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Evaluation of Prebiotic Fibers and Pectin on Probiotic Strains and Effect of Bacteriocin-like Substance on the Survival of Listeria Monocytogenes

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EVALUATION OF PREBIOTIC FIBERS AND PECTIN ON PROBIOTIC STRAINS AND EFFECT OF BACTERIOCIN-LIKE SUBSTANCE ON THE SURVIVAL OF *LISTERIA MONOCYTOGENES*

EVALUATION OF PREBIOTIC FIBERS AND PECTIN ON PROBIOTIC STRAINS AND EFFECT OF BACTERIOCIN-LIKE SUBSTANCE ON THE SURVIVAL OF *LISTERIA MONOCYTOGENES*

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ABSTRACT

Probiotics and prebiotics have been shown to provide beneficial effects on the host health and there are numerous research efforts being conducted on a variety of specific applications. However, most prebiotic carbohydrates are lower molecular weight oligosaccharides; and the health benefits of higher molecular weight pectins have not been fully evaluated. According to reviews, pectins are fermented in the large intestine due to the cooperative activities of colonic bacteria with complementary enzymatic activities. There are very few studies that demonstrate the fermentability of high methoxy pectin and low methoxy pectin by pure cultures of human isolates or probiotic bacteria. Thus, it is conceivable that lack of evidence/studies may be problematic for defining human health properties of pectin. In studying the fermentation of prebiotics by bacteria, optical density (OD) measurements can be used, but OD can be interfered with by other substances present in the media. Some studies have suggested the use of fluorescent dyes to observe the growth of bacteria which has the advantage of differentiating between live and dead cells. Finally, certain lactic acid bacteria or probiotics have the ability to inhibit or kill pathogens due to production of lactic acid or other antibacterial substance (eg: bacteriocin). Therefore, the objective of this research is to study the fermentability of fructooligosaccharides (FOS), galactooligosaccharides(GOS), inulin agave (IA), high methoxy pectin (HMP), and low methoxy pectin (LMP) with three strains of probiotic bacteria in order to find the suitable carbohydrates that could be used as synbiotics. In addition, we also aimed to explore the use of acridine orange as a method to monitor the growth response of bacteria. The antilisterial effects of *Lactobacillus acidilactici* are also studied using survival assays and spot-on-lawn assays.

We determined that FOS, GOS, and IA are good substrates to support the growth of probiotic bacteria and although HMP fermentability is still not clear, this carbohydrate could be of benefit to use as a synbiotic as well due to its ability to enhance the survival of probiotic bacteria both in normal media and simulated gastric conditions. In addition, we found that most bacteria grown in the presence of GLU exhibit better survival during exposure to bile solutions. However, the study on the use of acridine orange did not confirm that this dye can be used instead of OD. Finally, the last study has shown an effect of *Lactobacillus acidilactici* on the reduction of *Listeria* which could be partly due to pH.

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Chapter One: Overall introduction

The human large intestine contains up to 10^{12} bacteria per gram when enumerated from waste contents (Cummings 1981; Moore and Holdeman 1974) and hundreds of species of bacteria are present (Moore and Holdeman 1974). Colonic bacteria can be divided broadly into either harmful or beneficial bacteria. Harmful bacteria can cause diseases such as diarrhea, systemic infections, liver damage, cancer and intestinal putrefaction. Beneficial bacteria improve the health of the host by stimulating the immune system, synthesizing vitamins, lowering gas distention, inhibiting the growth of harmful bacteria and improving digestion and absorption of essential nutrients (Gibson and Roberfroid 1995). Most colonic bacteria derive part of their energy from undigestible dietary components such as polysaccharides and oligosaccharides which resist digestion in the upper intestinal tract. The main by-products of bacterial fermentation of these polysaccharides are short chain fatty acids (SCFA) which contribute to improving the host's health (Macfarlane and Macfarlane 1995). Furthermore, it has been found that changes in the diet can modify the microbial composition in the colon (Drasar and Roberts 1989). The colon contents of infants fed breast milk contain primarily *Bifidobacteria* (99%) and only approximately 1% of enterobacteria; however, the colon contents of infants fed formula are found to be predominated by *Bacterioides, Bifidobacteria, Clostridia* and *Streptococci* (Yoshita et al. 1991; Gibson and Roberfroid 1995). Probiotics and prebiotics can be employed to attain a better-balanced composition of microorganisms in the colon (Fuller 1989; Gibson and Roberfroid 1995). Bacteria used in probiotic products are from certain species of *Lactobacillus* and *Bifidobacteria* (Goldin and Gorbach 1992) and the potential prebiotics are fructo-oligosacharides, galacto-oligosaccharides, lactulose, lactosucrose, soybean oligosaccharides,

isomalto-oligosaccharides, palatinose, xylo-oligosaccharides, and gluco-oligosaccharides (Gibson et al. 2000; Manning and Gibson 2004).

Lactic acid bacteria (LAB) play a very important role as starter cultures in the fermentation of most food products such as dairy, meat, vegetable and bakery products. LAB can extend product shelf life and prevent the growth of spoilage and pathogenic microorganisms through the production of antibacterial by-products such as lactic acid, hydrogen peroxide, and bacteriocins (Ray and Daeschel 1992). Bacteriocins are proteins produced by bacteria that have antibacterial effects which can kill or inhibit the growth of other bacteria. Thus, they are used as biopreservatives in food (Abee et al. 1995). In most studies bacteriocins are pediocin-like bacteriocins which are active against pathogenic *Listeria* spp. as well as other bacteria (Klaenhammer et al. 1993).

Gut bacteria

The microflora in the gastrointestinal (GI) tract of infants originate from the vagina and feces of the mother during the birth of the child (Mevissen-Verhage et al. 1987) and are initially predominated by facultative anaerobes including *Escherichia coli* and enterococci (Rotimi and Duerden 1981). On the other hand, infants delivered by cesarean delivery have no contact with the vaginal or intestinal microflora; thus, their intestines are initially colonized by environmental microorganisms. Also, cesarean-delivered infants appear to have less diverse species of intestinal microbiota than the vaginally delivered infants and the *Bifidobacteria* species are absent from cesarean-delivered infants (Biasucci 2008). However, after weaning (greater than 2 years old), the microbial composition resembling adults' becomes established (Gibson and Roberfroid 1995). It has been shown that the gross differences of infant's microflora composition are due to

feeding (Drasar and Roberts 1989). Large intestine is the most colonized part of the human GI tract by microorganisms which derives a proportion of their energy from mucin and dietary components, which have not been degraded in the upper intestinal tract and reach the large intestine (Macfarlane and Gibson 1994). Dietary components consisting mainly of polysaccharide, oligosaccharide, protein, peptide and glycoprotein are fermented and produce short chain fatty acid (SCFA) as their major end products; the majority of these SCFA are acetate, propionate and butyrate (Macfarlane and Macfarlane 1995). The metabolism of intestinal microorganisms could also lead to the production of essential vitamins (eg, vitamin K, vitamin B12 and folic acid) and amino acids for the host (Hamer et al. 2008; Wong et al. 2006); and those microflora act against invading pathogens through the colonization resistance and the production of antimicrobial substances. In addition, the intestinal microbiota plays important role in the development, maturation and maintenance of the mucosal immune system, the intestinal barrier and the GI sensory and motoric functions (Gerritsen et al. 2011).

Probiotics

Probiotics are live microorganisms added into food to improve the host health by balancing the intestinal microbial composition (Fuller 1989). Certain species of *Lactobacillus* and *Bifidobacteria* are commonly used in the manufacture of probiotic products (Goldin and Gorbach 1992) because of their well-known beneficial effect to host health and they are generally recognized as safe (GRAS) (Chomarat and Espinouse 1991; Oakey et al. 1995). Many studies have shown that probiotics can stimulate the immune system, decrease serum cholesterol, alleviate lactose intolerance, decrease diarrheal incidence, control infections, act as antibiotics,

suppress tumors and protect against colon/bladder cancer by maintaining a healthy balance of microorganisms (Scheinbach 1998).

For most mammals, lactase activity declines after weaning; however, in some healthy humans, lactase activity persists at high levels throughout adult life due to the development of lactase persistence (Swallow 2003). Lactose intolerance among the population of the world varies depending on ethnicity and habit of consuming dairy products. Consequently, the northern European people who consume dairy foods frequently have as few as 2% of the population deficient in lactase. Lactase deficiency is prevalent in Hispanic people (80%), black and Ashkenazi Jewish people (60-80%) and almost 100% of Asian and American Indian people (Heyman 2006). When undigested lactose reaches the large intestine, it is metabolized by colonic microflora which subsequently produces methane, carbon dioxide, and hydrogen altering the osmotic balance in the colonic lumen. Symptoms of lactose intolerance include cramping, diarrhea, flatulence, and abdominal bloating (Suarez and Savaiano 1997). However, a number of human studies have shown that high-lactose milk products supplemented with certain starter cultures can be tolerated by lactose-intolerant individuals. For example, in fermented milk products such as yogurt, the lactose is digested during microbial fermentation and lactase in the starter microorganisms negates the enzyme deficiency of the host (Scheinbach 1998).

It is estimated that about two billion cases of diarrhea occur each year among children under 5 years old and more than half of these incidences happen in Africa and South Asia (www.who.com). In addition, the incidence of this disease varies greatly depending on the seasons and child's age. The youngest children are the most vulnerable to this illness with the highest incidence occurring in the first two years of their lives, then diarrhea incidence declines once a child get older (www.who.com). There are many causes responsible for this condition;

however, rotavirus is the most common one (Claeson and Merson 1990). The effect of *Bifidobacterium bifidum* and *Streptococcus thermophiles* to reduce rotavirus-caused diarrhea has been shown by Saavedra et al. (1994) through a trial on infants, aged 5-24 months, feeding formula with or without the above probiotics. This author reported the reduction of diarrhea incidences and rotavirus shedding within infants fed formula supplemented with *Bifidobacterium bifidum* and *Streptococcus thermophiles*.

Studies based on epidemiological information suggest that the incidence of colon cancer is associated with a high-fat diet as dietary fat stimulates bile acid turnover which can lead to an increase of bile acids in the colon. Secondary bile acids produced in the colon by bacterial metabolism have been implicated as factors promoting colon cancer (Reddy 1977). It is thought that indigenous microflora in the intestine could produce enzymes such as glucuronidase, nitroreductase and azoreductase which can convert procarcinogens into carcinogens. This has prompted research on the use of probiotic bacteria to reduce the risk of colon cancer. The mechanisms involved in the anti-tumor action of probiotics are proposed as following: preventing the growth of bacteria producing enzymes responsible for converting procarcinogens into carcinogens, decreasing pH which influences the activity of intestinal microflora and the solubility of bile acids, suppressing the carcinogen/procarcinogen by binding, blocking or removing it, altering colonic transit time to remove fecal mutagens more rapidly, and stimulating the immune system (Hawksworth et al. 1971).

However, there are challenges in probiotic application. In the GI tract of the host, probiotics must be active as they have to compete with indigenous microflora for nutrients and colonization sites, but they are likely to be in a stressed state when they reach the colon (Gibson and Roberfroid 1994) because they are exposed to diverse physical and chemical barriers such as

gastric acid and bile acids (Pochart et al. 1992; Gibson and Roberfroid 1995). Also, Bouhnik et al. (1992) observed that the probiotic bacteria are washed out rapidly from the colon if the host stops consuming the probiotic products.

Prebiotics

Prebiotics are employed to promote both beneficial bacteria which are already established in the colon and externally administered probiotic bacteria. Prebiotics are food ingredients which are undigestible in the upper GI tract and reach the colon to beneficially influence the host by selectively promoting the growth and/or activity of certain bacteria in the colon (Gibson and Roberfroid 1995). In fact, consumers receive moderate levels of prebiotics naturally from many fruits and vegetables such as leeks, Jerusalem artichokes, chicory, onion, garlic, bananas, and asparagus, but the levels of prebiotics in those food sources are generally too low to exhibit any significant effect (Manning and Gibson 2004) on the composition of intestinal microflora. Thus, prebiotics are commercially extracted and concentrated from fruits and vegetables through the hydrolysis of polysaccharides from dietary fibers or starch, or through enzymatic generation. Suggested potential prebiotics are fructo-oligosacharides, galacto-oligosaccharide, lactulose, lactosucrose, soybean oligosaccharides, isomalto-oligosaccharides, palatinose, xylo-oligosaccharides, and gluco-oligosaccharides (Gibson et al. 2000; Manning and Gibson 2004).

Furthermore, the prebiotic properties of carbohydrates may be dependent on the composition of monosaccharides of the molecules, and the glycosidic linkage between monosaccharide residues. Monosaccharides such as fructose, xylose, galactose and glucose are generally considered to be the main components of the known oligosaccharide prebiotics. Additionally, the fermentability and digestibility of prebiotics in the small intestine is determined

by the glycosidic linkage between monosaccharide residues; for example, *Bifidobacteria* contain enzyme β-fructofuranosidase which allows them to ferment prebiotic fructooligosaccharides (FOS) (Manning and Gibson 2004).

Short-chain fructooligosaccharides (sc-FOSs): Short-chain fructooligosaccharides are a group of linear Glucosyl $\alpha(1\rightarrow2)(\text{fructosyl})_n\ \beta(1\rightarrow2)$fructose polymers with a degree of polymerization (DP) between 1 and 5. They occur naturally in several plants such as asparagus, wheat, Jerusalem artichokes, rye and; particularly; onion, which contains the highest amount of FOS (25 to 40% of dry matter) and 97% of this content is sc-FOS (GFn, n<5; which G, F, and n represent a molecule of glucose, a molecule of fructose and number of fructose units respectively). The commercial production of sc-FOSs is mainly based on two processing methods which are partial hydrolysis of inulin by *endo*-inulinase (Orafti, Tienen, Belgium) and the conversion of sucrose by fungal fructosyltranserase (ACTI-LIGHT®, Beghin Meiji, Neuilly-sur-Seine, France). These sc-FOSs have similar test profiles to sucrose (30% lower in sweetness), are able to retain more water than sucrose, do not become involved in Maillard reaction, and are stable at temperatures up to 130°C and a pH greater than 3; They are often used in food applications such as breakfast cereals, biscuits, cakes, and dairy products. In addition, sc-FOSs has also gained popularity due to their nutritional benefits. These fructans are undigestible in the upper part of human GI tract and they are selectively fermented by bifidobacteria (Bornet et al. 2002).

Bifidobacteria commonly produce β-fructosidases and these enzymes selectively cleave the β-(1,2) glycosidic bonds of sc-FOSs. After the breakdown of the sc-FOS molecules, fructose is released and acts as a key substrate for the bifidus pathway of hexose fermentation which is mainly carried out by bifodobacteria (Scardovi 1965). This pathway is referred to as the fructose-

6-phosphate shunt (DeVries et al. 1967) and involves a series of cleavage and isomerization reactions that produce pentose phosphates which are subsequently metabolized to glyceraldehyde-3-phosphate (G-3-P) and acetyl phosphate. Acetyl phosphate is used to produce ATP and G-3-P is converted into pyruvate (Modler et al. 1990); and under some conditions for growth, the by-products, ethanol and formate are produced (DeVries and Stouthamer 1968). Bifidobacteria prefer fructans as a source of carbon and energy and the growth of different species of bifidobacteria is largely dependent on the types of fructan oligosaccharides. Oligofructose with a linear chain molecule comprised of glucose and fructose (degree of polymerization = 4), exhibits a higher bifidogenic effect than larger molecular weight carbohydrates and branched chain varieties (Gibson and Wang 1994); thus, it suggests that sc-FOS could induce β-fructosidase, and promote bifidobacteria growth more efficiently than long chain fructans (Bornet et al. 2002). In addition, human studies on the effect of this sc-FOS have yielded increases in bifidobacteria population and enumeration from fecal samples that correlate with the increases in activity of β-fructosidase (Bouhnik et al. 1996) and the ingested dose of sc-FOSs (Bouhnik et al. 1999).

Galactooligosaccharide (GOS): Human milk is composed of approximately 7% carbohydrates, 90% of which is lactose, and the rest being oligosaccharides (ESPGAN Committee on Nutrition 1977). The principal components of oligosaccharides present in human milk include sialic acid, N-acetylglucosamine, L-fucose, D-glucose and D-galactose. It is found that infants fed human breast milk contain high numbers of bifidobacteria compared to infants-fed formula (Yoshita et al. 1991; Gibson and Roberfroid 1995) and oligosaccharides in human milk may be the most relevant component for this prebiotic effect. However, oligosaccharides in human milk have very complicated structure; thus industry has not yet found carbohydrates from other sources which

have identical structures to these oligosaccharides (Boehm and Stahl 2007). GOS which has some similarities in their chemical structure to oligosaccharides in human milk (Boehm and Stahl 2007) is produced from lactose of cow's milk and has been shown to have prebiotic effects (Yanahira et al. 1995). The molecular structure of GOS is (Gal)n-Gal-Glu, where Gal, Glu, and n correspond respectively to a galactose molecule, a glucose molecule and an interval between 1 and 4 galactose molecules (Rivero-Urgell and Santamaria-Orleans 2001); and GOS is resulted from the cleavage of lactose molecules by β-galactosidase to produce many oligomers with different chain lengths (Prenosil et al. 1987). Due to the stability of GOS at high temperature and in acidic environments, they are used in many food applications such as infant formulas, dairy products, sauces, soups, breakfast cereals, snack bars, ice creams, beverages, bakery products, animal feeds, and as sugar replacements to increase texture and mouthfeel of foods, and act as bulking agents (Yang and Silva 1995). Also, the mixture of 90% GOS and 10% long-chain FOS (inulin) are used in infant formulas to simulate human milk (Boehm et al. 2002; Weaver 2003).

Inulin Agave: Many species of Agave plants originated from Mexico and the main products of their photosynthesis are fructans composed of fructose polymers which are generally linked to the moiety of a terminal glucose (López and Urías-Silvas 2007). Furthermore, these fructans are resistant to enzymes in the digestive tract of humans and are, therefore, considered as non-digestible oligosaccharides based on results from both *in vivo* and *in vitro* research. Owing to this resistance, fructans pass through the upper part of the human GI tract, reach the colon (Cummings et al. 2001), and are fermented by colonic microflora producing SCFA. In addition, the structure of fructans may vary depending on the plant species (López and Urías-Silvas 2007); however, fructans are divided into only five major groups based on the majority of structural units forming their molecules and the groups consist of inulin, levan, mixed levan, inulin

neoseries and levanneoseries as shown in table 1 (Vijn and Smeekens 1999; López and Urías-Silvas 2007). In the food industry, inulin and oligofructose are used for nutritional purposes based on their multi-functional properties; however, those two fructans are often used to improve the balance of nutritional composition and organoleptic quality of the products (López and Urías-Silvas 2007).

Pectin: Pectin is a complex galacturonic acid-rich polysaccharide which occurs naturally in the cell walls of higher plants and acts as a cement-like material for the cellulosic components of the plant cell wall (Thakur et al. 1997; Willats et al. 2001; Mohnen 2008). The major polysaccharides forming pectin include homogalacturonan (HGA), rhamnogalacturonan-I (RG-I), and rhamnogalacturonan-II (RG-II) which are believed to covalently link together (Thakur et al. 1997; Debra 2008). Among those, homogalacturonan (HGA) is the most abundant constituent of pectin comprising at least 65% to be considered "pectin". This HGA is formed by the linear linking of galacturonic acid (GalA) at C-1 and C-4. Furthermore, approximately 70 to 80% of the GalA on this domain undergoes methyl esterification at C-6 carboxyl. In addition to this reaction, the biosynthetic modifications and substitutions of HGA can also occur, but they are not frequent. For example, GalA residues in HGA can be O-acetylated at the C-2 and C-3 sites occurred particularly in sugar beet roots and potato tubers. These modifications on the domain can change the functional properties of HGA (Willats et al. 2001).

The other important component is RG-I which is often known as the hairy region of pectin. It is an acidic domain which represents 20 to 35% of pectin and contains approximately 100 disaccharides in repeating units of (1→2)-α-L-rhamnose-(1→4)-α-D-galacturonic acid (Mohnen 2008; Willats et al. 2001). Anywhere from 20 to 80% of the rhamnose residues in this domain are often substituted with side chains such as polymeric (1→4)-β-linked D-galactosyl

and (1→5)-α-linked L-arabinosyl residue. These side chains are predominantly neutral residues and they vary in size from a single glycosyl residue to 50 or more which result in a large and highly variable family of polysaccharides. However, it is unknown whether GalA on RG-I domain can be esterified with methyl groups as in the HGA domain (Thakur et al. 1997). The last known domain of pectin is RG-II which is the branched pectic domain containing an HGA backbone. RG-II is highly conserved and it has a backbone of approximately 9 GalA residues that are (1→4)-α-linked and RG-II is substituted by 4 well-defined side chains which often referred to as side chain A, B, C and D. These side chains contain sugar such as apiose, aceric acid and 2-keto-3-deoxy-D-manno-octulosonic acid (Beda 2011, Willats et al. 2001).

Pectin is a very important polysaccharide used in food, pharmaceuticals and other industries. The ability of pectin to form gels in the presence of Ca^{2+} ions or a solute at low pH makes it suitable for food applications and it is often used as a gelling agent, thickener, texturizer, emulsifier or stabilizer. Additionally, pectin has recently been used in low-calorie foods as a fat or sugar replacer. However, the commercial production of pectin is limited because the gel-forming ability of pectin is varied owing to the source of extraction and a number of other factors such as molecular size and degree of methoxylation. The main sources of commercial pectin are sugar beet, citrus peels, apple pomace and residues from the seed heads of sunflowers (Thakur et al. 1997). Furthermore, pectin as a functional food additive has been divided into 3 main types including high methoxy (HM), low methoxy (LM) and amidated pectin. High methoxy pectin has more than 50% of its acid units esterified with methyl and they can be further subdivided into rapid set and slow set pectin according to their gelling temperature. Due to the ability of HM pectin to form gels with acid in the presence of sugars, this form of pectin is often used in jam and jelly applications and occasionally in dairy foods as well.

The other type is LM pectin which is generated from the modification of the extraction process or continued acid treatment of HM pectin; it contains less than 50% of methyl ester groups and is referred to as low methoxy (LM) pectin. Low methoxy pectin has considerably more specialized applications than HM pectin including thickening, gelling, stabilizing and water binding functional properties to foods. The last type of pectin is amidated pectin which is produced by treating pectin with ammonia; this pectin is used in a range of foods such as lower sugar products, reduced sugar preserves, fruit preparations for yogurts, dessert gels and toppings (IPPA).

Pectin can also be extracted from soy hulls, a major co-product of soybean processing. These soy hulls are approximately 8% of the whole soybean and contain about 86% complex carbohydrates which make them a source of dietary fiber. The fraction of insoluble carbohydrates of soy hull cell walls consists of pectin (30%), hemicellulose (50%), and cellulose (20%) (Snyder and Kwon 1987). In addition, soy hulls in the diet have been reported to have ability in reducing blood serum cholesterol (Mahalko et al. 1984); and they have been added as a fiber supplement in bakery products (Johnson et al. 1985).

A fermentation study of high methylated citrus pectin (HMCP), low methylated apple pectin (LMAP), and pectic oligosaccharides derived from HMCP (POS I) and LMAP (POS II) were performed by using pure cultures selected from gut bacteria including *Bifidobacteria*, *Lactobacilli*, *Bacteroides*, and *Clostridia*. The result from this study indicated that all *Bacteroides* and *Clostridium* species could utilize pectins to grow, but some of them cannot consume the derived oligosaccharides. In addition, most bifidobacteria show better growth on the oligosaccharides rather than the pectins. Thus, it is suggested that oligosaccharides might have a prebiotic effect as pathogens fail to grow on this compound and bifidobacteria growth were

promoted (Olano-Martin et al. 2002). Additionally, Bayliss and Houston (1984) reported that pectins are fermented by many species of the human gut microflora including *Bacteroides distasonis, Bacterioides ovatus and Bifidobacterium infantis* , but both high methylated and low methylated pectin did not selectively promote the growth of bifidobacteria. Olano-Martin et al. (2002) have performed the follow-up experiment using POS I, POS II and their parent pectins (HMCP and LMAP) in controlled pH mixed fecal batch cultures to evaluate their potential prebiotic effects. They observed the increased number of *Bifidobacteria* when POS I and POS II were used as carbon source while the number of *Bacteroides* and *Clostridia* were not increased. Also, the prebiotic index (PI), which is the absolute increase in new cfu/g of *Bifidobacteria* in feces divided by daily-ingested dose of prebiotics (in gram) (Roberfroid 2007), increased as the fermentation of POS I and POS II progressed; on the contrary, the PI for the pectins was reduced. Thus, Olano-Martin et al. (2002) suggested that POS I and POS II are better candidate prebiotics than the parent pectins (HMCP and LMAP).

Gum Arabic: Gum Arabic or gum acacia is a dried exudate collected from acacia trees (*Acacia senegal* or *Acacia seyal*) which are commonly grown in tropical and subtropical geographical regions, particularly Africa. This gum is a heteropolysacchride with high molecular weight of approximately 350 to 850 kDa and it is composed of rhamnose, galactose, glucuronic acid, arabinose residues and other minerals such as magnesium, calcium and potassium (Williams and Phillips 2000; Calame et al. 2008). Gum arabic is used in the food industry with major applications in confectionery products such as toffees, marshmallow, pastilles and gums. It is also used as an emulsifier in soft drink production such as cola flavor oils and concentrated citrus. The other application is in flavor encapsulation to transform food volatile flavors in

liquids into flowable powders for incorporating into dried food products such as soups and dessert mixes (William and Phillips 2000).

Products of microbial fermentation in the colon and their effects on the host:

The fermentation of undigestible carbohydrates in the large intestines produced SCFA, mainly acetate, propionate, and butyrate (Roberfroid et al. 2010). The increased concentration of SCFA will result in pH reduction, which alters the composition of intestinal microflora (eg: reduces pathogens such as *Clostridia* as pH is very low), decreases bile acid solubility, increases mineral absorption and reduces the absorption of ammonia by the protonic dissociation of ammonia and other amines (eg: the formation of the less diffusible NH_4^+ compared with the diffusible NH_3) (Julia et al. 2006). Acetate is the main SCFA in the colon which is readily absorbed and transported to the liver (Cook and Sellin 1998); and has been shown to increase cholesterol synthesis (Wong 2006). However, propionate has been shown to inhibit cholesterol synthesis. The most important SCFA is butyrate which is used by colonic epithelial cells as an energy source; and also plays a major role in the regulation of cell proliferation and differentiation (Julia et al. 2006). Furthermore, butyrate may reduce the risk of colon cancer through stimulating apoptosis of cancer cells and it may also relieve patients from inflammatory bowel disease (Scheppach 1996).

Metabolism of Indigestible Food Components by Intestinal Microflora

Fermentation: The most important microbial-based metabolism in the large intestine is the fermentation of indigestible carbohydrates carried out by colonic microflora (Cummings and Macfarlane 1997). The undigestible carbohydrates enter the colon in the form of various complex polysaccharides (Cummings and Macfarlane 1991) and they are degraded by bacterial

enzymes including polysaccharidases (amylase, pectinase, and xylanase) and glycosidases (α-L-arabinofuranosidase, β-D-galactosidase and β-D-glucosidase). Furthermore, because the activities of these enzymes are found extracellularly and within the bacteria residing in different parts of the GI tract (Englyst et al 1987), Cummings and Macfarlane (1991) have suggested that the breakdown of these polysaccharides in the colon may result from the cooperative activity of enzymes produced from many different species of colonic bacteria. Some examples of the undigestible carbohydrates are plant polysaccharides, starch, and fructo-oligosaccharies (FOS).

The plant polysaccharides which could reach the colon are complex polymers of plant cell walls including cellulose, xyloglucan, mannan, pectins, lignin, arabinoxylan, and β-glucan. These cell wall materials are degraded in the colon by microbial enzymes such as hydrolases, esterases, and lyases (Louis et al. 2007). In addition, certain dietary starches which are not hydrolyzed by host amylases can also serve as substrates for colonic fermentation. The structure of this type of starch is composed of amylopectin and amylose, and the enzymes of colonic bacteria involved in the breakdown of starch molecules are α-amylases (hydrolyse α-1,4-linkages of amylose), type I pullulanases (cleave α-1,6-linkages of amylopectin) and amylopullulanases that are responsible for cleavage of both α-1,4- and α-1,6- linkage (MacGregor et al. 2001). Another example is the prebiotic fructo-oligosaccharides which are a mixture of fructose linearly linked together by β(2-1) bonds, which linkages prevent FOS from being digested like typical carbohydrates. However, bifidobacteria can produce intracellular enzymes, β-fructofuranosidases, which could breakdown the FOS molecules and, consequently, utilize these substrates (Warchol et al. 2002).

The primary products of colonic fermentation are the SCFA which can vary in amounts as well as types due to intestinal transit time, microflora composition, and substrate availability.

It is found that long transit times increase the SCFA concentration in the colon due to the shift of overall bacterial metabolism to the breakdown of proteins and fermentation of amino acids and the highest concentration of SCFA is in the proximal large intestine due to the greater amount of carbohydrates present in the region (Macfarlane and Macfarlane 2003). In addition, colonic fermentation is affected by chemical structure, physical form and quantity of the substrates available for colonic bacteria, number and types of microorganisms present, catabolite regulatory mechanisms, the availability of inorganic electron donors, for example, nitrate (Allison and Macfarlane 1988) and sulfate (Gibson et al. 1993), and competition and cooperation between different colonic bacteria species (Macfarlane and Gibson 1994). The main SCFA resulting from colonic fermentation are acetic, propionic and butyric acids (Cummings et al. 1987). Butyrate is the most important SCFA which provides energy to host epithelial cells, prevents carcinogenesis and inflammation, affects the intestinal barrier and plays a role in satiety and oxidative stress (Hamer et al. 2008). The fermentation of different polysaccharides produces different types and amounts of SCFA; for instance, considerable acetate is produced from the fermentation of xylan and pectin while substantial amounts of acetate and propionate originate from the breakdown of arabinogalactan molecules and, butyrate is produced mainly from starch breakdown (Cummings et al. 1987).

Enumeration Methods: The growth requirements of colonic bacteria are very complicated due to the richness of nutrients in the gut and cross-feeding among microorganisms. Also, surveys based on 16S rRNA genes reveals that greater than 75% of bacterial species in the GI tract do not match very closely to the ones that are cultivatable on anaerobic media and the detailed information on the growth requirements of most colonic bacteria is still unknown (Flint et al. 2007; Ricke and Pillai 1999). In addition, methods normally used to determine the number of

microorganisms in a culture or a sample are optical density (OD) measurement, microscopic enumeration, total plate count, biochemical measurement (such as protein and lipid), and flow cytometry (Martens-Habbena and Sass 2006). However, there are some constraints in using these methods; for example, monitoring OD is relatively simple and rapid to perform, but has low sensitivity is very low and detection can be interfered with by other substances present in the media. Microscopic enumeration is a more sensitive method compared to OD measurement, but it requires more time, bias can be introduced due to perceptions of the individual performing enumeration and the inability to distinguish dead from alive cells unless some sort of dye is used (Gasol et al. 1999; Gruden 2004). Total plate enumeration of colony forming units (CFU) is widely used and it has the advantage of further isolation of microorganisms of interest (Bollmann 2002). However, plate count methods are time-consuming as the time taken for microorganisms to form visible colonies is relatively long (Breeuwer and Abee 2000) and some microbial cells are viable but nonculturable due to cellular injuries, shortage of essential nutrients for replication, or adoption of cryptobiotic, dormant, moribund, or latent states (Kell et al. 1998) which can lead to erroneously low counts of viable bacteria. In fact, when the cells are sublethally injured by environmental conditions (Mossel and Netton 1984), they can enter a physiological state where specific repair mechanisms are required before they are able to regrow (Ray and Speck 1973). Also, in the case of starvation, microorganisms can reach the moribund state where they would stop replicating, but the cells are still functional (Postgate 1967). Dormancy of microorganisms is another state which is defined as a reversible state of metabolic shutdown. These dormant cells may not be grown by cultivation, but the regrowth can occur if specific stimuli are available (Kaprelyants et al. 1993).

Flow cytometry (FC): FC is a technology to quantitatively analyze and characterize the population of cells at single cell level based on illuminating the cells by a laser beam. The intensity of fluorescence or scattered optical signals correlates with the functional and/or structural cell parameters such as metabolic activity, membrane potential, and membrane integrity (Davey and Kell 1996; Shapiro 2003; Díaz et al. 2010). A common FC is composed of several basic parts including the light source, the flow cell and the hydraulic fluidic system, several optical filters, detector (a group of photodiodes or photomultipliertubes), and a data processing unit. The hydraulic fluid system is responsible for confinement of cells to ensure precise measurement and prevent obstruction of the nozzle (Díaz et al. 2010). The light sources produce a light beam to illuminate the samples (Álvarez-Barrientos et al. 2000) and current FC systems use several different varieties of lasers to emit light with different wavelengths (Shapiro 2000). However, the selection of lasers actually depends on the types of cells to analyze and the range of wavelengths suited for the excitation of the fluorescent markers used (Diaz et al. 2010). The scattered light is collected by a device which is composed of many dichroic mirrors and filters, with the collected light transmiting to detectors that can measure the magnitude of a pulse representing the extent of fluorescence or scattered light (Díaz et al. 2010).

FC techniques using fluorescent dyes would allow discrimination between viable and dead cells (Nebe-von-Caron et al. 2000) by measuring cellular properties such as metabolic activity, membrane potential and integrity, or macromolecule synthesis (Caron et al. 1998). The cell growth or viability is referred to the cells that are metabolically active and their membrane integrity is still present. In order to determine the cell growth, dyes can be used to bind to the cellular membranes of microorganisms by incorporating into the intracellular proteins or lipid bilayer, and this fluorescence would be halved to progenies of microorganisms during cell

division; carboxyfluorescein diacetate succinimidyl ester (CFSE) is an example of this dye which is effectively used to determine the growth of *Lactobacillus plantarum* (Ueckert et al 1997).

The detection of metabolic activity in the cells can be used to determine whether the cells are dead or alive, and the most common way to detect this metabolism is the measurement of esterase activity (Díaz et al. 2010) by using lipophilic, uncharged and non-fluorescent fluorogenic substrates. When these substrates enter the microbial cells, esterases cleave them to form polar fluorescent products which are retained inside the cells that possess an intact membrane. However, these products would be released rapidly from dead or dying cells with damaged membranes (Joux and Lebaron 2000).

Another parameter which is often measured to determine cell viability is the membrane potential (MP) (Dinsdale and Lloyd 1995). MP is created by the hydrolysis of ATP or cell respiration. In fact, the membranes of the cells are selectively permeable to a range of cations and anions, and this ion movement results in the electrical potential differences across the membranes with the negative charge inside the cells and positive charge outside the cells. Furthermore, this MP is very important in several cellular activities such as ATP synthesis, active transport, mobility, and regulation of intracellular pH (Joux and Lebaron 2000). Only live cells can maintain MP and the depolarization of the membranes means that cell activity is reduced, but it does not imply cell death. When the MP difference equals to zero, membranes are destroyed and ions can freely pass through membranes (Díaz et al. 2010). To measure MP of the cells, lipophilic dyes are used including cationic dyes (Rhodamine, $DioC_n(3)$, carbocianines) and anionic, lipophilic dye (oxonol). The cationic dyes are accumulated inside polarized cells (Díaz et al. 2010); however, with natural and environmental samples, a permeabilization step is needed to increase the permeability of the membranes prior to dye application and this can lead

to an application that is less conclusive. Also, these dyes are possibly able to interact with cell components or chemical compounds in the samples (Joux and Lebaron 2000; Porter et al. 1995) and Rh123 has been reported to be extruded by active pumps (Ueckert et al. 1995). These problems can lead to erroneous results. However, oxonols assemble inside non-viable cells and they increase their concentration by associating with intracellular compounds. Without applying permeabilization protocols, the uptake of the dyes is related more to the membrane integrity than to membrane potential and depolarization (Nebe-von-Caron et al. 2000; Ueckert et al. 1995, Díaz et al. 2010).

Additionally, it is suggested that the measurement of bulk nucleic acid content in the microbial culture is a sensitive and reliable way to determine microbial growth (Weinbauer et al 1998) and recently the determination of cellular nucleic acid contents was successfully applied by using fluorescent dye (Button and Robertson 2001; Gasol et al. 1999). In fact, this method is based on the assumption that all components inside the cells of microorganisms change at the same rate during balanced growth of microbial cultures (Koch 1994) and the ratio between cell biomass and their components such as proteins and nucleic acids are constant. Therefore, the measurement of cellular constituents such as proteins or nucleic acids can be used as a marker for determining biomass increase during the balanced growth of microorganisms (Button and Robertson 2001; Gasol et al. 1999). The use of nucleic acids markers in combination with scattering signals could allow a more accurate discrimination between microorganisms and other particles in samples or debris (Nebe-von-Caron et al. 2000; Vives-Rego et al. 2000; Díaz eat al. 2010). Some of the dyes that are used includes SYTO dye, which only becomes fluorescent when bound to nucleic acids, and DAPI (4',6-diamidino-2-phenylindole) ,or SYBR dyes, which enhance their fluorescence if bound to nucleic acids (Grégori et al. 2001; Berney et al. 2006).

Acridine orange (AO) is one of the fluorogenic dyes commonly used in environmental microbiology and microbial ecology in the direct count method of microorganisms (Tschech and Pfennig 1984). The AO dye has also been used to detect microorganisms in direct smears of clinical specimens (Kronvall and Myhre 1977). However, in an attempt to use AO as an alternative method instead of optical density, Martens-Habbena and Sass (2006) reported the unsuitability of AO for observing the growth of *E.coli* owing to the very high background fluorescence even at very low concentrations and the inability to discriminate between a cell-free control and 10^9 *E. coli* cells ml^{-1}. However, Chalova et al. (2004) reported the possibility of using fluorescence based on dye, SYTO 9, instead of optical density and it has advantage of improved detection sensitivity.

Antimicrobial compounds produced by beneficial bacteria

Bacteriocins

Lactic acid bacteria (LAB) are a very important starter cultures in the fermentation of foods such as dairy, meat, vegetables and bakery products. Fermentation contributes mainly to extended shelf life of the products and inhibits the growth of spoilage and pathogenic microorganisms through competition for nutrients and production of by-products such as bacteriocins, hydrogen peroxide and lactic acid (Ray and Daeschel 1992). Bacteriocins are proteins produced by bacteria which have antibacterial effects which can kill or inhibit the growth of other bacteria. Currently, certain chemicals have been used as preservatives to limit microbial growth; however, there is increasing awareness of consumers about the risks associated with some of these chemical preservatives (Abee et al. 1995). Thus, it prompted the research into exploiting bacteriocins produced by LAB as biopreservatives in food (Abee et al. 1995). Most bacteriocins produced by

LAB are classified into class I, II, II and IV. Class I (lantibiotic peptides), for example nisin, contain unusual amino acids and lanthionine rings, introduced by post-translational modifications. Pediocin PA-1 belongs to the class II bacteriocins and it is a small peptide which is heat-stable; and this class II can be subdivided into subclass IIa (pediocin-like bacteriocins) containing a consensus motif (YGNGV) and active against *Listeria* spp. The third and fourth classes of bacteriocins are large heat-labile proteins and complex proteins respectively which need a lipid or carbohydrate moiety for activity. However, most of the studies on bacteriocins focus on pediocin-like bacteriocins (Klaenhammer et al. 1993); and studies have shown that this bacteriocin is able to kill pathogens in meat products more effectively than nisin (Montville and Chen 1998). Bhunia et al. (1991) and Chikindas (1993) suggested that the mechanism of action of pediocin PA-1 (AcH) is that pediocin molecules attach to the surface of bacterial cells, followed by its binding to a receptor-like component of the cell membrane. Subsequently it inserts into the membrane and aggregates into oligomeric structures. These structures then form hydrophilic pores which allow the release of ions and small molecules from the target cells which ultimately leads to the cell death, with or without lysis. Bacteriocins have been used as preservatives in many food products such as dairy and meat products. For example, *Enterococcus facecium* DPC1146 produces a bacteriocin, enterocin 1146, which is heat-stable and extremely active against *L. monocytogenes* (Parente and Hill 1992a, b) and is used as a biopreservative in cheese making. For meat products, bacteriocins produced by LAB such as *Pediococcus, Leuconostoc, Carnobacterium* and *Lactobacillus* spp. are used (Abee et al. 1995).

Listeria monocytogenes

Listeria monocytogenes is a gram-positive, nonsporeforming, facultatively anaerobic rod which can survive or grow between -0.4 and 50°C (Farber and Peterkin 1991). *L. monocytogenes* is very tolerant to freezing, drying and heating; and most *L. monocytogenes* are pathogenic and cause a disease referred to as listeriosis (www.fda.org). The source and route of listeria infections are usually unknown; however, it is suggested that contaminated food is possibly the main source of the pathogens. There are a range of food products associated with listeria infection such as dairy products, meats, egg, vegetables and seafood (Farber and Peterkin 1991). *L. monocytogenes* has been detected in many dairy products, especially cheese; it can survive during the manufacture and ripening of many different cheeses and the level of contamination can be very high, as much as 10^7 cfu/g. However, the growth of *L. monocytogenes* seems to be slow once co-cultured with the lactic starter cultures used in making cheese (Griffith and Deibel 1990). *L. monocytogenes* also survives during the fermentation of skim milk with *Streptococcus cremoris* or *Streptococcus lactis* and during the storage of the products at refrigerated temperature. It is also found that the fermentation with thermophilic bacterium *Lactobacillus bulgaricus* causes more detrimental effects to the survival and growth of *L. monocytogenes* than fermentations with mesophilic lactic starter cultures (Schaack and Marth 1988). In addition, many kinds of meat have been reported to be contaminated with *L. monocytogenes* and most of the contamination occurred on the surface of meats. Johnson et al. (1990) found that in 5 out of 110 total samples from beef, pork, and lamb roasts, *L. monocytogenes* was present on the meats. Glass and Doyle (1989) suggested that the growth of *L. monocytogenes* was very dependent on meat types and pH of the products. However, on all test meats including ham, bologna, wieners, sliced chicken, sliced turkey, fermented semidried sausage, bratwurst, and cooked roast beef, this pathogen generally grew well at pH near or greater than 6.0, but poorly or not at all with pH near

or below 5.0. They also reported that poultry supported the growth of *L. monocytogenes* better than other meats. Another source of contamination is vegetables with frequent contamination occurred with potatoes and radishes (Heisick et al. 1989). This pathogen is also found in salad (2 out of 108 samples) and prepacked mixed salads (8 out of 42 samples) (Velani and Roberts 1991). It is suggested that sources of contamination of vegetables are soil, animal manure, water, decaying vegetation, and effluents from sewage treatment plants (Beuchat et al. 1990).

The infective dose of pathogenic *L. monocytogenes* to cause disease in the host is unknown; however, it is suggested that the number of *L. monocytogenes* cells required to cause disease mainly depends on strain differences of the pathogens and on host susceptibility. A low level of ingested *L. monocytogenes* would not cause illness to healthy susceptible individuals due to the reactivity of T cells present in most normal individuals (Munk and Kaufmann 1988). It is suggested that the infective dose of *L. monocytogenes* is approximately 10-100 million colony forming units (CFU) in a healthy host, and only 0.1-10 million CFU in people at high risk of infection (Bortolussi 2008; Farber et al. 1996).

Therefore, these studies aimed to investigate

1. Acridine orange to use as an alternative method to study the growth of bacteria instead of optical density.
2. The fermentability of FOS, GOS, IA, HMP and SBP by three probiotic strains for possible use as suitable prebiotics/carbohydrates for potential application as synbiotics.
3. The survival of *Listeria monocytogenes* in the presence of cell free supernatant (CFS) collected from three strains of lactic acid bacteria and the stability of the antibacterial effects of CFS treated with various heat and protease.

Table 1. Major classes of structurally different fructans in higher plants

Fructans	Chemical composition	Plant sources	Examples	Reference sources
Inulin	linear (2-1)-linked β-D-fructosyl unit (G1-2F1-2Fn)	plant species belonging to the order Asterales such as chicory and Jerusalem artichoke	trisaccharide 1-kestose (or isokestose) (the shortest inulin)	Bonnett et al. 1994; Koops and Jonker 1996
Levan	linear (2-6)-linked β-D-fructosyl units (G1-2F6-2Fn)	some grass; eg: *Dactylis glomerata*		Bonnett et al. 1997
Mixed levan	both (2-1)- and (2-6)-linked β-D-fructosyl units	most plant species belonging to the Poales, such as wheat and barley	Bifurcose	Carpita et al. 1989, Bonnett et al. 1997
Inulin neoseries	linear (2-1)-linked β-D-fructosyl units linked to both C1 and C6 of the Glu moiety of the Suc molecule (mF2-1F2-6G1-2F1-2Fn)	plants belonging to Liliaceae; eg onion and asparagus	Neoskstose (the smallest inulin neoseries)	Shiomi 1989
Levan neoseries	polymer of predominantly β(2-6)-linked fructosyl residues on either end of Glu moiety of the Suc molecule.	a few plant species belonging to the Poales; eg: oat		Livingstone et al. 1993

(adapted from Vijn and Smeekens 1999)

Table 2. Dyes used in detection of microbial activity

Dyes	Application	Reference sources
Fluorescein diacetate	Provide weak fluorescent signals as the retention of louorogenic substrates inside bacterial cells is very poor, consequently, weaker staining and increase background are obtained	(Diaper and Edwards 1994)
Calcein AM	Is found to inappropriate in the enumeration of viable bacteria in freshwater environment.	(Porter et al. 1995)
ChemChrome	Is considered an option that is workable for most microorganisms. In particular, ChemChrome V6 (CV6) is reported as a universal dye to stain both yeast and bacteria as it can overcome the limitations concerning the uptake of the dyes into the microbial cells and also their possible extrusion owing to the presence of active pumps.	(Catala et al. 1999, Díaz et al. 2010, Herrero et al. 2006, Parthuisot et al. 2003)

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Potential synbiotics, bile resistance and tolerance to simulated gastric juices of three *Lactobacillus* strains utilizing different prebiotic carbohydrates, inulin agave and pectin

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Abstract:

Aims: The objective of this study was to determine the fermentability of the prebiotic carbohydrates, inulin agave and pectin by three probiotic *Lactobacilli* and the effect of carbohydrate sources on bacteria growth and their tolerance to bile solution and simulated gastric juices (SGJ).

Methods and Results: The fermentation study of three probiotic strains was conducted by using carbohydrates including high methoxy pectin (HMP), sugar beet pectin (SBP), fructooligosaccharide (FOS), galactooligosaccharide (GOS), and inulin agave (IA) as sole carbon sources. Substrate fermentation was determined by changes in OD_{600}, log cfu/ml and pH during 48-h growth. In this study, we have introduced a method for studying the fermentation of polysaccharides, HMP and SBP. Normally, prebiotic solutions are sterilized by filtration, but; these large molecular weight polysaccharides are not filterable. Therefore, we dissolved pectin under low temperature conditions, approximately 55°C, and added 0.00125 g L^{-1} of sodium azide to prevent environmental contaminants. The results suggest that FOS, GOS, and IA are optimal prebiotic carbohydrates to support the growth of test probiotics. The fermentability of HMP was inconclusive; but indications are that HMP may help probiotic bacteria to survive longer. The study of gastric conditions indicated that the strains are tolerant to SGJ. However, significant reduction of log colony forming unit (cfu)/ml of all strains exposed to bile solution was observed. The survival of probiotic bacteria after exposure to bile solution is varied depending on bacteria strains and the carbohydrates utilized for its growth.

Conclusions: FOS, GOS, and IA may be appropriate carbohydrates to combine with probiotic products; however, HMP may also be a beneficial ingredient as well due to its ability to enhance the survival of bacteria.

Significance and impacts of the study: This study examined fermentation of pectin polysaccharides. We found that test carbohydrates could be fermented by probiotic bacteria and enhanced bacterial survival during gastric transit. This may lead to the development of symbiotic mixtures.

Introduction

The human gastrointestinal (GI) tract is composed of a number of microflora including pathogenic and beneficial bacteria (Manning and Gibson 2004). Harmful bacteria in the GI tract could cause health problems to the host such as diarrhea, intestinal putrefaction and carcinogenesis. However, beneficial bacteria such as *Bifidobacteria* and *Lactobacilli* could promote the host health by inhibiting the growth of harmful bacteria, stimulating the immune system, and enhancing the digestion and absorption of essential nutrients such as short chain fatty acids (Gibson and Roberfroid 1995). Probiotics are defined by the Food and Agriculture Organization and World Health Organization as live microorganisms which can improve host health when they are administered adequately. Certain species of *Lactobacillus* and *Bifidobacteria* have been used as probiotics to supplement food products (Goldin and Gorbach 1992) to provide better-microbial balances in the human GI tract and consequently improve the host health (Fuller 1989). However, probiotic application becomes limited as those bacteria are likely to be in a stressed state when they reach the colon owing to their exposure to diverse barriers in the host such as gastric acid and bile acids (Pochart et al. 1992; Gibson and Roberfroid 1995). This challenge is compounded by the requirement for probiotics to be active and able to compete with established microflora in the colon for nutrients and colonization sites (Gibson and Roberfroid 1995). Hernandez-Hernandez (2012) has shown that prebiotic carbohydrates could enhance the survival of probiotic bacteria during exposure to gastric conditions. Prebiotics are food ingredients which are indigestible in the human upper GI tract and reach the colon to beneficially influence the host by selectively promoting the growth and/or activity of certain bacteria in the colon (Gibson and Roberfroid 1995). The application of both

probiotics and their suitable prebiotics are predominant in food products beneficially influencing metabolic activities and stimulating the growth of administered probiotic bacteria through their synergistic effects (Zanoni et al. 2008). Normally, prebiotics are commercially extracted from fruits and vegetables through methods such as enzymatic hydrolysis of polysaccharides from dietary fibers or starch. Potential prebiotic carbohydrates include fructo-oligosaccharides (FOS), galacto-oligosaccharide (GOS), lactulose, lactosucrose, soybean oligosaccharides, isomalto-oligosaccharides, palatinose,xylo-oligosaccharides, and gluco-oligosaccharides (Gibson et al. 2000; Manning and Gibson 2004). Pectins, which also reach the colon (Englyst and Cummings 1987) and subsequently are fermented by intestinal microbiota do not necessarily function specifically as prebiotics (Olano-Martin et al. 2002). However, it has been reported that pectin can reduce serum cholesterol in both rats and humans (Gulfi et al. 2005) and is thought to have anti-carcinogenic effects (Scheppach et al. 1995). Olano-Martine et al. (2003) has shown that HM pectin, LM pectin, and their derived oligosaccharides could induce the *apoptosis* of human colonic adenocarcinoma *HT29* in vitro.

Therefore, in this study, we aimed to investigate the fermentability of FOS, GOS, inulin agave (IA), high methoxy pectin (HMP), and sugar beet pectin (SBP) by three probiotic strains for possible use as suitable prebiotics/carbohydrates for potential application as synbiotics. In addition, we examined the tolerance of the three probiotic strains grown in the presence of different test carbohydrates to bile solution and simulated gastric juices.

Materials and Methods

Carbohydrate Sources and Chemicals

High methoxy pectin (HMP, Tic Gums, Maryland, USA) and sugar beet pectin (SBP) extracted from soy hulls according to the procedure of Crandall and McCain (2002) were investigated for their fermentability by probiotic bacteria. Three prebiotic carbohydrates including fructooligosaccharide (FOS, FortiFeed,Golden, CO, USA, 95% short-chain fructooligosaccharide, 5% glucose, fructose, and sucrose), inulin agave (IA, Tic Gums, Maryland, USA), and galactooligosaccharide (GOS, GTC Nutrition, Golden, CO, USA, 90% pure) were also studied along with a non-prebiotic control, glucose (GLU, Sigma, USA). Chemicals used in the studies of bacteria tolerance to gastric conditions are pepsin from porcine stomach mucosa and bile extract porcine both purchased from Sigma Chemical Company, St. Louis, MO, USA.

Bacteria strains

Lactobacillus bulgaricus ATCC7517 (LB), *Lactobacillus casei* ATCC11578 (LC), and *Lactobacillus delbrueckii subsp. Lactis* ATCC 4797 (LD) were selected as probiotic bacteria from the culture collection of the Center for Food Safety, University of Arkansas, Fayetteville, AR, USA. Cultures were retrieved from storage at -80°C in Lactobacilli de Man, Rogosa and Sharpe (MRS) broth (EMD Chemicals, Gibbstown, NJ) supplemented with 20% glycerol.

Growth experiments

Basal MRS media supplemented with different carbohydrates was used throughout this fermentation study. The basal MRS media was prepared by mixing proteose peptone (10 g L^{-1}), beef extract (10 g L^{-1}) , yeast extract (5g L^{-1}), polysorbate 80 (1 g L^{-1}), ammonium citrate (2 g

L^{-1}), sodium acetate (5 g L^{-1}) , magnesium sulphate (0.1 g L^{-1}), manganese sulphate (0.05 g L^{-1}), dipotassiumsulphate (2 g L^{-1}), and cysteine-HCl (0.05 g L^{-1}) in deionized (DI) water and autoclaving at 121°C for 15 min. Carbohydrate sources GLU, FOS, GOS, and IA were dissolved in DI water and filtered through 0.45-µm filters. The HMP and SBP were dissolved slowly in DI water containing 0.00125 g L^{-1} of sodium azide (SA, J.T. Baker Chemical Co., New Jersey, USA) at 55°C while stirring constantly. Carbohydrate solutions were added to the autoclaved basal MRS to the final concentration of 10 g l^{-1} and then the pH was adjusted to 6.5 with 0.5 N NaOH or HCl (Fisher Scientific, Fair Lawn, NJ, USA).

All experiments were conducted at 37°C under anaerobic conditions created by flushing argon gas into anaerobic boxes (Mitsubishi Gas Chemical Co., Japan). Bacteria were inoculated in Lactobacilli MRS broth and incubated overnight. The overnight cultures were vortexed for 10 min to disrupt the chains of lactobacillus bacteria, and subsequently washed and resuspended with PBS to the same volume. One percent of resuspended cultures were inoculated into fresh basal MRS medium containing different carbohydrates and 250 µl of the inoculated medium was used to fill each well of a 96-well plate. The growth of bacteria was observed turbidometrically by a microtiter plate reader (Tecan Group Ltd, Grödig, Austria) for 48 h along with total plate count and pH measurement at the time intervals of 0, 6, 11, 14, 24, and 48 h. The specific growth rate (µ h^{-1}) was calculated over the exponential phase of the growth curve by fitting the linear line using Microsoft excel 2010 (Ricke and Schaefer 1996). For total plate count, bacteria were grown on MRS agar and incubated anaerobically at 37°C for 48h. Due to the observed growth of bacteria in basal MRS media without any added carbohydrates, the basal media was included as a negative control. Blank media were incubated in 96-well plates in every experiment to ensure

the absence of contamination. This experiment was conducted with duplicate samples and repeated 3 times.

Tolerance of probiotics grown in different carbohydrates to gastrointestinal stress

Simulated gastric juices (SGJ) and bile solution were prepared daily before the experimentation. The SGJ were prepared by suspending pepsin from porcine stomach mucosa (Sigma, St. Louis, MO, USA) in sterile saline (0.5% w/v, pH 2.0 adjusted with 1N and 0.1N HCL) to a final concentration of 0.22% and subsequently filtered through 0.45-µm syringe filters (Carrigtwohill, co., Cork, Ireland) (Charteris et al. 1998). Bile solution was prepared by dissolving porcine bile extract (Sigma, St. Louis, Missouri, USA) into DI water to a final concentration of 0.33% (Lian et al. 2002) and filtered through 0.22-µm syringe filters (VWR international, USA).

The methods described by Charteris et al. (1998), Lian et al. (2003), and Hernandez-Hernandez et al. (2012) were adopted with some modification for this study. Briefly, probiotic bacteria were grown anaerobically at 37°C in basal MRS media containing the different respective carbohydrates for 48h. The cultures were subsequently subjected to vortexing at 6s for 10 min to disrupt the *Lactobacillus* chains. One-ml aliquots of these cultures were centrifuged at 8000 rpm for 5 min (Brinkmann Centrifuge 5415C Eppendorf, Westbury, New York, USA), washed two times with phosphate-buffered saline (PBS, pH 6.59), and resuspended with PBS to the same volume of 1ml. An aliquot of 0.1-ml resupended cells was mixed with 0.9 ml of SGJ or bile solution, and incubated anaerobically at 37°C in anaerobic box (Mitsubishi gas chemical Co., Japan) flushed with argon gas. The 0-min samples were taken immediately after mixing the cells with bile solution or SGJ. The samples were taken at the time intervals of 0, 30, 90 and 180 min for bile solution experiment and for the SGJ experiment, the samples were taken only at 0,

120 and 240 min. All samples were plated on MRS agar and incubated anaerobically at 37°C for 48 h and blank media was included in every experiment to ensure the absence of contamination. This study was conducted with duplicate samples and repeated 3 times.

Statistical Analysis: Tukey honestly significant difference (HSD) was used to determine whether there were any significant differences between means of growth rate and viable counts in the study of kinetic growth and gastric conditions; for the gastric experiment, delta log cfu/ml was calculated by subtracting log cfu/ml at 0 min with the final log cfu/ml of bile solution (3h). The differences between the final biomass (OD_{600}) and pH (at 48 h) among all substrates and strains were also compared by Tukey HSD. The correlation between log cfu/ml and pH measured at 0, 6, 11, 14, 24, and 48h were also determined. All statistical analyses were performed using JMP package program ver. 9.0.0 (SAS Institute Inc, NC, USA), and the differences were considered significant if $P < 0.05$.

Results

Kinetic growth of LB, LC and LD in different carbohydrate substrates

The maximum growth rates of each probiotic strain in different substrates are shown in Table 1. It was observed that both LC and LD achieved the highest growth rates when they were grown in the presence of GLU, FOS, and IA; however, LB had a slower growth rate in these substrates compared to LC and LD. Utilization of GOS, LC and LD also exhibited similar growth rates which were higher than LB in GOS; noticeably diauxic effects were observed during the growth of LB in GOS. However, the growth rates of each probiotic strain in these substrates were statistically higher than their negative controls (WO). Each growth rate of LC

and LD in the presence of HMP and SBP were not significantly different from their WO, and the second growth rate phase of LB utilizing HMP, SBP, and WO were not available as LB grow very slow and it did not reach stationary phase even after 16 h. Diauxic effects are also found in LD in HMP, LC in WO, and LB in HMP, SBP, or WO (table 1).

According to the growth curves of LB, LC and LD depicted in figures 1 through 3, there were statistically significant increases of log cfu/ml of individual probiotic strains in each substrate which indicated that the bacteria were able to utilize the carbohydrates. However, it was observed that the basal MRS media without supplemented carbohydrates (WO) was able to support high viable counts of bacteria as well. Therefore, the maximum log cfu/ml (at 48 h) of probiotic bacteria among substrates for each strain was compared with the maximum log cfu/ml (at 48 h) of their individual WO to determine whether bacteria utilized the test substrates (figure 1 through 3). The analysis shows that the log cfu/ml of LB, LC, and LD in the presence of GLU, FOS, GOS, and IA was statistically higher than their negative control (WO). However, in MRS-HMP media, only LB (8.47) and LC (9.04) yielded a higher log cfu/ml than their WO, which were 7.86 and 8.27 respectively. The log number for all probiotic strains show that all probiotic bacteria cannot ferment SBP as there were no significant differences from their WO.

Relationship between bacteria cell mass (OD_{600}) and pH:

The comparison of final cell mass (OD_{600}) and pH at 48h is illustrated in figure 4. It is shown that the most abundant cell mass was obtained for all three probiotic strains grown in GLU, FOS and IA and followed by the OD of LC and LD in GOS; however, the OD of LB in GOS was not significantly higher than the OD of its WO. The final OD obtained from the growth of LB, LC and LD in HMP and SBP shows lower or similar cell mass to their WO. The

comparison of pH among substrates and probiotic strains has shown that LB, LC, and LD grown in media containing GLU, FOS, IA, and GOS have similar pH (at 48h) which is significantly lower than pH (MRS), except for LB in GOS. However, the pH of 48-h cultures of all three probiotic strains grown in HMP, SBP and WO are not significantly reduced from pH in MRS. Furthermore, the correlation between pH and log cfu/ml (table 2) illustrates a very strong negative relationship for all strains grown in the presence of GLU, FOS, GOS, and IA, except LB (GOS) (r^2 = -0.61). However, the correlation of each strain grown in media containing HMP, SBP, and WO was very slightly related with the exception of LB in HMP shows strong negative correlation between pH and log cfu/ml (r^2 = -0.89).

Survival of LB, LC and LD in bile solution and simulated gastric juice

The survival of probiotic strains grown in the presence of different carbohydrates after exposure to bile solution is shown in table 3. The results indicate that the number of viable cells for each treatment is significantly decreased over time. Furthermore, the comparison based on delta log cfu/ml (in 3h) shows that LD grown in HMP has the highest survival followed by LC and LD grown in GLU. The highest reduction of bacteria after exposure to bile solution is found with all strains grown in GOS and with LD in GLU. LD in FOS was also reduced dramatically to approximately 4.05. Then we observed the similar reduction rate among all test probitoics in basal MRS containing FOS or IA, LB in HMP and LC in HMP. For the SGJ study, the result shows that SGJ is not effective on the tested probiotic strains, except only LC in HMP, which show a significant decrease of log cfu/ml in 2- and 4h of exposure to SGJ (table 4).

Discussion

Prebiotics are carbohydrates which are not digestible in the upper part of the human intestines. The breakdown of these molecules occurs due to the metabolic activities of certain gut bacteria (Cummings and Macfarlane 1997), using specific enzymes such as polysaccharidases (amylase, pectinase, and xylanase) and glycosidases (β-D-galactosidase, α-L-arabinofuranosidase, and β-D-glucosidase) to digest these complex carbohydrates (Englyst et al. 1987). Plant polysaccharides such as pectin can also reach the colon and be degraded by microbial enzymes including hydrolases, esterases, and lyases (Louis et al. 2007). Therefore, to study the fermentability of HMP and SBP it is very important to retain the entire structures of the test pectin molecules to ensure accurate results on whether the probiotic bacteria have appropriate enzymes to break the test pectins. However, there are challenges in preparing the pectin solution as these polysaccharides tend to clump together during dissolving and they do not pass through 0.45-μm-vacuum filters (Nalgene, Rochester, New York, USA). Therefore, HMP and SBP were dissolved in DI water tempered at 55°C while stirring and, in order to prevent environmental contamination, 0.00125 g l^{-1} of sodium azide was used. The blank controls of all basal MRS media plus carbohydrates (HMP, SBP, FOS, GOS, IA, GLU) show no presence of microbial contaminants. Sodium azide is generally used in the concentration of 0.02% to prevent environmental contamination in the laboratory; however, owing to the finding that amount causes the test probiotic bacteria to produce very small colonies and grow very slowly, we reduced its concentration.

The study of kinetic growth observed the highest and similar growth rates among all test probiotics grown in MRS basal media containing GLU, FOS and IA. This result suggests that FOS and IA are substrates which could promote the bacteria growth as well as GLU; however,

the growth rates of LC and LD in MRS-GOS media were slightly lower (table 1). We also observed that all strains can grow in MRS basal media (WO), but the final biomass (OD_{600}) of FOS- and IA-based cultures for all strains was four times higher than the OD of their WO (figure 1). Similar observations have been reported by Kaplan and Hutkins (2000) that MRS basal media (or WO) alone could promote the growth of bacteria; however, they confirmed that only bacteria able to ferment prebiotic FOS reached the final OD_{600} which was twice as high as the OD of the MRS basal media (or WO) in 24-h growth and they also found that FOS can serve as optimal substrates as GLU in promoting bacteria growth. Furthermore, the above authors have suggested that the growth of bacteria in MRS basal media may be due to the background levels of carbohydrate in the basal MRS broth or the contaminated sugar in the test FOS. In our study, diauxic effects were observed during the growth of LB and LC in WO which suggest that there are two carbon sources available in the media. However, both the specific growth rates and final cell mass of LB, LC and LD utilizing HMP and SBP were not significantly different from their WO. Furthermore, the comparison of pH and OD at 48 h (figure 1) indicates that the pH of all cultures grown by utilizing GLU, FOS, and IA decreased to approximately 4 or below with its OD_{600} increased abundantly; and very strong negative correlation between pH and log cfu/ml are identified for all strains in MRS basal media containing GLU, FOS, IA, and GOS, except LB (GOS) which is only slightly related. This suggests that the more cells growth in the media, the lower the pH. However, cultures based on HMP, SBP and WO are somewhat related, except LB (HMP) which has strong negative correlation; and the pH of all these cultures are not significantly different from initial pH of the media. Rossi et al. (2005) indicated that the fermentation of FOS and inulin by *Bifidobacteria* resulted in an increase in OD_{600} while the pH of the cultures was reduced, but there was no strong correlation between these two parameters.

Also, Gulfi et al. (2005) reported that after, 24-h growth, pH of the cultures containing a range of the test HMP and LMP were only minimally reduced as compared to pH of lactulose-based cultures. The ability of *Lactobacilli* to lower the environmental pH is one of their mechanisms in preventing pathogen growth (Vadillo-Rodríguez et al. 2004) and, for the prebiotic concept, lowering pH results from the increase in the concentration of acidic by-products from substrate fermentation in the colon and could influence bacteria metabolism and competition (Flint et al. 2007). A study conducted by Walker et al. (2005) on the response of microflora in human feces to pH changes suggest that lowering pH may stimulate the production of butyrate, increase the number of bacteria responsible for its production, and restrict the growth of *Bacterioides* spp. Finally, based on the comparison of growth rates, final biomass, and pH, it suggests that FOS and IA are fermented by all three strains, and only LC and LD could utilized GOS; however, the maximum log cfu/ml of LB and LC in MRS-HMP media in 48-h growth are significantly higher than their WO which lead to the assumption that HMP may help the probiotic bacteria to survive longer in the media and the fermentability of the HMP is still inconclusive.

Each day the human stomach secretes about 2.5 L of gastric juice with pH approximately 2 (Hill 1990), which causes a barrier for probiotic application and when the probiotic bacteria reach the intestinal tract, the presence of bile can further reduce their survival (Kimoto-Nira et al. 2009). Therefore, it is necessary for probiotic bacteria to survive through the stomach and the small intestine to be effective in the GI tract of the host. Some studies have shown the enhanced survival of probiotic bacteria during their exposure to gastric conditions upon supplementation with milk protein (Charteris et al. 1998), whey cheese matrices (Madureira et al. 2011), or soymilk (Huang and Adam 2004) to the media. Encapsulation of microorganisms by gelatin, soluble starch, skim milk, or gum arabic could protect probiotic bacteria from gastrointestinal

stress as well (Lian et al. 2003). In our study, we have investigated the survival of 3 probiotic strains including LB, LC and LD grown in MRS basal media containing different carbohydrates including GLU, FOS, GOS, IA, and HMP. The result illustrates that LD grown in GLU was reduced to an undetectable level at 3 h of exposure to bile extract; however, the reduction of LB and LC were approximately 2.5 and 2.23 respectively. This finding suggests that the differences in bacteria survival are strain-dependent which is in agreement with Begley el al. (2005). Furthermore, all probiotics grown in basal MRS containing GOS were reduced drastically to undetectable level as well, followed by similar reductions observed among all strains grown in FOS and IA, LB in HMP, and LC in HMP. LD grown in HMP is found to survive longer than others; in addition, LB and LC grown in GLU could also maintain better survival after exposure to bile extract. Kimoto-Nira et al. (2010) and Hernandez-Hernandez (2012) also reported that the different levels of survival of bacteria exposed to bile extract for 3 h are varied according to strains and carbon sources utilized for bacteria growth. Furthermore, Kimoto-Nira et al. (2009) suggested that growth conditions could alter the fatty acid composition of bacteria cells which may enhance the survival of his test bacteria, lactococci, upon exposition to bile extract. The follow-up research of Kimoto-Nira et al. (2010) found that for all bile-sensitive bacteria grown in different carbon sources, only bacteria grown in lactose show different fatty acid composition; and they suggested that the highest bile tolerance of bacteria grown in lactose may be achieved due to the alteration of fatty acid composition. However, the mechanisms explaining these survival differences are still not clear. The result obtained from SGJ study demonstrated that the test probiotic bacteria are not sensitive to the treatment and only LC (HMP) exhibited slight reduction from its initial inoculum. Zanoni et al. (2008) suggested that although the acidic pepsin solution (pH 2) are used to simulate gastric transit, it may not be the most appropriate model as

he found that the loss of bacteria viable counts in human gastric juice is much lower than simulated gastric juices. The experimental conditions to study bacteria tolerance to gastric stress may vary according to the simplicity and complexity of the models, the differences in composition and concentration of components in gastric formulation, and pH ranges (Charteris et al. 1998; Madureira et al. 2011, Beumer et al. 1992, Tamplin 2005, Corcoran et al. 2005, Corcoran 2007, Fernández de Palencia et al. 2008, Kimoto-Nira 2010, Hernandez-Hernandez et al. 2012). However, the previous results from our lab conducted by Hernandez-Hernandez et al (2012) show the significant reduction of LB, LC, and LD, which are the same strains as our study, after exposition to low pH (2.5, without pepsin) for only 1 h. Thus, it is conceivable that the sensitivity of probiotic bacteria to gastric conditions may be varied depending on the experimental conditions. The similar question can be raised to the study of bacteria tolerance to bile solution whether the obtained result would truly reflect the ability of test bacteria to tolerate bile *in vivo*. The *in vivo* conditions are very difficult to simulate in the laboratory and the concentration of bile in the intestines is varied. In addition, the tolerance of bacteria to bile can be changed due to (1) bile-adaptation; eg: pre-exposure of bacteria to low-level bile may enhance their survival when placed in media containing high-level bile and (2) cross-adaptation; eg: pre-exposure of bacteria to certain pH, temperature, or growth conditions may increase either the tolerance or susceptibility of probiotic bacteria (Begley et al. 2005). Thus, this finding may only be of limited use and should be confirmed by appropriate *in vivo* studies.

The test FOS is a prebiotic fiber extracted from cane sugar which has application in the feed of monogastric animals (www.fortifeed.com), prebiotic GOS is a derivative of lactose which used in infant formula, baby foods, functional beverage and supplements (www.gtcnutrition.com), IA is a raw material used in beverages and other functional foods, and

HMP from citrus peel is an ingredient used in making jams and jellies (www.ticgum.com). Recently the first study on the fermentation properties of inulin agave (or IA) was conducted by Gomez et al. (2010) and they found an increase in the growth of bifidobacteria and lactobacilli in fecal samples which suggests that inulin derived from Agave plants has a potential prebiotic effect (Gomez et al. 2010).

In conclusion, the results from our study could have an impact on the expansion of probiotic products by incorporating the test prebiotic carbohydrates (FOS, GOS, IA) to enhance the survival of probiotic bacteria through the gastric transit and to have synergistic effects in the colon of the human host. Although the fermentability of HMP by the test probiotics was not shown, this compound could be still beneficial in probiotic application according to its ability to enhance the survival of bacteria both in normal media and bile solution. However, some studies have demonstrated that certain gut bacteria including bifidobacteria and lactobacilli could grow on HMP and LMP derived from apple and citrus (Olano-Martin et al. 2002, Sirotek et al. 2004, Gulfi et al. 2005). For the future study of the fermentability of HMP and SBP, human fecal samples or mixed cultures of beneficial bacteria including bifidobacteria and lactobacillus together with the pectin-fermented strains from Olano-Martin et al. (2002) and Gulfi et al. (2005) should be used because the breakdown of polysaccharides such as pectins are suggested as the result of cooperative activity of enzymes produced from many different species of colonic bacteria (Cummings and Macfarlane 1991).

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Table 1 Specific growth rates of *Lactobacillus bulgaricus*, *Lactobacillus casei*, and *Lactobacillus delbrueckii subsp. Lactis* utilizing different carbohydrates

Substrate	Specific growth rates		
	LB	LC	LD
GLU	0.16 ± 0.00^{a}	0.19 ± 0.00^{c}	0.19 ± 0.01^{c}
FOS	0.14 ± 0.00^{a}	0.17 ± 0.01^{c}	0.18 ± 0.01^{c}
GOS	$(0.09 + 0.06) \pm 0.01^{b}$	0.13 ± 0.01^{d}	0.12 ± 0.01^{d}
IA	0.14 ± 0.00^{a}	0.18 ± 0.00^{c}	0.17 ± 0.01^{c}
HMP	$(0.06 + NA) \pm 0.00$	0.06 ± 0.02^{e}	$(0.05 + 0.07) \pm 0.01^{e}$
SBP	$(0.27 + NA) \pm 0.03$	0.04 ± 0.00^{e}	0.04 ± 0.00^{e}
WO	$(0.10 + NA) \pm 0.00$	$(0.12 + 0.04) \pm 0.00^{e}$	0.06 ± 0.00^{e}

Different letters indicate significant differences ($P \leq 0.05$) of growth rates among all strains and substrates

GLU, glucose; FOS, fructooligosaccharide, GOS, galactooligosaccharide; IA, inulin agave; HMP, high methoxy pectin; SBP, sugar beet pectin; WO, basal MRS media; LB, *Lactobacillus bulgaricus*; LC, *Lactobacillus casei*; LD, *Lactobacillus delbrueckii subsp. Lactis*; NA: not available.

Table 2 Correlation between pH and log cfu/ml of individual strains grown in each substrate

	LB	LC	LD
GLU	-0.95	-0.92	-0.92
FOS	-0.97	-0.96	-0.96
GOS	-0.61	-0.99	-0.99
IA	-0.94	-0.98	-0.98
HMP	-0.89	0.31	0.31
SBP	-0.79	0.02	0.02
WO	-0.45	0.15	0.15

GLU, glucose; FOS, fructooligosaccharide, GOS, galactooligosaccharide; IA, inulin agave; HMP, high methoxy pectin; SBP, sugar beet pectin; WO, basal MRS media; LB, *Lactobacillus bulgaricus*; LC, *Lactobacillus casei*; LD, *Lactobacillus delbrueckii subsp. Lactis*

Table 3 Tolerance to bile solution of *Lactobacillus bulgaricus*, *Lactobacillus casei*, and *Lactobacillus delbrueckii subsp. Lactis* grown in different carbohydrates

Strain					
Carbon source	Viable counts (log cfu/ml) of survival bacteria				
	0 min	30 min	90 min	180 min	Delta log cfu/ml (in 3h)
LB					
GLU	7.45 ± 0.12	6.66 ± 0.24†	5.97 ± 0.42†	4.95 ± 0.69†	2.50 ± 0.59^{acde}
FOS	8.61 ± 0.10	7.22 ± 0.18	6.59 ± 0.46†	5.48 ± 0.35†	3.13 ± 0.25^{abcde}
GOS	7.85 ± 0.50	5.29 ± 0.41†	4.50 ± 0.28†	ND	ND
IA	8.60 ± 0.25	7.53 ± 0.13†	6.57 ± 0.56†	5.41 ± 0.38†	3.19 ± 0.42^{abcde}
HMP	7.00 ± 0.31	5.63 ± 0.44†	4.28 ± 0.47†	3.24 ± 0.31†	3.76 ± 0.50^{abc}
LC					
GLU	7.32 ± 0.17	6.52 ± 0.21	5.86 ± 0.32†	5.10 ± 0.52†	2.23 ± 0.69^{acde}
FOS	8.67 ± 0.22	7.19 ± 0.15†	6.24 ± 0.36†	5.37 ± 0.53†	3.29 ± 0.51^{abcde}
GOS	7.66 ± 0.22	4.84 ± 0.46†	ND	ND	ND
IA	8.63 ± 0.24	7.19 ± 0.18†	5.95 ± 0.26†	5.09 ± 0.33†	3.55 ± 0.21^{abcde}
HMP	7.13 ± 0.18	5.59 ± 0.32†	4.43 ± 0.23†	3.66 ± 0.58†	3.48 ± 0.62^{abcde}
LD					
GLU	6.25 ± 0.46	4.91 ± 0.24†	3.99 ± 0.28†	ND	ND
FOS	7.45 ± 0.27	5.35 ± 0.42†	4.37 ± 0.55†	3.40 ± 0.42†	4.05 ± 0.66^{b}
GOS	7.49 ± 0.23	5.01 ± 0.34†	4.31 ± 0.63†	ND	ND
IA	7.73 ± 0.21	5.67 ± 0.32†	5.00 ± 0.85†	4.83 ± 0.14†	2.90 ± 0.21^{abcde}
HMP	6.15 ± 0.15	5.52 ± 0.18	5.05 ± 0.17†	4.80 ± 0.45†	1.34 ± 0.43^{abcd}

† indicated the significant different from 0h for each strains and substrates.
Different letters indicate significant differences ($P \leq 0.05$) of delta log cfu/ml for all treatment
GLU, glucose; FOS, fructooligosaccharide, GOS, galactooligosaccharide; IA, inulin agave; HMP, high methoxy pectin; WO, basal MRS media; LB, *Lactobacillus bulgaricus*; LC, *Lactobacillus casei*; LD, *Lactobacillus delbrueckii subsp. Lactis*

Table 4 Tolerance to SGJ of LB, LC, and LD grown in different carbohydrates

Strains			
Carbon source	Viable counts (log cfu/ml) of survival bacteria		
	0 h	2 h	4 h
LB			
GLU	8.2 ± 0.16	7.95 ± 0.46	7.93 ± 0.49
FOS	9.29 ± 0.62	9.21 ± 0.60	9.16 ± 0.61
GOS	8.16 ± 0.07	8.20 ± 0.12	7.85 ± 0.29
IA	9.26 ± 0.65	9.23 ± 0.63	8.90 ± 0.41
HMP	7.41 ± 0.11	7.29 ± 0.22	6.99 ± 0.19
LC			
GLU	8.35 ± 0.31	8.03 ± 0.58	7.92 ± 0.67
FOS	9.54 ± 0.52	9.52 ± 0.51	9.44 ± 0.47
GOS	9.21 ± 0.47	9.34 ± 0.39	9.40 ± 0.54
IA	9.51 ± 0.44	9.51 ± 0.54	9.26 ± 0.43
HMP	8.09 ± 0.07	7.51 ± 0.25†	7.25 ± 0.16†
LD			
GLU	7.60 ± 0.65	6.89 ± 0.39	6.66 ± 0.19
FOS	8.62 ± 0.25	8.59 ± 0.27	8.30 ± 0.06
GOS	8.69 ± 0.11	8.68 ± 0.18	8.75 ± 0.20
IA	8.67 ± 0.20	8.59 ± 0.10	8.18 ± 0.27
HMP	6.89 ± 0.39	6.64 ± 0.78	6.15 ± 0.50

† indicated the significant different from 0h for each strains and substrates.
GLU, glucose; FOS, fructooligosaccharide, GOS, galactooligosaccharide; IA, inulin agave; HMP, high methoxy pectin; WO, basal MRS media; LB, *Lactobacillus bulgaricus*; LC, *Lactobacillus casei*; LD, *Lactobacillus delbrueckii subsp. Lactis*

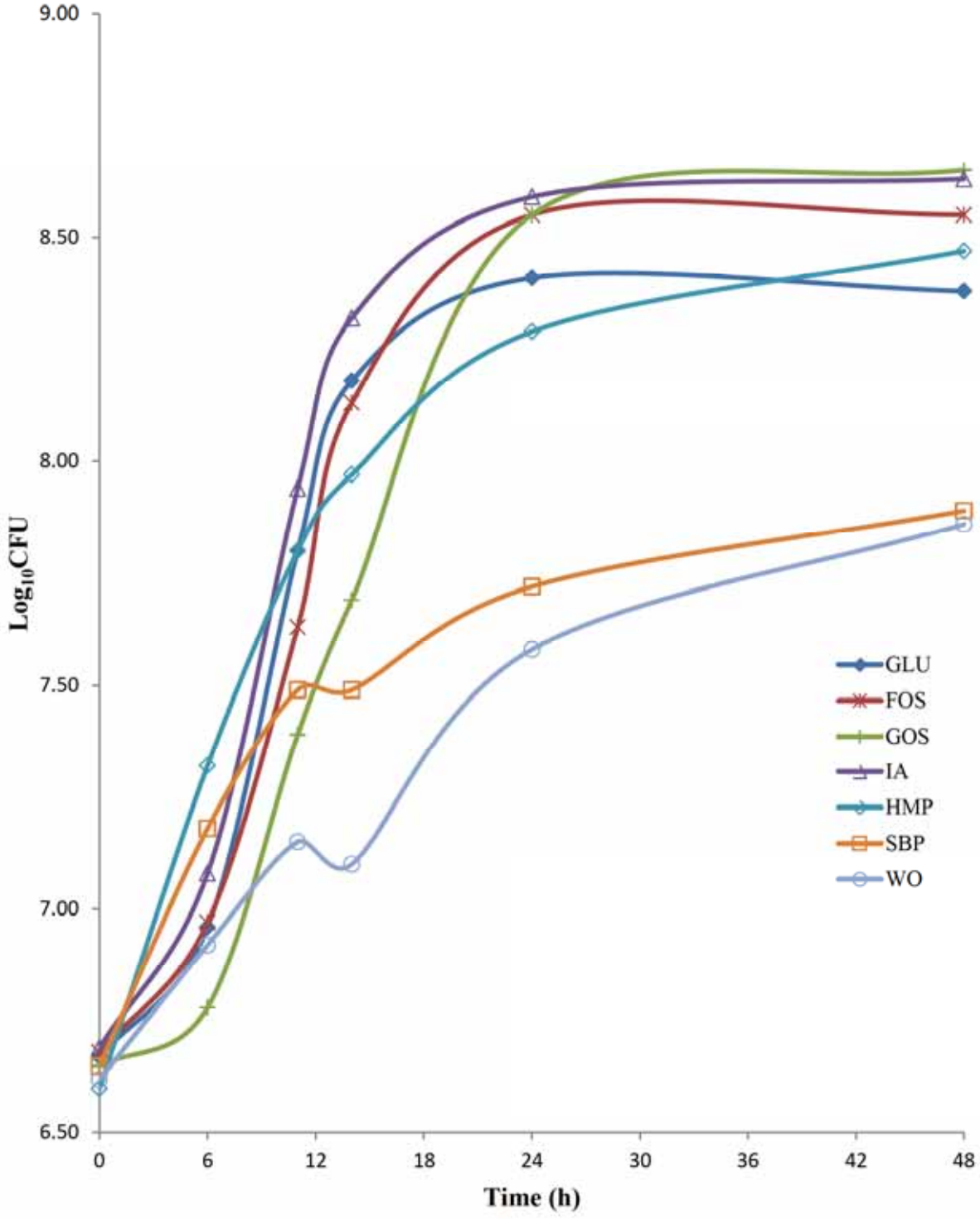

Figure 1 Kinetic growth of *Lactobacillus bulgaricus* based on $\log_{10}$ (CFU) in media containing different carbohydrates GLU, glucose; FOS, fructooligosaccharide, GOS, galactooligosaccharide; IA, inulin agave; HMP, high methoxy pectin; SBP, sugar beet pectin; WO, basal MRS media (the standard deviation of each $\log_{10}$(CFU) is ≤0.35)

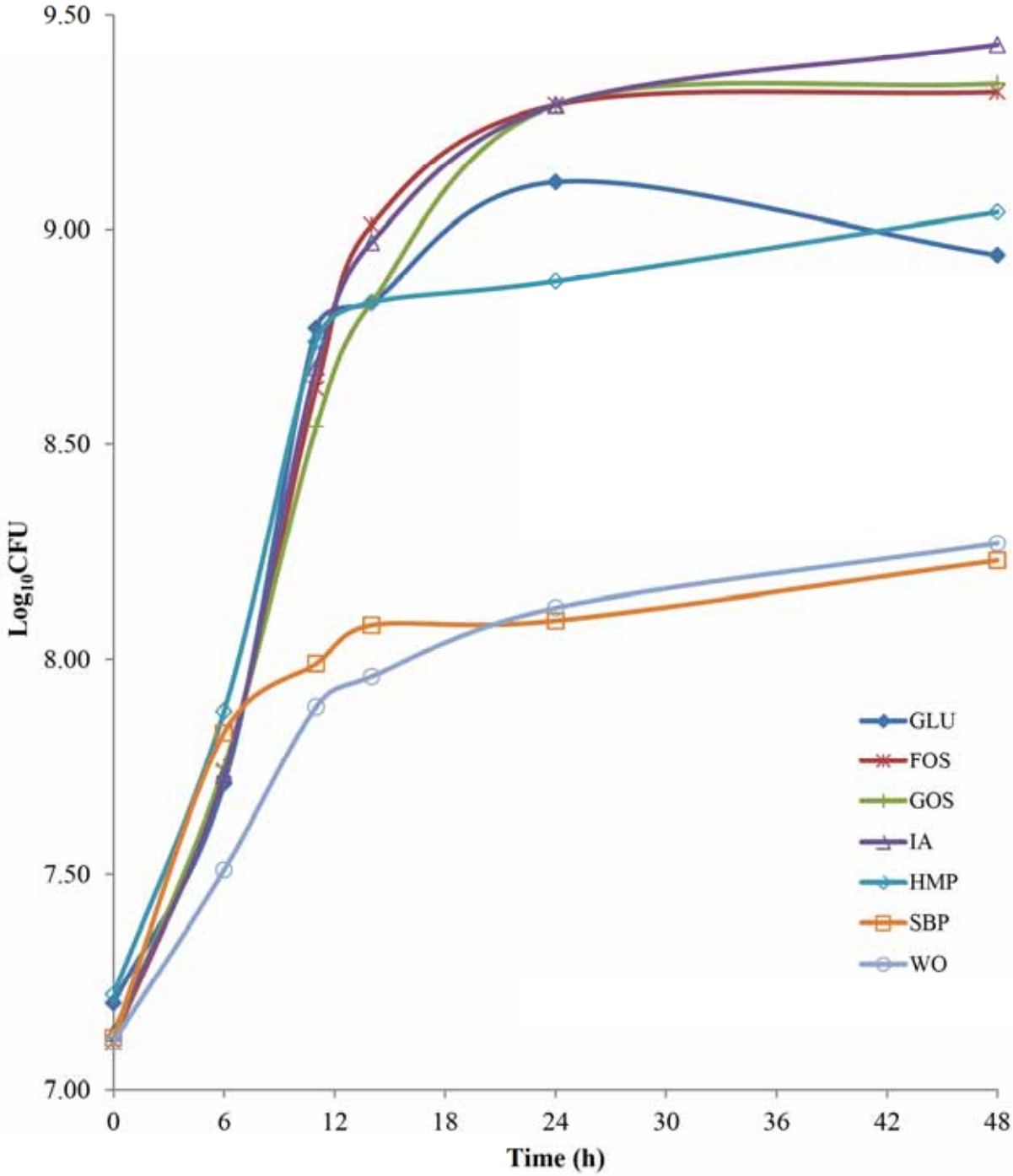

Figure 2 Kinetic growth of LC based on $\log_{10}$ (CFU) in media containing different prebiotic carbohydrates GLU, glucose; FOS, fructooligosaccharide, GOS, galactooligosaccharide; IA, inulin agave; HMP, high methoxy pectin; SBP, sugar beet pectin; WO, basal MRS media (the standard deviation of each $\log_{10}$(CFU) is $\leq$0.50)

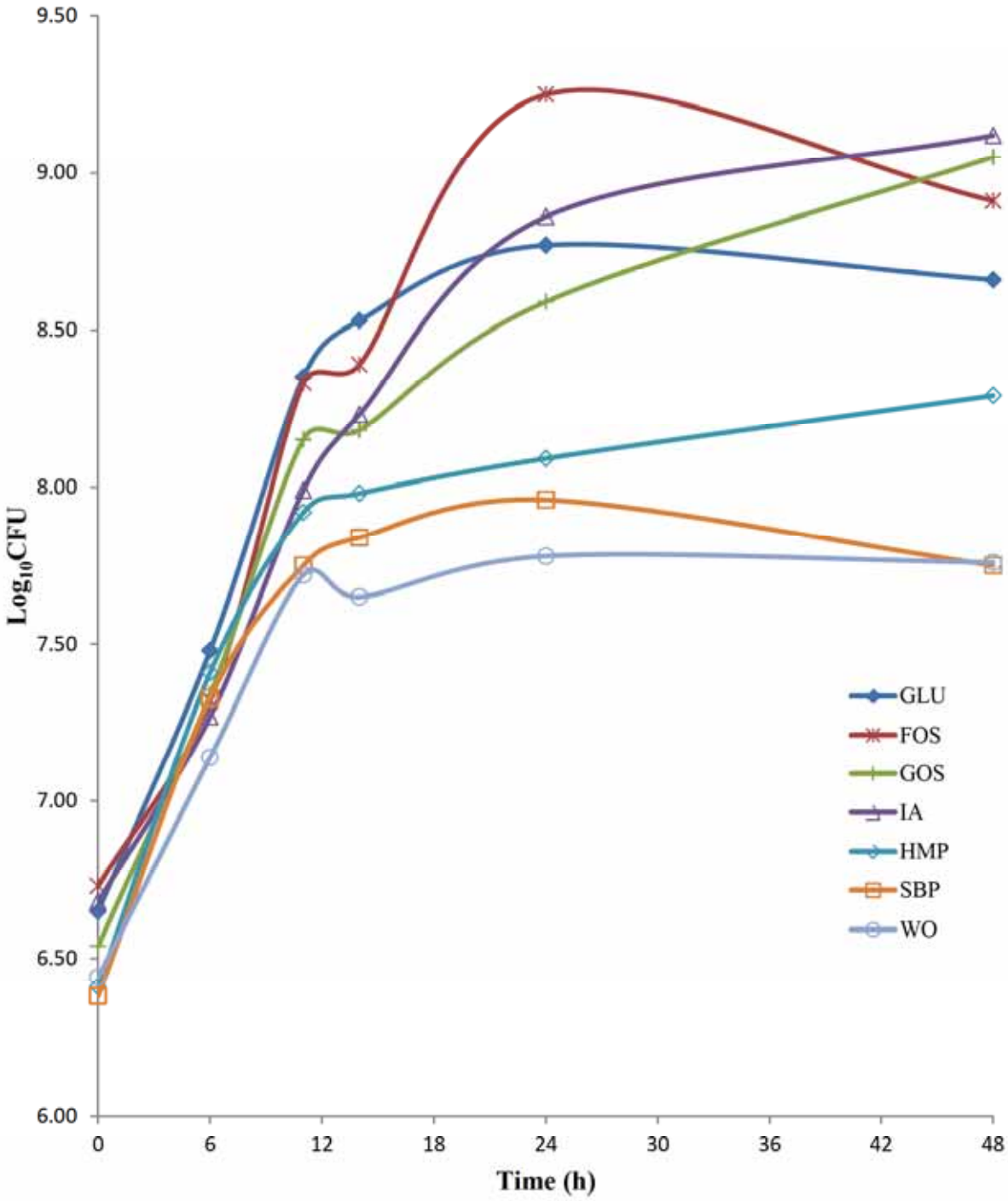

Figure 3 Kinetic growth of LD based on $\log_{10}$ (CFU) in media containing different prebiotic carbohydrates GLU, glucose; FOS, fructooligosaccharide, GOS, galactooligosaccharide; IA, inulin agave; HMP, high methoxy pectin; SBP, sugar beet pectin; WO, basal MRS media (the standard deviation of each $\log_{10}$(CFU) is ≤ 0.61)

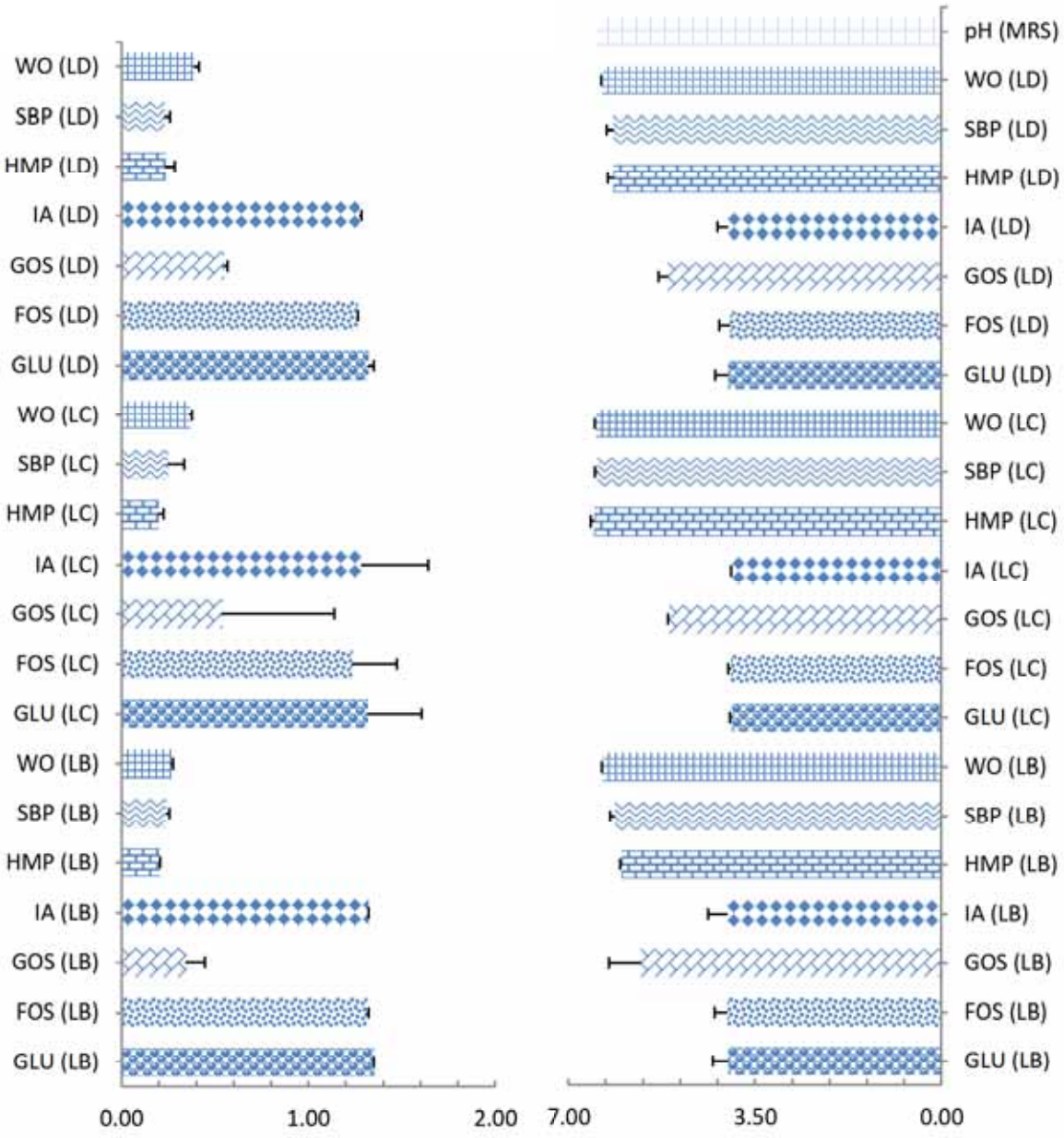

Figure 4 OD increase versus pH reduction in 48-h growth
GLU, glucose; FOS, fructooligosaccharide, GOS, galactooligosaccharide; IA, inulin agave; HMP, high methoxy pectin; SBP, sugar beet pectin; WO, basal MRS media; LB, *Lactobacillus bulgaricus*; LC, *Lactobacillus casei*; LD, *Lactobacillus delbrueckii subsp. Lactis*; pH (MRS), initial pH of MRS media

Survival of *Listeria monocytogenes* in cell-free supernatant containing bacteriocin

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Abstract:

Aims: To study the antibacterial effect of three lactic acid bacteria against *L. monocytogenes* and investigate its stability after treated with various heat and protease.

Methods and Results: The survival assay of *L. monocytogenes* was studied for 21 days at 7°C and it was observed that *P. acidilactici* CFS was the most effective against *L. monocytogenes* due to the observed drastic reduction of cells for only 24h. Thus, this strain was selected for further study of the treatment with heat and protease. In this second study, a spot-on-lawn assay was used to test the effect of treated and untreated CFS against *Listeria* and we observed the stability of this CFS at room temperature for 90 min and even autoclaving at 121°C for 30 min; however, it is noticeable that autoclaving at 90 min caused the CFS to lose its activity.

Conclusions: *P. acidilactici* effect on the reduction of *L. monocytogenese* is the combined effects of pH and possibly bacteriocin and this result could lead to the application of this strain as biopreservative in food products.

Significance and impacts of the study: This study has found a strain exhibiting anti-*Listeria* effects which is very heat stable and could be used in food application.

Introduction

Listeria monocytogenes is a significant threat to the safety of ready-to-eat (RTE) meat products as it is everywhere in the environment and it can proliferate at refrigerated temperatures (Zhu et al. 2005); in addition, this pathogen is also found in other food products such as meats, egg, vegetables, seafood, and dairy products (Farber and Peterkin 1991). The infection caused by *L. monocytogenes* is a rare human disease, but it can cause serious clinical manifestations for the susceptible population groups such as the elderly, immunocompromised individuals, fetuses, and neonates and approximately 20 to 30% of the cases are fatal (Doganay 2003; Jackson et al. 1993). Certain chemicals have been used in food products to prevent microbial growth; however, there is increasing awareness of consumers about the risks associated with some of these chemical preservatives. Thus, alternative methods such as bacteriocins, commonly produced by lactic acid bacteria (LAB), have been used to control *L. monocytogenes* in foods (Eijsink et al. 2002); for example, enterocin 1146 from *Enterococcus faecium* DPC1146 is used in cheese making (Parente and Hill 1992a, b) and bacteriocins produced by LAB such as *Pediococcus, Leuconostoc, Carnobacterium* and *Lactobacillus* spp. are used as biopreservatives in meat products (Abee et al. 1995). However, pediocin-like bacteriocins are the most studied bacteriocins which are active against *Listeria* spp. (Klaenhammer et al. 1993); also, studies have shown that the bacteriocins are able to kill pathogens in meat products more effectively than nisin (Montville and Chen 1998).

Therefore, in this study, we intended to examine the survival of *Listeria monocytogenes* incubated in the presence of cell free supernatant (CFS) collected from three different strains of LAB to see whether CFS contain some compounds (eg, bacteriocin) to kill/inhibit pathogen

growth. Also, we would further investigate the stability of the antibacterial activity CFS treated with various heat and protease.

Materials and methods

Bacterial growth

Bacteria used in this study are three strains of lactic acid bacteria (LAB) including *P. acidilactici* D3, *Lb. amylovorous* M35, *Lb. animalis* La51, and *L.monocytogenes* 10403S. All bacteria were stored at -80°C as glycerol stocks (15% glycerol in either de Man, Rogosa, Sharpe [MRS, EMD Chemicals, Gibbstown, NJ; LAB strains] or brain heart infusion [BHI, EMD Chemicals; *L. monocytogenes*] broth). Prior to the experiment, bacteria were grown to stationary phase for approximately 22 h at 37°C by using MRS (for LAB) or BHI (for *L. monocytogenes*). Then, cell free supernatant (CFS) was collected from overnight LAB by centrifuging following by filter-sterilization. The prepared CFS was stored in freezers at -20°C until use.

Survival assay of *L. monocytogenes* incubated in CFS

The survival of *L. monocytogenes* (initial inoculum of 10^6 cfu/ml) was determined by incubating in CFS from LAB, MRS and MRS (pH =4) at 7°C for 21 days. Samples were collected every 0, 1, 3, 7, 14, and 21 days and plated on BHI agar. The experiment was repeated 3 times.

Proteinase and heat treatment on bacteriocin-like substances:

All LAB strains were grown in MRS broth at 37°C for 22 h. CFS was subsequently collected by centrifuging at 7000 rpm for 15 min and filtered through 0.22-µm filters. To perform the heat treatment, CFS was subjected to heating at room temperature, 40, 60, 80, 100, and 121°C for 15, 30, and 90 min. For enzyme treatment, proteinase K at the concentration of 12.5 mg/0.5 ml was used to treat CFS. Basically, we conducted this experiment by inoculating 100µl of overnight *L. monocytogenes* 10403S into tempered semi-solid agar and poured on MRS agar as an overlay. Treated CFS was spotted on the overlay-BHI agar and finally incubated at 37°C for 24h. The triplicate experiment was repeated three times to determine the stability of CFS after treating with various heat and protease.

Statistical analyses:

One-way ANOVA with Tukey honestly significant difference (HSD) means comparison was performed where appropriate to determine statistical significance. Statistical analyses were performed using JMP 7 (SAS Institute Inc, Cary, NC).

Results and Discussion

Survival assays of *L. monocytogenes*:

Figure 1 illustrates the reduction of *L. monocytogenes* incubated with CFS from three LAB. The result shows that among all CFS-treated cells, *L. monocytogenes* exhibited drastic reduction when incubated with *P. acidilactici* CFS. However, pH effect was also observed as cells incubated in MRS (pH 4) were reduced at 14 and 21 days. CFS collected from *L.amylovorous and L. animalis,* and MRS show slight reduction of *L. monocytogenes* cells;

however *L. monocytogenes* recovers after incubation for 7 days in MRS medium. It has been shown that *L. monocytogenes* could develop resistance to bacteriocin produced by *P. acidilactici* (Gravesen et al. 2002); however, other studies have reported that bacteriocin produced by this organism affects *L. monocytogenes* (Neilsen et al. 1990, Uhart et al. 2004, Youssef et al. 1991). Thus, the observed reduction of *L. monocytogenes* in our study may be attributed to a lack of resistance of the cells incubated at low temperature (7°C) and the combined effect of pH as well.

Stability of CFS after heat or protease treatment:

As *P. acidilactici* was the most effective in reduction of *L. monocytogenes* cells, we further investigated the stability of its antibacterial effects after treating with heat and protease. The clear zones of CFS treated or untreated are depicted in figure 2. The results show that CFS has effect on inhibiting *L. monocytogenes* and its activity is lost after treatment with proteinase K (fig 2, B). CFS incubated at room temperature (RT) did not lose its activity (fig. 2, A) and heating at 121°C for 30 min also did not affect antimicrobial activity of the CFS. However, it is noticeable that autoclaving at 121°C for 90 min made the CFS lose its activity against *L. monocytogenes* (fig. 2, C). It has been shown previously that this isolate is susceptible to proteolysis once exposed to proteinase K, trypsin, and protease type XIV. Also, Miller et al. (2005) suggest that heat stability and protease sensitivity is a common characteristic of class II bacteriocins produced by *P. acidilactici.*

In conclusion, among all three strains of test LAB, *P. acidilactici* exhibited the most effect against *L. monocytogenes* as drastic reduction of the cells was observed even for 24 h. Further studies have suggested that it is the combined effect of pH and possibly bacteriocin.

Therefore, this study has indicated that *P. acidilactici* may act as biopreservative in food product to effectively control *L. monocytogenes*.

Acknowledgements

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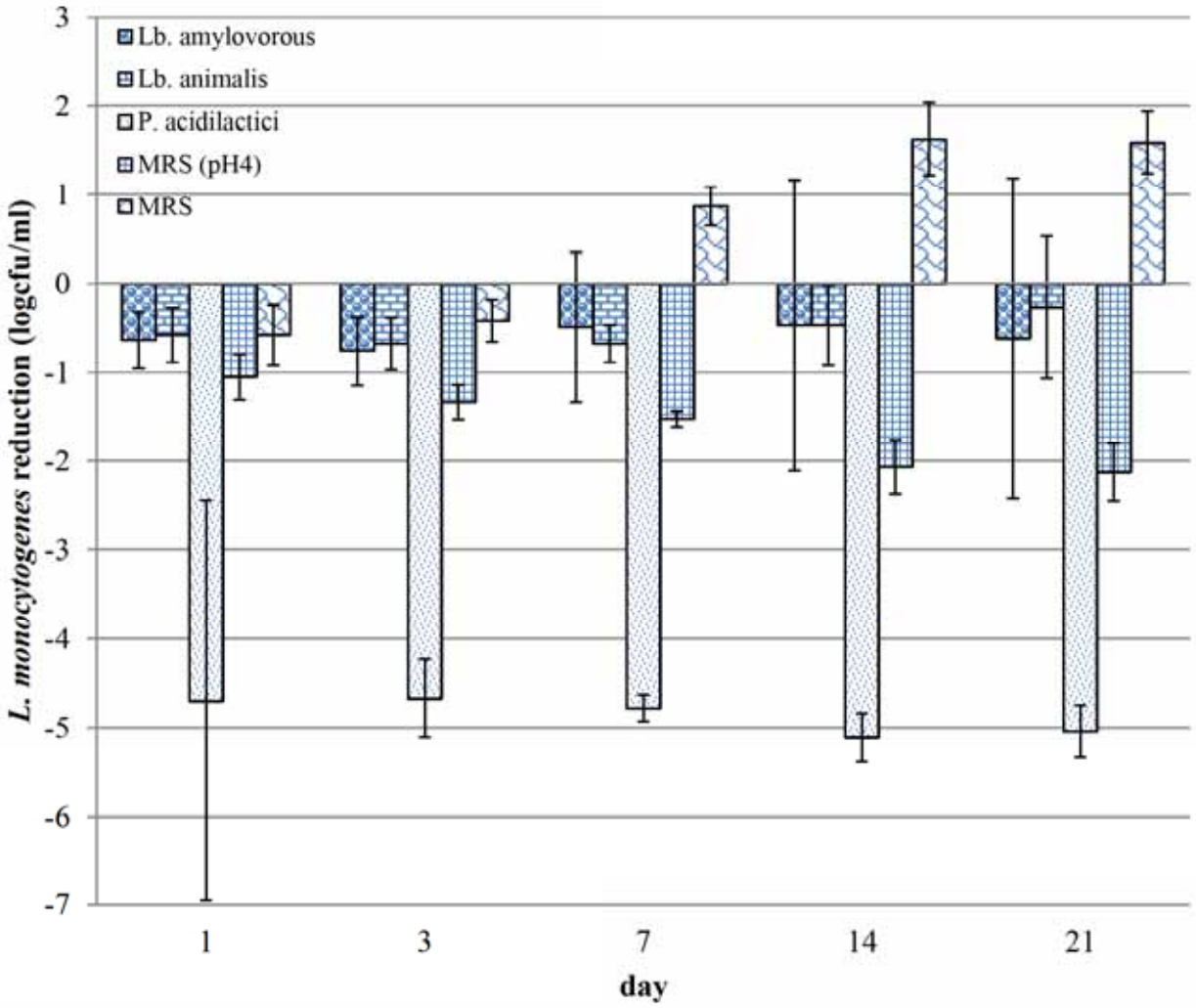

Figure 1 Reduction of log cfu/ml of *L. monocytogenese* upon exposition to cell free supernatant (CFS) collected from *Lactobacillus amylovorous, Lactobacillus animalis,* and *Pediococcus acidilactici*) for 21 days.

Figure 2 Stability of antilisterial effects of *P. acidilactici* cell free supernatant (CFS) after treated with protease and heat. (A) CFS incubated at room temperature (RT) for 90 min; (B) CFS: untreated CFS, Pro+cfs: CFS treated with proteinase K; (C) CFS autoclaved at 121°C (15 psi) for 15, 30, and 90 min.

Acridine orange as an alternative method to study kinetic growth of *Lactobacillus bulgaricus* ATCC7517 instead of optical density measurement

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Abstract:

Aims: To explore the use of acridine orange to study the growth of *Lactobacillus bulgaricus* ATCC7517 instead of optical density.

Methods and Results: The growth of bacteria in *Lactobacillus* MRS medium was checked by both methods, acridine orange and optical density measurement for 24h. The relationship between both methods was tested by looking at their correlations. The doubling time of bacteria based on the values of OD_{600} and AO obtained during 24-h growth were calculated. Although the result shows strong correlation of cell growth between OD_{600} and AO, we were unable to compare the doubling time between both methods because the AO value of cell growth exhibited very drastic fluctuations.

Conclusions: This study has failed to prove the AO could be used as a new method to checking bacteria growth instead of OD.

Significance and impacts of the study: This finding has shown that AO is not suitable for using to check the growth of bacteria although it has been used for microscopic count.

Introduction

A simple and rapid method normally used to determine the number of microorganisms in a culture or a sample is optical density (OD) measurement; however, there are some constraints in checking the optical density as its sensitivity is very low and the detection can be interfered with by other substances present in the media (Gasol et al. 1999; Gruden 2004). However, it is suggested that the measurement of bulk nucleic acid content in the microbial culture is a sensitive and reliable way to determine microbial growth (Weinbauer et al 1998) and recently the determination of cellular nucleic acid contents has been successfully applied by using fluorescent dye (Button and Robertson 2001; Gasol et al. 1999). Martens-Habbena and Sass (2006) have reported that SYBR green I which is used to stain nucleic acid can be used to check the growth of bacteria as it has excellent agreement with optical density measurement. Acridine orange (AO) is one of the fluorogenic dyes commonly used in environmental microbiology and microbial ecology in the direct count method of microorganisms (Tschech and Pfennig 1984). The AO dye has also been used to detect microorganisms in direct smears of clinical specimen (Kronvall and Myhre 1977). In studying basal de Man, Rogosa and Sharpe (MRS) media containing sugar beet pectin or low methoxy pectin we found it was very cloudy, possibly affecting the values of optical density.

Therefore, we aimed to investigate the measurement of the fluorescent dye, acridine orange (AO); as an alternative method instead of OD_{600} for studying the growth of *L. bulgariucs* ATCC7517. The validity of this new method (AO) will be confirmed by testing how strong the correlation between OD_{600} and AO is; and the doubling time obtained from both methods.

Materials and methods

Bacteria strains:

Lactobacillus bulgaricus ATCC7517 (LB) was purchased in lyophilized form and maintained at -80°C for long-term storage. Bacteria were cultured in Lactobacilli MRS broth (Difco Laboratories, Sparks, MD, USA) and incubated anaerobically at 37°C overnight prior to each experiment.

Comparison between acridine orange and optical density methods:

One percent of overnight LB culture was transferred into fresh MRS broth and incubated anaerobically at 37°C for 24 h. One-ml of the culture was centrifuged at 16×000 g for 2 min (Brinkmann Centrifuge 5415C Eppendorf, Westbury, New York, USA), the supernatant was discarded, and the pellet resuspended in 1-ml phosphate buffered saline (PBS). For fluorescent measurements, AO (Sigma, USA) was used to stain the DNA of bacteria. AO stock was prepared by adding 30 mg of AO powder in 1-ml sterile deionized water, protected from the light, and stored in refrigerator. Walpole's buffer was prepared by dissolving 27.2 g of sodium acetate trihydrate into 800 ml of deionized water, the pH was brought down to 4 by adding concentrated acetic acid, deionized water was added to a final volume of 1 L, and the solution autoclaved at 121°C for 15 min. Prior to each experiment, the AO solution was freshly prepared by adding 0.05 ml of AO stock into 5ml of Walpole's buffer. The above resuspended cells were incubated with 5-µl AO solution at room temperature in the dark for 15 min (Chalova et al. 2004), centrifuged, and resuspended with 1-ml PBS. Nuclon transparent 96-well plates were used to measure the fluorescent intensity at the excitation/emission wavelength of 485/520 nm (Martens-

Habbenaand Sass 2006) and the optical density at absorbance of 600 (OD_{600}) on a microplate reader (Tecan Group Ltd, Grödig, Austria). Both AO and OD measurement are performed with duplicate samples and repeated 3 times.

Statistical Analysis: The correlation between OD_{600} and AO values were determined by JMP package program ver. 9.0.0 (SAS Institute Inc, NC, USA), the values of OD_{600} and the AO values were converted into natural log value and the doubling time was calculated using $t_d = \ln 2/\mu$ where μ is the specific growth rate, and t_d is time at which the cell concentration had doubled.

Results and Discussion:

Comparison between acridine orange and optical density methods:

Figure 1 and 2 illustrate the growth curve of LB based on OD_{600} and AO respectively; and the correlation between AO and OD_{600} is shown in figure 3. However, the data obtained from the growth of LB cannot be used to calculate the doubling time due to the fluctuation of AO values during bacterial growth (figure 2). Actually, comparing doubling time between AO and OD methods could be the other evidence to proof that AO can be used as an alternative method instead of OD if the same doubling times are observed. However, the doubling time based on AO values cannot be calculated as these AO values are not very accurate. Also, the correlation between AO and OD methods (figure 3) shows weak correlation (r^2=0.86). Thus, we lack of evidences to proof that AO can be used instead of OD for checking bacterial growth. In fact, AO is one of the fluorogenic dyes commonly used in environmental microbiology and microbial ecology in the direct count method of microorganisms (Tschech and Pfennig 1984). However,

AO has a disadvantage as it cannot differentiate between live and dead cells (Mcfeters et al. 1991). There are some studies in exploring the use of AO to microscopically enumerate microorganisms in blood cultures (McCarthy and Senne 1980), and in clear and humic water (Bergström et al. 1986). However, in an attempt to use AO as an alternative method instead of optical density, Martens-Habbena and Sass (2006) reported the unsuitable use of AO for observing the growth of *E. coli* owing to the very high background fluorescence even at very low concentrations and did not allow discrimination between a cell-free control and 10^9 *E. coli* cells ml^{-1}. Furthermore, the use of fluorescent dyes may have advantage over optical density measurement due to the ability of some dyes to distinguish between physiological active and live cells and also in turbid media. However, AO yield a very strong background color of the cells after staining, so the use of AO requires some washing steps which could introduce errors due to the loss of cells. Also, the procedure is very laborious and time-consuming which would not be practical for experiment associated with several samples.

In conclusion, AO is not an appropriate fluorescent dye for studying the growth of bacteria and other dyes might be of interest to test as the previous work done by Chalova et al. (2004) has shown that dye, SYTO 9, can be used instead of optical density and it has advantage of improved detection sensitivity. In addition, further experiments should include the turbid media which have interfering substances besides bacterial cells present in the media in order to see whether the dyes could have advantages in distinguishing cells from other compounds.

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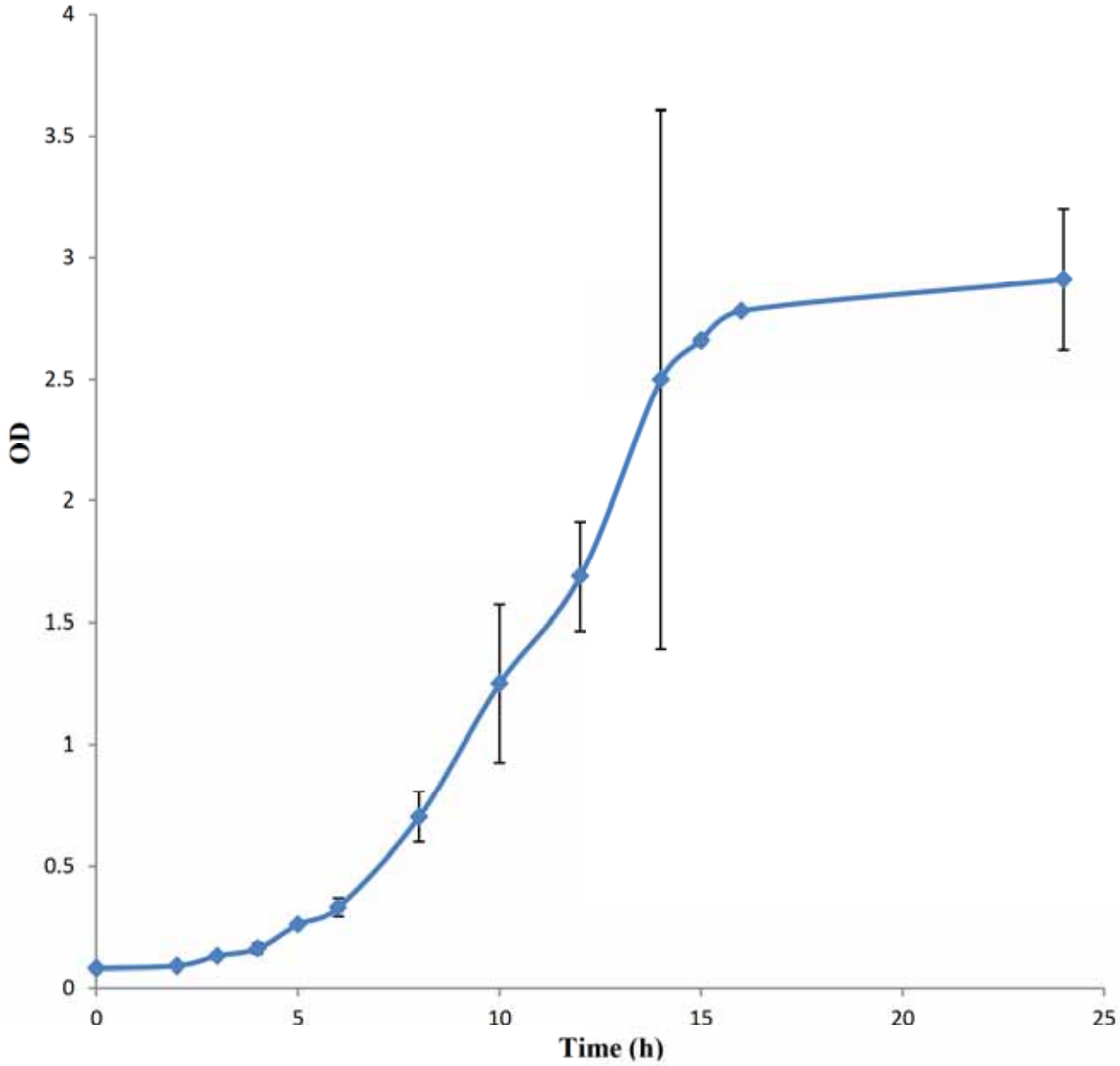

Figure 1 Growth of LB in Lactobacilli MRS broth over time measured by OD

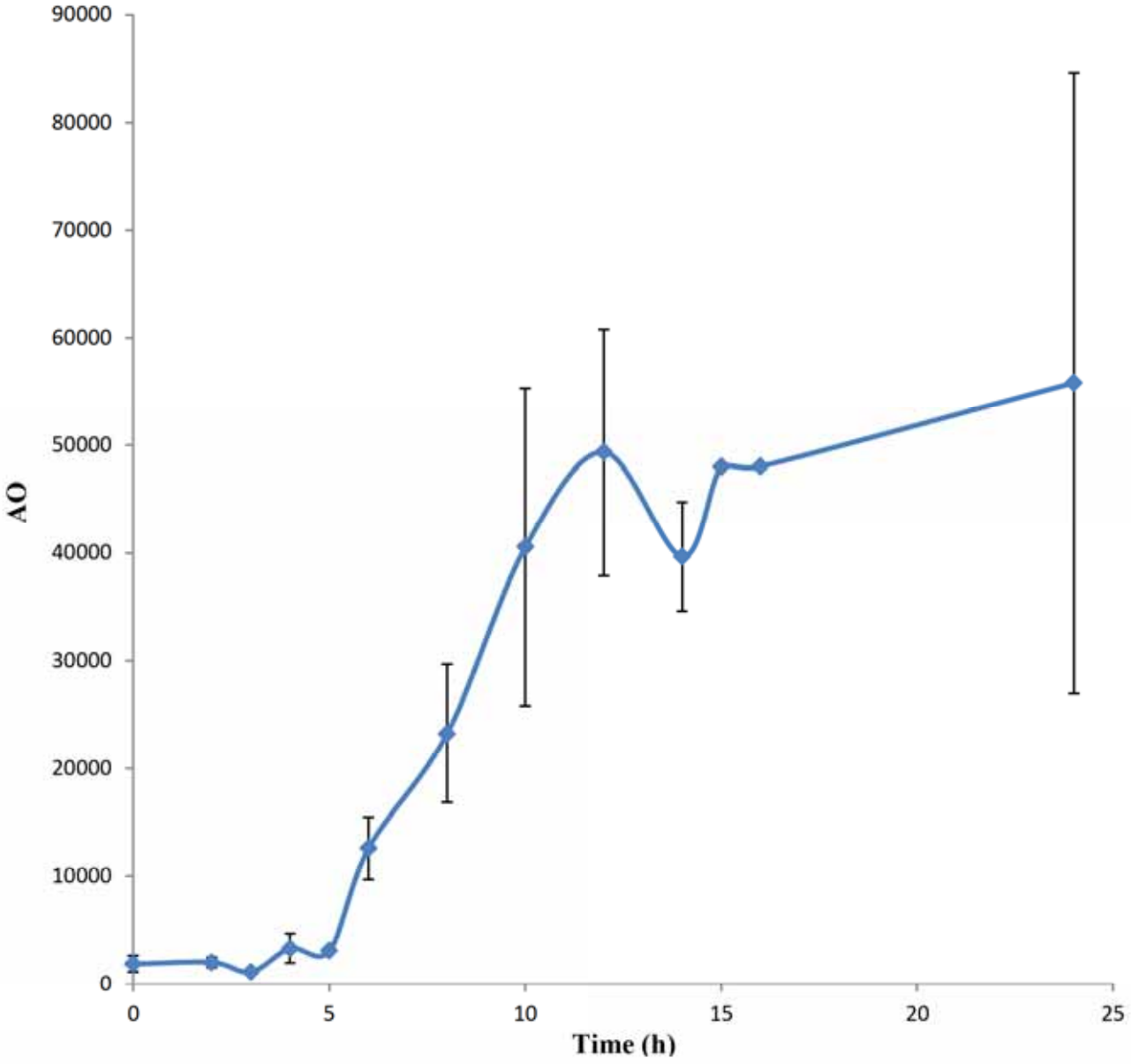

Figure 2 Growth of LB in Lactobacilli MRS broth over time measured by AO

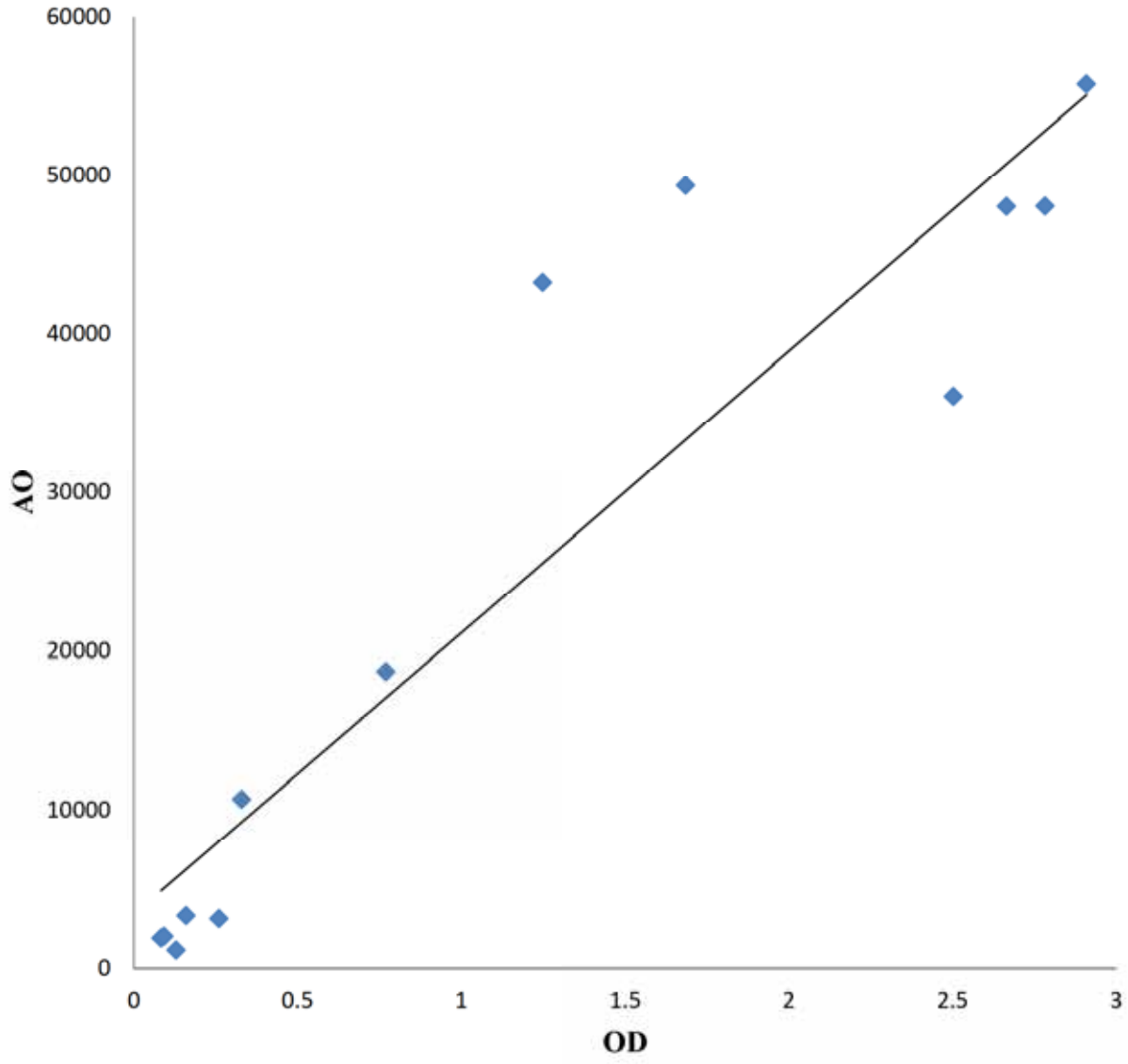

Figure 3 Correlation between OD and AO (R^2=0.86)

Chapter Five: Overall Conclusions

The microbial metabolism in the large intestine is very important as it could beneficially affect the host health (Cummings and Macfarlane 1997). Thus, probiotic bacteria such as bifidobacteria and lactobacilli are introduced into food products to gain better-balanced of intestinal microflora in the colon (Goldin and Gorbach 1992; Fuller 1989). However, there are challenges in probiotic application due to stresses that probiotics encounter in the stomach such as gastric acid and bile acid (Pochart et al. 1992; Gibson and Roberfroid 1995), which often cause the organisms to become weak. Therefore, the effectiveness of probiotics in the host GI tract is uncertain as probiotics need to compete with indigenous microorganisms for colonization sites and nutrients to be effective in the colon (Gibson and Roberfroid 1995). Therefore, prebiotics are incorporated into probiotic products to have synergistic effects in the large intestine through enhancing the survival and implanting of probiotics in the GI tract of the host (Andersson et al. 2001). Certain LAB used in food fermentation could produce antibacterial substances such as lactic acid, peroxides, and bacteriocins (Ray and Daeschel 1992). There are four different classes of bacteriocins with subdivisions, but the most studied one is pediocin-like bacteriocins which are active against *Listeria* spp (Klaenhammer et al. 1993). *L. monocytogenes* is a relatively rare human pathogen, but it could cause serious clinical manifestations for the susceptible population groups such as the elderly, immunocompromised individuals, fetuses, and neonates and approximately 20-30% of the cased are fatal (Doganay 2003; Jackson et al. 1993).

Study on the growth of probiotic bacteria (LB, LC and LD) in the presence of different carbohydrates has shown that FOS, GOS, and IA are utilized by test bacteria. FOS and IA are as good substrate as glucose for promoting probiotic growth. Also, the pH of FOS and IA was reduced drastically to below 4 along with its final biomass, based on OD_{600} at 48 h, increased

abundantly. The strong correlation between pH and log cfu/ml at 48 h was observed for each probiotic bacteria grown in the presence of FOS and IA which suggested that the more the cells grow, the lower the pH. Thus, pH should be another indicator for carbohydrate utilization; however, based on the results of growth rate, final biomass, log cfu/ml and pH, we failed to prove that HMP and SBP are digested by all three strains of probiotic bacteria. However, due to the high log cfu/ml of LC and LD in HMP after 48-h growth, we assume that HMP may have the ability to enhance the survival of probiotic bacteria.

The studies on the tolerance of probiotic bacteria to gastric conditions have shown that all of the test bacteria are very sensitive to bile solution and the resistance is varied depending on the strains and carbohydrate utilized for bacteria growth. It is found that all strains grown in GOS show the least resistance and LD grown in HMP has the highest survival. LB and LC grown in GLU show better survival compared to others. However, all test probiotics were not sensitive to SGJ, except LC grown in HMP. The previous work done in our lab using these same strains has observed the reduction of viable count even after 1 h of exposure to low pH as the gastric stress. Therefore, we suggest that the test of probiotic tolerance to gastric conditions are varied in results possibly due to different experimental conditions such as gastric constituents, pH ranges, and the models simplicity and complexity.

In the attempt to find new methods for checking the growth of probiotic bacteria, AO was suggested and tested compared to OD. We have observed strong correlation (R^2=0.86) between the values obtained from AO and OD_{600} for 24-h growth of LB in Lactobacillus MRS media. However, the growth curves of LB based on AO show high fluctuation which did not allow the calculation of doubling times. Thus, we did not have enough data to prove the validity of this AO as the alternative method instead of optical density measurement.

The study on the survival of *L. monocytogenes* has shown that among all three LAB, only *Pediococcus acidilactici* is very effective against *Listeria*. Thus, we have further investigated the stability of its antilisterial effect after the treatment with various temperatures and enzymes. We have found that its activity is very sensitive to proteinase K and it is very heat-stable; however, it is noticeable that its antibacterial effects were lost after autoclaving at 121°C for 90min. This finding suggests that *Pediococcus acidilactici* may produce a class II bacteriocin.

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Appendix

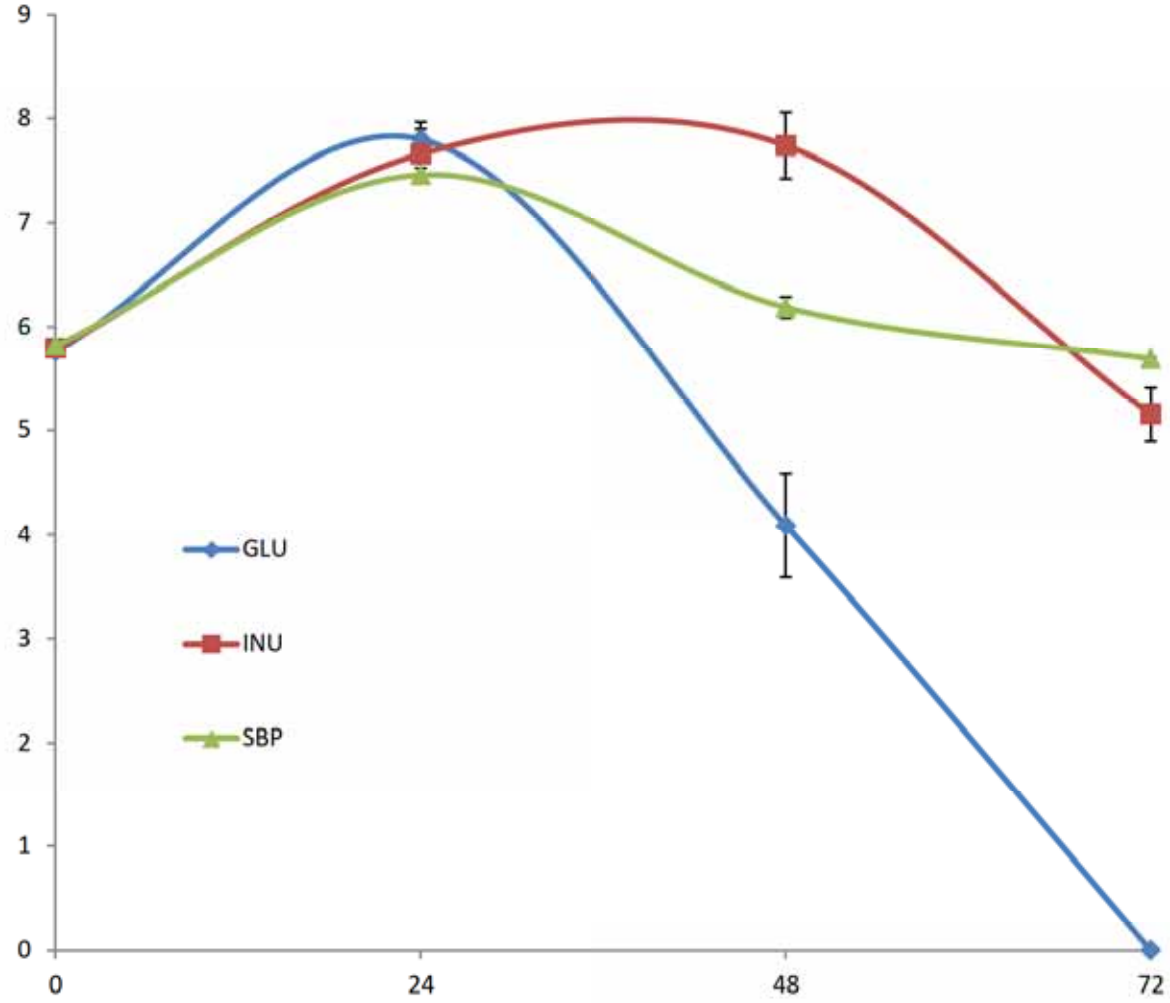

Figure 1 Kinetic growth of streptococcus bovis JB1 based on $\log_{10}$ (CFU) in media containing different carbohydrates GLU, glucose; INU, inulin; SBP, sugar beet pectin