COMPARISON OF MICROBIAL QUALITY OF COMMERCIAL PROBIOTIC DIETARY SUPPLEMENTS

by

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ABSTRACT

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Probiotics provide positive health benefits and potentially can be used as a treatment and prevention for foodborne diseases. To provide such health effects, probiotic microbes must survive before and after consumption and successfully colonize the gastrointestinal tract in the human body and display antimicrobial properties. There is lacking of studies comparing survival and antimicrobial effects of probiotic bacteria in dietary supplements sold in USA. Therefore, 11 probiotic supplements were compared for their microbial quality. Viable counts of five supplements exceeded or closely met the counts listed on the label. Two supplements did not contain any live bacteria in one of the two tested lots and the remaining four had viable counts about 1-2 log lower than the claimed viable counts.

Nine products, containing viable counts in both tested lots, were further analyzed for their tolerance of simulated gastrointestinal (SGI) condition. The results show that the survival of probiotic bacteria in SGI condition depended on encapsulation and bacteria strains. Probiotic bacteria in the form of pearl exhibited better survival in simulated gastric juice than those in capsule form. Nine probiotic bacteria including seven *Lactobacillus* and two *Bacillus coagulans* were isolated from the nine products and identified. The nine isolates were resistance to 4 -7 out of eight tested antibiotics. Culture filtrates of the seven *Lactobacillus* isolates inhibited the growth of *Escherichia coli* O157:H7 and *Salmonella* Typhimurium but not *Listeria monocytogenes*. However, after adjusting pH to 6.5, none of the culture filtrates showed any growth inhibition effect. Five probiotic isolates, namely *L. acidophilus* La-5 and La-14, *L. plantarum* Lp-115 and 299v and *L. rhamnosus GG*, which had relatively higher viable counts after exposure to SGI were compared for their ability to adhere to HT-29 cells and to reduce adhesion of the three pathogens to HT-29 cells. After incubation for 1 h, *L. plantarum* Lp-115 displayed the highest mean adhesion ratio (25.9 ± 3.4 CFU/cell) whereas *L. acidophilus* La-5 and La-14 had the lowest two mean adhesion ratios which were 0.8±0.1 and 1.9±0.5 CFU/cell respectively. Adhesion reduction of the

three pathogens on HT-29 cells varied depending on the probiotic strains, the pathogens, and the method for analysis (exclusion, competition, and displacement). Among the five, *L. plantarum* Lp-115 showed the strongest pathogen inhibition ability. It excluded >97% *E. coli* O157:H7 and >91% *S.* Typhimurium and displaced >96% *L. monocytogenes* on HT-29 cells. *Lactobacillus plantarum* v299G and *L. rhamnosus* GG also reduced adhesion of the three pathogens on HT-29 cells by the same mechanisms; however, the percentages of reduction were slightly lower. The *L. acidophilus* La-5 reduced > 93% *E. coli* O157:H7 on HT-29 cells by competition or displacement, and displaced about 94% *L. monocytogenes* on the cells. Nevertheless, it only reduced <28% *S.* Typhimurium on HT-29 cells by the three mechanisms. The *L. acidophilus* La-14 showed similar effects on adhesion reduction of the three pathogens on HT-29 cells. Overall, Nature's way® Pearls was the best probiotic supplements since the form of pearl made the probiotic bacteria more resistant in SGI condition. Additionally, the *L. plantarum* Lp-115 in this supplement had the highest adhesion ratio and the best antimicrobial efficacy.

CHAPTER 1. INTRODUCTION

1.1 Overview of Probiotics

Probiotics are defined as "live microorganisms which when administered in adequate amounts confer a health benefit on the host (Dinan and Quigley 2011)". In recent years, the popularity of probiotic products has grown rapidly worldwide. Probiotics have been widely incorporated into a variety of foods including yogurt, cheese, fermented dairy products, chocolate, ice cream and juice (Fredua-Agyeman and Gaisford 2014). Probiotics are also sold as food supplements to enhance human health. Most of the international organizations have recommended that the minimum viable count of probiotic bacteria products should be at least 10^6 CFU/mL at expiration (FAO/WHO, 2002). It is also believed that $10^8 – 10^9$ CFU of live probiotic bacteria should be consumed daily to convey health benefits. In addition, after consumption, the probiotic bacteria have to survive low pH condition in the stomach and tolerate bile salts and digestive enzymes found in the small intestine (Akın et al., 2007).

Probiotic microbes may benefit human health by decreasing risks of colon cancer, improving lactose tolerance, reducing serum cholesterol, alleviating allergy, stimulating immune system, decreasing depression and combating intestinal infectious diseases (Al-Tawaha and Meng, 2018). There are abundant evidences supporting the use of probiotic in treatment of acute diarrhea as well as prevention of antibiotic-associated diarrhea (Kechagia et al., 2013). After antibiotic therapy, the normal microflora tends to be suppressed and imbalanced, thus increasing the risk of infection by enteric pathogens. The presence of probiotics can restore the balance of microflora in the gut and prevent infection by pathogens (Madsen, 2001). Probiotic microorganisms can also produce inhibitory substances such as organic acids, hydrogen peroxide, short chain fatty acids and bacteriocins to make an unfavorable environment for pathogens or kill them. Furthermore, they can inhibit the pathogens by competitive exclusion, immunity enhancement and mucosal barrier protection (La Fata et al., 2018). Probiotic bacteria such as *L. reuteri*, *L. rhamnosus GG*, and *L.* casei have been reported to significantly reduce the duration of diarrhea in children (Huang et al., 2002, Shah, 2007).

Probiotics also play a role in modulating innate and adaptive immunity of the host (Yan and Polk, 2011). Colonization of probiotic bacteria in the gut can stimulate host's immunity. Recently,

Lactobacillus paracasei SD_1 have been found to promote salivary IgA production and reduce *Streptococcus mutans* in salivary samples (Pahumunto et al.,2019). Another study showed that mucosal responses to *L. casei* modulated the balance of T helper cells. *Lactobasillus casei* promoted cells to TH_2 type with upregulating IL-17D and IL-21, which promoted the development of NK cells (van Baarlen et al., 2011). One study evaluated the antimicrobial effects of probiotic strains isolated from commercial Greek yogurt and observed that *L. plantarum* ACA-DC 2640 was able to improve anti-inflammatory modulation by increasing IL10 expression (Zoumpopoulou et al., 2018)

1.2 Probiotic Microbes

Probiotic microbes include multiple species from the genera of *Streptococcus, Lactococcus, Pediococcus* and *Leuconostoc* but the most commonly used are *Lactobacillus* and *Bifidobacterium spp*. (Maldonado et al., 2012). Moreover, a growing number of spore-forming bacteria *Bacillus* and some yeast such as *Saccharomyces* have also been used in probiotic products. These microbes were reported to have prophylactic and therapeutic effects against foodborne pathogens (Kopp-Hoolihan, 2001). Table 1 summarizes the commonly used probiotic microorganisms.

1.2.1 *Lactobacillus* species

Lactobacillus spp. are gram positive, facultative anaerobic rod-shaped bacteria, which have been extensively studied on their health benefits (Fijan, 2014). They are commonly found in fermented products as well as in human digestive and genital tracts. They are also widely used in yogurt, fermented milk and supplement (Mitropoulou et al., 2013). *Lactobacillus gasseri* CECT 5714 has been reported to support vaginal homeostasis, prevent infection by *Helicobacter pylori* and regulate immune system (Selle and Klaenhammer, 2013, Olivares et al., 2006). *Lactobacillus plantarum* 299v has been shown to alleviate irritable bowel disease and maintain the balance of gut flora (Molin, 2018). Some *Lactobacillus acidophilus* strains such as L-55 (Sunada et al., 2008, Fujii et al., 2016) and L-92 (Shah et al.,2012) have been indicated to mitigate nasal symptoms of allergic rhinitis and atopic dermatitis. *Lactobacillus rhamnosus* GG, which is one of the most widely used probiotics, has been shown to improve gastrointestinal disorder and prevent respiratory pathogens (Gorbach et al., 2017).

1.2.2 *Bacillus*

Bacillus coagulans is a gram positive, facultative anaerobic, rod-shaped bacterium which also produces lactic acid (Corona-Hernandez et al., 2013). Unlike *Lactobacillus spp.*, *B. coagulans* is able to generate endospores, which makes it more resistance in adverse environments and thus maintain viability for long shelf period. Several studies reported that *B.* coagulans not only maintained its viability under food processing and food storage (Konuray and Erginkaya, 2018), it also resisted gastric acid and exhibited antimicrobial activity (Majeed et al., 2016). Wang et al., (2013) reported that *B. coagulans* TQ33, which was isolated from skimmed milk powder, was able to produce antifungal compounds against pathogenic fungi. Dolin, (2009) found that *B. coagulans* GBI-30 was able to significantly reduce the number of daily bowel movement in patients with Irritable Bowel Syndrome (IBS). Since *B. coagulans* have ability to resist high temperatures and dry environment, they have incorporated into various dietary supplements and foods such as tea, coffee, chocolate, gummy and cookies (Keller et al., 2010)

1.2.3 *Bifidobacterium*

Bifidobacterium is a pleomorphic gram-positive obligate anaerobe and a natural inhabitant of the gastrointestinal tract and vagina in mammals. Viability of *Bifidobacteriums* spp. is affected by the O_2 concentration. Therefore, the manufacturing process, transportation and the storage condition of probiotic product containing *Bifidobacterium* should be really meticulous (Charteris, 1998). It is reported that the colonization of *B. longum* was able to enhance antiviral immunity by eliciting the expression of type-I IFN-induced GTPases (Buffie and Pamer, 2013). Silva et al., (2004) also found that *B. longum* Bb46 was able to reduce *Salmonella* infection by modulating inflammatory response. Similar to *Lactobacillus*, *Bifidobacterium* have been widely used in probiotic products such as dietary supplement and yogurt (Luchansky and Tsai,1999).

Table 1 Probiotic microorganisms found in commercial products and fermented foods

(Source: Irfan-maqsood, 2016)

Lactobacillus	*Bifidobacterium*	Other bacteria	Yeast
L. acidophilus	*B. bifidum*	*Enterococcus faecalis*	*Saccharomyces boulardii*
L. bulgaricus	*B. breve*	*Enterococcus faecium*	*Saccharomyces cerevisiae*
L. casei	*B. infantis*	*Lactococcus lactis*	
L. gasseri	*B. lactis*	*Leuconostoc mesenteroides*	
L. lactis	*B. longum*	*Pediococcus acidilactici*	
L. paracasei		*Pediococcus pentosaceus*	
L. plantarum		*Streptococcus thermophilus*	
L. reuteri		*Bacillus cereus*	
L. rhamosus		*Bacillus coagulans*	
L. salivarius		*Bacillus subtilis*	
		Escherichia coli	

1.3 Antibiotic Resistance of Probiotics

Antibiotics have been introduced to treat microbial infections for decades. Since then, a major problem of the treatment has been the development of antibiotic resistance in pathogens. A resistant gene can be vertically inherited within a resistant strain, and horizontally transferred between the same or different species by conjugative plasmids, transposons, integrons and bacteriophages (Davies et al., 1994, Mathur and Singh, 2005). One study indicated that gene transfer often occurred in gastrointestinal tract, between gut microbiota and pathogens (Scott, 2002). There is a potential risk for transferring antibiotic resistance genes from probiotic bacteria to other gut flora or pathogens (Sanders et al., 2010). However, antibiotic resistant probiotic bacteria may be useful to treat antibiotic associated diarrhea (Diep et al.,2009).

It has been reported that the antibiotic susceptibility of *Lactobacillus* was species-dependent and even strain-dependent (Danielsen and Wind, 2003). Many *Lactobacillus* species have been reported to have a resistance to vancomycin, a glycopeptide antibiotic used to block the construction of cell well against gram-positive microbes (Gorbach et al., 2017, Sharma et al., 2016), and gram-negative spectrum antibiotics, gentamicin, kanamycin and streptomycin (Shao et al., 2015). While, most *Lactobacillus* were still found to be sensitive to the other gram-positive spectrum antibiotics such as erythromycin, and broad-spectrum antibiotics, tetracycline,

rifampicin, and the β-lactam antibiotics, penicillin, ampicillin and cephalothin (Zhou et al., 2005). Konuray and Erginkaya (2018) reported that *B. coagulans* was more resistant to antibiotics than other lactic acid bacteria (LAB) while Cano Roca, (2014) showed that *B. coagulans* was susceptible to most antibiotics.

1.4 Acids and Bile Tolerance of Probiotics

To provide positive health effects, probiotic microbes must survive before and after consumption and successfully colonize the gastrointestinal tract in the human body. Thus, the ability to survival in the harsh physiological environment of the gastrointestinal tract is an important criterion for selecting probiotic bacteria. In vivo studies have been conducted in mice and human (Murphy, 1999, Dunne et al.,1999). Since in vitro studies are expensive, labor intensive and involving ethical issues, they are often used for initial selection of probiotic strains (Sahadeva et al., 2011). In vitro studies have been conducted to, evaluated the resistance of potential probiotic strains to sodium chloride (NaCl), low pH and biliary salts (Aroleva et al., 2011, Chenoll et al., 2011), as well as simulated gastric and bile juice (Campana et al., 2017, De Palencia et al.,2008). Reliability of the in vitro studies could be a concern because the complex nature of real human system is hard to duplicate and the bile concentration level in human fluctuates. Furthermore, the food matrices in stomach may help probiotic bacteria survive.

Bacillus coagulans has been widely reported for their ability to survive in a harsh environment because it is spore-forming bacteria (Corona-Hernandez et al., 2013). The spores not only allow the bacteria survive during the storage and manufacturing conditions but also resist the low pH in stomach and thus reach to the intestine (Gu et al., 2015). The survival of LAB in acid and bile salts depends on species as well as strains. Shah and Lankaputhra (1995) found that among five yogurts containing *L. acidophilus*, only three yogurts containing *L. acidophilus* were resistant to acids and bile salts. Zoumpopoulou et al (2017) tested 20 strains of LAB isolated from Greek dairy products, and found that the reduction of viable counts of nine *L. plantarum* strains under SGI condition varied ranging from 0.3 to 2.7 log. It has been widely reported that encapsulation, which can protect the enclosed bacteria from adverse conditions, effectively enhanced the survival of probiotics in the harsh gastrointestinal environment (Ding and Shah, 2008, Talwalkar and Kailasapathy, 2004b). The common materials used for encapsulation include alginate, chitosan, gelatin, cellulose and starch (Sreeja and Prajapati 2013). Numerous supplements have been using

the encapsulation technique to enhance the viability of probiotics. It is reported that *L. acidophilus* and *Bifidobacterium* spp. encapsulated with alginate-starch showed a lower reduction in viable counts in yogurt as compared to cultures without encapsulation (Vidhyalakshmi et al., 2009).

1.5 Adhesion of Probiotic to Human Intestinal Cells

The probiotics with good adhesive ability to intestinal epithelial cell could easily colonize the intestine tract and exert their health benefits. Since it is difficult to study bacterial adhesion in vivo, especially in humans, many researchers have used in vitro models simulating the in vivo intestinal conditions. The disadvantages of in vitro adhesion assay include (1) the absence of normal microbiota in human intestines, (2) the cultured cancer cell model which might be different from the normal epithelial cells and (3) the lack of enterocytes underlying the mucus model (Das et al., 2016). However, some studies have found that the probiotic strains with higher adhesion ability in vitro also had a better performance in vivo. Balgir et al., (2013) observed a good correlation between in vitro adhesion and in vivo persistence in human gut *of Pediococcus acidilactici*. Similarly, Krishnamoorthy et al., (2018), examined the adhesion of *L. fermentum* by in vivo using an aquatic fish mode and by in vitro using microbial adhesion to hydrocarbon assay. They found that *L. fermentum* had good adhesive properties both in vitro and in vivo.

Singh et al., (2017) found that the *L. reuteri* strain with highest adhesion ability generally showed much better ability to inhibit the adhesion of pathogens to Caoco-2 cell. Feng et al. (2015) have reported that *Lactobacillus* strains with the highest adhesion abilities showed a high expression of tumor necrosis factor-α and IL-12 by splenic monocytes and significantly inhibited the invasion of *Salmonella enteritidis* to Caco-2 cells. Ouwehand and Salminen, (2003) showed that high adhesive *L. rhamnosus* GG was able to enhance immune responses while such effect was not observed with low-adhesive *L. rhamnosus* strains, highlighting the importance of adhesion ability. Similarly, Juntunen et al., (2001), observed that the high adhesive ability of probiotic strain such as *Lactobacillus rhamnosus GG* can strengthen immunoglobulin A response to rotavirus.

1.6 Antimicrobial Activity of Probiotics

Increasing evidences supported that the consumption of adequate amounts of probiotics may inhibit growth of enteric pathogens (Campana et al., 2017). Probiotic bacteria may exert multiple

antimicrobial mechanisms, such as secreting antimicrobial molecules, decreasing pH in gastrointestinal tract, competing with pathogens for colonization sites and nutrients (Boirivant, and Strober, 2007). Numerous studies also demonstrated that probiotics can influence several aspects of immune responses such as enhancing phagocytosis, increasing production of IgA and modulating T cell responses. Probiotics has been proposed as an alternative for antibiotics or anti-inflammatory agent (Oelschlaeger, 2010).

1.6.1 Secretion of Antimicrobial Substances

Producing antimicrobial substances is one of the key properties of probiotic bacteria to compete with pathogens. Many studies have reported that probiotic bacteria can produce several kinds of antimicrobial substances such as organic acids, bacteriocins, hydrogen peroxide, and short chain fatty acids to inhibit the growth or kill bacteria in the intestinal tract (Florou-Paneri et al., 2013). Several *Lactobacillus* and *Bifidobacterium* strains have been reported to produce antimicrobial substances against pathogenic bacteria (Campana et al., 2017, Makras et al., 2006). In the food industry, bacteriocins produced by *Lactococcus lactis* have been widely used as substitutes for chemical preservatives to inhibit Gram-positive pathogens, such as *Listeria monocytogenes* (Cosentino et al.,2012). Similarly, *L. salivarius* strains were found to produce the bacteriocin called Abp118, which can efficiently decrease the growth of *L. monocytogenes (*Diep et al.,2009). *Lactobacillus plantarum* were also frequently used in dairy industry to inhibit gram negative bacteria such as *E. coli* and *Salmonella* by producing a bacteriocin called plantaricin NC8 (Jiang et al.,2016). Zoumpopoulou et al (2017) found that *Streptococcus thermophilus* ACA-DC 26 isolated from dairy products might produce some proteinaceous compounds against *Streptococcus mutans* LMG 14558, the main bacteria in developing dental cavities.

The secreted molecules from probiotic bacteria such as capric acid, phenyl lactic acid, 3-hydroxylated fatty acids and cyclic dipeptides have also been shown to have potential antifungal activities (Al-Tawaha and Meng, 2018). Murzyn et al., (2010) indicated that *Saccharomyces boulardii* can reduce the virulence factors such as hyphae and biofilm formation of *C. albicans* SC5314 by producing capric acid. Similarly, Vilela et al. (2015) demonstrated that cell-free culture filtrate (CFCS) of *L. acidophilus* ATCC 4356 can reduce the growth and hyphae formation of *C. albicans* ATCC 18804, suggesting that *L. acidophilus* ATCC 4356 might produce anti-fungal compounds against *Candida* species.

1.6.2 Antagonistic Activity against Pathogens

In addition to the secretion of antimicrobial substances, probiotic bacteria have been reported to prevent the adhesion of pathogens by competing for nutrients and binding sites (Singh et al.,2017). Some *Lactobacillus* and *Bifidobacterium* have adhesins that are similar to enteric pathogens (Singh et al.,2017, Hütt et al.,2006, Jankowska et al., 2008), thus, they can compete with pathogens for the receptor sites on the host cells.

Probiotic bacteria may reduce the adhesion of pathogens on intestinal epithelial cells by exclusion, competition and displacement (Singh et al.,2017). Exclusion is the probiotic bacteria adhered to the intestinal epithelial cells first and block the adhesion of pathogenic bacteria. Competition is the probiotic bacteria compete with pathogens for the specific receptors or binding sites on the intestinal epithelial cells. Displacement occurs when probiotic bacteria with high affinity to intestinal epithelial cell replace the pathogens adhered to the epithelial cells. Several studies reported that the antagonistic activity of probiotic bacteria is strain specific. Sribuathong et al., (2014) reported that *L. plantarum* PD 110 was the most effective strain than the other three LAB to reduce the adhesion of *S.* Typhimurium and *L. monocytogenes* to Caco-2 cells ranging from 85 to 97% and from 94 to 99% respectively by exclusion, competition and displacement. In contrast, some probiotic bacteria were reported to increase the adhesion of pathogens to Caco-2 cells or human intestinal mucus (Collado et al., 2007). It is a safety concern to consume these probiotic bacteria. Therefore, the mechanisms and reasons for such increases should be further investigated (Gueimonde et al., 2006).

1.7 Desirable Characteristics of Probiotic Microbes

Since the use of probiotic has become widely accepted, demands for probiotic products continuously increase. There is a continuous effort to search for new strains of probiotic microbes with diverse functional characteristics (Kosin and Rakshit, 2006). Probiotic microbes can be isolated from breast milk and feces of animals and humans and from existing fermented foods. The isolated microorganisms must be identified at genus, species and strain level and be deposited in an international recognized culture collection (Fontana et al., 2013). For safety concerns, probiotic microorganisms must be non-pathogenic and non-toxic. The antibiotic resistance pattern of the strain should also be assessed (Sanders et al., 2010). To offer positive health effects to the

host, the selected probiotic strain should be able to tolerate the harsh environment in the stomach and the intestines, and colonize the intestinal epithelial cells (Sahadeva et al., 2011). Although adhesion ability of probiotic bacteria is important, the colonization of probiotics seems to be transient. Once the intake stopped, probiotic bacteria usually disappear in feces within one or two weeks (Vinderola et al., 2017). Therefore, the consistent consumption of probiotic is necessary. For functional characteristics, probiotic bacteria should be able to inhibit pathogens by producing antimicrobial molecules such as acids, hydrogen peroxide, or bacteriocin and compete with pathogens. In addition, it is highly desirable for probiotic strains to provide other health benefits such as modulating the immune response, and reducing cholesterol level (Florou-Paneri et al., 2013, Tsai et al., 2012). Furthermore, to incorporate probiotic bacteria into food products, a selected strain should not affect the flavor and texture and be able to survive during food processing and storage. Many in vitro tests can initially be performed to predict the outcome in human. In vivo tests in animals should further confirm their functional and health characteristics. Lastly, clinical trials in human should be conducted before release as the safety commercial probiotic products (Charteris et al., 1998).).

1.8 Microbial Quality of Probiotic Dietary Supplements

The quality of probiotic dietary supplements has always been a concern since they are not regulated by US Food and Drug Administration. Marinova et al., (2019) evaluated 16 commercially available probiotic supplements and found that none of the 16 supplements fully met the viable counts on their labels and some samples were contaminated with unacceptable microbes. Similarly, Goldstein et al. (2014) investigated five commercial probiotic supplements from the US and found that one of the probiotic supplements did not meet the viable counts claimed on label. They also observed inconsistency of viable counts in different lots. It is critical for probiotic products including probiotic supplements to provide sufficient live probiotic microbes to benefit the host. There are many factors such as manufacturing process and storage conditions can affect the viable counts of probiotic microbes in probiotic supplements. Therefore, the quality control of products is very important.

1.9 Foodborne Pathogens

Escherichia coli O157:H7, Salmonella typhimurium, and *Listeria monocytogenes* are three most common foodborne pathogens which are frequently involved in foodborne illness outbreaks. They can cause illnesses ranging from mild gastrointestinal disorders to server life-threatening illnesses including hemolytic uremic syndrome, hemorrhagic colitis, meningitis, septicemia, and deaths around the worlds (Van Cauteren, 2017). The Centers for Disease Control and Prevention (CDC) estimated that 48,000,000 people get sick from a foodborne illness every year in the US and about 265,000 infections by *E. coli*, 1,000, 000 infections by *Salmonella* and 1,600 infections by *Listeria.* Consequently, this would lead to negative impact on human health and economics. Scharff, (2012) reported that foodborne illness results in 77.7 billion annual costs including medical costs, productivity losses and illness mortality.

1.9.1 *Escherichia coli* O157:H7

Escherichia coli is a gram negative, rod-shaped facultative anaerobic bacterium. Most *E. coli* strains are harmless and can be found in the gut of humans and animals. However, some strains such as *E. coli* O157:H7 can cause severe illnesses. *Escherichia coli* O157:H7 is a pathogen with high virulence. It can cause disease with an infectious dose of 5-50 cells. Cattles are the major reservoir of *E. coli* O157:H7 (Beauvais et al., 2018). The bacteria are primarily associated with undercooked ground meat and dairy products. It can also be transmitted from contaminated vegetables and fruits by manure or the improper handling of carcass. *Escherichia coli* O157:H7 infection can be asymptomatic sometimes. The major virulence factor of this pathogen is Shiga toxins which can cause severe bloody diarrhea and abdominal cramps. The illness often lasts for 5-10 days. It can also cause kidney failure and even death with a high dose infection. In immunocompromised, young-aged or elderly individuals, the infection can lead to the life-threatening complications such as hemolytic uremic syndrome (HUS).

1.9.2 *Salmonella* Typhimurium

Similar to *E. coli, Salmonella* also belongs to Enterobacteriaceae family. It is a gram-negative, rod-shaped facultative anaerobic bacterium. It is the most frequently reported cause of foodborne disease **(**Iglesias et al., 2017**)**. Animals and humans are the main reservoir of

Salmonella. Transmission commonly occurs when bacteria present during food processing including improperly managing carcass, poor hygiene at working place and contaminated underground water, thus allow bacteria to multiply in food. The organism can also be transferred through direct and indirect contact with infected humans or animals, and the fecal contaminated environments.

Salmonella Enteritidis and *Salmonella* Typhimurium are the two major serotypes responsible for half of all human salmonellosis (Guibourdenche et al., 2010, Threlfall, 2002). Symptoms of salmonellosis generally include acute diarrhea, abdominal cramp, fever and sometimes vomiting after a 12-36 h incubation period. However, severe symptoms and sepsis may develop when the infection spread to the surrounding tissues or the bacteria enter into the blood. Antibiotic treatment is normally used for treating severe salmonellosis. Overall the mortality of infection is low. The illness caused by *Salmonella* can be reduced by thermal processing and proper sanitation (El-Gazzar and Marth, 1992). The illness commonly through consumption of salads, raw meat, poultry and seafood.

1.9.3 *Listeria monocytogenes*

Listeria monocytogenes is a gram-positive, rod-shaped, facultative anaerobic bacterium. It is commonly found in environments like water, soil, and sewage, as well as processed meat products (Buchanan et al., 2017). It is known to have the ability to survive under adverse conditions. It can grow at refrigeration temperatures and survive in frozen storage temperatures. In addition, it is more resistant to acidic environments and the heat than many other pathogens. Although it can be killed by proper heat processing, there is still a concern for Ready-to-eat food which does not require heating prior to consumption.

The infection by *Listeria* can cause illness from mild symptoms to severe or fatal infections. Initially, it usually shows flu-like symptoms such as fever and muscle pain. It can invade the blood stream and develop into septicemia. If the infection spreads to the central nerve system, it can induce meningitis. Additionally, it can cause preterm delivery, neonatal infection, or infant death in pregnant women (Arqués et al., 2015). The occurrence of Listeriosis has remained constant since the last decade. The outbreak of Listeriosis frequently associated with ready-to-eat food, fruit, ice cream or dairy products.

1.10 Scope of This Study

There are a wide variety of commercial probiotic supplements available on today's US market. However, there is lack of study evaluating microbial quality of these products. Several previous studies indicated that not all the probiotic supplements contained viable counts as stated on their packages (Goldstein et al., 2014, Marinova et al., 2019). It is unknown whether the bacteria in the probiotic supplements can survive in the gastrointestinal conditions, successfully colonize on intestinal cells, and effectively inhibit enteric pathogens. This study investigated (1) viable bacterial counts of 11 commercial available probiotic supplements, (2) the survival of the probiotic bacteria in these commercial products in a simulated gastrointestinal (SGI) environment and (3) ability of nine isolated probiotic bacteria to inhibit three common enteric pathogens, *E. coli O157:H7, Salmonella* Typhimurium, and *Listeria monocytogenes* by secreting soluble antimicrobial substances. Five probiotic isolates which showed the best survival in SGI were further compared for their ability to adhere to cultured intestinal cells, HT-29, and to reduce adhesion of the three pathogens to HT-29 cells by exclusion, competition, and replacement.

CHAPTER 2. MATERIAL AND METHODS

2.1 Probiotic Products

A total of 11 commercial probiotic dietary supplements were analyzed in this study (Table 2). Seven products were in the form of capsule; four of these contained one or two species of *Lactobacillus* while the other three contained both *Lactobacillus* and *Bifidobacterium* spp. Two products are in the form of pearl which contained both *Lactobacillus* and *Bifidobacterium* spp. Two were in the form of gummies containing *Bacillus coagulans*. Two packages with different lot numbers of each product were purchased from local supermarkets. None of the samples exceeded its expiration date. All samples were stored at room temperature until analysis.

2.2 Isolation and Identification of Probiotic Bacteria

Lactobacillus spp. and *B. coagulans* were isolated using Man Rogosa Sharpe (MRS) agar and Tryptic Soy agar (TSA) plates respectively. *Bifidobacterium* spp., which are obligate anaerobes, were not isolated and analyzed in this study. Each unit of supplements was dissolved in 0.85% sterile saline with a 1:9 ratio, streaked on MRS or TSA and incubated aerobically at 37 ^{o}C for 48 h. Bacteria were preliminarily isolated based on colonial morphology, such as size, shape, and color. All the selected colonies were streaked on MRS plates or TSA plates for isolation. Each probiotic isolate was gram stained and sent to a commercial laboratory (GENEWIZ, NJ, USA) for final identification by gene sequencing.

2.3 Bacterial Cultures

Three major human foodborne pathogens, *Escherichia coli* O157:H7, *Listeria monocytogenes*, and *Salmonella* Typhimurium were used. Stock cultures of *E. coli* O157:H7 and *S.* Typhimurium were maintained on TSA slants and *L. monocytogenes* was maintained on TSA with 0.6% Yeast Extract (TSAYE) slants at 4 °C. Stock cultures of *Lactobacillus* spp. were maintained on MRS slants and *B. coagulans* were maintained on TSA slants at 4 °C. All *Lactobacillus* stock cultures were sub-cultured every two weeks whereas the rest were sub-cultured every three weeks. All the bacteria were also stored in cryopreservative beads (Microbank^{TM}, Texas, US) at -80^{o}C. To prepare working cultures, *E. coli* O157:H7 and *S.*

Typhimurium were grown in Tryptic Soy Broth (TSB) and *L. monocytogenes* was grown in TSB with 0.6% Yeast Extract (TSBYE) at 37 °C for 24 h. *Lactobacillus* and *Bacillus* isolated from probiotic products were grown in MRS broth and TSB respectively at 37 °C for 48 h.

2.4 Viable Counts of Probiotic Bacteria in Commercial Probiotic Supplements

Three samples randomly selected from each package were tested for viable counts. To prepare 10^{-1} dilution, each pill (capsule or pearl) sample was weighed aseptically and added into 0.85% sterile saline with a 1:9 ratio by weight. To prepare a 10^{-1} dilution of a gummy sample, a gummy sample was weighed, added into 0.85% sterile saline with a 1:9 ratio by weight, mixed by a vortex and placed in a 50 °C water bath to dissolve the gummy. After the products were dissolved, a serial 10-fold dilution of each was prepared. Then 100 µl of diluted sample was spread plated on triplicate plates. Viable counts of *Lactobacillus* and *Bacillus* were enumerated on MRS and TSA plates respectively. All plates were incubated aerobically at 37°C for 48 h. To determine the total viable counts of probiotic pills containing both *Lactobacillus* and *Bifidobacterium spp.*, all the MRS plates were incubated anaerobically at 37 °C for 48 h. Viable counts were expressed as log CFU/ml (Figure 1).

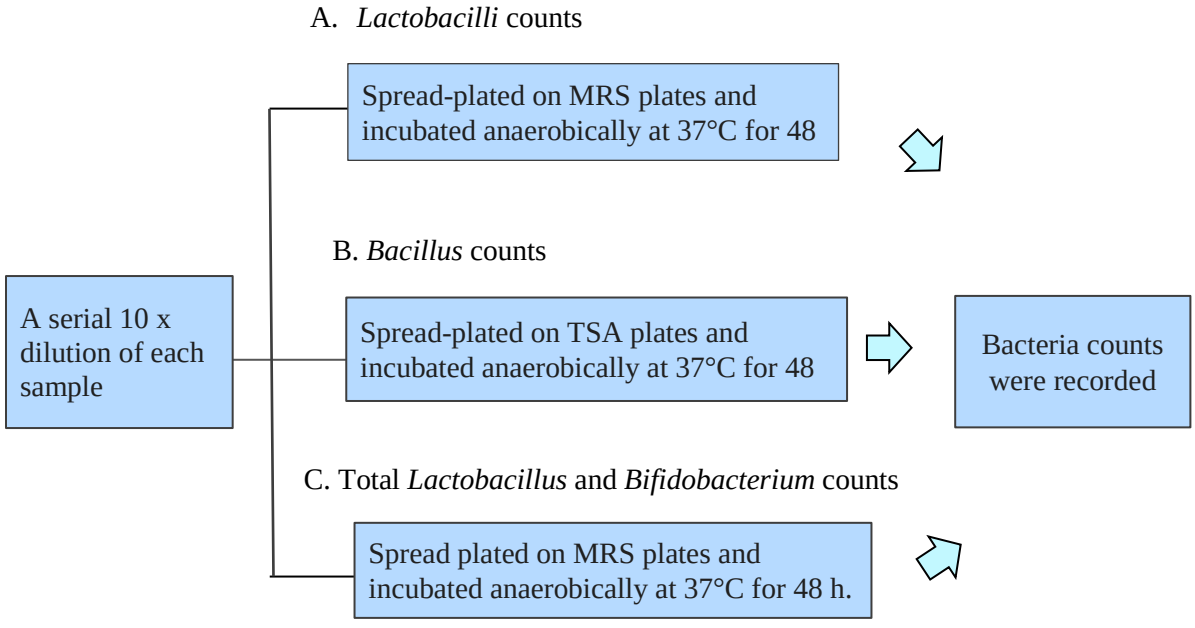


Figure 1 Preparation and enumeration of probiotic bacteria in probiotic supplements

Table 2 Active ingredients of eleven probiotic dietary supplements.

Probiotic Product	Claimed Probiotic Bacteria	Log CFU/unit on label	Unit Weight (g)	Dosage unit/day	Lot	Lot #	Expiration Date	Months to Expiration
Products containing *Bacillus* spp.								
Align® Prebiotic + Probiotic	*B. coagulans* unique IS2™	9.0	2.8	4	1	8005C369A1	07/31/2019	7
					2	8310C369A1	05/31/2020	13
OLLY® Purely Probiotic	*B. coagulans* MTCC 5856	9.0	2.3	2	1	8144M1071519	08/31/2019	8
					2	9059M1090812	07/31/2020	15
Products containing *Lactobacillus* spp.								
Culturelle®	*L. rhamnosus* GG	10.0	0.5	1	1	17284CLG2	09/30/2019	12
					2	18351CLG7	11/30/2020	18
Nature Made®	*L. plantarum* 299V	10.0	0.5	1	1	2244551	02/28/2019	6
					2	2305337	07/31/2020	15
Meijer® Digestive Health	*L. acidophilus* La-14	10.0	0.5	1	1	01/06/2020	934010	15
					2	02/17/2021	934014	21
Meijer® Wellness Probiotic	*L. rhamnosus* GG *L. acidophilus*	9.2	0.5	1	1	10/31/2019	815022872	10
					2	06/30/2020	906428291	14
Products containing *Lactobacillus* spp. and *Bifidobacterium* spp.								
Meijer® Probiotic Pearls	*L. acidophilus* La-14 *B. longum*	8.0	0.5	1	1	Not Available	12/31/2019	11
					2	300017	03/31/2020	10
Nature's Way Pearls®	*L. plantarum* Lp-115 *L. acidophilus* *B. lactis* BI-04 *B. longum* BB536	9.7	0.5	1	1	2008504301	09/30/2019	12
					2	2010340601	08/31/2020	16
Meijer® Digestive Care	*L. acidophilus* *B. bifidum, B. lactis*	9.7	0.5	1	1	733121874	02/28/2019	6
					2	833826839	05/31/2020	13
Philip's Colon Health®	*L. gasseri,* *B. bifidum, B. longum*	9.2	0.5	1	1	7G20B	07/31/2019	10
					2	8E09C	5/31/2020	13
TruBiotics®	*L. acidophilus* La-5, *B. animalis* BB-12	9.3	0.4	1	1	52088	04/31/2019	7
					2	55438	06/30/2020	15

[1]The recommended daily serving for all probiotic supplement are 1 unit except for Align is 4 units and OLLY is 2 units.
[2] NS means not stated on the label.

2.5 Antibiotic Susceptibility

Antibiotic resistance of each probiotic isolate was determined by a disc diffusion assay. Eight antibiotic discs including erythromycin (15 μg), rifampin (5 μg), clindamycin (2 μg), cephalothin (30 μg), linezolid (30 μg), amdinocillin (10 μg), tetracycline (30 μg), and penicillin (10 IU) were used in this study. The probiotic isolates were grown on agar plates at 37 °C for 48 h. Bacterial suspension of each isolates was prepared using sterile saline and turbidity of each suspension was adjusted to match 0.5 McFarland standard. To test antibiotic resistance, 100 μl of bacterial suspension was spread on MRS or Muller-Hinton agar plate using a sterile cotton swab and four antibiotic discs were put on the surface of each agar plate. After incubation at 37°C for 48 h, inhibition zones were measured. *Bacillus* isolates were tested on Muller-Hinton agar plates and results were interpreted according to the standards for disc susceptibility tests (NCCLS, 2009); whereas, *Lactobacillus* isolates were tested on MRS agar plates and results were interpreted as suggested by Charteris et al. (2001).

2.6 Antimicrobial Activity Assay

Antimicrobial effect of each probiotic isolate was determined by an agar well diffusion assay described by Campana et al., (2017) with some modifications. Instead of using nutrient agar plates, TSA plates were used to grow *E. coli* O157:H7 and *S.* Typhimurium, and TSAYE was used to grow *L. monocytogenes* in this study. Probiotic CFCS were prepared by centrifuging 15 ml working cultures at 17,900 x g for 10 min. Each supernatant was divided into two tubes. The supernatant in one tube was adjusted to pH 6.5 using 1M NaOH. Both the supernatant and the supernatant adjusted to pH 6.5 were then filtered with sterile 0.22 μm pore size polyethersulfone membranes (Whatman, USA) to remove residual bacterial cells. Antimicrobial effect of both the filtrates and the filtrates that were adjusted to pH 6.5 were tested to determine if the inhibition was due to the low pH values of the filtrates. An agar plate containing a pathogen was prepared by mixing 100 μl of a working culture (about 10^7 CFU/ml) and 25 ml melted TSA or TSAYE (approximately 50 ^{o}C) in a petri dish. After the agar plate was solidified at room temperature, 6 mm wells were made on the agar plate aseptically using a sterile cork borer and 50 μl of a probiotic CFCS was added into each well. Antimicrobial agent (1.5 % BacDown, Decon, USA) and MRS broth adjusted to pH 6.5 were used as positive and negative controls respectively. To determine if

the antimicrobial effect was due to lactic acid in the probiotic CFCS, 1M lactic acid solutions diluted to the same pH as the probiotic CFCS were also tested. After incubation at 37 °C for 24 h, the diameter of the inhibition zone surrounding each well was measured. The results were recorded as follows: – , no inhibition; +, diameter >6-10 mm; ++, diameter >10-12 mm; +++, diameter >12 mm. Each experiment was repeated three times and each assay was performed in triplicate.

2.7 Survival of Probiotic Bacteria in Simulated Gastric and Bile Conditions

To evaluate survival of probiotic bacteria in SGI condition, the procedure described by Arboleya et al., (2011) was used with some modification. Instead of using pure cultures of probiotic isolates, single unit of each probiotic product was tested in the study. A unit of each probiotic sample was added into a simulated gastric juice (SGJ) solution (125 mM NaCl, 7 mM KCl, 45 mM NaHCO3, and 3 g/L pepsin, adjusted to pH 2.5 with1M HCl) with a 1:9 ratio by weight and incubated at 37°C for 90 min. The samples that dissolved in SGJ were centrifuged at 17,900 x g for 10 mins at room temperature and transferred into a simulated bile juice (SBJ) solution (45 mM NaCl, 1 g/L pancreatin and 3 g/L Oxgall, adjusted to pH 8.0 with1M NaOH) with a 1:9 ratio by weight. The samples that did not dissolve in SGJ were directly transferred into the SBJ solution. All samples in SBJ were incubated at 37 °C for 180 min. Viable counts of *Lactobacillus* and *Bacillus* in SBJ were determined on MRS and TSA plates respectively prior to and after incubation in SGJ solution as well as after incubation in SBJ.

2.8 Cell Cultures

Human colorectal adenocarcinoma cells, HT-29, were maintained in 56.7 cm^2 petri dishes with approximately 10 mL of Dulbecco's Modified Eagle Medium (DMEM) (Sigma, USA) supplemented with 10% of fetal bovine serum (FBS) (Sigma, USA) and 1% of antibiotics (10,000 Units/mL penicillin and 10,000 μg/mL streptomycin, Life Technology Corporation, NY, USA) at 37 °C in a humidified atmosphere containing 5% CO_2. Culture media were changed every three days. For all the assays, each well of 24-well plates was seeded with 10^4 cells/ml cells and incubated until confluence (approximately 10^6 cells/well). Before the experiments, each well with confluent cells was washed twice with sterile phosphate buffered saline (PBS) and incubated in DMEM with 1% FBS at 37 °C with 5% CO_2 for 1 h.

2.9 Adhesion Assay

Adhesion ability of five probiotic strains including *L. acidophilus* LA-5 and La-14, *L. plantarum* Lp-115 and 299V, and *L. rhamnosus GG* on HT-29 cells were compared. Suspension of each was prepared by centrifuging a working culture at 17,900 x g for 10 mins and the pallet was washed twice with sterile PBS, and resuspended in DMEM with 1% FBS. The OD_{600} of *L. acidophilus*, *L. plantarum* and *L. rhamnosus* GG suspensions were adjusted to 1.2~1.3, 0.9~1.0 and 0.9~1.0 respectively (approximately 10^8 CFU/ml). Before inoculation, the DMEM with 1% FBS was removed from each well. Each well of HT-29 cells was inoculated with 1ml bacterial suspension and incubated at 37 °C with 5% CO_2 for 1 h. After incubation, the supernatant in each well was removed and the cells were gently washed twice with sterile PBS to remove unattached bacteria. Following the last wash, HT-29 cells in each well were detached by adding 500 μl 0.25% (V/V) trypsin (Sigma, USA) at 37 °C for 15 min. The number of *Lactobacillus* adhered to HT-29 cells (adhesion ratio) was enumerated by plating a serial 10-fold dilution of the HT-29 cell suspension on MRS agar plates. The number of HT-29 cells were counted by a cell counter (Bio-Rad, CA, USA). Adhesion results were expressed as adhesion ratio (CFU/cell) and adhesion percentage by the following formula:

$$\text{Adhesion ratio (CFU/cell)} = \frac{\text{Bacteria adhered to HT-29 cell (CFU/mL)}}{\text{HT-29 cells (cells/mL)}}$$

$$\text{Adhesion \%} = \frac{\text{Bacteria adhered to HT-29 cell (CFU/mL)}}{\text{Initial bacteria inoculum (CFU/mL)}} \times 100\ \%$$

2.10 Inhibition of Pathogens Adhered to HT-29

Three inhibitory mechanisms, exclusion, competition and displacement, of the five probiotic bacteria against the three pathogens on HT-29 cells were evaluated in this study. Confluent HT-29 cells (approximately 10^6 CFU/well) without antibiotics were prepared in each well of 24-well plates as described previously. Each probiotic inoculum was prepared as described in the adhesion assay. The OD_{540} of each working culture of pathogens was adjusted to 0.15~0.18, which contained approximately 10^7 CFU/ml. Each pathogen inoculum was prepared by centrifugation of 1 ml of pathogen working culture at 17,900 x g for 10 min, then the pallet was

washed twice with sterile PBS, and resuspended in 1 ml DMEM with 1% FBS. The following assays were performed to determine how each of the five probiotic bacterial suspension influenced adhesion of the three pathogens on HT-29 cells (Figure 2).

2.10.1 Exclusion Assay

Confluent HT-29 cells were incubated in 1 ml of a probiotic inoculum for 1 at 37 °C with 5% CO_2. After incubation, each well was washed twice with sterile PBS to remove probiotic bacteria that were not adhered to the HT-29 cells. Then 1 ml of a pathogen inoculum was added to the HT-29 cells and incubated for another 1 h at 37 °C with 5% CO_2. In control wells, confluent HT-29 cells were first incubated in 1 ml of DMEM without probiotic inoculum for 1 h before exposure to 1 ml of a pathogen inoculum.

2.10.2 Competition Assay

One milliliter of a probiotic inoculum and a pathogen inoculum were mixed, centrifugation at 17,900 x g for 10 min, and resuspended in 1 ml of DMEM supplemented with 1% FBS. The confluent HT-29 cells were cultured with 1 ml of the mixed suspension for 2 h at 37 °C with 5% CO_2. In control wells, confluent HT-29 cells were incubated in 1 ml of a pathogen inoculum for 2 h.

2.10.3 Displacement Assay

Confluent HT-29 cells were incubated with 1 ml of a pathogen inoculum for 1 h at 37 °C with 5% CO_2. After incubation, each well was washed twice with sterile PBS to remove pathogens that were not adhered to HT-29 cells in the well. Then the HT-29 cells were incubated with 1 ml of a probiotic inoculum for another 1 h at 37 °C with 5% CO_2. In control wells, confluent HT-29 cells were first incubated in 1 ml of a pathogen inoculum for 1 h and then in 1 ml of DMEM without probiotic bacteria for another hour.

2.10.4 Viable Counts of Pathogens on HT-29 cell

At the end of each assay, HT-29 cells in each well were washed twice with sterile PBS to remove bacteria that were not attached to the HT-29 cells in the well. Then the HT-29 cells were

detached from each well by incubating the cells in 0.25% (V/V) trypsin at 37 °C for 15 min. A serial 10-fold dilution of the cells were prepared in sterile saline. Viable counts of *E. coli* O157:H7 and *S.* Typhimurium were enumerated on McConkey, and Xylose Lysine Deoxycholate (XLD) agar respectively and incubated at 37^{o}C for 24h. Viable counts of *L. monocytogenes* were enumerated on Polymyxin Acriflavin Lithium-chloride Ceftazidime Esculin Mannitol (PALCAM) and incubated at 37^{o}C for 48h.

2.11 Statistical Analysis

Results were presented as means ± standard deviations (n=6). Mean results from survival in SGJ and SGJ, adhesion assay and antagonistic activity were individually compared using one-way analysis of variance (ANOVA) followed by Tukey HSD test. Differences were considered significant when $P<0.05$.

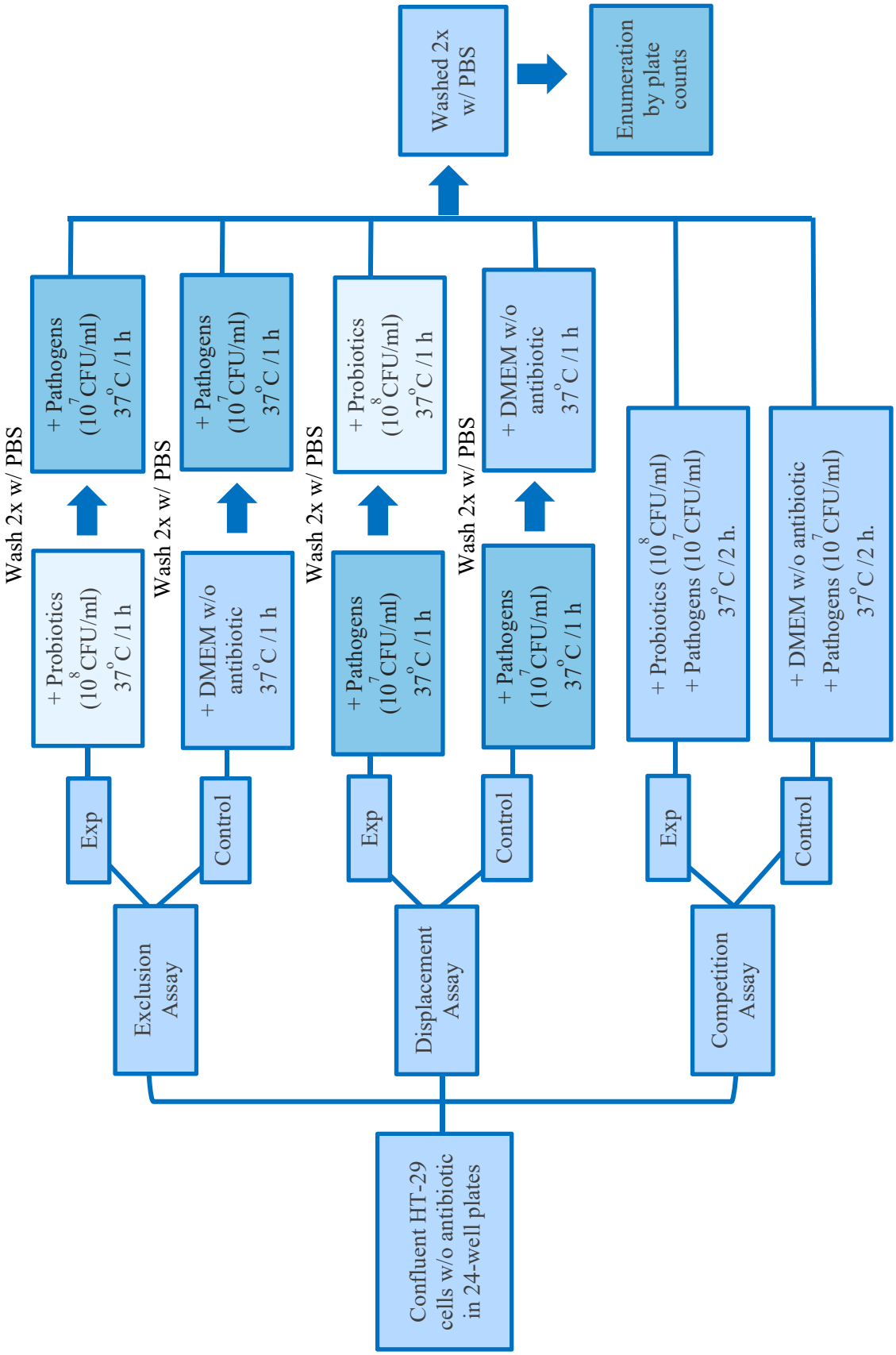


Figure 2 Experimental design of antagonistic activity of probiotic isolates

CHAPTER 3. RESULTS

3.1 Isolation and Identification of Probiotic Bacteria from Probiotic Supplements

Thirteen bacteria were isolated from 11 probiotic supplements based on their colonial morphology on MRS or TSA plates (Figure 3). Twelve isolates including two *Bacillus coagulans,* five *Lactobacillus acidophilus, one Lactobacillus gasseri,* two *Lactobacillus plantarum* and two *Lactobacillus rhamnosus* were identified by gene sequencing and the results matched genes and species claimed on the label. However, one isolate from Meijer Digestive Care® failed to be identified by the gene sequencing method. According to the package, Meijer Digestive Care® contains two *Bifidobacterium* spp. and *L. acidophilus*. The colony morphology of this isolate, which was isolated on MRS agar plates incubated aerobically, was similar to the other three identified *L. acidophilus* isolates. None of the 11 supplements showed any microbial contamination.

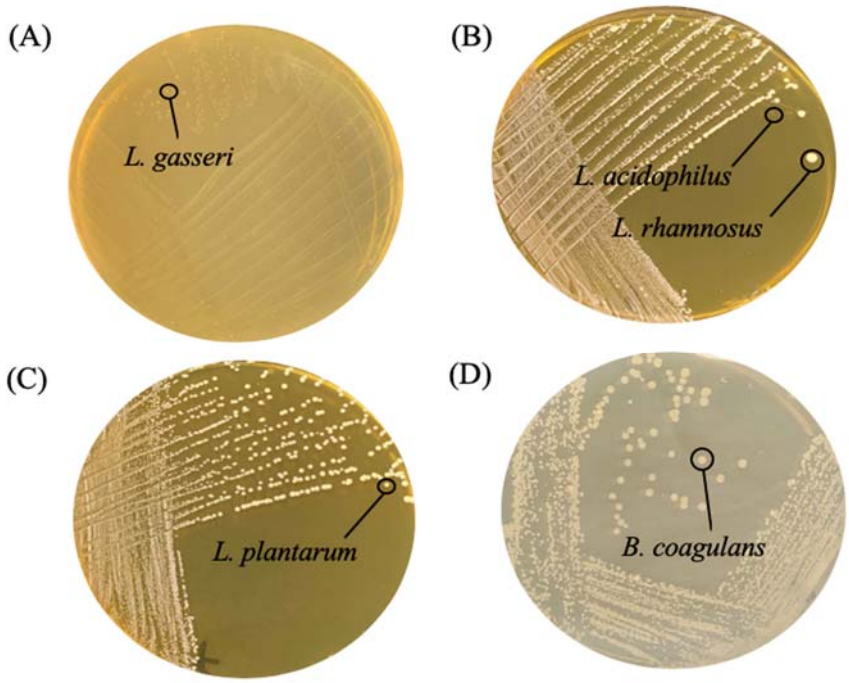


Figure 3 Colonial morphologies of probiotic isolates from probiotic supplement grown on agar plates. (A) *Lactobacillus* gasseri KS-13 on MRS agar plate, (B) *Lactobacillus* acidophilus and *Lactobacillus rhamnosus* on MRS agar plate, (C) *Lactobacillus plantarum* on MRS agar plate, (D) *Bacillus coagulans on* TSA agar plate.

3.2 Initial Viable Probiotic Counts in Supplements

Viable counts of 11 probiotic supplements from two different lots were determined. Two products contained *B. coagulans*, four contained *Lactobacillus spp.* and five contained both *Lactobacillus* spp. and *Bifidobacterium* spp. (Table 2). *Lactobacillus* spp. and *B. coagulans* are facultative anaerobes which grew on plates incubated aerobically and anaerobically; *Bifidobacterium* spp., are obligate anaerobes which only appeared on MRS plates incubated anaerobically (Figure 4a). To compare mean viable counts of these products with the claimed viable counts on their labels, anaerobic viable counts /unit were used (Table 3).

(a)

(b)

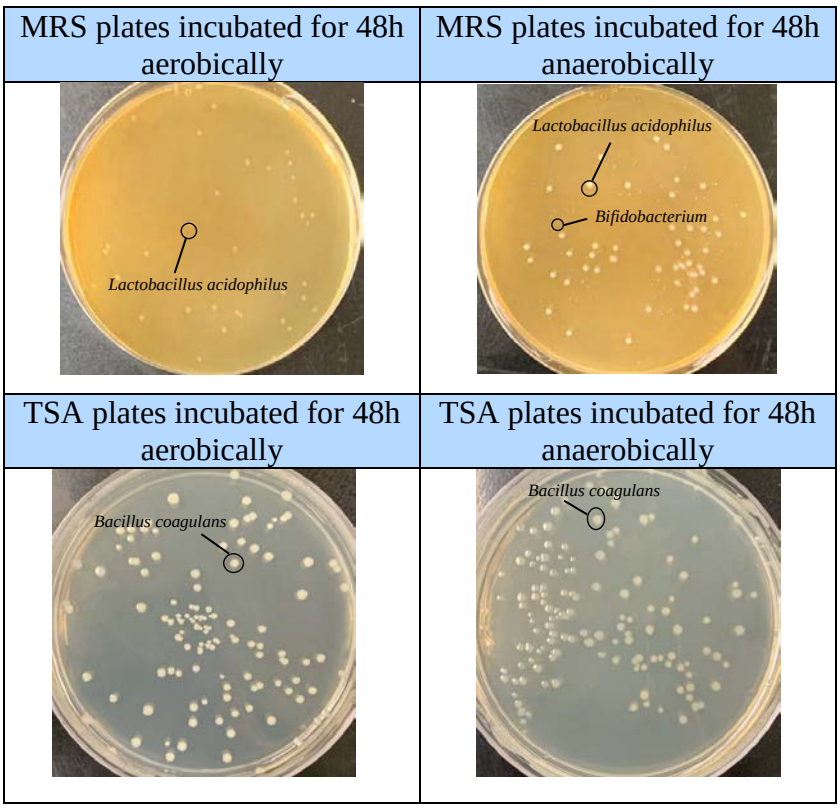


Figure 4 Viable counts of a probiotic product contained (a) both *Lactobacillus acidophilus* and *Bifidobacterium* spp. determined on MRS plates after 48h incubation at 37°C under aerobic and anaerobic condition (b) *Bacillus coagulans* determined on TSA plates after 48h incubation at 37°C under aerobic and anaerobic condition.

Align® Prebiotic + Probiotic and OLLY® Purely Probiotic contained *B. coagulans* and both products are in the form of gummy. Mean aerobic and anaerobic viable counts of *B. coagulans* ranged from 6.5 ± 0.1 to 7.1 ± 0.2 log CFU/g and 6.7 ± 0.1 to 7.4 ± 0.0 log CFU/g respectively.

There was no significant difference between the mean aerobic and anaerobic viable counts since *B. coagulans* can grow in both incubation conditions. Each unit of Align® Prebiotic + Probiotic is 2.8 g; therefore, each unit had mean viable counts 7.0 ± 0.1 and 7.8 ± 0 log CFU/unit for the first and second lots respectively. Each unit of OLLY® Purely Probiotic is 2.3 g, thus the mean viable counts were 7.0 ± 0.1 log CFU/unit for the first lot and 7.1 ± 0 log CFU/unit for the second lot. Neither one met their claims on the label which is 9 log CFU/unit (Table 3).

Four products contained *Lactobacillus* species and their mean aerobic and anaerobic viable counts were about the same. All the four products are in the form of capsule, which is 0.5 g/capsule. Culturelle® and Nature Made® had mean aerobic viable counts ranging from 10.0 ± 0.1 to 10.4 ± 0.1 log CFU/g and mean anaerobic viable counts per unit ranged from 9.7± 0.1 to 10.1± 0.1 log CFU, which closely matched their claimed 10 log CFU/unit on the labels (Table 3). No viable counts were detected in the samples from the first lot (Lot # 934010) of Meijer® Digestive Health and the first lot (Lot # 815022872) of Meijer® Wellness Probiotic, (Table 2 and 3). However, mean aerobic and anaerobic viable counts of the samples from the second lot (Lot # 934014) of Meijer® Digestive Health were 9.6 ± 0.2 and 9.8± 0.2 log CFU/g respectively. Each capsule contained mean anaerobic counts 9.5 ± 0.2 log CFU, which was slightly lower than the claimed 10 log CFU/unit. Mean aerobic and anaerobic viable counts of the samples from the second lot (Lot# 906428291) of Meijer® Wellness Probiotic were 10.5 ± 0.2 and 10.6 ± 0.1 log CFU/g respectively. Each capsule contained mean anaerobic counts 10.3 ± 0.1 log CFU, which exceeded the claimed 9.2 log CFU/unit.

Five products contained both *Lactobacillus* and *Bifidobacterium* species. Aerobic viable counts ranged from 8.0 ± 0.1 to 9.5 ± 0 log CFU/g and anaerobic viable counts ranged from 8.4 ± 0.2 to 10.3 ± 0.3 log CFU/g. As expected, anaerobic counts, which included both *Lactobacillus* spp. and *Bifidobacterium* spp., were significantly higher than the aerobic counts, which only included *Lactobacillus* species. Meijer® Probiotic Pearls and Nature's Way Pearls® are in the pearl form, which are protected probiotic bacteria in triple-layer soft gel. Each pearl is 0.5 g, therefore, each unit of Meijer® Probiotic Pearls contained mean anaerobic counts 8.6 ± 0.2 1 log CFU in the samples from the first lot and 8.7 ± 0.1 log CFU in the samples from the second lot. Each unit of Nature's Way Pearls® contained mean anaerobic counts 9.9 ± 0.2 log CFU/unit for the first lot and 10.0 ± 0.3 log CFU/unit for the second lot. Both supplements met their claimed on the labels, which are 8 and 9.7 log CFU/unit respectively. Meijer® Digestive Care and Philip's Colon Health®

are in the capsule form and each capsule is 0.5 g. Meijer® Digestive Care had mean viable counts 8.8 ± 0.1 and 9.0 ± 0.0 log CFU/unit for the first and second lot respectively. Philip's Colon Health® had mean viable counts 8.2 ± 0.2 log and 8.5 ± 0.2 log CFU/unit for the first and second lot respectively. Neither one met their claimed on the labels, which are 9.7 and 9.2 log CFU/unit respectively. TruBiotics® is also in the capsule form and each capsule is 0.4 g thus the mean viable counts per unit were 9.5 ± 0.2 log CFU and 9.7 ± 0.1 log CFU for the first and second lot respectively. Both exceeded the claimed 9.3 log CFU/unit.

Most of the products showed that the sample with a longer shelf life tended to have higher viable counts. However, the difference was not significant ($P>0.05$). All samples except for the samples from the first lots of Meijer® Digestive Health and Meijer® Wellness Probiotic, met the minimum requirement count of 6.0 log CFU/g recommended by FAO/WHO guideline (2002).

3.3 Antibiotic Susceptibility of Probiotic Isolates

Nine probiotic strains isolated from dietary supplements were examined for their susceptibility to eight antibiotics. As shown in Table 4, all nine probiotic isolates were resistant to cephalothin, amdinocillin, and penicillin, and all except *L. gasseri* KS-13, were also resistant to rifampin. *Lactobacillus gasseri* KS-13 was moderately susceptible to rifampin. None of the nine isolates showed any resistance to erythromycin. Susceptibility to the remaining three antibiotics, clindamycin, linezolid, and tetracycline, varied among the nine probiotic isolates. The two *B. coagulans* isolates were susceptible to the three antibiotics. The three *L. acidophilus* strains were all susceptible to tetracycline. The *L. acidophilus* isolated from Meijer® Digestive Care was moderately susceptible to clindamycin, while the other two were resistant to clindamycin. The *L. acidophilus* La-5 was moderately susceptible to linezolid, whereas the other two were resistant to linezolid. *Lactobacillus gasseri* KS-13 was susceptible to linezolid and tetracycline and resistant to clindamycin. *Lactobacillus plantarum* 299v was susceptible to linezolid and was resistant to clindamycin and tetracycline. *Lactobacillus plantarum* Lp-115 was the most resistant strain among nine isolates and, was resistant to clindamycin, linezolid and tetracycline. *Lactobacillus rhamnosus GG* was susceptible to clindamycin and resistant to tetracycline and linezolid.

Table 3 Aerobic and anaerobic viable counts of probiotic bacteria in probiotic supplements.

Probiotic Product weight (g) /unit	Unit	Claimed Probiotic Bacteria	Lot	Aerobically Bacteria Counts (log CFU/g)	Anaerobically Bacteria Counts (log CFU/g)	Log CFU/unit on label	Months to expiration	Mean Total (Anaerobic) Viable Counts (log CFU/unit)
Products containing *Bacillus coagulans*								
Align® Prebiotic + Probiotic	Gummy	*Bacillus coagulans* unique IS2™	1	6.5 ± 0.1	6.8 ± 0.2	9	7	7.2 ± 0.2
			2	7.1 ± 0.2	7.4 ± 0	9	13	7.8 ± 0.0
OLLY® Purely Probiotic	Gummy	*Bacillus coagulans* MTCC 5856	1	6.6 ± 0.2	6.7 ± 0.1	9	8	7.0 ± 0.1
			2	6.6 ± 0.1	6.8 ± 0	9	15	7.1 ± 0.0
Products containing *Lactobacillus* species								
Culturelle®	Capsule	*Lactobacillus rhamnosus* GG	1	10.2 ± 0.3	10.3 ± 0.2	10	12	10.0 ± 0.2
			2	10.2 ± 0.3	10.4 ± 0.1	10	18	10.1 ± 0.1
Nature Made®	Capsule	*Lactobacillus plantarum* 299V	1	10.2 ± 0.2	10.2 ± 0.1	10	15	9.9 ± 0.1
			2	10.0 ± 0.1	10.0 ± 0.1	10	6	9.7 ± 0.1
Meijer® Wellness Probiotic	Capsule	*Lactobacillus rhamnosus* GG, *Lactobacillus acidophilus*	1	< 1	< 1	9.2	10	< 1.0
			2	10.5 ± 0.2	10.6 ± 0.1	9.2	14	10.3 ± 0.1
Meijer® Digestive Health	Capsule	*Lactobacillus acidophilus* La-14	1	< 1	< 1	10	15	< 1.0
			2	9.6 ± 0.2	9.8 ± 0.2	10	21	9.5 ± 0.2
Products containing *Lactobacillus* and *Bifidobacterium* species								
Meijer® Probiotic Pearl	Pearl	*Lactobacillus acidophilus* La-14 *Bifidobacterium longum*	1	8.4 ± 0	8.9 ± 0.2	8	11	8.6 ± 0.2
			2	8.4 ± 0.2	9.0 ± 0.1	8	10	8.7 ± 0.1
Nature's Way Pearls®	Pearl	*Lactobacillus plantarum* Lp-115, *Lactobacillus acidophilus*, *Bifidobacterium lactis* BI-04, *Bifidobacterium longum* BB536	1	8.8 ± 0.4	10.2 ± 0.2	9.7	12	9.9 ± 0.2
			2	9.2 ± 0.3	10.3 ± 0.3	9.7	16	10.0 ± 0.3
Meijer® Digestive Care	Capsule	*Lactobacillus acidophilus*, *Bifidobacterium bifidum*, *Bifidobacterium lactis*	1	8.2 ± 0.3	9.1 ± 0.1	9.7	6	8.8 ± 0.1
			2	8.8 ± 0.1	9.3 ± 0	9.7	13	9.0 ± 0.0
Philip's Colon Health®	Capsule	*Lactobacillus gasseri* KS-13, *Bifidobacterium bifidum*, *Bifidobacterium longum*	1	8.0 ± 0.1	8.4 ± 0.2	9.2	10	8.2 ± 0.2
			2	8.3 ± 0.2	8.8 ± 0.2	9.2	13	8.5 ± 0.2
TruBiotics®	Capsule	*Lactobacillus acidophilus* La-5, *Bifidobacterium animalis* BB-12	1	9.5 ± 0	9.9 ± 0.2	9.3	7	9.5 ± 0.2
			2	9.4 ± 0.2	10.1 ± 0.1	9.3	15	9.7 ± 0.1

Table 4 Susceptibility of nine probiotic bacteria isolated against eight antibiotics

Probiotic strains	Mean Diameter of Inhibition Zone diameter (mm)							
	Antibiotics							
	AMD [1] (10 μg)	CEF (30 μg)	CLI (2 μg)	ERY (15 μg)	LZD (30 μg)	PEN (10 IU)	RIF (5 μg)	TET (30 μg)
Bacillus coagulans unique IS2™	0 (R)	12 (R)	30 (S)	38 (S)	25 (S)	0 (R)	9 (R)	30 (S)
Bacillus coagulans MTCC 5856	0 (R)	0 (R)	40 (S)	46 (S)	50 (S)	0 (R)	8 (R)	40 (S)
Lactobacillus acidophilus	10 (R)	7 (R)	10 (MS)	26 (S)	20 (R)	0 (R)	0 (R)	18 (S)
Lactobacillus acidophilus La-5	0 (R)	8 (R)	0 (R)	24 (S)	22 (MS)	0 (R)	7 (R)	17 (S)
Lactobacillus acidophilus La-14	0 (R)	0 (R)	7 (R)	22 (S)	20 (R)	0 (R)	0 (R)	18 (S)
Lactobacillus gasseri KS-13	0 (R)	0 (R)	0 (R)	26 (S)	25 (S)	0 (R)	15 (MS)	22(S)
Lactobacillus plantarum Lp-115	0 (R)	0 (R)	7 (R)	14 (MS)	20 (R)	0 (R)	0 (R)	10 (R)
Lactobacillus plantarum 299V	0 (R)	0 (R)	7 (R)	23 (S)	23 (S)	0 (R)	0 (R)	12 (R)
Lactobacillus rhamnosus GG	0 (R)	0 (R)	12(S)	17 (MS)	20 (R)	0 (R)	4 (R)	10 (R)

[1] Susceptibility of bacteria expressed as R (resistant), MS (moderately susceptible), or S (susceptible); AMD: Amdinocillin, CEF: Cephalothin, CLI: Clindamycin, ERY: Erythromycin, LZD: Linezolid, PEN: Penicillin, RIF: Rifampin, TET: Tetracycline

3.4 Antimicrobial Activity of Cell Free Probiotic CFCS against Pathogens

The antimicrobial effects of nine probiotic CFCS were tested against three enteric pathogens, *E. coli* O157:H7, *Salmonella* Typhimurium, and *Listeria monocytogenes* (Table 5 and Figure 5). All of the seven *Lactobacillus* CFCS, which had pH ranging from 3.9-4.6, were able to inhibit *E. coli O157:H7* and *S.* Typhimurium. Particularly, two *L. plantarum* and *L. rhamnosus* GG showed strong inhibition against *E. coli O157:H7.* However, the *Lactobacillus* CFCS adjusted to pH 6.5 did not show any inhibition against the three pathogens. *Bacillus coagulans* CFCS, which had pH ranging from 4.9-5.0, did not inhibit the growth of the three pathogens before or after pH adjustment to 6.5. In addition, none of the nine probiotic CFCS was able to inhibit *L. monocytogenes* before or after pH adjustment to 6.5.

To determine if the antimicrobial effect was due to lactic acid in probiotic CFCS , 1 M lactic acid was diluted with sterile dH_2O to pH 3.9, 4.0, 4.4, 4.6, 4.9 and 5.0 corresponding to pH values of the probiotic CFCS (Table 6 and Figure 6). It was found that the diluted 1 M lactic acid solutions showed much stronger growth inhibitions effects against the three pathogens than did the probiotic CFCS at the same pH. *Escherichia coli* O157:H7 was slightly inhibited by lactic acid adjusted to pH 5.0 (zone of inhibition ben < 10 mm), moderately inhibited by lactic acid adjusted to pH 4.9 (zone of inhibition between 10 - 12 mm) and strongly inhibited by lactic acid adjusted to pH 3.9 - 4.6 (zone of inhibition >13 mm). *Salmonella* Typhimurium was not inhibited by lactic acid adjusted to pH 5.0, slightly inhibited by lactic acid adjusted to pH 4.9, moderately inhibited by lactic acid adjusted to pH 4.6 and strongly inhibited by lactic acid adjusted to pH 3.9-4.4. *Listeria monocytogenes* was moderately inhibited by lactic acid adjusted to pH 5.0 and strongly inhibited by lactic acid adjusted to pH 3.9 - 4.9 (Table 6).

Table 5 Antimicrobial effects of cell-free supernatants of nine probiotic strains on the growth of *E. coli O157:H7*, *L. monocytogenes* and *S.* Typhimurium by well-diffusion agar assay.

Probiotic Strains	pH before adjusted	Inhibition zone without pH adjusted	Inhibition zone after adjusted to pH 6.5	Inhibition zone without pH adjusted	Inhibition zone after adjusted to pH 6.5	Inhibition zone without pH adjusted	Inhibition zone after adjusted to pH 6.5
		E. coli O157:H7		*S.* Typhimurium		*L. monocytogenes*	
Bacillus coagulans unique IS2™	4.9	– [1]	–	–	–	–	–
Bacillus coagulans MTCC 5856	5.0	–	–	–	–	–	–
Lactobacillus acidophilus	4.4	++	–	++	–	–	–
Lactobacillus acidophilus La-5	4.6	++	–	++	–	–	–
Lactobacillus acidophilus La-14	4.6	++	–	++	–	–	–
Lactobacillus gasseri KS-13	3.9	+++	–	++	–	–	–
Lactobacillus plantarum Lp-115	3.9	+++	–	++	–	–	–
Lactobacillus plantarum 299V	3.9	+++	–	++	–	–	–
Lactobacillus rhamnosus GG	4.0	++	–	++	–	–	–
BacDown (+ control)	7.1	+		+		+++	
MRS (- control)	6.5	–		–		–	

[1] – , no inhibition; +, diameter between >6 - 10 mm; ++, diameter between >10 - 12 mm; +++, >12 mm.

Table 6 Antimicrobial effects of lactic acid on the growth of *E. coli O157:H7*, *L. monocytogenes* and *S.* Typhimurium by well-diffusion agar assay.

Lactic acid pH adjusted	Inhibition zone[1]		
	E. coli O157:H7	*S.* Typhimurium	*L. monocytogenes*
3.9	+++ [1.]	+++	+++
4.0	+++	+++	+++
4.4	+++	+++	+++
4.6	+++	++	+++
4.9	++	+	+++
5.0	+	−	++

[1.] –, no inhibition; +, diameter > 6 - 10 mm; ++, diameter 10 - 12 mm; +++, >12 mm

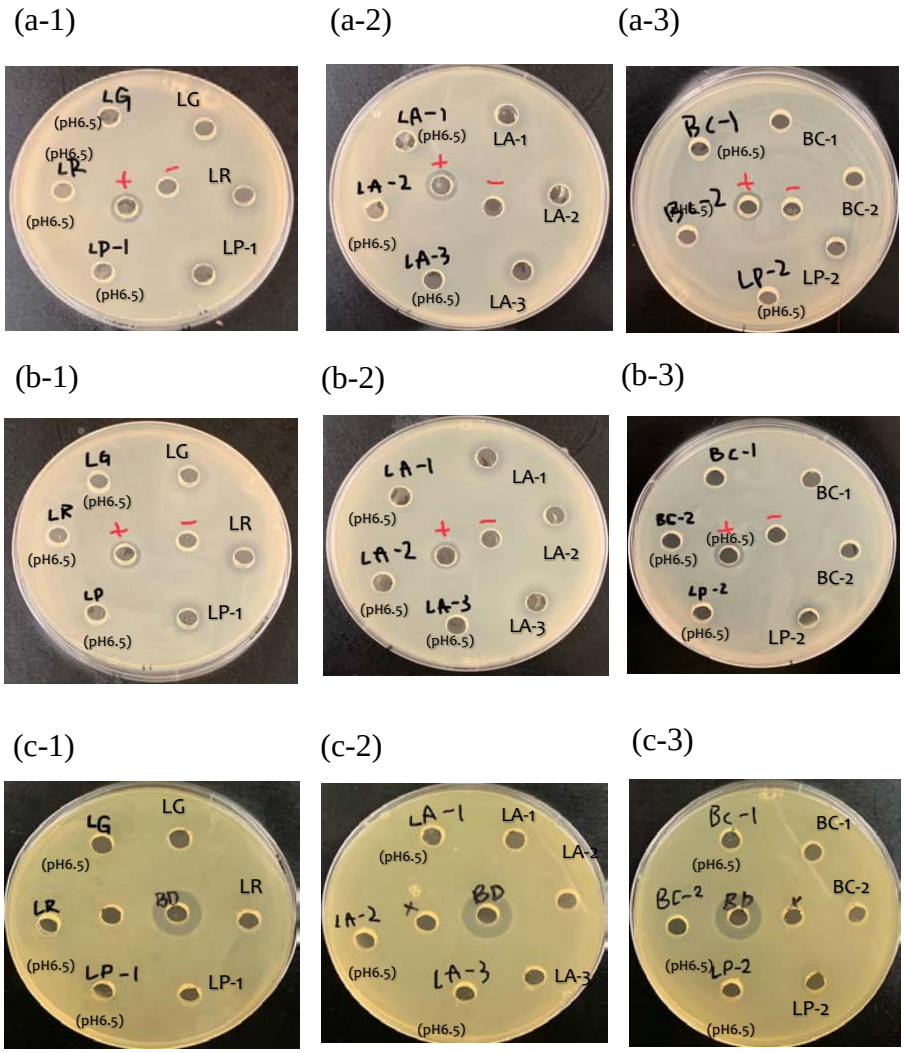


Figure 5 Antimicrobial activity from probiotics supernatant and supernatant adjusted pH to 6.5 against (a) *E. coli* O157:H7, (b) *S.* Typhimurium and (c) *L. monocytogenes* by agar-well diffusion assay.

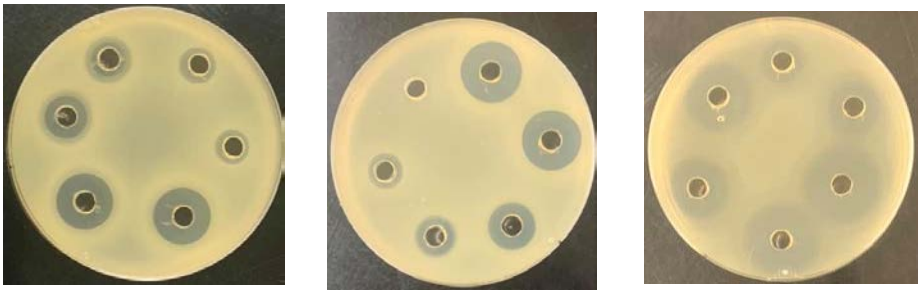


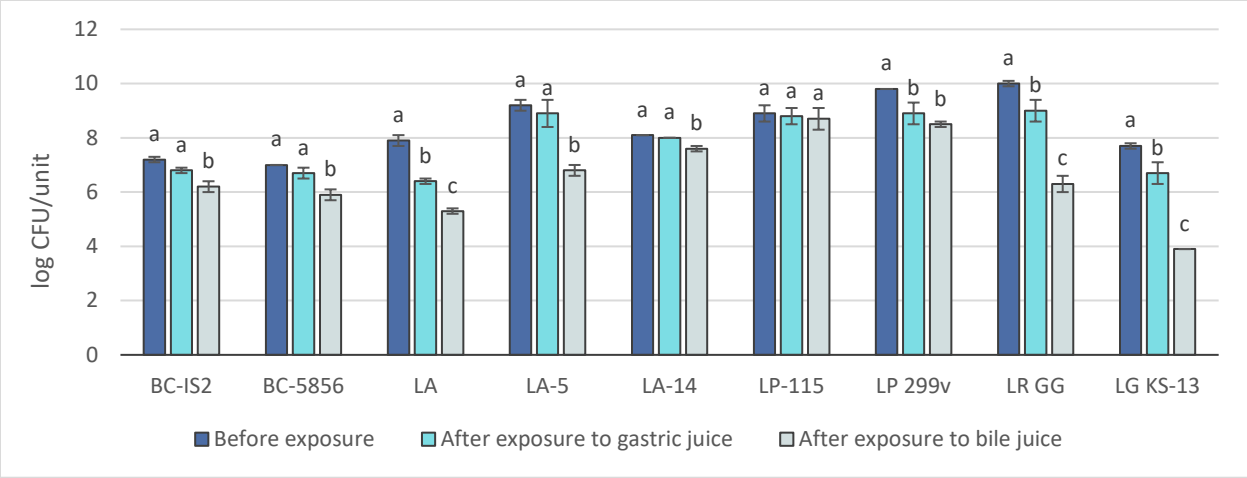
Figure 6 Antimicrobial activity from different pH of lactic acid against *E. coli* O157:H7, *S.* Typhimurium and *L. monocytogenes* by agar-well diffusion assay.

3.5 Tolerance to Simulated Gastrointestinal Tract Conditions

Nine probiotic products, which had viable counts in both tested lots, were selected for this study. One unit (gummy, capsule, or peal) of each product was incubated in SGJ for 90 min and then in SBJ for 180 min at 37°C, Viable counts of each product were evaluated after exposure to SGJ and SBJ and the results are shown in Figure 7.

Two *B. coagulans* strains in the gummy form, showed no significant reduction in viable counts after incubation in SGJ and a significant reduction of 0.4 - 0.8 log CFU/unit after incubation in SBJ. Mean viable counts of the *L. acidophilus* in the capsule of Meijer® Digestive Care reduced 1.5 log CFU/unit and 1.1 log CFU/unit in SGJ and SBJ respectively. *Lactobacillus acidophilus* La-5, in the capsule, was stable in SGJ with no significant decrease but not in SBJ. The mean viable counts decreased 2.1 log CFU/unit after 1 h in SBJ. Mean viable counts of *L. acidophilus* La-14 in the pearl form, remained stable in SGJ and reduced 0.4 log CFU/unit in SBJ. *Lactobacillus plantarum* 299v in the capsule form had a mean viable count reduction of 0.9 log CFU/unit in SGJ but no significant reduction in SBJ. *Lactobacillus plantarum* Lp-115 in the pearl form, was the most resistant product in SGJ and SBJ with no significant reduction in mean viable counts. Mean viable counts of *L. rhamnosus* GG and *L. gasseri* KS-13 showed 1 log CFU/ml reduction in SGJ and 2.7 – 2.8 log CFU/unit reduction in SBJ. Both *L. acidophilus* La-14 and *L. plantarum* 299v which are in the form of pearl, showed the higher survival rates in SGJ than did their counterparts in capsule form.

Five probiotic isolates, *L. acidophilus* La-5, *L. acidophilus* La-14, *L. rhamnosus* GG, *L. plantarum* 299v, and *L. plantarum* Lp-115, with relatively higher viable counts after exposure to SGI were selected for further analysis.



*BC-IS2: *B. coagulans unique* IS-2, BC-5856: *B. coagulans* MTCC 5856, LA: *L. acidophilus*, LA-5: *L. acidophilus* La-5, LA-14: *L. acidophilus* La-14, LP-115: *L. plantarum* Lp-115, LP-299v: *L. plantarum* 299v, LR GG: *L. rhamnosus* GG, LG KS-13: *L. gasseri* KS-13

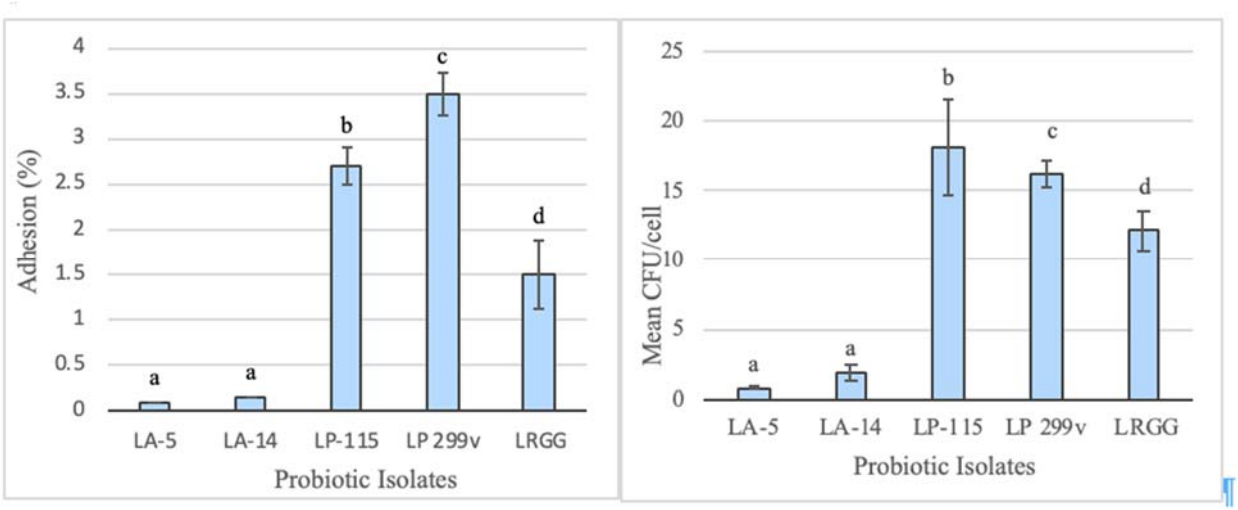
Figure 7 Mean viable of nine probiotic bacteria after incubating in a SGJ conditions for 90 mins and SBJ conditions for 180 mins.

3.6 Adhesion Ability of Probiotic Isolates to Human Colorectal Cells (HT 29)

Five selected probiotic isolates were examined for their ability to adhere to monolayer of HT-29 cells. Considering probiotic inoculum sizes and cells numbers in a well could vary among different wells, the results are expressed as two different ways, adhesion percentages and adhesion ratios (mean viable counts of probiotic bacteria adhered to each HT-29 cell). As shown in Figure 8, the mean adhesion percentages of probiotic isolates ranged from 0.1 ± 0.0 to 3.5 ± 0.2%. *Lactobacillus plantarum* 299v demonstrated the highest adhesion (3.5 ± 0.2 %), followed by *L. plantarum* Lp-115 (2.7 ± 0.2%) and *L. rhamnosus* GG (1.5 ± 0.4 %). The differences among the three were significant (P<0.05). *Lactobacillus acidophilus* La-5 and La-14 showed significantly lower adhesion percentages, which were 0.1 ± 0 % and 0.2 ± 0 % respectively, as compare to the other three. There was no significant different between the adhesion percentages of the two *L. acidophilus.*

Lactobacillus plantarum Lp-115 showed the highest mean adhesion ratio (25.9 ± 3.4 CFU/cell), followed by *L. plantarum* 299v (11.4±1 CFU/cell) and *L. rhamnosus* GG (10.9±1.4 CFU/cell). The differences among the three adhesion ratios were significant (P<0.05). Both *L. acidophilus* La-5 and La-14 had significantly lower mean CFU/HT-29 cell which were 0.8±0.1

and 1.9±0.5 respectively and there was no significant difference between CFU/HT-29 cell ratios of the two *L. acidophilus* strains.



* LA-5: *L. acidophilus* La-5, LA-14: *L. acidophilus* La-14, LP-115: *L. plantarum* Lp-115, LP-299v: *L. plantarum* 299v, LR GG: *L. rhamnosus* GG

Figure 8 Adhesion abilities of five probiotics to HT-29 cell at 37 °C with 5% CO_2 for 1 h (n=6). Values shown are the mean ± SD. Results are expressed as (a) adhesion % (CFU bacteria adhered to HT-29 cells/CFU bacteria added) x 100% and (b) adhesion ratio CFU bacteria adhered/cell number) Values with different letters (a, b, c and d) were significantly different (P<0.05).

3.7 Reduction of Pathogens' Adhesion to HT-29 Cells by Exclusion

To investigate if the probiotic isolates adhered to the HT-29 cells for 1 h could block the adhesion of pathogens to the cells, an exclusion assay was performed. The results are summarized in Table 7. All five isolates were able to reduce the adhesion of *L. monocytogenes* to HT-29 cells by 37.0 ± 1.9 to 67.4_+ 12.8 % and there was no significance among the mean reduction percentages. The two *L. acidophilus* strains were less effective than the other three probiotic bacteria to exclude *E. coli* O157:H7 and *S.* Typhimurium from adhesion to HT-29 cells. *Lactobacillus acidophilus* LA-5 and LA -15 only reduced adhesion of *E. coli O157:H7* and *S.* Typhimurium to HT-29 cells by <37%and <19% respectively. On the other hand, *L. plantarum* Lp-115 299v, and L. rhamnosus GG were able to reduce adhesion of *E. coli O157:H7* and *S.* Typhimurium to HT-29 cell s by >63% and >73% respectively.

Table 7 Exclusion of *E. coli* O157:H7, *S.* Typhimurium and *L. monocytogenes* from adhesion to HT-29 cells by different strains of probiotic bacteria.

	Mean Adhesion Reduction (%) by Exclusion[1]		
Probiotic Strains	*E. coli O157:H7*	*S.* Typhimurium	*L. monocytogenes*
L. acidophilus La-5	$36.5 \pm 21.5^{b\ xy\ 2}$	$18.7 \pm 5.5^{b\ x}$	$67.2 \pm 12.2^{a\ y}$
L. acidophilus La-14	$31.2 \pm 29.5^{b\ x}$	$9.0 \pm 4.3^{b\ x}$	$43.2 \pm 10.4^{a\ x}$
L. plantarum Lp-115	$97.7 \pm 0.4^{a\ x}$	$91.5 \pm 3.6^{a\ x}$	$67.4 \pm 12.8^{a\ y}$
L. plantarum 299v	$96.3 \pm 1.5^{a\ x}$	$86.8 \pm 5.8^{a\ x}$	$37.0 \pm 1.9^{a\ y}$
L. rhamnosus GG	$62.7 \pm 11.8^{ab\ x}$	$73.5 \pm 13.7^{a\ x}$	$53.5 \pm 24^{a\ x}$

1. Mean percentage of adhesion reduction to HT-29 cells = 100% - [(mean viable counts of pathogen in duplicate test wells/mean viable counts of pathogen in duplicate control wells) x100%], data shown is mean ± standard deviation of three trials of the experiment.

2. Different letters (a, b and c) means significant difference ($P<0.05$) within the same column. Different letters (x, y and z) means significant difference ($P<0.05$) within the same row.

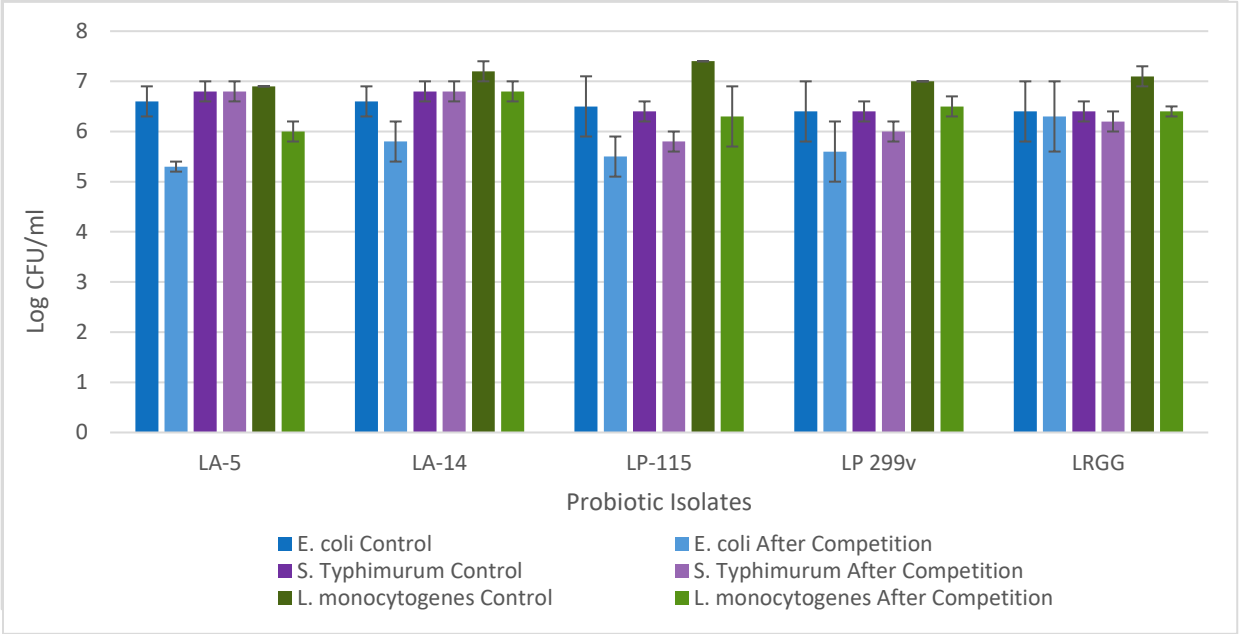


Figure 9 Numbers of pathogens' reduction adhered to HT-29 cells by exclusion.

3.8 Reduction of Pathogens' Adhesion to HT-29 Cells by Competition

To determine if adding probiotic isolates and pathogens (at a ratio of 10:1) to HT-29 cells at the same time could reduce the adherence of pathogens to the HT-29 cells, a competition assay was performed. The five probiotics decreased adhesion of *L. monocytogenes* to HT-29 cells by 54.4 ± 2.5 % to 86.9 ± 4.8% and there was no significant difference among the reduction

percentage. The two *L. acidophilus* (LA-5 and LA-14) were more effective in reducing adhesion of *E. coli* O157:H7 and less effective in reducing adhesion of *S.* Typhimurium to HT-29 cells. The mean reduction percentages of *E. coli* O157:H7 and *S.* Typhimurium on HT-29 cells were >80% and <9 % respectively and the difference was significant. The two *L. plantarum* strains (Lp-115 and 399v) were equally effective against the three pathogens and the mean reduction percentages ranged from 62.0 ± 7.6 to 88.1 ± 6.5%. There were no significant differences among these. Although *L. rhamnosus* GG was effective in decreasing adhesion of *L. monocytogenes*, it was not very effective in reducing adhesion of the other two pathogens. It only reduced adhesion of *E. coli* O157:H7 and *S.* Typhimurium to HT-29 cells by 23.5 ± 5.8% 33.0 ± 18% respectively. (Table 8)

Table 8 Competition between pathogens and different strains of probiotics to adhere to HT-29 cells for 2 h.

	Mean Adhesion Reduction (%) by Competition[1]		
Probiotic Strains	*E. coli* O157:H7	*S.* Typhimurium	*L. monocytogenes*
L. acidophilus La-5	$94.1 \pm 2.9^{b\ x\ 2}$	$8.2 \pm 6.8^{a\ y}$	$86.9 \pm 4.8^{a\ x}$
L. acidophilus La-14	$80.6 \pm 8.3^{b\ x}$	$5.2 \pm 4.7^{a\ y}$	$54.4 \pm 2.5^{b\ z}$
L. plantarum Lp-115	$88.1 \pm 6.5^{b\ x}$	$76.3 \pm 3.8^{b\ x}$	$83.6 \pm 11.8^{a\ x}$
L. plantarum 299v	$84.5 \pm 6.0^{b\ x}$	$62.0 \pm 7.6^{b\ x}$	$62.7 \pm 11.5^{ab\ x}$
L. rhamnosus GG	$23.5 \pm 5.8^{a\ x}$	$33.0 \pm 18^{a\ x}$	$79.2 \pm 10.7^{ab\ y}$

1. Mean percentage of adhesion reduction to HT-29 cells = 100% - [(mean viable counts of pathogen in duplicate test wells/mean viable counts of pathogen in duplicate control wells) x100%], data shown is mean ± standard deviation of three trials of the experiment.

2. Different letters (a, b and c) means significant difference ($P<0.05$) within the same column. Different letters (x, y and z) means significant difference ($P<0.05$) within the same row.

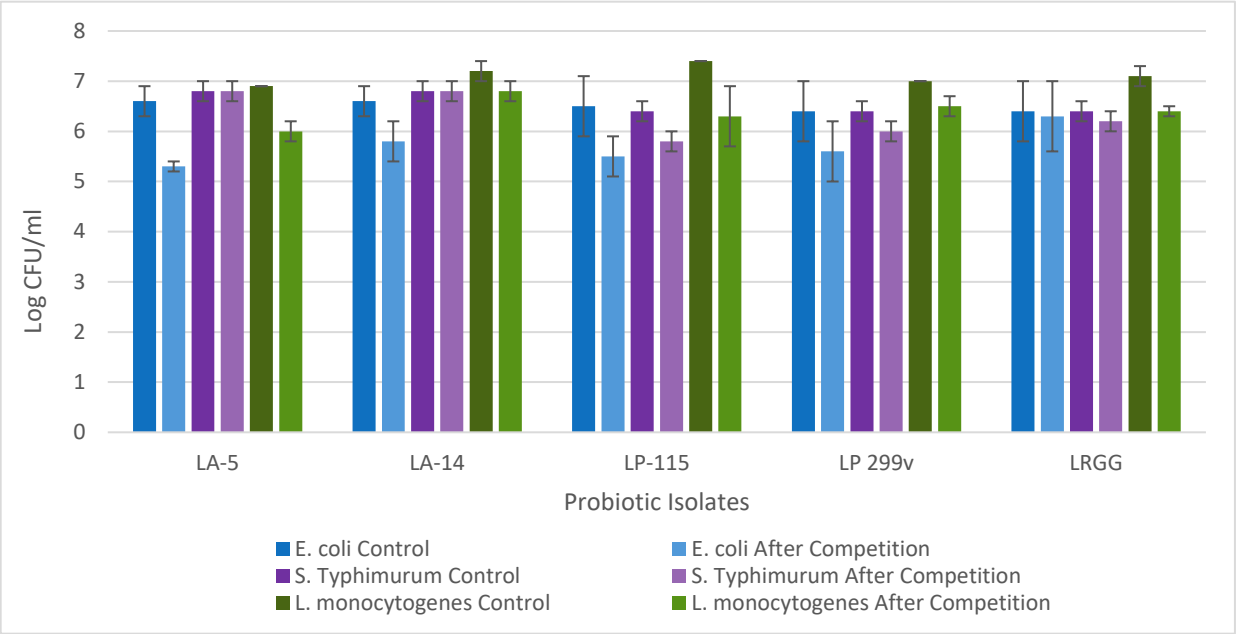


Figure 10 Numbers of pathogens' reduction adhered to HT-29 cells by competition.

3.9 Reduction of Pathogens' Adhesion to HT-29 Cells by Displacement

To determine the ability of each of the five probiotic isolates to displace pathogens adhered to the HT-29 cells, a displacement assay was performed. The results of pathogens displacement by probiotic isolates are shown in Table 9. The two *L. acidophilus* (LA-5 and LA-14) isolates were able to significantly reduce the adhesion of *E. coli* O157:H7 and *L. monocytogenes* to HT-29 cells by 93.8 ± 5.2 - 95.9 ± 2.9 % and 83.3 ± 4.6 - 94.0 ± 2.3% respectively, while they only reduced 27.5 ± 5.2 - 30.1 ± 4.8% adhesion of *S.* Typhimurium. Similarly, two *L. plantarum* were able to significantly reduce the adhesion of *E. coli* O157:H7 and *L. monocytogenes* with range 71.7 ± 12.9 - 82.2 ± 13.3% and 89.1 - 96.1± 2.9% respectively, while they only reduced 38.8 ± 7.3 - 56.8 ± 7.8% adhesion of *S.* Typhimurium. *Lactobacillus rhamnosus* GG was able to significantly reduce 89.2 ± 5.1 % adhesion of *L. monocytogenes*, whereas it only reduced 7.0 ± 2.1% adhesion of *E. coli* O157:H7 and 3.3 ± 7.0% adhesion of *S.* Typhimurium to HT-29 cells.

Table 9 Displacement of pathogens adhering to HT-29 cells by adding different strains of probiotics for 1 h after incubating with pathogens for 1 hr.

	Mean Adhesion Reduction (%) by Displacement[1]		
Probiotic Strains	*E. coli* O157:H7	*S.* Typhimurium	*L. monocytogenes*
L. acidophilus La-5	$93.8 \pm 5.2^{b\ x}$ [2]	$27.5 \pm 5.2^{b\ y}$	$94.0 \pm 2.3^{a\ x}$
L. acidophilus La-14	$95.9 \pm 2.9^{b\ x}$	$30.1 \pm 4.8^{b\ y}$	$83.3 \pm 4.6^{b\ z}$
L. plantarum Lp-115	$82.2 \pm 13.3^{b\ xy}$	$56.8 \pm 7.8^{c\ x}$	$96.1 \pm 2.9^{a\ y}$
L. plantarum 299v	$71.7 \pm 12.9^{b\ x}$	$38.8 \pm 7.3^{bc\ y}$	$89.1 \pm 0.3^{ab\ x}$
L. rhamnosus GG	$7.0 \pm 2.1^{a\ x}$	$3.3 \pm 7.0^{a\ x}$	$89.2 \pm 5.1^{ab\ y}$

1. Mean percentage of adhesion reduction to HT-29 cells = 100% - [(mean viable counts of pathogen in duplicate test wells/mean viable counts of pathogen in duplicate control wells) x100%], data shown is mean ± standard deviation of three trials of the experiment.

2. Different letters (a, b and c) means significant difference ($P<0.05$) within the same column. Different letters (x, y and z) means significant difference ($P<0.05$) within the same row.

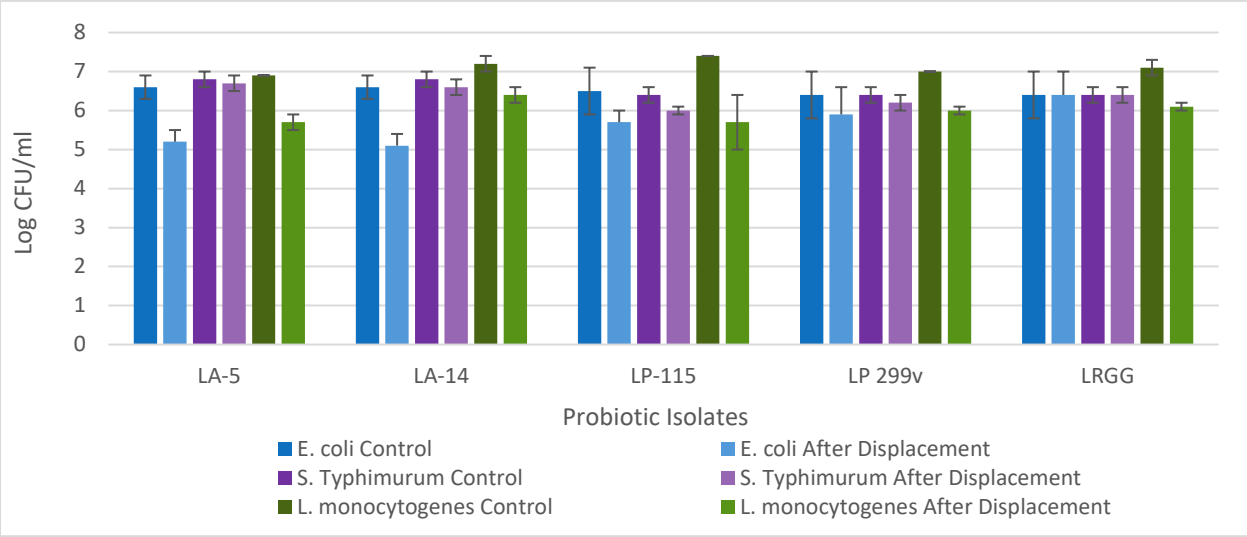


Figure 11 Numbers of pathogens' reduction adhered to HT-29 cells by displacement.

3.10 Summary Performance of Five Probiotic Isolates

Five probiotic isolates, *L. acidophilus* La-5, *L. acidophilus* La -14, *L. plantarum* Lp-115, *L. plantarum* 299v, and *L. rhamnosus* GG were isolated from TruBiotics®, Meijer® Probiotic Pearl Nature's Way® Pearl, and Nature Made® and Culturelle® respectively. were chosen for further analysis because they had relatively high viable counts after exposure to SGI condition. The following is a summary of the performances of the five probiotic bacteria (Table 10):

A. Survival in SGI Condition:

After exposure to SGI condition, mean viable counts of *L. plantarum* Lp115 in Nature's Way® Pearl and *L. acidophilus* La-14 in Meijer® Probiotic Pearl, reduced 0.2 and 0.5 log CFU/unit respectively; whereas *L. plantarum* 299v, *L. acidophilus* La-5, and *L. rhamnosus* GG, which were in capsule form, reduced 1.3, 2.4, and 3.7 log CFU/unit respectively. This result suggests that Pearl effectively protected the two probiotic bacteria in SGI. In capsule form, *L. plantarum* 299v was the most resistant while *L. rhamnosus* GG appeared to be least resistant to the harsh environment in the SGI condition.

B. Antibiotic Resistance

All five probiotic isolates showed multiple resistance against eight tested antibiotics. Among these, *L. plantarum* Lp-115 showed resistance to seven out of eight tested antibiotics. *Lactobacillus plantarum* 299V and *L rhamnosus* GG were resistance to six; *L. acidophilus* La-5 was resistant to five; *and L. acidophilus* La-14 was resistant to four antibiotics.

C. Antimicrobial Effect of probiotic CFCS

Probiotic culture filtrates of the five probiotic bacteria inhibited growth of both *E. coli* O157:H7 and *S.* Typhimurium but not *L. monocytogenes*. The two *L. plantarum* showed a strong inhibitory effect against *E. coli* O157:H7. However, after adjusting pH to 6.5, none of the CFCS had any antimicrobial activity against the three pathogens.

D. Adhesion to Intestinal Epithelial Cells

Two *L. plan*tarum, Lp-115 and 299v, had the two highest adhesion percentages (2.7 and 3.5% respectively) as well as the two highest adhesion ratios (25.9 and 16.2 CFU/HT-29 cell respectively). While the two *L. acidophilus*, La-5 and La14, exhibited the lowest adhesion percentages (0.1 – 0.2% respectively) and adhesion ratios (0.8 and 1.9 CFU/HT-29 cell respectively).

E. Reduction of Adhesion of Pathogens to HT-29 Cells by Exclusion, Competition, and Displacement

All five probiotics were able to reduce adhesion of *L. monocytogenes* on HT-29 cells and the most effective mechanism was through displacement which resulted in 83.3 ± 4.6 to 96.1 ± 2.9% viable count reduction of *L. monocytogenes* on HT-29 cells. However, the efficacy and the mechanism used to reduce the adhesion of *E. coli* O157:H7 and *S.* Typhimurium on HT-29 cells varied among the five probiotic bacteria. The two *L. plantarum* strains, Lp-115 and 299v were able to excluded >96% *E. coli* O157:H7 and >86% *S.* Typhimurium on HT-29 cells. *Lactobacillus rhamnosus* GG, however, only excluded 62.7 ± 11.8% of *E. coli* O157:H7 and 73.5 ± 13.7% of S. Typhimurium on HT-29 cells. The two *L. acidophilus* strains. La-5 and La-14, were able to displace >94% *E. coli* O157:H7 on HT-29 cells but were ineffective in reducing adhesion of *S.* Typhimurium on HT-29 cells. The reduction percentages were <31%.

Overall, the results of this study showed that the best probiotic performer of the five was *L. plantarum* Lp-115 from Nature's Way Pearl which had the highest survival rates after exposure to the SGI environment, the highest adhesion ratio on HT-29 cells, the highest reduction percentage of the three pathogens on HT-29 cells although *L. plantarum* 299v from Nature Made® was a close second.

Table 10 Summary performances of *L. acidophilus* La-5, *L. acidophilus* La-14, *L. plantarum* Lp-115, *L. plantarum* 299v and *L. rhamnosus* GG from survival study, antibiotic susceptibility, antimicrobial activity and antagonistic activity.

		L. acidophilus La-5	*L. acidophilus* La-14	*L. plantarum* Lp-115	*L. plantarum* 299v	*L. rhamnosus* GG
Initial viable counts CFU/unit		9.2 ± 0.2	8.1 ± 0.0	8.9 ± 0.3	9.8 ± 0.0	10 ± 0.1
Log reduction after exposure in SGI condition		2.4 (capsule)	0.5 (pearl)	0.2 (pearl)	1.3 (capsule)	3.7 (capsule)
Log CFU/unit after exposure in SGI condition		6.8 ± 0.2	7.8 ± 0.1	8.7 ± 0.4	8.5 ± 0.1	6.3 ± 0.3
# Resistant antibiotic out of 8 antibiotics		5/8	6/8	7/8	6/8	6/8
Adhesion ability	Adhesion (%)	0.1 ± 0.0 ^{d}	0.2 ± 0.0 ^{d}	2.7 ± 0.2 ^{b}	3.5 ± 0.2 ^{a}	1.5 ± 0.4 ^{c}
	CFU/HT-29 cell	0.8 ± 0.1 ^{d}	1.9 ± 0.5 ^{d}	25.9 ± 3.4 ^{a}	16.2 ± 1.0 ^{b}	10.9±1.4 ^{c}
E. coli O157:H7	Inhibition by CFCS	++	++	+++	+++	++
	Inhibition by exclusion	36.5 ± 21.5 ^{b}	31.2 ± 29.5 ^{b}	97.7 ± 0.4 ^{a}	96.3±1.5 ^{a}	62.7 ± 11.8 ^{ab}
	Inhibition by competition	94.1±2.9 ^{a}	80.6 ± 8.3 ^{a}	88.1 ± 6.5 ^{a}	84.5 ± 6.0 ^{a}	23.5 ± 5.8 ^{b}
	Inhibition by displacement	93.8±5.2 ^{a}	95.9±2.9 ^{a}	82.2±13.3 ^{a}	71.7±12.9 ^{a}	7.0 ± 2.1 ^{b}
S. Typhimurium	Inhibition by CFCS	++	++	++	++	++
	Inhibition by exclusion	18.7 ± 5.5 ^{b}	9.0 ± 4.3 ^{b}	91.5 ± 3.6 ^{a}	86.8 ± 5.8 ^{a}	73.5 ± 13.7 ^{a}
	Inhibition by competition	8.2 ± 6.8 ^{b}	5.2 ± 4.7 ^{b}	76.3 ± 3.8 ^{a}	62.0±7.6 ^{a}	33.0 ± 18 ^{b}
	Inhibition by displacement	27.5 ± 5.2 ^{b}	30.1 ± 4.8 ^{b}	56.8 ± 7.8 ^{a}	38.8 ± 7.3 ^{ab}	3.3 ± 7.0 ^{c}
L. monocytogenes	Inhibition by CFCS	–	–	–	–	–
	Inhibition by exclusion	67.2 ± 12.2 ^{a}	43.2 ± 10.4 ^{a}	67.4 ± 12.8 ^{a}	37.0 ± 1.9 ^{a}	53.5 ± 24 ^{a}
	Inhibition by competition	86.9 ± 4.8 ^{a}	54.4 ± 2.5 ^{b}	83.6 ± 11.8 ^{a}	62.7 ± 11.5 ^{ab}	79.2 ± 10.7 ^{ab}
	Inhibition by displacement	94.0 ± 2.3 ^{a}	83.3 ± 4.6 ^{b}	96.1 ± 2.9 ^{a}	89.1 ± 0.3 ^{ab}	89.2 ± 5.1 ^{ab}

CHAPTER 4. DISCUSSION

4.1 Isolation and Identification of Probiotic Bacteria from Probiotic Supplements

Based on gene sequencing analysis, 13 bacteria from 11 probiotic supplements were identified and the results showed that 12 out 13 (92%) isolates were identified to match genes and species claimed on the label. Although some supplements listed specific strains on label, the probiotics at strain-level were not determined in this study. In a recent study, Ansari et al. (2019) examined 21 commercial probiotic supplements and beverages and found that 82% of species identifications matching the product label by 16S rRNA sequencing. Their study also highlighted the difference among different probiotic strains and suggested that the strains of probiotic bacteria should be clearly labeled on the packages to convey strain diversity. A previous study reported that more than 42% of 26 tested probiotic supplements were contaminated with unacceptable microbes and some strains were misidentified on the labels (Marinova et al., 2019). Similarly, another study summarized that probiotic products were occasionally contaminated with unknown microbes and some were even potential pathogenic species (Kolacek et al., 2017). In this study, no contaminated bacteria were detected in any of the probiotic supplement samples.

4.2 Viable Counts of Probiotic Supplements

Probiotic products are growing popular rapidly. Consumers who bought probiotic supplements are actively searching for beneficial effects from probiotic bacteria and expect the products containing live bacteria claimed on the label by the expiration date. However, many factors such as different species and strains (Yeung, 2016), manufacturing process (Grześkowiak et sl., 2011), transportation (Sahadeva et al., 2011), storage time and conditions (Eratte et al., 2016) as well as preservation methods (Kharchenko et al., 2017) may affect viable counts of probiotics. Numerous studies have reported poor quality control of probiotic products. One study showed that only 27.0 % of 15 probiotic products had viable counts that met or exceeded the claims on their labels (Weese and Martin, 2011). Results of this study also indicated that 12 (54.5%) of 22 tested samples met or closely met viable counts as claimed on their labels, and two (9.1%) (from two different supplements) of 22 tested samples did not contain any live bacteria, indicating that quality control of probiotic supplements might be lacking. These results agreed with Marinova et al., (2019)

who investigated microbiological quality of 16 commercially available probiotic supplements and 10 directly obtained from a local manufacturer. The authors found that none of the 16 commercially available supplements fully met the viable counts on their labels and 11.5% of the samples did not contain any of the live bacteria. However, the 10 probiotic supplements from the local manufacturer matched the viable counts on labels ranging from 10^8-10^{10} CFU/g. Their results suggest that besides quality control, transportation and storage conditions might also be reasons affecting the quality of probiotic supplements. The quality issue also occurred in the study by Goldstein et al. (2014) who investigated five commercial probiotic supplements with three different lots obtained in the US. The authors found that four of the five (80.0%) of probiotic supplements had viable counts that met the claims on label and probiotic counts of one brand from two different lots were lower than the counts stated on the label, suggesting the inconsistency in microbial quality of probiotic supplements. They also found some lot to lot variations among products and the viable counts did not correlate with the time to expiration. The results of the study showed that two of the 11 probiotic supplements had no viable counts in one of the two lots tested and there was no significant difference between the two lots of the remaining nine products. However, the samples with longer shelf life tended to have higher viable counts.

4.3 Antibiotic Susceptibility of Probiotics

Bacteria possessing antibiotic resistance genes have the potential to pass these genes to gut bacteria via horizontal gene transfer (Sornplang et al., 2011). Due to this concern, antibiotic resistance of bacteria is one of the criteria to select safe probiotic strains. However, the resistance to broad spectrum antibiotics can also be a desirable characteristic to restore the gut microbes during antibiotic therapy.

In the present study, Kirby Bauer method (Bauer et al., 1996) was carried out for testing antibiotic resistance of *Bacillus* and the diameter ranges for “susceptible” or “resistant” were determined according to the National Committee for Clinical Laboratory Standards (NCCLS, 2009). However, *Lactobacillus* grew poorly on Müller Hinton agar plates thus MRS agar plates were used for testing antibiotic resistance of *Lactobacillus* spp. and the results were interpreted based on the range for susceptibility described by Charteris et al. (1998)

Among the eight tested antibiotics, amdinocillin, cephalothin, and penicillin belong to beta-lactam antibiotics which inhibit bacterial cell wall synthesis. Penicillin was used for a wide range

of infections, especially against gram positive bacteria. All nine probiotic isolates were resistant to 10 IU penicillin, although susceptibility toward penicillin has been found in *Lactobacillus* spp. such as *L. acidophilus* and *L. gasseri* (Liasi et al., 2009; Temmerman et al., 2003; Klare et al., 2007). Aquilanti et al., (2007) also found that *L. plantarum* was resistant to penicillin, the β-lactam antibiotics, and the resistance might be attributed to β-lactamases coding genes in the bacteria. Similarly, Sharma et al. (2016) showed that commercially available *Lactobacillus* isolates including two strains of *L. rhamnosus* and one strain of *L. plantarum* were resistant to penicillin by disc diffusion soft agar overlay method.

Cephalothin is a broad-spectrum antibiotic against both Gram-positive and Gram-negative bacteria in surgery, blood or skin infection. This study showed that all isolates were resistant to 30 μg cephalothin (Table 4). Several previous studies also found high resistance of *Lactobacillus* spp. against cephalothin (Ammor et al., 2007; Danielsen and Wind, 2003; Temmerman et al., 2003). For example, Danielsen and Wind (2003) reported that probiotic bacteria including *L. rhamnosus, L. plantarum, L. gasseri* and *L. acidophilus* showed a high resistance to cephalothin by E-test method.

Amdinocillin, a semisynthetic antibiotic, used to treat urinary infection. Our results showed that all nine probiotic isolates were resistant to 30 μg amdinocillin. Similarly, Dixit et al., (2013) demonstrated that two strains of *L. acidophilus,* NCIM 2903 and NCIM 2285, were resistant to 33 μg amdinocillin by disc diffusion assay. Liasi et al., (2009), also observed *L. plantarum* La-22 isolated from a fermented fish product was resistant to 25 μg amdinocillin.

Rifampin is an antimycobacterial agent which inhibits bacterial RNA synthesis. The results showed that eight out of nine probiotic isolates tested in this study were resistant to 5 μg rifampin. Many probiotic bacteria have resistance to 5 and 30 μg rifampin (Chang et al., 2009; Charteris et al., 1998; Modzelewska-Kapituła et al., 2008; Ocaña et al., 2006). However, Charteris et al. (1998) stated that 46 *Lactobacillus* strains were sensitive to 5 μg rifampin. Zhou et al., 2005 also found that 10 strains of LAB including *L. rhamnosus, L. plantarum* and *L. acidophilus* were sensitive to 5 μg rifampin.

Clindamycin, linezolid, tetracycline, and erythromycin are broad spectrum antibiotics which inhibit bacteria by interfering protein synthesis. It was found that the resistance to clindamycin, linezolid, and tetracycline varied among the nine probiotic bacteria tested. Two of the three *L. acidophilus* strains (La-5 and La-14), *L. gasseri* KS-13, and both *L. plantarum* strains

were found to be resistant to 2 μg clindamycin. Martín et al. (2005) showed that *L. gasseri* isolated from breast milk was resistant to 2 μg clindamycin. Klare et al., (2007), investigated the antibiotic susceptibility of 383 *Lactobacillus* isolates and found that only three isolates including one strain of *L. rhamnosus* were resistant to clindamycin (0.032 – 32 mg/L).

Lactobacillus acidophilus, L. acidophilus La-14, *L. rhamnosus GG* and *L. plantarum* Lp-115 were found to be resistant to 30 μg linezolid. In contrast, several studies have showed that most lactobacilli were sensitive to linezolid (Meini et al., 2015; Sharma et al., 2016, Guo et al.,2017). Specifically, Sharma et al., (2016) observed that *L. acidophilus, L. rhamnosus GG* and *L. plantarum* were sensitive to 30 μg linezolid. Meini et al., 2015 found that the minimum inhibitory concentration (MIC) of clinical isolate *L. rhamnosus and L. rhamnosus GG* were 0.5 and 1 μg/ml, which suggested that they are susceptible to linezolid.

In this study, both *L. plantarum* strains and *L. rhamnosus* GG were found to be resistant to 30 μg tetracycline, whereas the other six probiotic isolates were sensitive to this antibiotic. Guidone et al (2014), also found tetracycline resistance in *L. plantarum* strains isolated from dairy products. Ocaña et al., (2006) reported that resistance to tetracycline depended on particular strains. Several *Lactobacillus* species had been detected with tetracycline resistance genes (Zoumpopoulou et al., 2017).

Lactobacillus plantarum Lp-115 and *L. rhamnosus* GG were moderately susceptible to 15 μg erythromycin, whereas the rest of the probiotic isolates were all susceptible. Most studies found LAB to be sensitive or moderately sensitive to 15 μg erythromycin (Sharma et al., 2016; Ocana et al., 2006; Liasi et al., 2009). Sornplang et al., (2011) found 10 tested LAB from fermented fish products were moderately susceptible to 15 μg erythromycin. However, Rajoka et al., (2018) observed that 13 LAB including *L. gasseri* isolated from poultry intestine were all resistant to 30 μg erythromycin.

Two B. coagulans isolates were resistant to 10 μg amdinocillin, 30 μg cephalothin, 10 IU penicillin, and 5 μg rifampin, and susceptible to 15 μg erythromycin, 30 μg tetracycline, 30 μg linezolid and 2 μg clindamycin. Cano Roca (2014), observed that *B. coagulans* was susceptible to all tested antibiotics including clindamycin and erythromycin. Gu et al., (2015) indicated that *B. coagulans* CGMCC 9951 did not carry any resistance genes to 15 common clinical antibiotics including erythromycin and tetracycline and claimed the strain is at a high level of safety.

Among the nine probiotic isolates, *L. plantarum* Lp-115 showed resistance to seven out of eight antibiotics tested. *Lactobacillus plantarum* 299V and *L rhamnosus* GG were resistance to six; two *L. acidophilus* strains were resistant to five; one *L. acidophilus* strain (La-14) and the two *B. coagulans* strains were resistant to four antibiotics. Using antibiotic resistant bacteria in commercial probiotic products could be a safety concern, although it could also be an advantage when using them for balancing gut microbes in patients who are taking antibiotics.

Antibiotic susceptibility of probiotic strains is a concern for the probiotic application, However, due to the lacking of standard methods for testing probiotic bacteria, it is difficult to determine the antibiotic susceptibility of probiotic strains (Choi et al., 2018).

4.4 Antimicrobial Activity

Secretion of antimicrobial compounds such as organic acids, short chain fatty acids and bacteriocin is an important attribute for probiotics to outcompete with pathogens in the intestine (Hawaz, 2014). Among these, bacteriocins have been studied the most in the food industry due to the potential applications for food preservation (Saranraj et al., 2013). Probiotics can produce acids to decrease the pH leading to an unfavorable environment for the growth of pathogens (Karami et al., 2017). The antimicrobial effect of cell free probiotic culture against the three pathogens are shown in Table 5. The pH values of overnight probiotic cultures ranged from 3.9 to 5.0 due to acid production. In this study, all of the untreated CFCS of *Lactobacillus* (pH 3.9 – 4.6*)* was able to inhibit *E. coli O157:H7* and *S.* Typhimurium. However, when the CFCS was adjusted to pH 6.5, the inhibition was not observed. This result suggested that the acids secreted by these *Lactobacillus* bacteria played an important role in inhibiting the two pathogens. Mirzaei et al., (2018) also observed that the CFCS of seven *Lactobacillus* bacteria including *L. rhamnosus* and *L. plantarum* species isolated from yogurt and milk had inhibitory activity against *Shigella* strains by producing organic acids or hydrogen peroxide but not bacteriocin. Similarly, Zoumpopoulou et al., (2018) reported that the CFCS of 53 *Lactobacillus* isolates including *L gasseri* and *L. plantarum* strains were not able to inhibit 25 indicator pathogens after adjusting the pH to neutral (pH 6.5). Nevertheless, Monteiro et al., (2019) showed that both the pH 4.5 and pH 6.5 CFCS of *L. plantarum* ATCC8014 were able to inhibit *Clostridium* species. Campana et al., (2017) also found that *L. rhamnosus* W71 had a strong inhibitory activity against five intestinal pathogens including *C. jejuni*, *C. sakazakii*, *E. coli* O157:H7, *L. monocytogenes*, and *S. enteritidis* by their

CFCS adjusted to pH 6.5. These results indicated that the antimicrobial activities against pathogens were due to the production of antibacterial molecules which were not acids.

The CFCS of the two strains of *B. coagulans* tested did not show any antimicrobial effects. However, Abada (2008) found that *B. coagulans* isolated from industrial wastewater drainage secreted bacteriocin against *E. coli, Pseudomonas aeruginosa, Klebsiella pneumoniae, B. subtilis, Staphylococcus aureus*, and *Candida albicans.* Abdhul et al., (2015) also reported that the CFCS of *B. coagulans* SDU3 adjusting to pH 7 was able to inhibit *Bacillus cereus* and *Staphylococcus aureus*, indicating the production of bacteriocin.

It has been reported that LAB can secret large amount of lactic acid during the growth. Therefore, we further analyzed the antimicrobial activity of lactic acids adjusted to different pH and the results was showed in Table 6. An increasing inhibition zone was observed as the pH decrease. Similarly, Choi et al., (2018) examined the sensitivity of pathogens to a range of lactic acid and found that the pathogens were significantly inhibited at 64 mM of lactic acid and completely killed at 125 mM of lactic acid. This indicated that lactic acid produced by the LAB strains in their study may be important against pathogens. However, the CFCS of the nine probiotic bacteria, which had pH 3.9 – 5.0, showed weak antimicrobial effect. This result suggested that lactic acid was not the only acids contributed to the acidic pH of the probiotic CFCS.

4.5 Acid and Bile Resistance of Probiotic

To be effective, probiotic bacteria must be able to survive the transit through stomach and intestine and to colonize on epithelium cells in animal gastrointestinal tract. It was found that the two *B. coagulans* strains were resistant to the SGI condition and the viable counts reduced only 0.9 – 1.0 log CFU/ml after exposure. It has been widely reported that *Bacillus* species were able to survive under adverse environments due to their spore forming attribute (Palop et al.,1999; Ripamonti et al., 2009). Gu et al., (2015) found that *B. coagulans* CGMCC 9951 was resistant to low pH (pH 1-pH 3) and high concentrations of bile salts (0.1-0.9% w/v). Similarly, Sudha et al., (2010) revealed that *Bacillus coagulans* IS2 was able to survive at pH 2 and pH 3 for 3 h as well as in 1% and 2% bile for 2 h with only one log reduction.

In this study, *L. gasseri* KS-13 and *L. rhamnosus* GG were the two least resistant isolates in SGI condition. The mean viable counts of these two probiotic isolates reduced 3.9 and 3.8 log CFU/ml respectively after 1 h exposure to SGJ and 1 h exposure to SBJ. *Lactobacillus. rhamnosus*

GG has been reported to survive well in gastrointestinal tract and often used as positive control. Chenoll et al., (2011) observed only one log decrease of *L. rhamnosus* GG after anaerobic incubation in SGJ adjusted to pH 3.0 for 2 h and pancreatin juice adjusted to pH 8.0 for 4 h. Charteris et al., (1998) also showed one log decrease in viable counts after 90- and 180-min treatments with pancreatin. Similarly, it has been reported that *L. gasseri* ACA-DC 85a and *L. gasseri* ACA-DC 222 reduced only 1.5 and 1.7 log CFU/ml respectively in in pH 2.5 for 2 h and 0.12 and 0.05 log CFU/ml respectively in 1% bile salts for 3h (Zoumpopoulou et al., 2018). It appears that survival of the two bacteria might be influenced by different SGI conditions as well as different strains of the bacteria.

The two strains of *L. plantarum* survived well in the SGI condition with a total of 0.3 – 1.3 log CFU/ml reduction in the mean viable counts. Specifically, the viable counts reduced 0.1 – 1.5 log after 1 h incubation in SGJ and reduced 0.1 – 2.8 log after 1 h incubation in SBJ respectively. Similar results were reported by Campana et al., (2017) who found that among seven tested LAB, *L. plantarum* W21 had the lowest viable count reduction (1.5 log CFU/ml). They also observed that the LAB were more resistant in acid than in bile salts conditions. Zoumpopoulou et al. (2018) showed that viable counts of 20 strains of LAB isolated from dairy products decreased < 2 log in phosphate buffered saline (PBS) of pH 2.5 for 2 h and reduced < 0.5 log in PBS of pH 8 containing 1% bile salts for 3h.

Encapsulation of probiotics can positively affect viability of probiotic bacteria in SGI condition *(*Sreeja and Prajapat, 2013). Based on this study, Probiotic Pearl^{TM} with a triple layer encapsulation maintained the highest viability of probiotic bacteria with only 0.2 – 0.5 log reduction as compared to capsule or gummy forms of probiotic supplements. The Probiotic Pearl^{TM} package claims that the unique triple layers of soft gels protect probiotic microbes from stomach acid and release them in the intestine. However, the in vitro model might not adequately duplicate the actual in vivo condition since food matrices might moderate the harsh condition and thus help bacteria survive. In vivo studies should be conducted in the future.

4.6 Adhesion Ability of Probiotic Isolates to HT-29 cells

In addition to the production of antimicrobial substances, there are various mechanisms used by probiotic to inhibit the pathogens. Five probiotic isolates with minimum viable count reduction in SGI were selected for further analysis. Their ability to adhere to cultured intestinal

cells (HT-29) and their interactions with three enteric pathogens on the cultured HT-29 cells were studied.

The adhesion of probiotic bacteria to intestinal epithelial cells is essential to exert their health benefits such as modulating immune response, stimulating host-microbe interaction and inhibiting colonization of pathogens in intestines (Campana et al., 2017; Rajoka et al., 2018; Singh et al., 2017). Most studies expressed adhesion ability as adhesion percentage, which calculated by the ratio of the bacteria attached to the cells to the bacterial inoculum. The results of adhesion percentage varied depending on the experimental design. There are many factors can affect the adhesion results such as size of inoculum, contact time, type and number of epithelial cells used, and washing process after adhesion. Therefore, it is important to keep those factors consistent in the experiments.

It is reported that adhesion ability of probiotic bacteria is strain specific (Campana et al., 2017; Singh et al., 2017). The results of this study showed that the adhesion to HT-29 cells of the five probiotic isolates at a multiplicity of exposure of 100:1 (bacterial count: cell count) ranging from 0.1 – 3.5% and varied among species and strains. Malcata et al., (2016) used the similar process to investigate the adhesion of four LAB isolated from fermented olive brines including *L. rhamnosus* GG and *L. plantarum.* They inoculated 10^8 CFU/well to HT-29 cells at the same multiplicity of exposure of 100: 1 and incubated for 1 h. Their results showed that the attachment to HT-29 cells of the four LAB ranged from 0.7 – 1.8% with no significant differences.

The two *L. plantarum* strains, Lp-115 and 299v, showed the highest adhesion percentages which were 2.7 and 3.5% respectively. Strains of *L. plantarum* have been widely researched and found to be a promising probiotic (Ahmad et al., 2018; Dimitrov et al., 2014). Sharma and Kanwar (2017) also stated that among 11 tested bacteria isolated from fermented food, three strains of *L. plantarum* showed the highest adhesion (9.36 – 12.88%) to HT-29 cells after incubation with 10^9 CFU/well inoculum for 2 h. Similarly, Oguntoyinbo and Narbad, (2015) reported that *L. plantarum* ULAG24 isolated from fermented cereal had 7.5 – 8% adhesion rates to HT-29 cells after incubation with 10^8 CFU/well inoculum for 2 h. It is reported that percentage adhesion observed is dependent on the experimental design, therefore, the percentage should not be literally interpreted (Letourneau et al., 2011). The adhesion ability of bacteria might associate with the adhesive factors on their cell surface, such as adhesion proteins or lectin-like complex (Ahmad

et al., 2018). Zoumpopoulou et al., (2018) found that *L. plantarum* not only possessed high adhesion ability but also had higher adhesion rates to HT-29 cells than Caco-2 cells. This might be due to the presence of mucus layer on HT-29 cells but not on Caco-2 cells (Gagnon et al., 2013).

The results of this study showed that *L. rhamnosus* GG had 1.5% adhesion to HT-29 cells after 1 h, which is lower than the results of other studies. *Lactobacillus rhamnosus* GG have been widely used in commercial products and regarded as a commercial reference strain due to their probiotic properties (Ouwehand et al., 2004). It is reported that *L. rhamnosus* GG, had 13.5% adhesion to HT-29 cells after the incubation with 10^9 CFU/well inoculum for 2 h (Sharma and Kanwar, 2017). Collado et al., (2007), reported that among 12 commercial probiotic strains including *L. plantarum, L. acidophilus* and *L. rhamnosus* strains, *L. rhamnosus* GG possess the highest adhesion ability (19.7%) to human intestinal mucus after the incubation with 10^7 CFU/well for 1 h.

The two *L. acidophilus* strains were found to have the lowest adhesion ability ranging from 0.1 – 0.2% or 800 – 1900 CFU/1000 HT-29 cells. Similarly, Gopal et al., (2001) reported that *L. acidophilus* LC-1 had 1210 CFU/1000 epithelial cells adhesion after 1 h incubation. While Das et al., (2016) stated that *L. acidophilus* NCFM possessed high adhesion ability to epithelial HT-29 MTX cells. Instead of plate count method, they stained the cells with Cell Tracker Orange staining dye and counted numbers of adhered bacteria in 20 random microscopic fields. They observed that among four *Lactobacillus, L. acidophilus* NCFM showed the maximum adherence of 12% to the cells after the incubation with 10^8 CFU/well inoculum for 1 h.

Again, the value of adhesion percentage varied depending on many factors. The inoculum size can range from 10^7-10^9 CFU/well, time period can range from 1 – 2 h and wash times can range from 1 – 3 times in previous studies (Malcata et al., 2016; Letourneau et al., 2011; Ouwehand et al., 2004).

4.7 Reduction of Adhesion of Pathogens to HT-29 Cells by Exclusion, Competition, and Displacement

Since probiotics and pathogens have similar adherence proteins on their surfaces, probiotic can inhibit pathogens by competing with pathogens for the adhesion sites on intestinal cells (Lee and Puong, 2002). In this study, five probiotic isolates were evaluated for their ability to exclude,

displace and compete with three enteric pathogens, *E. coli* O157:H7, *S. Typhimurium* and *L. monocytogenes*, on HT-29 cells. Reduction of the three pathogens on HT-29 cells varied among probiotic species and strains, pathogen species, as well as antagonistic mechanisms (displacement, competition or replacement). Overall the results agreed with those of previous studies (Campana et al., 2017; Singh et al., 2017).

In the exclusion assays, probiotic isolates were investigated if they could exclude colonization of the three pathogens by pre-occupying the binding site on HT-29 cells in vitro. Although some studies have reported that no clear correlation between adhesion ability of probiotic strains and pathogen inhibition (Collado et al., 2006; Bibiloni et al., 1999), the results showed that the exclusion of *E. coli* O157:H7 and *S.* Typhimurium to HT-29 cells seemed to be correlated with adhesion ability of the probiotic bacteria used. Whereas, there was no significant difference in exclusion of *L. monocytogenes* on HT-29 by the five probiotic isolates. *Lactobacillus rhamnosus* GG was found to reduce adhesion of *E. coli* and *L. monocytogenes* by 67.2 ± 12.2% and 53.5 ± 24 % respectively (Table 7), which were similar to the results reported by Campana et al., 2017. They found that *L. rhamnosus* was able to reduce adhesion of *E. coli* O157:H7 and *L. monocytogenes* by 66.1% and 86.2% respectively. The two *L. plantarum* strains which exhibited the highest two adhesion ability (16.2 – 25.9 CFU/HT-29 cell) also exhibited the highest exclusion rates of *E. coli* O157:H7 and *S.* Typhimurium. The two strains were able to excluded 96.3 ± 1.5 to 97.7 ± 0.4% of *E. coli* O157:H7 and 86.8 ± 5.8 to 91.5 ± 3.6% of *S.* Typhimurium to HT-29 cells (Table 7). Similarly, Sribuathong et al., (2014) found that among three LAB, *L. plantarum* PD 110 was the most effective strains to reduce the adhesion of *S.* Typhimurium and *L. monocytogenes* to Caco-2 cells by 85 to 97% and 94 to 99% respectively through exclusion. Malcata et al., (2016) also reported that pre-exposure of HT-29 cells to *L. plantarum* LB95 resulted in a significant reduction of *L. monocytogenes* adhered to HT-29 cells relative to pre-exposure to other LAB such as *L. rhamnosus* GG or *L. casei* Shirota. However, Collado et al., (2007) observed that *L. plantarum* Lp-115 and *L. acidophilus* NCFM increased 5.6 – 46.2% adhesion of *S.* Typhimurium and *E. coli* to epithelial cells and decreased only 7.4 – 15.4 % adhesion of *L. monocytogenes* to epithelial cells after the pre-incubation of LAB. Singh et al., (2017) who observed a similar trend, showed that the *L. reuteri* strains with the highest adhesion ability showed much higher pathogen inhibition, indicating that the pathogen inhibition capacity of *L. reuteri* strains may be related to their adhesion ability to Caco-2 cells.

In the competition assays, probiotic isolates were examined for their ability to reduce the number of the three pathogens adhered to HT-29 by co-incubation of a probiotic and a pathogen with HT-29 cells for 2 h (Table 8). *Lactobacillus rhamnosus* GG reduced adhesion of *E. coli* O157:H7 to HT-29 cells by 23.5 ± 5.8% which was significantly lower than the reduction rates of 80.6 ± 8.3 to 94.1 ± 2.9 yielded by the other four probiotic bacteria. However, Campana et al., (2017) found that *L. rhamnosus* was able to reduce 52.8% adhesion of *E. coli* O157:7 by competition. Collado et al., (2007) observed that the adhesion of *E. coli* to epithelial cells increased 5.0 – 5.5% after co-incubating the *E. coli* with *L. plantarum* Lp-115 or *L. rhamnosus* GG for 1 h. Similarly, Lee et al., (2003) showed that *L. rhamnosus* GG increased 40.0% adhesion of *E. coli* O157:H7 to Caco-2 cells after 1 h co-incubation. In this study, all five probiotic isolates were found to reduce the adhesion of *L. monocytogenes* ranging from 54.4 ± 2.5 to 86.9 ± 4.8 %. A previous study showed that *L. acidophilus* NCFM *L. plantarum* Lp-115 and *L. rhamnosus* GG reduced the adhesion of *L. monocytogenes* on human colonic mucus by 37 to 53% (Collado et al., 2007). It is reported that inhibitory capability by competition was strain-specific and might be related to the affinity of adhesins on the surface of probiotic isolates and pathogens for the binding sites (Lee et al., 2003).

The displacement of pathogens was also found to be strain and pathogen dependent and no correlation was observed between adhesion ability and inhibition capability. The displacement of pathogens by probiotic bacteria indicates that affinity of probiotic isolates for the binding sites is higher than the pathogens (Coman et al., 2015). The results showed that all five probiotic isolates were able to displaced *L. monocytogenes* on HT-29 cells with reduction rates ranging from 83.3 ± 4.6 to 96.1 ± 2.9. All except *L. rhamnosus* GG also effectively displaced 71.7 ± 12.9 to 95.9 ± 2.9 % *E. coli* O157:H7 cells adhered to HT-29 cells (Table 9). The four probiotic bacteria were less effective in displacing *S.* Typhimurium on HT-29 cells. *Lactobacillus plantarum* displaced 38.8 ± 7.3 to 56.8 ± 7.8 % while *L. acidophilus* displaced 27.5 ± 5.2 to 30.1 ± 4.8 % *S.* Typhimurium on HT-29 cells. *Lactobacillus rhamnosus* GG was the least effective in replacing *E. coli* O157:H7 or S. Typhimurium on HT -29 cells. It only reduced adhesion of *E. coli* O157:H7 and S. Typhimurium to HT 29 cells by 7.0 ± 2.1 and 3.3 ± 7.0 % respectively. This agreed with the results of Lee et al., (2003) who reported that *L. rhamnosus* GG displaced 1.2% adhesion of *E. coli* O157:H7 to Caco-2 cells. The results showed that two strains of *L. acidophilus* were able to significantly reduce *E. coli* O157:H7 and *L. monocytogenes*

by 93.8 – 95.9% and 83.3 – 94% respectively. While Collado et al., (2007) found that *L. acidophilus* NCFM increased 27.7% adhesion of *E. coli* and reduced only 51.9% adhesion of *L. monocytogenes.* Lee et al., (2003) showed that the displacement of GI bacteria by *L. rhamnosus* GG and *L. casei* was a very slow process. Since the probiotics were not able to displace an adhered GI bacterium unless the bacterium detaches from the receptor, then the binding probiotics would block the reattachment of bacterium to the receptor.

Results of this study show that the two *L. acidophilus* effectively reduced adhesion of *E. coli* O157:H7 on HT-29 cells by competition and displacement and less effective by exclusion. However, the two *L. acidophilus* was less efficient in reducing *S.* Typhimurium on HT-29 cells by any of the three mechanisms. The two *L. plantarum* strains however could effectively reduce adhesion of both *E. coli* O157:H7 and *S.* Typhimurium by exclusion or competition. Additionally, they significantly displaced more *E. coli* O157:H7 cells than *S.* Typhimurium on HT-29 cells. *Lactobacillus rhamnosus* GG reduced adhesion of *E. coli* O157:H7 and *S.* Typhimurium more effective through exclusion but not through competition or displacement. All five effectively displaced *L. monocytogenes* on HT-29 cells.

The mechanisms of exclusion, competition and displacement might be different and involved many other factors such as co-aggregation ability with pathogens (Campana et al., 2017). Some studies observed the increases in the adhesion of pathogens by probiotic strains. Although the mechanisms and reasons of these increases is unknown, those strains, which helped the pathogens to adhere to cells, should be further investigated (Gueimonde et al., 2006; Collado et al., 2007).

CHAPTER 5. CONCLUSION

The goal of this study was to investigate survival of probiotic bacteria isolated from commercial dietary supplements in SGI environment and their antimicrobial efficacy against three common foodborne pathogens, *Escherichia coli* O157:H7, *Listeria monocytogenes* and *Salmonella* Typhimurium.

According to the results of survival study and antimicrobial activities assay, the conclusions can be drawn as following:

- Not all the probiotic supplements contain live probiotic bacteria. Two probiotic supplements did not have any viable counts in one of the two lots tested, suggesting the quality control is lacking.
- Not all the probiotic supplements met the counts claimed on their labels. Only 12 (54.5%) of 22 tested samples met or closely met the viable counts printed on their labels.
- Ability of probiotic bacteria to survive in SGI condition varied among different probiotic strains and methods of encapsulation. Bacteria in the form of pearl showed minimum reduction in viable counts after exposure to SGI condition. *Bacillus* coagulans, spore-forming bacteria, also survive well after incubation in SGI condition. Among the seven probiotic bacteria in capsules, *L. plantarum* 299v had the lowest viable count reduction after exposure to SGI.
- The nine probiotic bacteria isolates tested in this studied resisted multiple (four to seven) antibiotics out of eight. This indicates a safety concern of using these bacteria in supplement since probiotic bacteria carrying multiple drug resistant genes have potential to pass the genes to other intestinal flora.
- Culture filtrates of the *Lactobacillus* were able to inhibit growth of both *E. coli* O157:H7 and *S.* Typhimurium. However, after adjusting pH to 6.5, the culture filtrate had no antimicrobial activity against the two pathogens, suggesting there was no antimicrobial substances other than acids in the culture filtrates.
- Ability of probiotic bacteria to adhere to HT-29 cells varied among different probiotic bacteria.
- Antimicrobial efficacy of probiotic isolates against the three pathogens varied depending on the probiotic strains, the pathogens, and the method for analysis. Ability of probiotic

isolates to exclude the adhesion of *E. coli* O157:H7 and *S.* Typhimurium on HT-29 cells seemed to be related to their adhesion ability to the cells.

- According to all the results in this study, Nature's way® Pearls containing *Lactobacillus plantarum* Lp-115 was the best probiotic supplements since the form of pearl made it more resistant in SGI condition and *L. plantarum* Lp-115 in this supplement had the highest adhesion ratio and the best antimicrobial efficacy.

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