - 500BP Identification

Customer: Stratton

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D16M2 Library Revision: 2.22

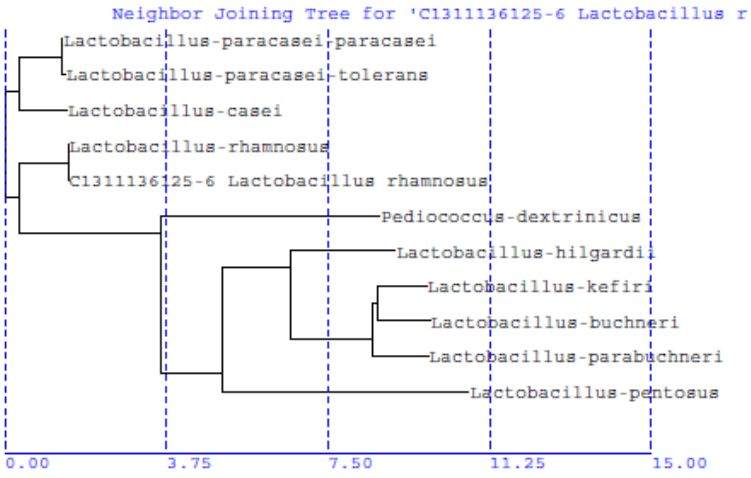
Created: 11/18/2013 1:43:41 PM
Sample ID: C1311136125-6 Lactobacillus rhamnosus

16S DNA: 553 base pairs

D16M2 DNA Match Report

Match	%Diff	Length	Library Entry Name
1	0.00	553	Lactobacillus-rhamnosus
2	2.80	553	Lactobacillus-paracasei-paracasei
3	2.89	553	Lactobacillus-casei
4	2.89	553	Lactobacillus-paracasei-tolerans
5	9.71	558	Pediococcus-dextrinicus
6	10.18	559	Lactobacillus-hilgardii
7	10.37	560	Lactobacillus-kefiri
8	10.55	561	Lactobacillus-parabuchneri
9	10.78	561	Lactobacillus-buchneri
10	11.01	552	Lactobacillus-pentosus

Exact match with Lactobacillus-rhamnosus



Closest Match: **Lactobacillus-rhamnosus**
Confidence level: **Species**

Reviewer's Signature: 11-18-13

Cliff Poindexter, Laboratory Manager

Alignment Report - 500BP Identification

Customer: Stratton

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D16M2 Library Revision: 2.22

Created: 11/18/2013 1:43:43 PM
Sample ID: C1311136126-7 Lactobacillus salivarius

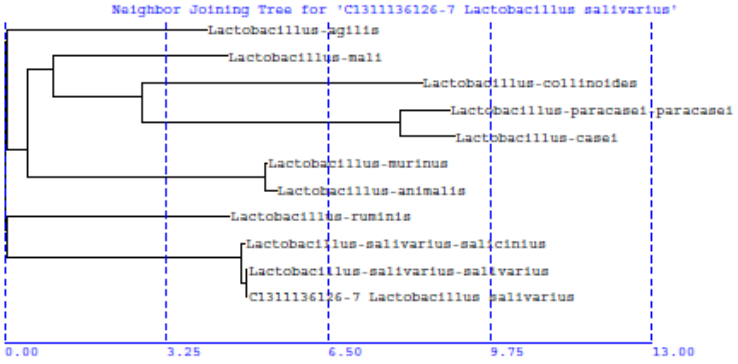
16S DNA: 548 base pairs

D16M2 DNA Match Report

Match	%Diff	Length	Library Entry Name
1	0.00	548	Lactobacillus-salivarius-salivarius
2	0.18	548	Lactobacillus-salivarius-salicinius
3	8.88	548	Lactobacillus-agilis
4	9.22	545	Lactobacillus-mali
5	9.38	549	Lactobacillus-ruminis
6	10.64	550	Lactobacillus-murinus
7	10.91	550	Lactobacillus-animalis
8	12.59	550	Lactobacillus-collinoides
9	13.04	553	Lactobacillus-paracasei-paracasei
10	13.06	553	Lactobacillus-casei

Concise Alignment (maximum difference 0.36):

```
                197|  210|
Sample:            G    C
Lib Match 1:       G    C
Lib Match 2:       R    Y
                   *    *
```



Closest Match: Lactobacillus-salivarius
Confidence level: Species

Reviewer's Signature: 11-18-13

Cliff Poindexter, Laboratory Manager

Alignment Report - 500BP Identification

Customer: Stratton

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D16M2 Library Revision: 2.22

Created: 11/18/2013 1:43:48 PM
Sample ID: C1311136131-12 Lactococcus lactis

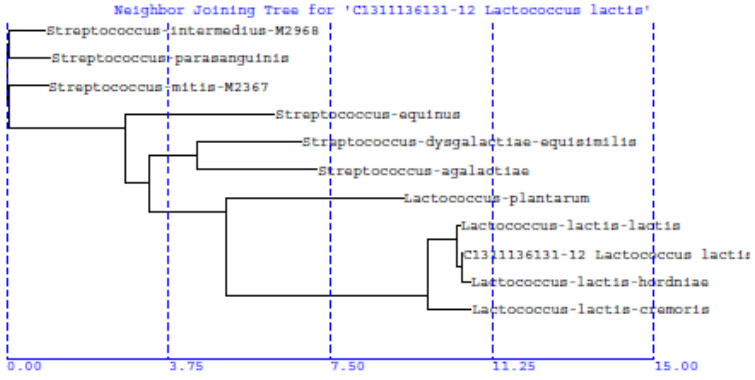
16S DNA: 535 base pairs

D16M2 DNA Match Report

Match	%Diff	Length	Library Entry Name
1	0.19	535	Lactococcus-lactis-hordniae
2	0.19	535	Lactococcus-lactis-lactis
3	1.68	535	Lactococcus-lactis-cremoris
4	9.31	535	Lactococcus-plantarum
5	10.78	535	Streptococcus-agalactiae
6	11.15	535	Streptococcus-parasanguinis
7	11.34	535	Streptococcus-equinus
8	11.50	536	Streptococcus-mitis-M2367
9	11.59	535	Streptococcus-dysgalactiae-equisimilis
10	11.62	535	Streptococcus-intermedius-M2968

Concise Alignment (maximum difference 0.37):

```
                 86|  138|
Sample:            A    C
Lib Match 1:       A    T
Lib Match 2:       G    C
                   *    *
```



Closest Match: Lactococcus-lactis
Confidence level: Species

Reviewer's Signature: 11-18-13

Cliff Poindexter, Laboratory Manager

Alignment Report - 500BP Identification

Customer: Stratton

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D16M2 Library Revision: 2.22

Created: 11/18/2013 1:43:46 PM
Sample ID: C1311136129-10 Bifidobacterium longum

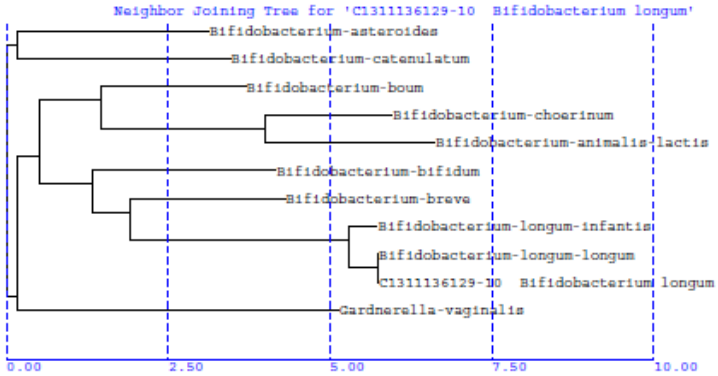
16S DNA: 505 base pairs

D16M2 DNA Match Report

Match	%Diff	Length	Library Entry Name
1	0.00	505	Bifidobacterium-longum-longum
2	0.89	506	Bifidobacterium-longum-infantis
3	6.56	511	Bifidobacterium-breve
4	6.69	508	Bifidobacterium-bifidum
5	7.84	508	Bifidobacterium-catenulatum
6	9.07	506	Bifidobacterium-asteroides
7	9.41	510	Bifidobacterium-boum
8	10.35	509	Gardnerella-vaginalis
9	10.92	521	Bifidobacterium-choerinum
10	11.13	520	Bifidobacterium-animalis-lactis

Concise Alignment (maximum difference 0.99):

```
                74|      274|  450|
Sample:         AAGC-     C     A
Lib Match 1:    AAGC-     C     A
Lib Match 2:    GGGCT     M     T
                **  *     *     *
```



Closest Match: Bifidobacterium-longum
Confidence level: Species

Reviewer's Signature: 11-18-13

Cliff Poindexter, Laboratory Manager

Alignment Report - 500BP Identification

Customer: Stratton

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D16M2 Library Revision: 2.22

Created: 2/21/2014 10:36:41 AM
Sample ID: C1402180164-7. Bacillus coagulans

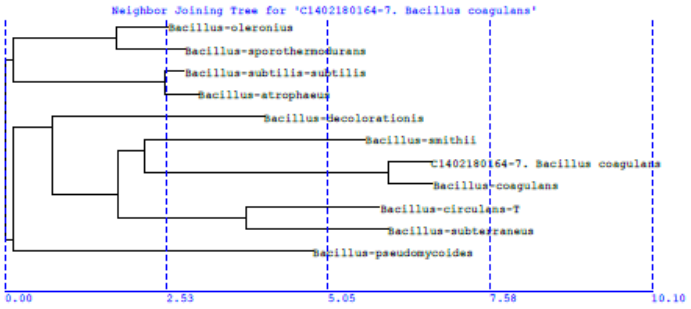
16S DNA: 537 base pairs

D16M2 DNA Match Report

Match	%Diff	Length	Library Entry Name
1	1.40	537	Bacillus-coagulans
2	7.99	537	Bacillus-smithii
3	9.03	537	Bacillus-decolorationis
4	9.12	534	Bacillus-subterraneus
5	9.28	532	Bacillus-circulans-T
6	9.78	537	Bacillus-oleronius
7	10.04	537	Bacillus-pseudomycoides
8	10.06	535	Bacillus-subtilis-subtilis
9	10.13	537	Bacillus-sporothermodurans
10	10.15	535	Bacillus-atrophaeus

Concise Alignment with Bacillus-coagulans

```
Sample:     (139) TC (172) G (209) ACGGCTTYKGCTGT (260) T (272) T
            -----    -----   -----  ||||||  ||||  -----   -----
LibEnt 1:   (139) CT (172) R (209) GCGGCTTCGGCTGC (260) C (272) C
```



Closest Match: Bacillus-coagulans
Confidence level: Genus

GenBank Match: 99%, Bacillus coagulans DQ297925*
**GenBank is a public database supported by The National Center for Biotechnology Information. GenBank is used for reference purposes only and is not a validated database.*

Reviewer's Signature: 02-21-14

Cliff Poindexter, Laboratory Manager