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Effect of Resistant Starch on Microbial Content of the Intestinal Tract

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EFFECT OF RESISTANT STARCH ON MICROBIAL CONTENT OF THE INTESTINAL TRACT

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DEDICATION

- To my parents Mr. Sunil De Alwis and Mrs. Ranjani Amarasinghe: without your sacrifices any of this would not be possible.
- To the center of my life, my husband Dr. Indrajith C. Senvirathne: thank you for being my best friend.

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ABSTRACT

Resistant starch (RS) increases beneficial gastro-intestinal bacterial populations simultaneously increasing short-chain fatty acids (SCFA) such as butyrate. Butyrate acts as energy source for epithelial cells of colonic mucosa which stimulates intestinal cell proliferation and has been implicated as important in reducing obesity, diabetes and cancer. The *Lactobacillus spp, Bifidobacterium spp, Bacteroides thetaiotaomicron,* Clostridium cluster IV and Clostridium cluster XIV are mainly involved in the production of butyrate by fermenting RS in the large intestine. With age there is a decline in these gut bacteria, but in the present study RS and diet restriction both enhanced the bacteria involved in butyrate production. In addition, RS reduces body fat in some types of obesity but not all. For example, RS reduced body fat in an endocrine model of obesity caused by ovariectomy (OV). In this study RS significantly increase bacterial populations involve in butyrate production. However, in high fat diet induced obesity the results were different. The high fat diet (41% dietary energy) prevented fermentation of RS and reduced bacterial populations in the ceca compared to a low fat diet (18% dietary energy). The RS failed to reduce obesity in these rodents fed a high fat diet while increasing Bacteroides group population. To follow up these studies and determine if type of fat was important in directly altering gut fermentation, an *in-vitro* fermentation model of rat large intestine was used. Both Corn oil and Lard reduced bacterial populations which are involved in fermentation of RS. However, if the fat used was fish oil there were no negative effects on the fermentation of RS or the bacterial population. These studies illustrate the need to control the type of fat when studying the effects of prebiotics or other sources of resistant starch. With the age *Bifidobacterium spp, Bacteroides spp,* Clostridium cluster IV and Clostridium cluster XIV decreased both calorie restricted diet and RS diets were able to improve these bacterial populations.

CHAPTER 1: INTRODUCTION

1.1 General Introduction

Our gastro-intestinal system is sterilised when we are in the mother's womb, then rapid bacterial proliferation begins soon after birth. Although within 24 hours after birth a newborn baby's gut is proliferated with *Coliform bacteria, Enterococcus, Lactobacilli, Staphylococci* and some *Clostridia spp*, during the next 3-4 days after birth, *Bifidobacterium spp* become dominant and remain throughout the infancy (Mitsuoka, 1978; Tannock *et al.*, 1990). After the weaning stage, the infant's intestinal micro-flora changes rapidly and becomes similar to that of adults. A normal intestinal tract has around 10^{14} bacteria with more than 1000 different species (Rajilic-Stojanovic *et al.*, 2007). This wonderful ecosystem plays a vital role in safeguarding the health of the gut (Tlaskalova-Hogenova *et al.*, 2004; Matsuki *et al.*, 2004; Tomotari, 1978). As a consequence of the digestive microbial ecosystem being very complex, it is not very well characterized. Dietary changes regulate both quantitative and qualitative changes in microbial communities (Louis *et al.*, 2007; Rudi *et al.*, 2009). This modulation of host gut microbiota may help to regulate host health.

Prebiotics such as resistant starch stimulate the growth of beneficial bacteria in the colon, with the potential to increase fat oxidation and reduce body fat while maintaining bowel health (Ferguson *et al.*, 2000; Higgins, 2004; Higgins *et al.*, 2004; Higgins *et al.*, 2006; Keenan *et al.*, 2006; Shen *et al.*, 2009; Zhou *et al.*, 2009). Overweight and obesity are a major health concerns among developed countries; this has caused rapid growth of the weight reducing industry. Research into the beneficial effects of RS in controlling overweight and obesity has recently become a high priority of NIH (http://www.nih.gov/). To understand the real picture of lowering body fat microbial analyses are needed.

Short chain fatty acids (SCFA) such as acetate, propionate, and butyrate have been found in greater concentration in the feces of those consuming a diet containing RS (Hold *et al.,* 2003). Benefits of and physiology of butyrate metabolism in the large intestine have been intensively studied by several investigators (Mortensen & Clausen, 1996; Hijova & Chmelarova, 2007). It is the major SCFA in providing protection against cancer and ulcerative colitis by reducing cell proliferation, by blocking the absorption of cancer-causing substances and by making the colon less vulnerable to DNA damage. It also helps to boost the absorption of calcium to maintain a healthy epithelium (Hagopien *et al.*, 1977 ,Cummings & Macfarlane, 1991, Gibson *et al.*, 1998).

Fermentation of prebiotics such as RS in the large intestine, and production of butyrate is a complex process. Production of butyrate from RS requires the involvement of several groups of bacteria. The study of the fermentation of RS has demonstrated initial bacterial adherence to starch molecules especially by *Bacteroides thetaiotaomicron, Bifidobacterium longum* and some *Lactobacillus spp* (Bird *et al.*, 2000; Xu *et al.*, 2003; Louis, 2007). The main acidic fermentation products from *Bacteroides thetaiotaomicron* are acetate, propionate and succinate. Lactate and acetate are produced by *Bifidobacterium spp* and *Lactobacillus spp*. Thus, these bacterial species adhere to the surface of starch molecules and ferment RS into intermediate products that are converted by other species to butyrate (Duncan *et al.*, 2002; Duncan *et al.*, 2004a; Duncan *et al.*, 2004b; Louis *et al.*, 2007; Louis, 2007). Those species fall under the Clostridium clusters I, III, IV, XI, XIVa, XV and XVI. The majority of the bacterial species that are butyrate producers are included in two Clostridium clusters - Clostridium cluster IV and Clostridium cluster XIV.

Thus, resistant starch is a prebiotic and its fermentation results ultimately in production of butyrate through actions of several bacteria. Production of butyrate from fermentation of dietary resistant starch is associated with reduced body fat in rats on a low fat diet (Keenan et al., 2006,

Shen et al., 2009). However, it is known that adding higher than usual fat to the diet of the ruminant (Ferguson et al., 1990; Harvatine & Allen, 2006) or adding high fat to an *in vitro* model of the rumen (Ferguson et al., 1990) reduces fermentation. The objectives of these studies were to investigate the effect of different diets, levels and types of fat on gut microflora involved with fermentation of RS. To attain these goal three C57bl/6J mice studies, one rat study and one *in-vitro* study were conducted.

CHAPTER 2: REVIEW OF LITERATURE

2.1 Gastro-Intestinal Tract Microbiota

2.1.1 Development of Gastro-Intestinal Tract Microbiota

The digestive system microbiota is a complex ecosystem system with the combination of bacteria, archaea, yeasts and filamentous fungi (Rajilic-Stojanovic *et al.*, 2007)**.** Before birth the gastro-intestinal system is bacteria free. During the birth in short period of time infant has contact with the mother's vagina, feces, skin and the environment, resulting in a rapid bacterial proliferation in the infant's gastrointestinal system soon after the birth (Edwards & Parrett, 2002). In the caesarean babies gastrointestinal can inoculate during nursing and the handling. However cesarean infants seem to have a reduced number of bacteria compared with those naturally delivered infants (Morelli, 2008). Kurokawa and his co-workers have found 136-unique infant intestinal microbiota, and out of those 78 were characteristic to infants (Kurokawa *et al.*, 2007). Within 24 hours of birth newborn baby's faeces contain variety of bacteria such as *Coliform bacteria, Enterococcus, Lactobacilli, Staphylococci* and some *Clostridia spp*. During the next 3-4 days after birth *Bifidobacterium spp* start to colonize and become dominant. Breast-fed infant digestive tracts are dominated with both *Bifidobacterium spp* and *Lactobacilli spp,* whereas those who formula-fed infant digestive tracts prominent with more *Bacteroides spp, Clostridia spp* and Enterobacteriaceae family (Tannock *et al.*, 1990; Edwards & Parrett, 2002). After the weaning stage the intestinal microflora changes rapidly similar to adults. During weaning introduction of solid food to the breast-fed infant causes a significant increase in the number of *Enterobacteria* and *Enterococci*, *Bacteroides spp*., *Clostridium spp.* and anaerobic *Streptococci* but addition of solid food to the diet of the formula-fed infant does not have such an impact on the gastro-intestinal flora (Stark & Lee, 1982).

2.1.2 Gastro-Intestinal Tract Microbiota in Adult

In adults, the combined microbial populations in the human body exceed 100 trillion cells, and are about 10 times more than total number of the human cells (Kurokawa *et al.*, 2007). Our body can be known as 'superorganisms' which is made up of 'host' human cells and a complex ecosystem of indigenous microbes. Hence the human genes are naturally mixed with trillions of microbes which colonized in our bodies. The isolation of human genes from the microbe genes is impossible. Metagenome is the term applied to the complex interactions of the human genome with the microbial genome (Turnbaugh *et al.*, 2006; Hattori & Taylor, 2009). The gastro-intestinal tract harbors a vast majority of microbiota more than anywhere else in the human body. A normal intestinal tract has around 10^{14} bacteria with close to 1000 different species (Cani & Delzenne, 2007). Even though the bacterial community in the gastro-intestinal tract is extremely diverse; the majority of species (98%) living in gastro-intestinal tract belongs to the few bacterial divisions or pyla. Those bacterial pyla are namely Firmicutes, Bacteroidetes Actinobacteria and Proteobacteria (Backhed *et al.*, 2005). The rests of species belong to minor taxonomic divisions that are secondary to the majority. The phylogenetic distributions of the human gastrointestinal prokaryotic phylotypes are clearly organized in Table 2.1.

As explained in Rajilic-Stojanovic and coworkers 2007, other than bacteria Archaea, Eukarya and Viruses are also identified in the adult gastrointestinal tract. The major Archaeal communities that have been found in the human gut are *Methanosphaera stadtmanae, Methanobrevibacter ruminantium* and *Methanobrevibacter smithii.* The major eukaryote fungi in the human intestine are *Candida*, *Aspergillus* and *Penicillium,* and more than 1200 viral genotypes have also been identified in human feces (Rajilic-Stojanovic *et al.*, 2007; Hattori & Taylor, 2009).

Table 2.1 The Phylogenetic Distribution of the Human Gastrointestinal Prokaryotic Phylotypes Based on SSU rRNA Gene Sequence (Rajilic-Stojanovic et al., 2007)

Phylum	Abundance	Class	Order	Family/Cluster
Firmicutes	10^{11}	Asteroleplasma	Anaeroplasmatales	Anaeroplasmataceae
		Bacilli	Bacillales	Bacillaceae
				Staphylococcaceae
			Lactobacillales	Aerococcaceae
				Carnobacteriaceae
				Lactobacillaceae
				Leuconostocaceae
				Lactococcaceae
				Streptococcaceae
		Clostridia	Clostridiales	Cl. cluster I
				Cl. cluster III
				Cl. cluster IV
				Cl. cluster IX
				Cl. cluster XI
				Cl. cluster XIII
				Cl. cluster XIVa
				Cl. cluster XV
				Unclassified
		Mollicutes	Unclassified	Cl. cluster XVI
				Cl. cluster XVII
				Cl. cluster XVIII
Bacteroidetes	10^{11}	Bacteroidetes	Bacteroidales	Rikenellaceae
				Bacteroidaceae
				Prevotellaceae
				Porphyromonadaceae

(Table 2.1 Con'd)

Phylum	Abundance	Class	Order	Family/Cluster
Actinobacteria	10^{10}	Actinobacteria	Actinomycetales	Actinomycetaceae 6
				Corynebacteriaceae 6
				Micrococcaceae 4
				Propionibacteriaceae
			Bifidobacteriales	Bifidobacterium
			Coriobacteriales	Coriobacteriaceae
Proteobacteria	10^{8}	*Alphaproteobacteria*	Rhizobiales	Unclassified
			Sphingomonadales	
		Betaproteobacteria	Burkholderiales	Alcaligenaceae
				Oxalobacteriaceae
				Burkholderiaceae
				Incertae sedis
				Unclassified
			Neisseriales	Neisseriaceae
		Gammaproteobacteria	Aeromonadales	Aeromonadaceae
				Succinivibrionaceae
			Enterobacterales	Enterobacteraceae
			Pasteurellales	Pasteurellaceae
			Pseudomonadales	Moraxellaceae
				Pseudomonadaceae
			Vibrionales	Vibrionaceae
			Xanthomonadales	Xanthomonadaceae
		Deltaproteobacteria	Desulfovibrionales	Desulfovibrionaceae
		Epsilonproteobacteria	Campylobacterales	Campylobacteraceae
				Helicobacteraceae

It has been found people with the gastrointestinal conditions such as inflammatory bowel disease (IBD), allergy and cancer have reduced microbial diversity using 16S analysis compared to those of healthy controls (Penders *et al.*, 2007a; Penders *et al.*, 2007b; Xavier & Podolsky, 2007; Hattori & Taylor, 2009).

2.1.3 Composition of Intestinal Bacteria in Each Part of Digestive Tract

The various different parts of the gastro-intestinal tract have different type of bacterial populations according to environmental condition. The mouth has the second largest population of bacteria that is influenced by large intestine having the largest population. The human oral cavity harbors around 10^{10} bacterial population with more than 500 bacterial species. The major inhabitant microbial divisions that inhibit the mouth are Firmicutes, Bacteroidetes, Proteobacteria, Fusobacteria and Actinobacteria (Tlaskalova-Hogenova *et al.*, 2004). Due to the high acidity and digestive enzymes, the stomach contains the least amount of bacteria. Bacteria present in the stomach are attached to the gastric-epithelia or mucus (Tlaskalova-Hogenova *et al.*, 2004). The stomach is mostly dominated by Gram positive and Gram negative aerobic bacteria and the overall bacterial population in the stomach is about 10^{3}-10^{4}. The most common micro-organism in the stomach is Gram negative *Helicobacter pylori*. This organism is present in 30- 80% of healthy humans. This organism is an opportunistic pathogen and can cause gastritis, gastric ulsers and gastric cancers. The small intestine harbors a relatively higher concentration of bacteria than the stomach, that are mainly Firmicutes such as *Lactobacilli, Bacilli* and Gram positive Coci but some Bacteroidetes, Proteobacteria, Fusobacteria and Actinobacteria. The bacterial population of the small intestine varies from 10^{6}-10^{8}. The large intestine contains an average of 10^{11}-10^{12}, and most of them are anaerobic or facultative anaerobic. The major divisions are Firmicutes, Bacteroidetes, Actinobacteria and Proteobacteria

(Table 2.1). The colonic microbiota changes due to a response in the nutritional shifts such as weaning, progressive changes such as aging or variation of food intake (Topping & Clifton, 2001).

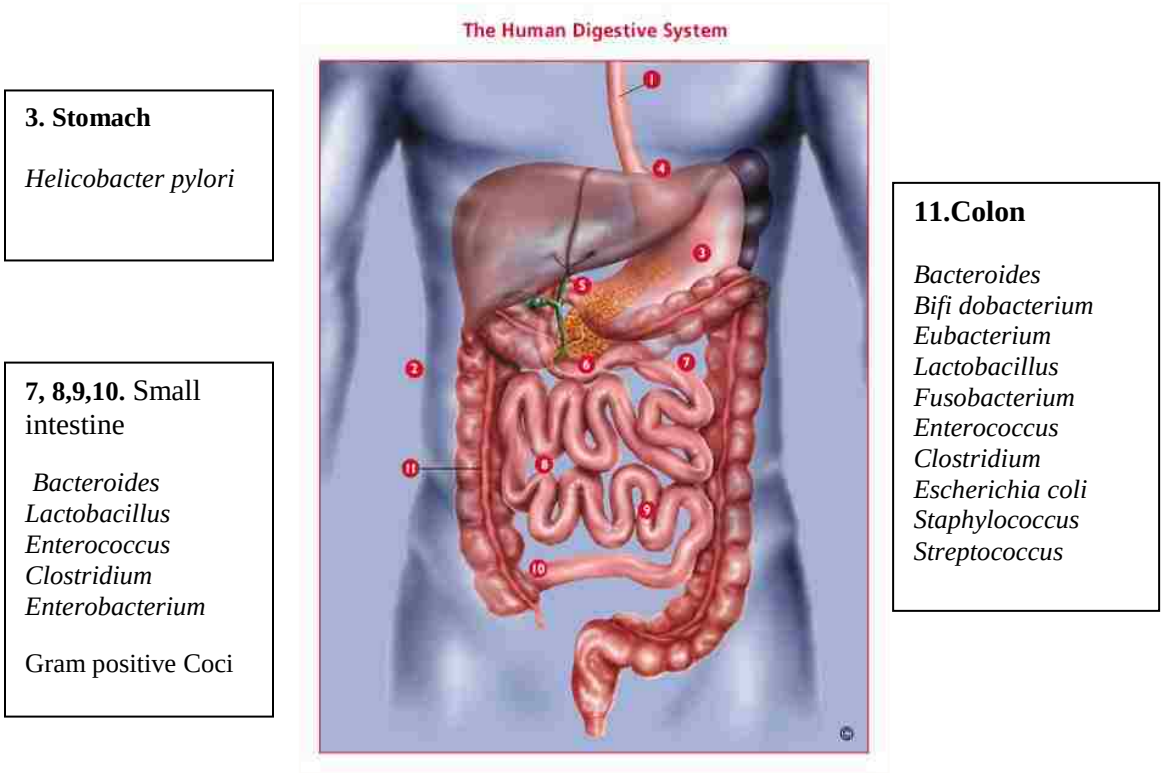


Figure 2.1 Human Gastro-Intestinal Tract Microbiota (Rajilic-Stojanovic *et al.*, 2007)

2.1.4 Digestion in Simple Stomach Animals and Involvement of Microbiota

The digestive system is one of the most complex and complicated biological system. The four major regions of the digestive system are the oral cavity, stomach, small intestine and large intestine. Digestion of food starts in the mouth. During the oral phase of digestion food starts with mechanical breakdown, then salivary impregnation, α amylase hydrolysis and finally bolus formation occurs prior to swallowing (Woolnough *et al.*, 2008). In the stomach hydrolysis of protein, carbohydrate and fat occurs. Protein digestion is initiated by pepsin and hydrochloric acid resulting in peptide formation; Carbohydrate digestion is initiated by salivary amylase and lipid digestion occurs with gastric lipase.

The food passing from the stomach is further hydrolyzed in the small intestine. Proteins and peptides passing from stomach expose to pancreatic enzymes such as trypsin, chymotrypsin, elastate, peptidase, carboxypeptidase and finally convert into free amino acids and smaller peptides. Carbohydrates are also further hydrolyzing by pancreatic amylase to maltose and to dextrin. Those are again exposing to maltase, lactase, sucrose, and isomaltase convert to monosaccharide. Lipid is also exposed to three different enzymes such as pancreatic lipase, carboxylic ester hydrolase, phospholipase and one coenzyme named colipase. The pancreatic lipase breakdown triacylglycerols into monoacylglycerols and fatty acids, the carboxylic ester hydrolase hydrolyses carboxylic esters, and phospholipase hydrolyses fatty acids in the 2-position of glycerophospholipid. The presence of bile salts enhanced activity of lipase and absorption of long-chain fatty acids and monoacylglycerols. The amino acids, peptides simple sugar molecules and digested fatty acid are absorbed in small intestine (Boisen & Eggum, 1991). In the small intestine 98% of the fat is digested and absorbed. The reminders of digested fat (2%) carry over into the large intestine (Saunders & Sillery, 1988).

In the large intestine endogenous microflora are directly involved with the digestion of dietary proteins and carbohydrates. From 10-20 % of endogenous nitrogen from protein intake in the small intestine is recovered by microflora in the large intestine. Dietary protein (ex. mucin) and dietary carbohydrates (ex. Resistant starch (RS), Fructo oligo sacharides (FOS), Inulin) are further hydrolyzed by bacterial enzymes. But fatty acids cannot be fermented by bacterial enzymes, so lipid is only marginally influenced during passage through the large intestine. The major outcome of the dietary fibers and protein are Short chain fatty acids (SCFA), which can be absorbed and act as an energy source for humans (Boisen & Eggum, 1991).

2.2 Benefits of Microbiota in the Gut

The first bacterial species recovered from a human gastrointestinal sample and identified was *Escherichia coli*, which was isolated in 1885 from children's diarrhoeal faeces (Rajilic-Stojanovic *et al.*, 2007). Microbes in the digestive system have a homeostatic symbiosis relationship in which the host provides a stable environment with nutrients and the microbes provide benefits to the host (Leser & Molbak, 2009). This wonderful ecosystem is playing a vital role by safeguarding the health of the gut (Tomotari, 1978 ;Tlaskalova-Hogenova *et al.*, 2004). Gut microbiota are directly involved with several mechanisms including defense against pathogens, synthesis of vitamins, fermentation of dietary fibers and dietary proteins; priming the immune system early in life; Stimulating the gut motility (Topping & Clifton, 2001; Cani *et al.*, 2007a; Cani *et al.*, 2007b ;Leser & Molbak, 2009). The gut microbiota helps to increase thickness of the villi in the intestinal wall. Germ free mice that are absences of gut microorganism resulted in a thinner villi and shorter crypt as well as low motility in the gastrointestinal tract (McCullogh *et al.*, 1998; Langlands *et al.*, 2004). The animals with gut microbiota conditions also exhibit fast epithelial cell turnover and it is twice faster compared with Germ free mice (Leser & Molbak, 2009). The microbiota in the gut contributes to the development of healthy conditions within the intestinal tract by fighting against colonization of pathogenic organisms suppressing. For example gut microbes such as Lactic acid bacteria produce antimicrobial peptides called bacteriocins which suppress the growth of potentially enteric pathogenic organisms (Itoh *et al.*, 1995; Spinler *et al.*, 2008). *Faecalibacterium prausnitzii* provides protection against a major gastrointestinal condition named inflammatory bowel disease (IBD), Crohn's disease and ulcerative colitis (Sokol *et al.*, 2008). Probiotics, live microorganisms that improve health on the host, are also using as antibiotic therapies (Mellon *et*

al., 2000; Kajander *et al.* 2008; Surawicz, 2003). For example *Lactobacillus* species, *Enterococcus* species, and *S. boulardii* have used as probiotics to treat infective diarrhea in both adults and children and that have shown to be effective in the treatment of (Boyle *et al.*, 2006).

Dietary changes also regulate both quantitative and qualitative changes in microbial communities. The main factors affecting the GI microbial communities are: bacterial metabolism, competition and gut environment such as pH, gases (oxygen and hydrogen), metabolite concentrations, the duration that food stays in the gastrointestinal tract, and host secretions such as acids, enzymes and hormones (Louis, & Others, 2007). Production of Short chain fatty acids (SCFA) is one of the most useful benefits of gut microbes.

2.2.1 Benefits of SCFA Produced by Microbiota in the Gut

Even though human beings are omnivorous the basic fermentative reaction in the human colon is similar to that in obligate herbivores (Topping & Clifton, 2001). Various bacterial populations involved with the process of SCFA production and those populations involves with conversion of polysaccharides, oligosaccharides, and disaccharides to their constituent sugars. This fermentation also yields metabolizable energy for microbial growth and maintenance and some metabolic end products. The fermentation of dietary carbohydrates and dietary protein in the mammalian gut is the results in production of high concentrations of SCFA. Intestinal microbial communities hydrolyze non-digestible plant polysaccharides such as lignin, hemicelluloses, pectin, cellulose and RS into SCFA. Other organism metabolise are carbon dioxide, hydrogen and methane. The acetate, propionate, and butyrate have been found in greater concentration in the feces of those who consume a diet containing dietary fibers including RS (Morita *et al.*, 1999). The typical ratios of SCFA in feces are proportion of 3:1:1 in acetate: propionate: butyrate (Duncan *et al.*, 2002). These SCFA help regulate the colonic physiological

processes and maintain normal bowel function. Reduction of pH values from SCFA help to reduce the growth of pH sensitive pathogenic organisms, reduce absorption of toxic alkaline compounds with carcinogenic potential in the gut (Bird *et al.*, 2000). In Inflammatory Bowel Disease (IBD) patients have reported lower levels of SCFA when compared to healthy individuals (Galvez *et al.*, 2005).

(a) Butyrate

Benefits of butyrate and physiology of butyrate metabolism in the large intestine have been intensively studied by several investigators. Butyrate is a major energy source for epithelial cells of colonic mucosa which stimulates cell proliferation (Scheppach *et al.*, 2001; Sato *et al.*, 2008). Butyrate is the major SCFA that in providing protection against cancer and ulcerative colitis by reducing cell proliferation, blocking the absorption of cancer-causing substances and making the colon less vulnerable to DNA damage (Pitcher & Cummings, 1996). It also helps to boost the absorption of calcium to maintain a healthy epithelium (Hagopien *et al.*, 1977 ,Cummings & Macfarlane, 1991, Gibson *et al.*, 1998). Keenan and his group have shown that dietary resistant starch was associated with increased gene and hormone expression for peptide YY (PYY) and glucagon-like peptide-1(GLP-1), which are also associated with increased butyrate in the cecum in rats (Keenan *et al.*, 2006; Zhou *et al.*, 2006).

(b) Propionate

Propionate is the primary precursor for gluconeogenesis and may inhibit liponeogenesis and protein synthesis (Louis *et al.*, 2007; Schwiertz *et al.*2009). Propionate is believed to inhibit the synthesis of fatty acids in the liver and also involved in the control of hepatic cholesterol synthesis. It helps to lowers plasma cholesterol concentrations by inhibiting hepatic cholesterogenesis through colonic fermentation (Cheng & Lai, 2000). The propionate

concentration and proportion of total SCFA increases significantly in lean to obese subjects (Schwiertz *et al.*2010). Production of propionate could be important factor that contributes to weight gain in obesity subjects.

(c) Acetate

Acetate is the major SCFA produced by the colonic microflora. It is around 60–75% of the total SFCA detected in feces and it is formed by many of the colonic microflora and about one-third coming from reductive acetogenesis which is produced by anaerobic bacteria (Miller & Wolin, 1996; Louis *et al.*, 2007). It is quickly absorbed soon after production and transport to the liver. Because of that it is not metabolized in the colon. Remaining acetate is further utilized by colonic microbiota and covert in to butyrate. Acetate is essential for cholesterol synthesis in the body (Hijova & Chmelarova, 2007). The major benefit of acetate is it acts as an energy substrate for muscles and systemic circulation (the portion of the cardiovascular system which carries oxygenated blood away from the heart, to the body, and returns deoxygenated blood back to the heart). Acetate has been shown to suppress harmful bacteria (Araya-Kojima *et al.*, 1995).

2.3 Gut Hormones and Benefits

Peptide YY (PYY) and glucagon-like peptide 1 (GLP-1), are two main hindgut hormones that are produced in greater amounts when increased food material passes through the small intestine in to the large intestine (Cani *et al.*, 2005; Keenan *et al.*, 2006; Zhou *et al.*, 2006; Shen *et al.*, 2009). The gut hormone PYY is produced by L endocrine cells mainly located in the rectum, ileum, and large intestine (McGowan & Bloom, 2004). GLP-1 is also produced by L endocrine cells that are located in the ileum and large intestine (Kreymann *et al.*, 1987; Kreymann *et al.*, 1988). GLP-1 acts through binding to the GLP-1 receptors that are found on many cell types including, beta cells of the pancreas, neuronal cells in the brain, adrenal,

pituitary, kidney, and throughout the gastrointestinal tract (Gotthardt *et al.*, 2006). GLP-1 enhances both early and late phase of insulin secretion stimulated by glucose because of this GLP-1 is important in the treatment of diabetes mellitus (Wicki *et al.*, 2007). GLP-1 controls feeding behavior in the brain that GLP-1 is more important on energy intake and energy expenditure make it a logical candidate for weight control (Perez-Tilve *et al.*, 2006).

Peptide YY (PYY) is also play a role in modulating energy balance and adiposity through control of food intake and nutrient partitioning. It helps to inhibit both food intake and gut motility and fat oxidation (Adams *et al.*, 2004; Adams *et al.*, 2006). PYY is also important in the control of insulin sensitivity and controlling obesity (Boey *et al.*, 2006a; Boey *et al.*, 2006b; Boey *et al.*, 2007).

Increase of butyrate in the intestinal tract by fermentation of prebiotics may help to increase PYY and GLP-1 (Keenan *et al.*, 2006; Zhou *et al.*, 2006). PYY and GLP-1 are associated with reduced body fat and decreasing the blood glucose level, reducing body weight and improving insulin sensitivity in mammalians (Young *et al.*, 1999). Lower respiratory exchange ratio is prominent in mice fed RS, indicating a partitioning of fat to oxidation rather than storage (Zhou et al., 2009).

2.4 Probiotic, Prebiotic, Synbiotic and Gut Microbiota

The term probiotic firstly defined by Parker then it modified by Fuller in 1989 as "A live microbial feed supplement which beneficially affects the host by improving its intestinal microbial balance" (Parker, 1974; Fuller, 1986, 1989). The live microbial cultures that are present in dairy products sauerkraut, fermented cereals and other plant-based foods, and salami are known to be probiotic (Schrezenmeir & de Vrese, 2001). The main probiotic microorganisms are in the genera of *Lactobacillus*, *Bifidobacterium,* and certain strains of *Enterococcus* and

Saccharomyces spp. Some of the species that are include *Lactobacillus plantarum, Lactobacillus delbrüecki, Lactobacillus bulgaricus , Bifidobacterium breve , B. longum, B. bifidum* and *Streptococcus thermophilus*.

Probiotics should maintain following features:

1. There should be the food products in addition to microorganisms.
2. Microbial numbers should be at a sufficient number to exert health effects.
3. Probiotics consumed in foods and dietary supplements are generally recognized as safe status (GRAS).

Probiotics have shown vast range of health benefits including inhibiting the attachment and growth of pathogenic microbes in the epithelium of the intestinal tract. For example Bifidobacteria are known to be involved in resisting the colonization of pathogens in the gut by producing bacteriocins, hydrogen peroxide, and biosurfactants (Macfarlane & Cummings, 1999).

In-vitro and *in-vivo* studies showed that when pH-controlled co-culture of *Bifidobacterium infantis* was inoculated together with *Escherichia coli,* and *Clostridium perfringens,* with the presence of oligo-fructose the Bifidobacteria has expressed an inhibitory effect on the growth of the other two species (Cummings *et al.,* 2001).The *B. bifidum* and *Streptococcus thermophilus* have also been used in studies of the prevention and treatment of rotavirus and diarrhea in children in the hospital (Saavedra *et al.,* 1994).

Lactobacillus probiotics can decrease intestinal mucosal permeability and prevent pathogenic activity (Reid, 1999). *Lactobacillus GG* (*Lactobacillus case*i sps. *rhamnosus*) exerted a beneficial effect on allergic reaction such as development of eczema by improving mucosal barrier function in pregnant women (Boyle *et al.,* 2006). Probiotics are known to be enhanced the

immune response, increased ability to digest food, and alleviate many common digestive disorders such as constipation, diarrhea and Irritable bowel syndrome IBS.

The term prebiotic was firstly defined by Gibson and Roberfroid as "a non-digestible food ingredient that are beneficial to the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon." this is overlaps with the definition of dietary fiber (Gibson & Roberfroid, 1995; Schrezenmeir & de Vrese, 2001).

Fiber can be divided in to three main categories which are dietary, functional and total fiber. Dietary fiber includes non-digestible carbohydrates and lignin that are intrinsic and intact in plants, but functional fiber includes isolated, non digestible carbohydrates that have beneficial physiological effects in humans. Total fiber is the combination of both dietary and functional fiber. Some of the well recognized fermentable fibers that increase the beneficial bacterial loads in the large intestine are or Resistant starches (RS), and inulin, trans-galactosylated oligosaccharides, soybean-oligosaccharides and oligo-fructoses or Fructo-oligosaccharides (FOS) (Cani *et al.*, 2004; Wang *et al.*, 2004; Falony *et al.*, 2006; Topping & Clifton, 2001). Dietary fiber which increase the beneficial bacterial loads in the large intestine are known as prebiotic.

Resistant starch is considered as a prebiotic. The definition for the RS is starch which is resistant to digestive tract amylase. The RS can be divided in to four categories:

1. Type1 or RS1 - found in whole grains with intact plant cell walls
2. Type2 or RS2- consists of ungelatinized starch in high amylose cornstarches
3. Type3 or RS3- retrograded starch formed from cooking and cooling or extrusion (Ex: Cooked potatoes).

4. Type 4or RS4 - chemically modified starch due to addition of esters, ethers and cross bonding.

RS1 and RS2 can be digested by α-amylase the longer they remain in the small intestine, but RS3 and RS4 are not being digested in small intestine (Englyst *et al.*, 1992; Cummings *et al.*, 1996).

Resistant starch (RS) has been observed to benefit human health in numerous ways, including the potential decrease in metabolizable energy to reduce body weight while maintaining the bowel health (Ferguson *et al.*, 2000; Keenan *et al.*, 2006). Overweight and obesity are major health concerns among developed counties; overweight related maternal diabetes and hypertension in women can cause serious pregnancy-related complications, this has caused rapid growth of the weight reducing industry and in pregnant women. Research into the beneficial effects of RS in controlling weight has been a high priority.

Synbiotics are defined as probiotic bacteria plus complex carbohydrates as prebiotics. When use combination of live probiotics with specific prebiotics as a symbiotic, that combination will help for the survivability of probiotics. Prebiotics provides specific substrate which required for probiotic bacterial growth (de Vrese *et al.*, 2001; Schrezenmeir & de Vrese, 2001). From this combination host is getting benefits from both probiotics and prebiotics. For example FOS and probiotic *Bifidobacterium* or RS and *Bifidobacterium* or *Lactobacillus* would fulfill the definition.

Synbiotic therapy is widely using to cure active inflammatory bowel disease *(*IBD), ulcerative colitis (UC) and Crohn disease (CD). The major advantage of using a synbiotic is that the prebiotic component would promote the growth of indigenous beneficial organisms in the gut with the help of probiotic properties. Furrie and colleagues conducted human clinical trials with

consumption of synbiotic (*Bifidobacterium longum*/Synergy 1) twice daily over four weeks. They have found significant reduced effect in mucosal inflammatory markers in active ulcerative colitis (Furrie *et al.*, 2005).

2.4.1 Fermentation of RS in the Large Intestine

Fermentation of RS in the large intestine, and production of butyrate is a complex process. Production of butyrate requires several groups of bacteria. The bacteria involved include *Lactobacillus spp*, *Bifidobacterium spp,* and *Bacteroides spp.* The majority of the bacterial species that are butyrate producers are included in two Clostridium clusters - Clostridium cluster IV and Clostridium cluster XIV (Louis *et al.*, 2007a; Sato *et al.*, 2008). However, some butyrate producers are included in other Clostridium clusters I, III, XI, XV and XVIa.

During the fermentation of RS, bacteria will attach to starch molecules (Figure 2.2) especially *Bacteroides thetaiotaomicron, Bifidobacterium longum* and some *Lactobacillus spp* (Bird *et al*., 2000; Xu *et al*., 2003; Louis *et al.*, 2007b*)*. However, *Bacteroides spp*, *Lactobacillus spp* and *Bifidobacterium spp* do not produce butyrate as a final product from RS. The end fermentation products for *Bacteroides spp* are acetate, propionate and succinate. Lactate and acetate are produced by *Bifidobacterium spp* and *Lactobacillus spp* when fermenting RS. Thus, these bacterial species adhere to the surface of starch molecules and ferment RS into intermediate products that are converted by other species to butyrate (Duncan *et al.*, 2002; Duncan *et al.*, 2004a; Duncan *et al.*, 2004b; Louis *et al.*, 2007a).

The butyrate producing bacteria are mainly species in Clostridium cluster IV and Clostridium cluster XIV. Duncan and his collaborators initially discovered *Coprococcus* spp. and *Roseburia spp.* classified in Clostridium cluster XIV and the species *Faecalibacterium prausnitzii* that is classified in Clostridium cluster IV, that produce butyric acid from acetate. However, these

bacteria do not utilize lactate for production of butyrate (Duncan *et al.*, 2002; Cani & Delzenne, 2007). Later this same research group discovered lactate utilizing bacteria from human feces that produce butyrate as their main fermentation end product. *Eubacterium hallii* and *Anaerostipes caccae* from Clostridium cluster XIV were able to utilize both the D and L isomers of lactic acid while *C. indolis* from Clostridium cluster XIVa was only able to use D lactic acid (Duncan *et al.*, 2004b). *Eubacterium limosum* from Clostridium cluster XIVb also was able to convert lactate into acetate and butyrate in the presence of *Bifidobacterium longum* (Sato *et al.*, 2008).

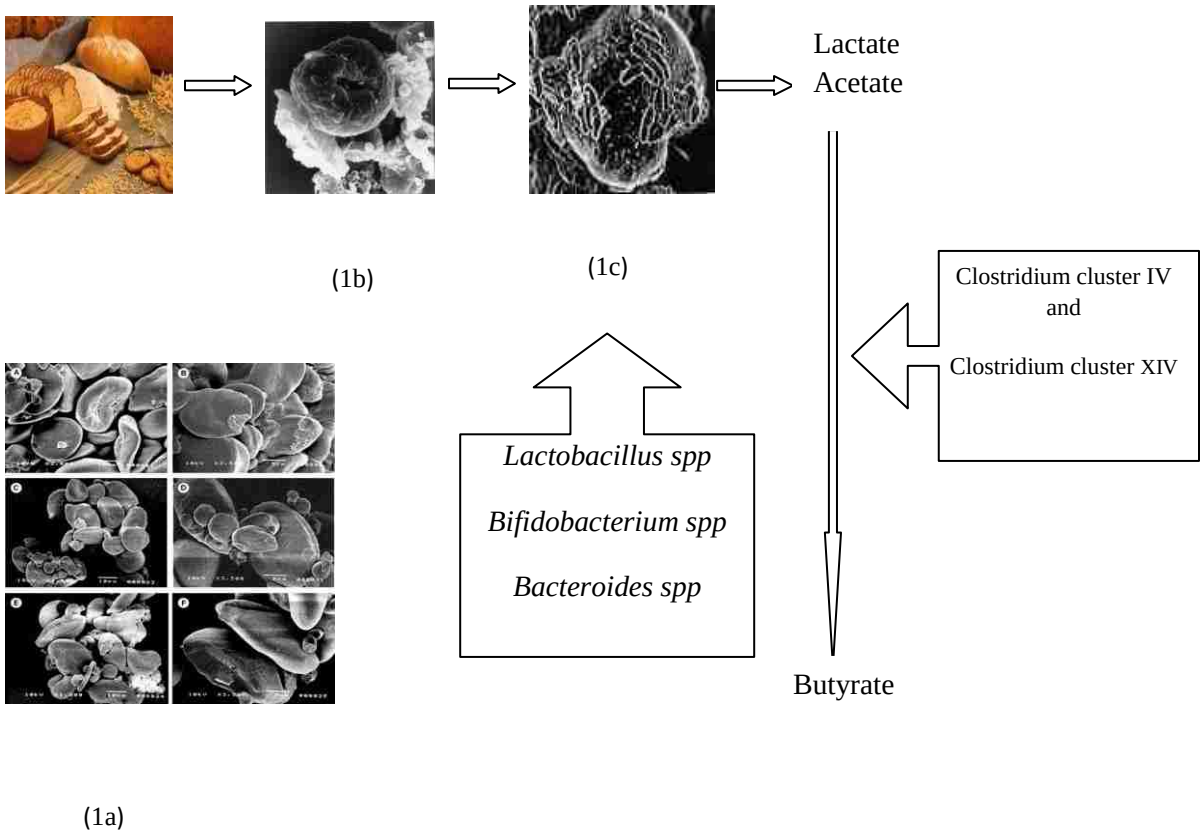


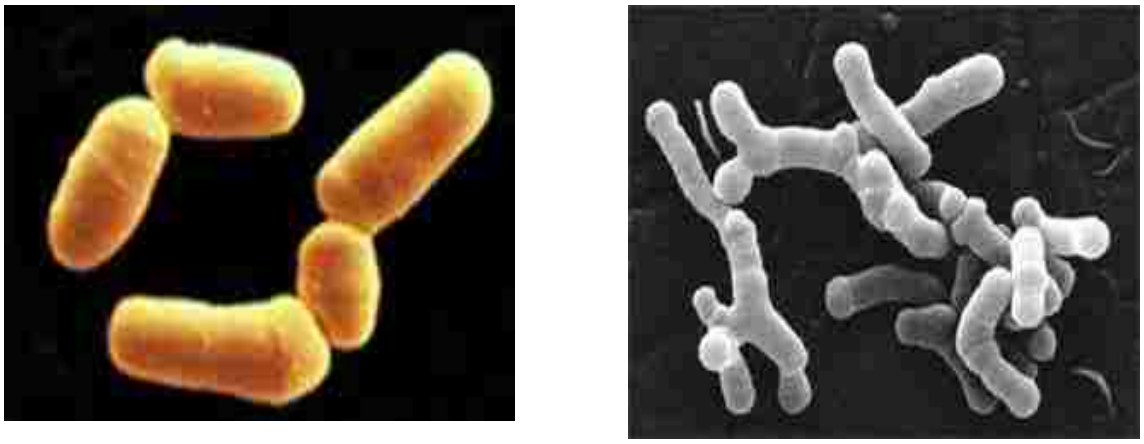
Figure 2.2 Bacteria Associated with Resistant Starch for the Production of Butyric Acid. (1a) High amylose starch granule (1b) after passage though small intestine and (1c) showing adherent bacteria (Bird et al., 2000; Topping & Clifton, 2001)

2.4.2 Bacteria involved with Fermentation of RS to Butyric Acid

2.4.2.1 *Bifidobacterium spp.*

Bifidobacterium spp is classified under phylum Actinobacteria; it is a Gram-positive strictly anaerobic branched rod that produces lactic and acetic acid without generation of CO_2. The genus *Bifidobacterium* is the third most numerous bacterial populations in the human intestine after the genera *Bacteroides* and *Eubacterium. Bifidobacterium* is about 6% of total fecal bacteria (Matsuki *et al.*, 2004). *Bifidobacterium spp* start to colonize the infants gut and becomes the predominant bacterial population during infancy then it gradually decreases during weaning stage becoming stable until adult hood but further decrease during old age (Edwards & Parrett, 2002; Mitsuoka, 1978; Mitsuoka, 1992). *Bifidobacterium spp* can be used as a probiotic because it improves the digestion absorption and immune system. Because of that it helps to decrease the side-effects of antibiotic therapy. It also provides protection against enteric pathogens, putrefactive substances, and believed to be involved with mechanisms of reduction of cholesterol levels and anti-tumoral activity (Leahy *et al.*, 2005). *Bifidobacterium spp.* is involved in vitamin production and antagonic products production, like organic acids and bacteriocins. This organism shows synbiotic relationship with complex carbohydrates.

It is well-known that *Bifidobacterium spp* promoted by the fermentation of dilatory fibers such as Resistant Starches, Fructo-oligosaccharides and inulin, transgalactosylated oligosaccharides and soybean oligosaccharides (Ito *et al.*, 1993; Gibson *et al.*, 1995; Gibson & Roberfroid, 1995; Cani *et al.*, 2004; Wang *et al.*, 2004; Falony *et al.*, 2006; Topping & Clifton, 2001). *Bifidobacterium spp* is adhere to prebiotic and translocate starch molecules (change the position of starch molecules) and produce acetate and lactate as an end product, which help to promote butyrate.



(a) (b)

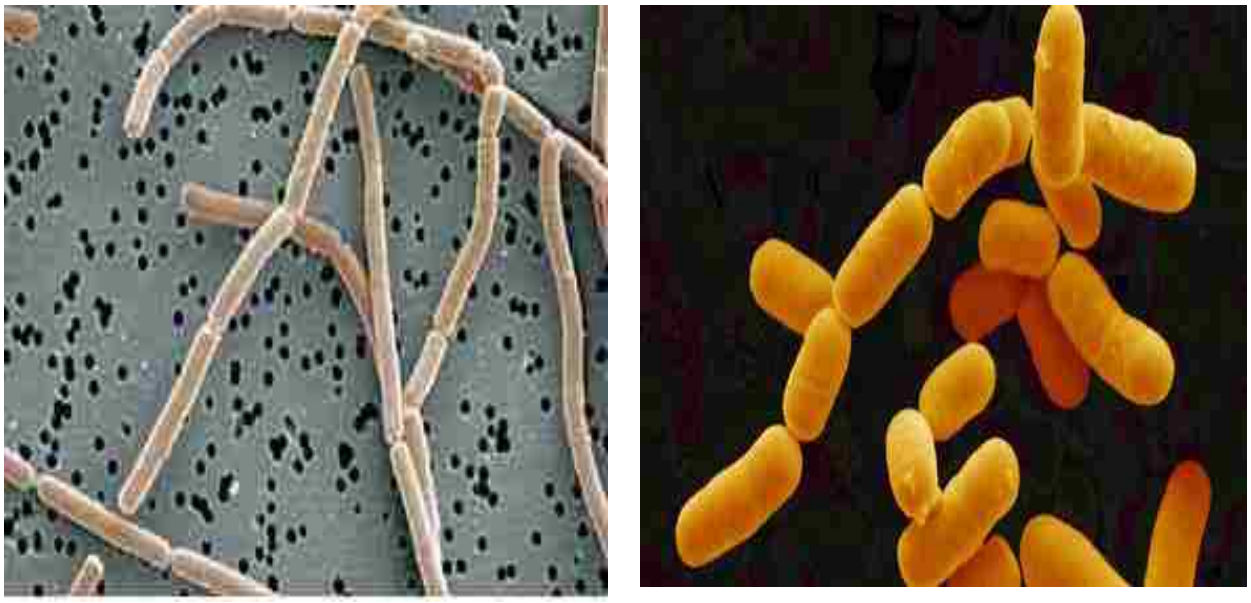
Figure 2. 3 (a) *Bifidobacterium bifidum*, (b) *Bifidobacterium longum*. ; Photograph: From Nutrinibbles and Mark Schell, University of Georgia

2.4.2.2 *Lactobacillus spp*.

The members of the genus *Lactobacillus* are Gram-positive non-spore-forming rods (ranging from coccobacilli to long, slender bacilli organisms) that belong to the general category of lactic acid bacteria under phylum *Firmicutes. Lactobacillus* strains could grow under aerobic conditions, or even intestinal under anaerobic conditions. *Lactobacillus spp.* produces lactic acid as the major acid during fermentation of glucose with acetic, succinic and formic acids in minor quantities.

Lactobacillus spp. helps to decrease intestinal mucosal permeability that prevents entering pathogenic organisms by reducing pathogen adhesion. Some strains of *Lactobacillus spp.* can prevent adhesion of pathogens by steric hindrance and by producing biosurfactants (Velraeds *et al.*, 1996). *Lactobacillus fermentum* has evidence of reduction adhesion and competitive exclusion of pathogenic *Escherichia coli, Listeria monocytogenes, Shigella sonnei,*

and *Salmonella typhimurium* in both *in-vitro* and animal studies (Reid, 1999). *Lactobacillus GG* (combination of *Lactobacillus casei and L. rhamnosus*) *L. acidophilus, L. casei, L. plantarum, L. delbrueckii,* and *L. rhamnosus* have shown beneficial effect on allergic reaction by modulation of immune system by increasing phagocyte activity and secretory immunoglobulin (Perdigon *et al.*, 1988a; Perdigon *et al.*, 1988b). *L. acidophilus* and *L. casei* are known to be balancing of gut microbiota and have shown anti-tumor effect. *L. acidophilus, L. casei, L. gasseri, L. delbrueckii and L. plantarum* are well studied for Prevention of diarrhea caused by *Clostridium difficile,* Shigellae, Salmonellae, Campylobacter and *E. coli*. *L. acidophilus* effects have been studied in humans as reducing problems associated with lactose intolerance *(*Reid, 1999).

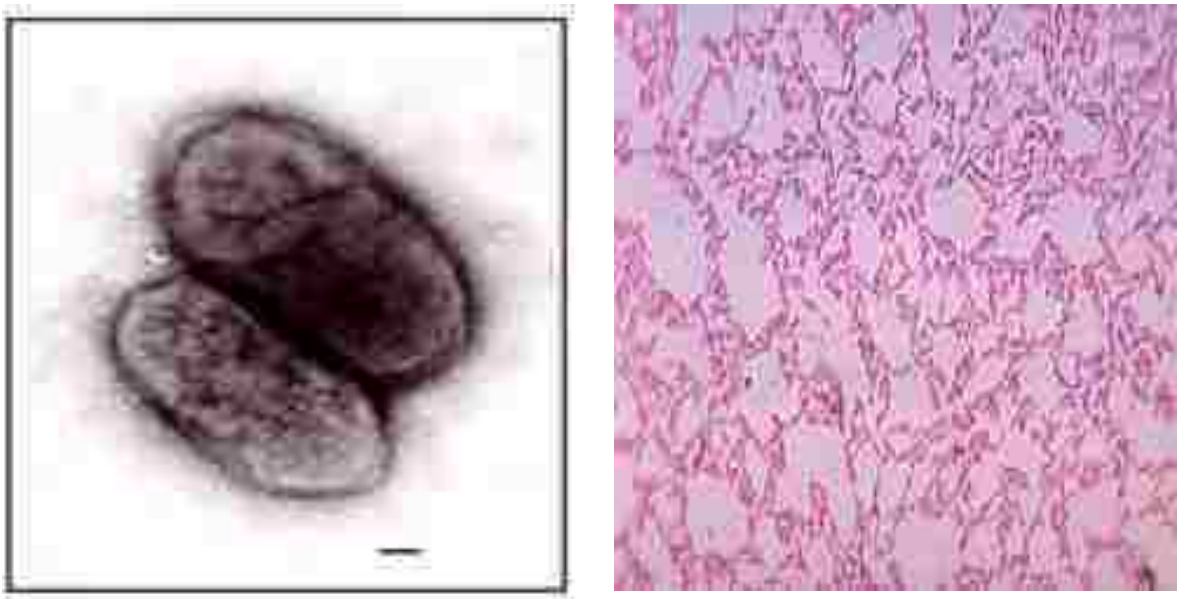


(a) (b)

Figure 2.4 (a) *Lactobacillus Bulgaricus* , (b) *Lactobacillus rhamnosus* :Photograph: Visuals Unlimited/Corbis; bioweb.uwlax.edu

2.4.2.3 *Bacteroide spp*

Bacteroides spp. is strictly anaerobic Gram-negative, dominant bacillus bacterial group in human gastrointestinal tract. *Bacteroides* spp. could break down a wide variety of otherwise indigestible dietary carbohydrate by producing acetate and succinate as the major metabolic end products. *Bacteroides thetaiotaomicron* have been extensively studied by several investigators (Cani & Delzenne, 2007; Ley *et al.*, 2008; Turnbaugh *et al.*, 2009). This organism increased rapidly soon after the introduction of prebiotics such as Resistant Starch. This bacterium attaches to starch molecules and starts the fermentation process (Wang & Gibson, 1993; Brown *et al.*, 1997; Bird *et al.*, 2000). During young age higher population of *Bacteroides spp*. are seen in the intestinal tract compared to the elderly age. But the diversity among the genus Bacteroides increases with age. The highest diversity of genus Bacteroides could be seen in elderly population with compared to younger populations.



(a) (b)

Figure 5 (a) *Bacteroides thetaiotaomicron* (b) *Bacteroides fragilis*; Photograph: From MicrobeWiki

2.4.2.4 Clostridium Clusters

Clostridium clusters categorization has done based on16S rRNA sequence analysis, According to Phylogenetic analysis of 16S rRNA genes shows that the group is very diverse, it includes non-clostridial species in deeply branching clusters. The main two butyrate producing Clostridium clusters groups that present in healthy humans are *C. leptum* group or Clostridium clusters IV (Figure 2.6) and *Clostridium coccoides*–Eubacterium rectale group or Clostridium clusters XIVa –XIVb (Figure 2.7).

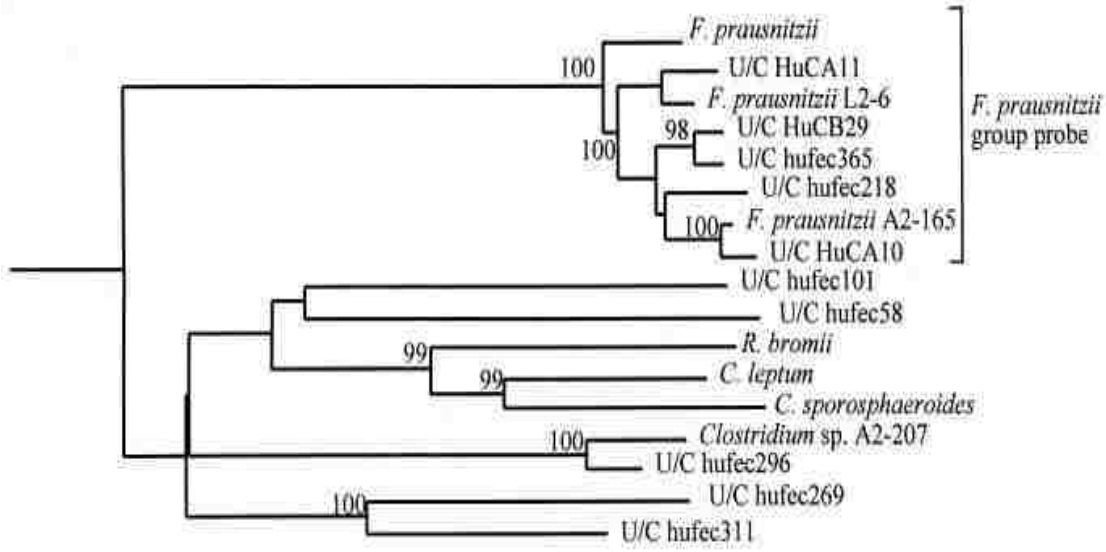


Figure 2.6 *C. leptum* group or Clostridium clusters IV (Hold et al. 2003)

2.5 Gut Microbes and Human Health

2.5.1 Age and Related Effects in Gut Microbiota

Our digestive tract microbial ecosystem changes with our age and the life pattern. Mother's vaginal birth canal introduces the very first bacterial inocula in to the babies during natural birth. During a cesarean section birth bacteria introduce in to babies during feeding and cleaning etc. The first bacteria in the gastrointestinal-tract harbours are *Coliform bacteria, Enterococcus, Lactobacilli, Staphylococci* and some *Clostridia spp.*

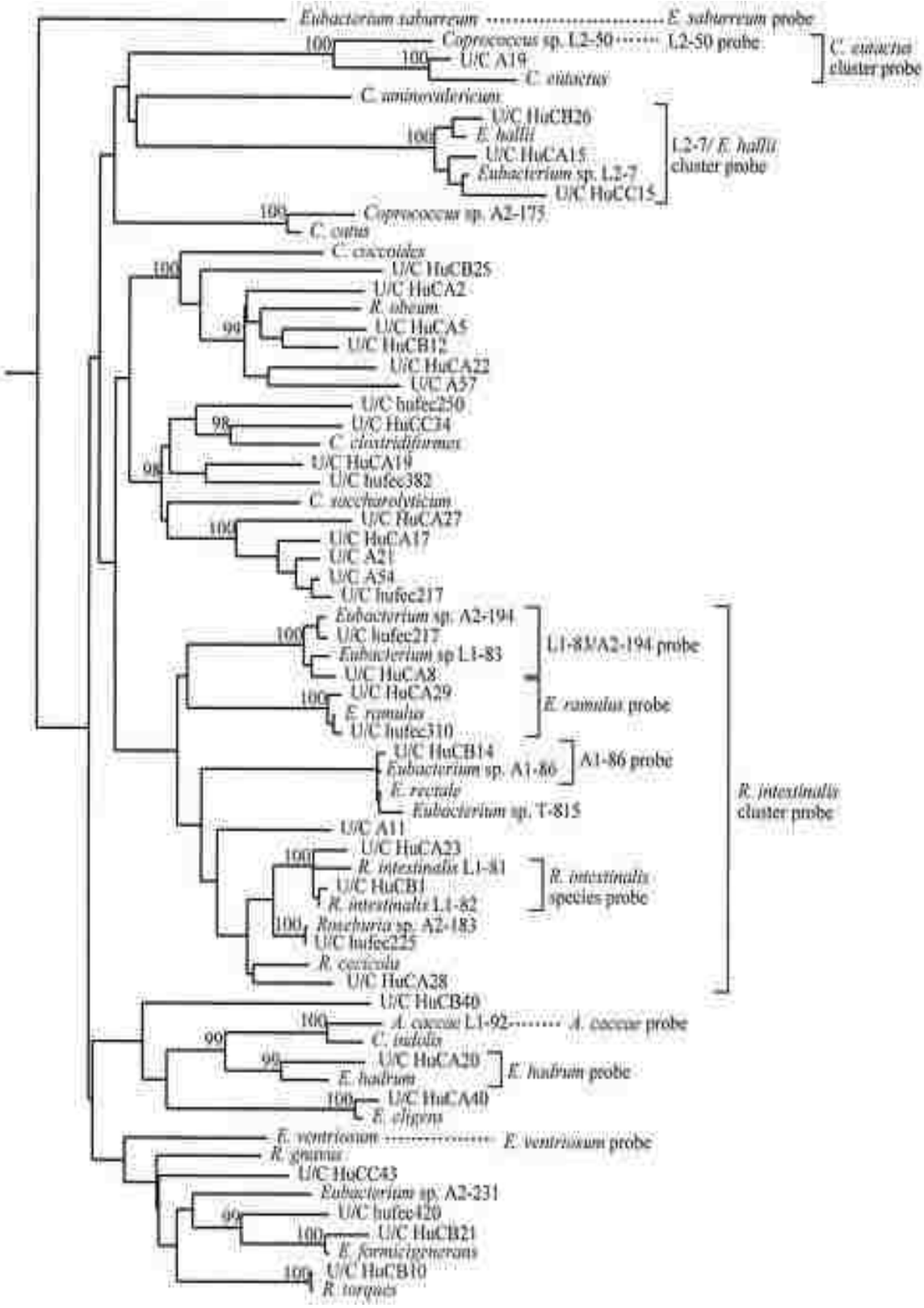


Figure 2.7 Clostridium coccoides–Eubacterium rectale group or Clostridium clusters XIVa-b (Hold et al. 2003)

These organisms appeared in newborn baby's feces within 24 hours after birth. Soon afterword *Bifidobacterium spp* starts to colonize the gut and become dominant during the next 3-4 days (Mitsuoka, 1978; Tomotari, 1978; Rajilic-Stojanovic *et al.*, 2007). During the age of 2, after the weaning stage of babies, gut microbial diversity become similar to adults. Hence health and age-dependent changes in gut microbiota could be of major significance. The gut microbiota continues to evolve throughout the lifespan of the host. Children's digestive tract organisms are less complex bacteriologically than those of the adult (Tuohy, 2007). During aging elderly populations, indicated a decrease in *Bifidobacterium spp.* and an increase in Fungi, members of family Enterobacteriaceae, *Lactobacillus spp.* and *Clostridia spp.*(*C. difficile* group) compared to adults (Hopkins *et al.*, 2001; Mueller *et al.*, 2006; Tuohy, 2007).

Most of the age related gut microbial studies have been conducting in South East Asia and Europe for several decades (Rhodes *et al.*, 1993; Sharma *et al.*, 1995; Fukushima *et al.*, 1999; Mueller *et al.*, 2006; Commane *et al.*, 2009). A Japanese group of scientist has found *Clostridium coccoides subgroup or Clostridium* cluster XIVa tended to be lower in elderly than in younger Japanese subjects. Eventhough the *Clostridium* cluster XIVa including *Clostridium leptum /Faecalibacterium prausnitzii* and relatives shows controversial results according to geographical location of the host (Mueller *et al.*, 2006), several investigators have noticed reduction of *Clostridium* cluster XIVa with age.

In all age group fecal samples contain E. *coli* and its occurrence is independent of age, whereas *Enterobacter* and *Klebsiella spp* have high counts in children and the elderly than in adults (Tuohy, 2007). *Bacteroides thetaiotaomicron, B. ovatus,* and *Prevotella tannerae* are commonly isolated *Bacteroides spp.* from younger adults. Although age-related reduction in the *Bacteroides* group appeared, *Bacteroides* species richness was increased with age. The

Firmicutes/Bacteroidetes ratio increases with age that is from birth to adulthood and is further altered with advanced age (Mariat *et al.*, 2009).

2.5.2 Obesity and the Dominant Groups of Digestive Tract Bacteria

Obesity is a growing epidemic in many developed western countries. Only in the United States more than half of the population is overweight and one third of the population obese. Obesity is a major concern because it's directly related to serious health consequences such as type 2 diabetes mellitus, cardiovascular diseases, pulmonary hypertension, obstructive sleep apnea, gastro esophageal reflux disease, musculoskeletal disorders, a variety of cancers, and even an increase risk of mortality. A major reason for obesity is high energy intake with less physical activity, but resent findings suggest that the gut microbiota have direct effect on obesity (Ley; Ley *et al.*, 2005; Ley *et al.*, 2006; Turnbaugh *et al.*, 2006; DiBaise *et al.*, 2008). According to these articles gut microbiota is influencing energy harvest from dietary substances (Fiaf) of host as well as affecting genes that regulate how energy is expended and stored.

Lay and coworkers found variation in the microbial populations in the gut of obese and lean people. They suggest that obese people and mice demonstrated a lower percentage of *Bacteroidetes* and proportionally more *Firmicutes* than in lean. When the obese people lost weight their microflora reverted back to that observed in a lean person, suggesting that obesity may have a microbial component (Ley *et al.*, 2006; Turnbaugh *et al.*, 2006). Resent findings from Touhy and others suggested that obese animals have significantly lower *Bifidobacterium spp.* levels than their lean animals. Cani and co-workers had a controversial idea regarding metabolic endotoxaemia (ME) theory and Firmicutes to *Bacteroidetes* ratio. According to this theory obese people and mice should demonstrated a higer percentage of *Bacteroidetes* and proportionally less *Firmicutes* than in lean (Cani & Delzenne, 2007). Similar results were

presented by Schwiertz and co-workers with the body Mass index (BMI). This article shows increase ratio of *Firmicutes to Bacteroidetes* with the obesity as well (Schwiertz *et al.*).

2.5.3 Diabetics and the Dominant Groups of Digestive Tract Bacteria

Diabetes also another growing epidemic is in the United States. Around 8% of adults are affected by this problem. This has potential of likely increase with the increase of child hood obesity (Robertson *et al.*, 2005). Modulating gut microbial population could be beneficial for controlling diabetic by improving glycemic control and insulin sensitivity. The effect of gut microbiota on glycemia and insulinemia are not yet fully understood. Moderate increase of plasma concentration of the bacterial lipopolysaccharide (LPS) may be responsible for the onset of metabolic diseases (Cani *et al.*, 2008; Knauf *et al.*, 2008). Modulation of gut microbiota by antibiotics such as norfloxacin and ampicillin improves whole body glucose tolerance and reduces hepatic steatosis/ fatty liver diseases. This could be used as a therapeutic strategy on controlling gut microbiota for treating or managing type 2 diabetes by reducing plasma LPS levels (Membrez *et al.*, 2008).

2.6 Analyzing the GI Microbiota

2.6.1 Culture Based Techniques

Plate based culture phenotypic characterization method is the traditional method for the identification and quantification of bacteria. Even though most of the data available on the gut bacteria have been generated by cultivation and enumeration, it has some drawbacks. Those are inability to detect non-cultivatable bacteria and unknown species. Only 10% to 40% of complex bacterial communities cultured through the selective growth media and special growth conditions (Ott *et al.*, 2006).

2.6.2 Nucleic Acid Based Methods for Analysing the GI Microbiota

Molecular techniques based Methods for analyzing the GI Microbiota have become popular in recent studies, since they do not require cultivation of microbes. It is evident that 10% to 40% or less of complex gut bacterial communities cultured through the selective growth media with special growth conditions. So that majority of the GI tract bacterial species cannot be cultivated. Due to that molecular methods based on 16S rRNA or 16S rDNA are more popular for analysis of gut microbiota. Those are namely fluorescent in situ hybridization, rRNA-targeted dot-blot hybridization with probes, denaturing gradient gel electrophoresis, temperature gradient gel electrophoresis, and cloning and sequencing of rDNA (Rinttila *et al.*, 2004; Rajilic-Stojanovic *et al.*, 2007; Zoetendal *et al.*, 2008).

Early 2006, almost 900 rRNA gene based phylotypes originating from the human GI tract were available in public sequence databases (Rajilic-Stojanovic *et al.* 2007). The main phyla recognized by 16S rRNA gene sequencing-based studies are Firmicutes, *Bacteroidetes, Proteobacteria, Actinobacteria, Fusobacteria* and *Verrucomicrobia (*Eckburg *et al.*, 2005; Wang *et al.*, 2005; Dethlefsen *et al.*, 2006).

2.6.2.1 Real-time Polymerase Chain Reaction (PCR)

Real-time PCR have several names. Those are kinetic PCR, qPCR, qRT-PCR and RT-qPCR, which is quantitative PCR method for the determination of cope number of PCR templates such as DNA or cDNA in a PCR reaction. Higuchi and colleagues invented Quantitative real-time PCR in 1993. The first application was done using fluorescence of double-stranded DNA (dsDNA)-bound ethidium and detect the accumulation of amplified DNA in the reaction (Higuchi *et al.*, 1993).

Real-time PCR is based on the ability of quantifying the PCR product. There are several ways to detect the fluorescent PCR product. Those are Taqman PCR method and SYBER green method.

(a) TaqMan Real-time PCR Method

The principle of TaqMan real-time PCR is based on Thermus aquatic DNA polimarase cleaved fluorescence probe (TaqMan probe), which is designed to be complementary to a specific sequence spanned by the PCR primers. The TaqMan probe has a reporter dye at its 5́ end and a quencher dye at its 3́ end. The Quencher dyes usually FAM (6-carboxyfluorecein) and TAMARA (6- carboxyl- tetra methyl -rhodamin). Using DNA binding dye more DNA copied created and florescence increased.

SYBR Green method is an alternate method for TaqMan, which is used to perform real-time PCR analysis. SYBR green is the most frequently used DNA binding dye in RT-PCR. SYBR Green is a dye that binds the minor groove of double stranded DNA, then intensity of the fluorescent emissions increases with more double stranded amplicons. Figure2. 9 shows the entire process of each type of realtime chemistry.

Running dissasociation curve is important for SYBER green based real-time PCR Method. In SYBR Green RT-PCR, the SYBR Green dye can bind and detect any double stranded DNA as well as primer dimers. It is also able to detect contaminating DNA, and PCR product from misannealed primer. To prevail over these errors after the 40 reaction cycles, perform a dissociation curve following the real time PCR is needed.

At the melting temperature, melting of the double-stranded DNA is indicated by a significant reduction in fluorescence .The real-time instruments generally plot disassociation

curves as a first derivative of decreasing the fluoracenc over Time. The Single peaks indicate a specific targeted product, without primerdimer artifact or contaminants, as in Figure 2.10

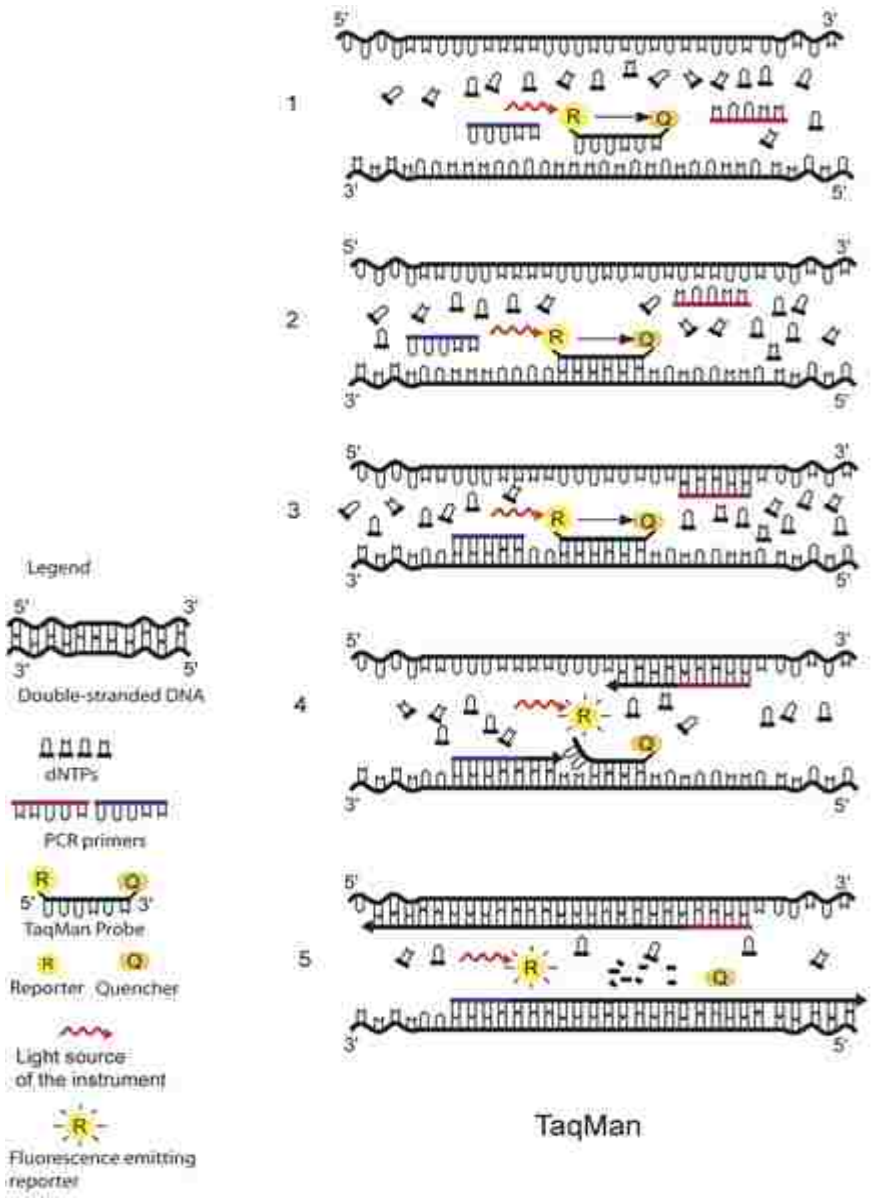


Figure 2. 8 The Principle of TaqMan Real-time PCR: (1)Reporter and the quencher dyes are in close proximity, no fluorescence signal is emitted due to the quenching effect (black arrow in 1, 2, and 3), (2) Soon after the annealing of the TaqMan probe (3) and the primers (4), the primers are extended by the DNA polymerase. As the polymerase reaches the TaqMan probe, it uses its exonuclease activity to remove the probe one nucleotide at the time (5). This releases the reporter from the proximity of the quencher and allows for the release of a fluorescence signal from the reporter (http://www.dpd.cdc.gov).

(b) SYBR green Real-time PCR Method

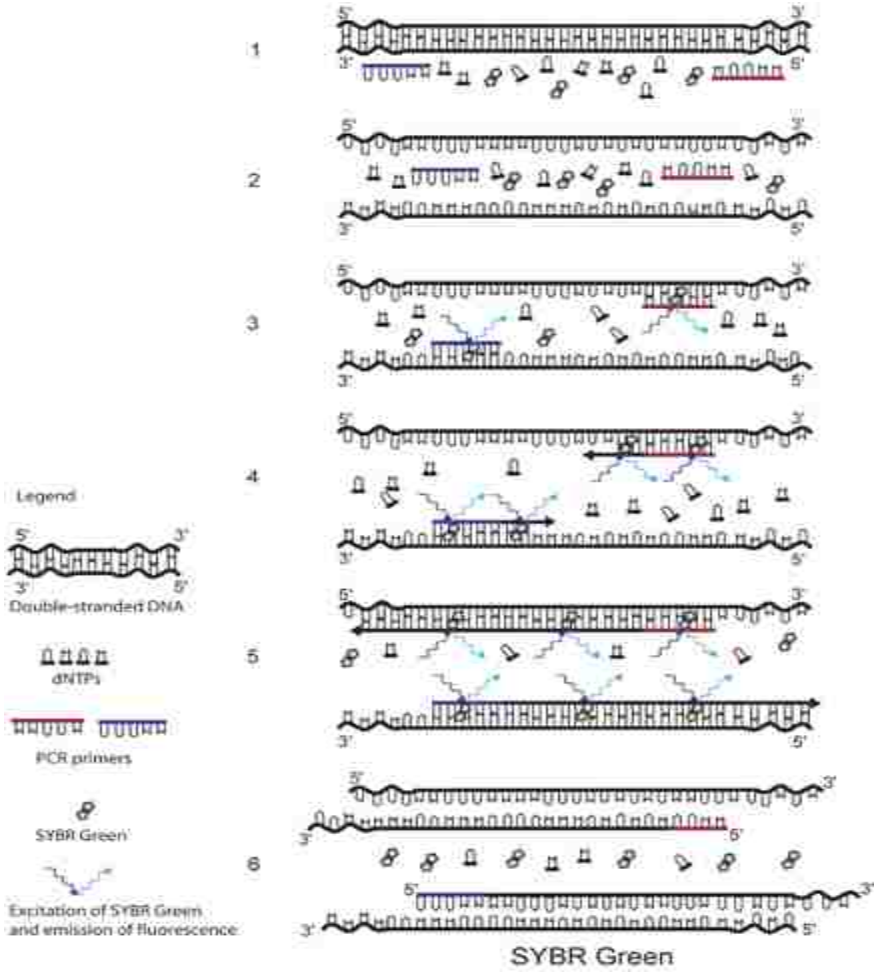


Figure 2. 9 The Principle of SYBR Green Real-time PCR:(1-2). The fluorescent dye SYBR Green is added to the PCR mixture (3). SYBR Green fluoresces strongly when bound to double-stranded DNA. (4-5). As the reaction proceeds and PCR product accumulates, the amount of double-stranded DNA increases and with it the fluorescence signal. (6). The signal is only detectable during annealing and extension, since the denaturation step contains predominantly single-stranded DNA. (http://www.dpd.cdc.gov).

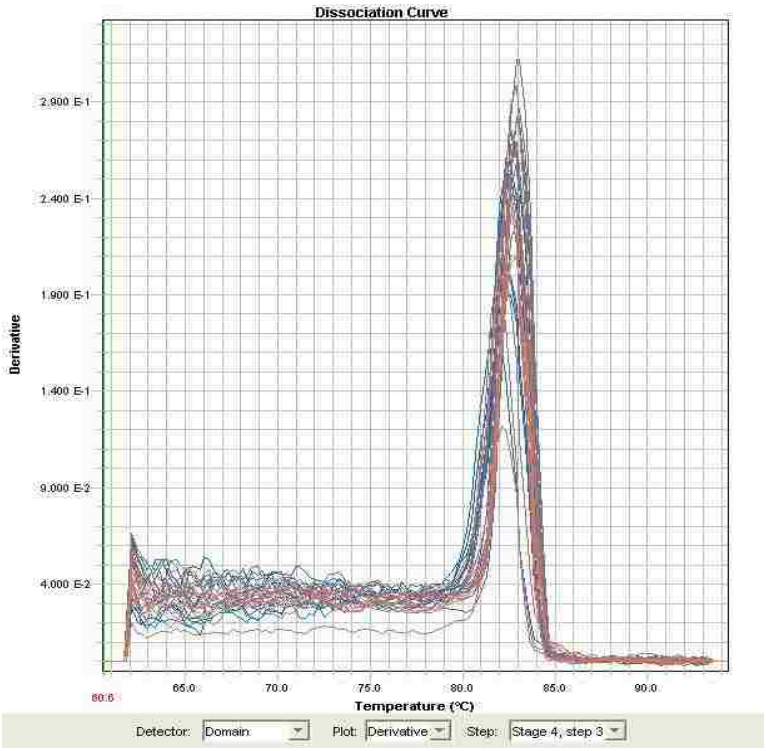


Figure 2.10 Dissasociation Curve Profile: the inflection point occurs at 82.5 ^{o}C

2.7 References

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CHAPTER 3: CHANGES IN GUT MICROBIOTA BY DIETARY RESISTANT STARCH VERSUS ENERGY CONTROL DIET WITH LOW OR HIGH FAT LEVELS

3.1 Introduction

Overweight and obesity are major health concerns among developed countries; this has caused rapid growth of the weight reducing industry. Research into the beneficial effects of RS in controlling overweight and obesity has recently become a high priority of NIH (http://www.nih.gov/).

Like other ecosystems, the digestive microbial ecosystem is a complex system. Even though our gastro-intestinal system is bacteria free when we are in the mother's womb, rapid bacterial proliferation begins soon after birth (Tannock *et al.*, 1990). Within 24 hours after birth a newborn baby's feces contains a variety of bacteria such as *Coliform bacteria*, *Enterococcus*, *Lactobacilli*, *Staphylococci* and some *Clostridia spp.* Within the next 3-4 days after birth, *Bifidobacterium spp* starts to colonize the gut and become dominant (Mitsuoka, 1978). After the weaning stage, the intestinal micro-flora change rapidly due to addition of different prebiotics (especially if breast fed) such as different carbohydrates and other dietary components in weaning foods that promote a baby's growth and the continued development of intestinal function (Edwards & Parrett, 2002). Gradually the microbial ecosystem becomes similar to adults. A normal intestinal tract has around 10^{14} bacteria with more than 1000 different species (Rajilic-Stojanovic *et al.*, 2007). This wonderful ecosystem plays a vital role in safeguarding the health of the gut (Tlaskalova-Hogenova *et al.*, 2004; Mitsuoka, 1978). As a consequence of the digestive microbial ecosystem being very complex, it is not very well characterised. Dietary changes regulate both quantitative and qualitative changes in microbial communities. The main factors affecting the microbial communities are: bacterial metabolism, competition and gut environment such as pH, oxygen and hydrogen; metabolite concentrations; the host's physical and chemical environment in the gastro-intestinal tract such as the duration that food stays in the

gastrointestinal tract; and host secretions such as acids, enzymes and hormones (Louis *et al.*, 2007; Rudi *et al.*, 2009). Intestinal anaerobic and facultative anaerobic microbial communities help us in numerous ways; mainly by hydrolyzing non-digestible plant polysaccharides such as hemicelluloses, pectin, cellulose, resistant starch, lignin and proteins, by chemically altering conjugated bile acids, and by the synthesis of vitamins B12 and K.

Prebiotics such as resistant starch stimulate the growth of beneficial bacteria in the colon, with the potential to increase fat oxidation and reduce body fat while maintaining bowel health (Ferguson *et al.*, 2000; Higgins, 2004; Higgins *et al.*, 2004; Higgins *et al.*, 2006; Keenan *et al.*, 2006; Shen *et al.*, 2009; Zhou *et al.*, 2009). Overweight and obesity are major health concerns among developed countries; this has caused rapid growth of the weight reducing industry. Research into the beneficial effects of RS in controlling overweight and obesity has recently become a high priority of NIH (http://www.nih.gov/). To understand the real picture of lowering body fat microbial analysis is needed.

Short chain fatty acids (SCFA) such as acetate, propionate, and butyrate have been found in greater concentration in the feces of those consuming a diet containing RS (Hold *et al.*, 2003). These three SCFA account for 90- 95% of the total fatty acids produced by gut microflora (Wang *et al.*, 2004). Colonic physiological processes and maintenance of normal bowel function are affected in part by SCFA. Increases in SCFA reduce pH values in the colon. This acidic environment reduces the growth of pH sensitive pathogenic organisms, the absorption of toxic alkaline compounds and the carcinogenic potential in the gut (Bird, Brown, & Topping, 2000). For example, in Inflammatory Bowel Disease (IBD) patients have lower levels of SCFA when compared to healthy individuals (van Nuenen *et al.*, 2004).

Benefits of butyrate and physiology of butyrate metabolism in the large intestine have been intensively studied by several investigators (Mortensen & Clausen, 1996; Hijova & Chmelarova, 2007). It is the major SCFA in providing protection against cancer and ulcerative colitis by reducing cell proliferation, by blocking the absorption of cancer-causing substances and by making the colon less vulnerable to DNA damage. It also helps to boost the absorption of calcium to maintain a healthy epithelium (Hagopien *et al.*, 1977 ,Cummings & Macfarlane, 1991, Gibson *et al.*, 1998).

The butyrate producing bacteria fall under the Clostridium clusters I, III, IV, XI, XIVa, XV and XVI. The majority of the bacterial species that are butyrate producers are included in two Clostridium clusters - Clostridium cluster IV and Clostridium cluster XIV. Duncan and his collaborators initially discovered *Coprococcus* spp. and *Roseburia spp.* classified in Clostridium cluster XIV and the species *Faecalibacterium prausnitzii* which is classified in Clostridium cluster IV. These species are associated with production of butyric acid from acetate. However, these bacteria do not utilize lactate for production of butyrate (Duncan *et al.*, 2002; Cani & Delzenne, 2007). Later this same research group discovered lactate utilizing bacteria from human feces that produce butyrate as their main fermentation end product. *Eubacterium hallii* and *Anaerostipes caccae* from Clostridium cluster XIV were able to utilize both the D and L isomers of lactic acid while *C. indolis* from Clostridium cluster XIVa was only able to use D lactic acid (Duncan *et al.*, 2004b). *Eubacterium limosum* from Clostridium cluster XIVb also was able to convert lactate into acetate and butyrate in the presence of *Bifidobacterium longum* (Sato *et al.*, 2008).

Thus, resistant starch is a prebiotic and its fermentation results ultimately in production of butyrate through actions of several bacteria. Production of butyrate from fermentation of dietary

resistant starch is associated with reduced body fat in rats on a low fat diet (Keenan et al., 2006, Shen et al., 2009). However, it is known that adding higher than usual fat to the diet of the ruminant (Ferguson et al., 1990; Harvatine & Allen, 2006) or adding high fat to an *in vitro* model of the rumen (Ferguson et al., 1990) reduces fermentation. Therefore, the aim of this experiment was to investigate the effect of a high fat diet on gut microflora involved with fermentation of RS. Female C57bl/6J mice were fed either a low (18% of dietary energy) or high fat (41% of dietary energy) diet with RS either presents (27% w/w) or absent in the diet.

3.2 Material and Methods

3.2.1 Animals and Diets

Thirty-six C57bl/6J female mice ranging in age from 10 to 15 weeks were stratified by age and body fat and fed one of four diets, Low fat (Lo fat) Energy Density Control, High fat (Hi fat) Energy Density Control, Lo fat RS, or Hi fat RS, for two weeks with two levels of energy as fat (18% and 41%) and two levels of RS (0 and 27% w/w). Control diet contained cellulose to equalize the energy density to the RS diet. The diet table is shown in Table 3.1. Mice ceca were tied with threads, separated from the rest of the GI tract, and aseptically transferred to separate Whirl-pak bags. Whirl-pak bags were placed in a double Zip lock bag with an anaerobic GasPak™ EZ Gas generating Pouch System (BBL GAS PAK, Voiglobal Distribution INC. P.O. Box 1130, Lawrence, Kansas 66044-8130 USA) and immersed in ice.

3.2.2 Microbial Analysis of the Cecal Contents

3.2.2.1 Direct Plating

Anaerobic and aerobic bacterial counts were enumerated using plate count methods. Ceca and contents were diluted 1:4 with peptone buffer solutions (PBS) and serial dilutions were made.

Table 3.1 Diet Compositions

Ingredients	Energy Density Control				RS diet			
	High Fat		Low Fat		High Fat		Low Fat	
	grams	kcal	grams	kcal	grams	kcal	grams	kcal
Amioca® Cornstarch[1] 100% amylopectin	384.7	1346.5	450.7	1577.5	0	0	70	245
Hi-Maize® Cornstarch[1] 60% Amylose/ 40% Amylopectin	0	0	0	0	480.7	1346	480.7	1346
Sucrose	100	400	100	400	100	400	100	400
Casein	170	608.6	170	608.6	170	608.6	170	608.6
Soybean Oil[2]	100	845	70	591.5	100	845	70	591.5
Lard[2]	100	880	0	0	100	880	0	0
Cellulose	96	0	160	0	0	0	60	0
Mineral Mix	35	30.8	35	30.8	35	30.8	35	30.8
Vitamin Mix	10	38.7	10	38.7	10	38.7	10	38.7
Choline Chloride	1.3	0	1.3	0	1.3	0	1.3	0
L-Cystine	3	12	3	12	3	12	3	12
Total	1000 g/kg	3.3 Kcal/g	1000 g/kg	4.2 Kcal/g	1000 g/kg	4.2 Kcal/g	1000 g/kg	3.3 Kcal/g

1. **Amioca®and Hi-Maize® cornstarches were gifts from National Starch and Chemical Company (Bridgewater, NJ). Hi-Maize® cornstarch consists of 56% resistant starch determined by the Englyst method (Englyst et al., 1992) as provided by National Starch and Chemical Company.**
2. **The fat levels in the low and high fat diets were 18% and 41% of energy, respectively.**

3M Petrifilms were used to detect total *E. coli*-coliform and the Total Enterobacteriaceae family (3M Microbiology.St. Paul, MN).One milliliter of each dilution was plated onto *E. coli*-Coliform Count Plates, and Enterobacteriaceae Count Plates, and plates were aerobically incubated at 37°C for 48 hrs. Lactic acid bacteria (LAB) were enumerated by using de Man-Rogosa-Sharpe Agar (MRS agar) (Difco, Laboratories, Detroit, Michigan, USA). Reinforced Clostridial agar (Oxoid, Basingstoke, UK) plates were used to quantitate total anaerobic and Clostridial counts. The MRS agar plates were anaerobically incubated at 30°C for 48 hrs and reinforced Clostridial agar plates were anaerobically incubated at 37°C for 3-4 days in a chemically generated anaerobic system using anaerobic GasPak™ EZ in an anaerobic box (Mitsubishi Gas Chemical America, Inc., New York, N.Y.). Then the totals Colony Forming Units (CFU) were determined.

3.2.2.2 DNA Extraction

The above mentioned 1:4 diluted ceca samples which were used for direct plating were also used for DNA extraction. DNA was extracted using a QIAamp DNA Stool Mini kit (QIAGEN, Valencia, CA) using the manufacturer's instructions with slight modifications. After adding stool lysis (ASL) buffer to samples of the diluted cecal contents (200 µl), the samples were subjected to three cycles of freeze-thaw in liquid nitrogen and 5 min at 95°C in water bath to break the thick gram positive bacterial cell walls. Purified DNA was quantified using a NanoDrop Spectrophotometer and all DNA sample extracts were diluted to 1ng/µl. Purified DNA was stored in a -80 ^{o}C freezer.

3.2.2.3 Quantitative Real-time PCR

The SYBR® Green method of quantitative real–time PCR (qRT-PCR) assay was performed using an ABI Prism 7900HT Sequence Detection System (serial 100151) (Applied

Biosystems, Foster City, CA). The information of the targeted bacterial groups, primer sequences, annealing temperatures and literature references are given in Table 3. 2. All reactions were performed in sterile MicroAmp® optical 384-well reaction plates with barcode sealed with MicroAmp® optical adhesive film (Applied Biosystems, Foster City, CA). All reactions consisted of 5µl of 2X SYBR Green Master Mix (Applied Bio systems, Foster City, CA,USA), 0.5 µl of each primer at 10 µM, 0.5 µl of bovine serum albumin (BSA) (final concentration 250 mg /ml), 0.5 µl of nuclease free water and 3 µl of DNA template in a 10 µl total volume. The cycling conditions were 50°C for 2 min, 95°C for 10 min, then 40 cycles of 95°C for 15 s, primer-specific annealing temperature (Table 3. 2) for 1 min, then 78°C for 30 s. Following amplification, a dissociation step was included to analyze the melting curve of the amplified product to determine the specificity of the amplification.

3.2.2.4 Preparation of PCR Standards for DNA Quantification

To construct a standard curve for qRT PCR bacterial dilutions were prepared, Ct values (fractional cycle number) and CFU/ml for each dilution were determined for the following bacterial species *Lactobacillus acidophilus* ATCC 4646, *Clostridium leptum* ATCC 29065, *Clostridium coccoides* ATCC 29236, *Bacteroides fragilis* ATCC 25285, and *Bifidobacterium longum* ATCC 15708. *Escherichia coli* ATCC 25947 was used to construct the total bacterial (16S universal) domain standard curve (Table 3. 2).

Bacterial cultures were grown overnight and serial dilutions were made. Then for each dilution the Ct value was measured by qRT- PCR and plated onto appropriate media to determine the actual bacterial cells present in the overnight culture (CFU/ml). For real-time PCR, 200 µl of each dilution tube was used to isolate DNA templates from the bacterial standard species listed above. DNA was isolated by the commercial QIAamp method as explained above.

qRT- PCR primers were used to amplify the16S rDNA (Table 3. 2). To determine CFU/ml unknown Ct values were compared to the Ct of the standard curves.

Table 3.2 16S rDNA qRT-PCR Primers Used to Profile Intestinal Samples

Targeted bacteria	Annealing Temp (^{0}C)	Sequence of oligonucleotide	Reference	standard curves	
				Slope	R^2
Firmicutes		F -TGG ATG CCT TGG CAC TAG GA		-3.5	0.98
Lactobacillus spp	60	R- AAA TCT CCG GAT CAA AGC TTA CTT AT	Haarman & Knol, 2006		
Clostridial cluster IV (*Clostridium leptum* subgroup, includes *Faecalibacterium prausnutzii*)	60	F- TTA CTG GGT GTA AAG GG R- TAG AGT GCT CTT GCG TA	Wise & Siragusa, 2007	-3.5	0.99
Clostridium cluster XIVa and XIVb (*Clostridium coccoides – Eubacterium rectale* subgroup)	50	F- AAA TGA CGG TAC CTG ACT AA R- CTT TGA GTT TCA TTC TTG CGA A	Matsuki et al. (2002)	-3.6	0.99
Bacteroidetes		F -GAA GGT CCC CCA CAT TG	Wise & Siragusa, 2007	-3.5	0.99
Bacteroides group including *Prevotella* and *Porphyromonas*	60	R- CAA TCG GAG TTC TTC GTG			
Actinobacteria		F –GGG TGG TAA TGC CGG ATG	Bartosch et al. (2004)	-3.6	0.99
Bifidobacterium spp	60	R- TAA GCC ATG GAC TTT CAC ACC			
Bacterial Domain	60	F - TGSTGCAYGGYYGTCGTCA R -ACGTCRTCCMCNCCTTCCTC	Belenguer, et al., 2006	-3.2	0.99
16S universal primers					

To assess the correct annealing of primers during PCR melting curves profile of each bacterium was obtained and analyzed.

3.2.3 Statistical Analysis

This study was analyzed as a completely randomized factorial with two independent variables, diet (two levels, RS or Energy control) and fat (low and high) using Two-way ANOVA to determine the main and interactive effects. Results were considered significant at $p<0.05$. Statistical analyses were done using SAS version 9.13 (SAS Institute Inc., Cary, NC). All data are presented as least square means (Ls means) with pooled SE.

3.3 Results

Using only culture based methods to examine samples from either cecal or fecal material gives a limited representation of the microbiota. Culture based methods will represent about 10 - 40% of the microbiota in the gut (Sharp & Macfarlane, 2000). Most of the anaerobic colonic bacteria need special requirements such as substrates, the colonic environment, including co-culture with other bacterial species, for proper growth in culture. Several of the culturable bacterial populations that we investigated in the current study are given in Table 3.3 Total culturable anaerobic bacteria and the total culturable LAB were incubated anaerobically; total coliform bacteria and total Enterobacteriaceae family bacteria were incubated aerobically.

Total culturable anaerobic bacteria and total culturable Lactic Acid Bacteria (LAB) were significantly affected by the dietary fat level because animals fed low fat diets had greater numbers of bacterial populations in the cecal contents than those fed high fat diets. For these two types of bacteria there was also a significant diet effect as well because animals fed RS diets had greater numbers of bacteria in cecal contents than those fed the EC diet. These results emphasize anaerobe populations and facultative anaerobes such as LAB populations increased with the RS

diets. These diet effects confirmed previous findings (Kleessen *et al.*1997; Louis, Scott, Duncan, & Flint, 2007). Total Coliform and Enterobacteriaceae family bacteria were significantly greater in the two low fat diet groups in cecal contents than for the two high fat diet groups. However, there was a significant interaction as EC diet fed animals had higher Total Coliform and Enterobacteriaceae family bacteria than the RS fed with low fat diet, but the reverse was true with animals fed the high fat diets.

Table 3.3 Total Culturable Anaerobic/Aerobic Bacterial Population

	Diets			
Bacterial Population	**EC**		**RS**	
(Log CFU)	**High Fat**	**Low Fat**	**High Fat**	**Low Fat**
Total culturable anaerobic bacteria	6.4 ± 0.07	6.98 ± 0.07	8.5 ± 0.07	9.2 ± 0.07
Total Culturable LAB	7.18 ± 0.10	7.65± 0. 10	7.88 ± 0. 10	8.42± 0.10
Total Coliform bacteria	3.99 ± 0.11	6.36 ± 0.11	5.12 ± 0.11	4.49 ± 0.07
Total Enterobacteriaceae family bacteria	3.99 ± 0.11	6.77± 0.11	5.04 ± 0.11	5.32 ± 0.11

RS, resistant starch; EC, energy density control; Factorial statistical analysis demonstrated the following results: Total culturable anaerobic bacteria (Diet, $p < 0.001$; Fat, $p < 0.053$; Interaction, NS), Total Culturable LAB (Diet, $p < 0.001$; Fat, $p < 0.014$; Interaction, NS), Total Coliform (Diet, NS; Fat, $p < 0.002$; Interaction, $p<0.0001$); Total Enterobacteriaceae family (Diet, NS; Fat, $p < 0.0002$; Interaction, $p<0.0007$).

One of our major interests for this study was the determining the changes in bacterial populations involved in the process of fermenting resistant starch (RS) to butyric acid in the ceca.

Table 3.4 Quantitative RT- PCR Results

Bacterial Populations (Log CFU)	Diets: EC High Fat	EC Low Fat	RS High Fat	RS Low Fat
Firmicutes *Lactobacillus spp*	7.99 ± 0.15	8.31 ± 0. 15	8.67 ± 0. 15	9.4 ± 0. 15
Clostridial cluster IV (*Clostridium leptum* subgroup, includes *Faecalibacterium prausnutzii*)	9.12 ± 0.22	9.49 ± 0.22	9.69 ± 0.22	10.26 ± 0.22
Clostridium cluster XIVa and XIVb (*Clostridium coccoides – Eubacterium rectale* subgroup)	8.95 + 0.32	9.00 + 0.32	9.74 + 0.32	10.31+ 0.32
Bacteroidetes Bacteroides group including Prevotella and Porphyromonas	10.06 ± 0.07	9.55 ± 0.07	9.81 ± 0.07	9.38 ± 0.07
Actinobacteria *Bifidobacterium spp*	7.56 ± 0.32	7.35 ± 0.32	8.34± 0.32	9.33 ± 0.32
Bacterial Domain 16S universal primers	10.15 ± 0. 15	10.68 ± 0. 15	10.55 ± 0. 15	11.11 ± 0. 15
Ratio of Fermicute/ Bacteroidetes	0.217	1.58	3.04	11.12

RS, resistant starch; EC, energy density control; Factorial statistical analysis demonstrated the following results: *Lactobacillus spp* (Diet, $p < 0.0004$; Fat, $p < 0.008$; Interaction, NS), Clostridial cluster IV (Diet, $p < 0.01$; Fat, $p < 0.06$, Interaction, NS), Clostridium cluster XIVa and XIVb (Diet, $p < 0.01$; Fat, NS, Interaction, NS); Bacteroides group (Diet, NS; Fat, $p < 0.02$, Interaction, NS), *Bifidobacterium spp* (Diet, $p < 0.002$; Fat, NS, Interaction, NS), Bacterial Domain (Diet, $p < 0.01$; Fat, $p < 0.003$; Interaction, NS); Ratio of Fermicutes/ Bacteroidetes (Diet, $p < 0.01$; Fat, $p < 0.03$; Interaction, NS). All primers were sensitive and specific for the group that they were targeted as demonstrated by amplification.

The total culturable anaerobic bacteria population counts are reported in Table 3.3, we used Reinforced Clostridial agar plates. In these plates different anaerobic cultures have different growth rates and appearance. For example most anaerobic cultures such as *Bacteriodes spp.* and *Clostridium spp* on this medium take relatively long periods (4-5 days) to appear on the plates while others appear within 2-3 days. Even though they eventually appear, several of these species could be hard to recognize in mixed cultures because some colonies are often tiny and colorless. These colorless tiny colonies would not be visible during counting with other colonies. However, our results were similar to previous studies with these culture techniques. For better confirmation of results, molecular methods may be more appropriate.

Bacterial populations involved in the process of fermenting resistant starch to butyric acid were further analyzed using qRT- PCR (Table 3.2) and the rsults were given in Table 3.4. Results for the Bacterial Domain and *Lactobacillus spp* showed significant effects for fat and diet because animals fed low fat diets versus high fat diets or RS diets versus EC diets had greater numbers of bacteria in the cecal contents.

Bacteria from Clostridial clusters IV and Clostridial clusters XIVa - b were significantly greater in cecal contents of mice fed RS versus EC diets. However, the effect of fat only approached significance for Clostridial cluster IV as bacteria from cecal contents from mice fed the low fat diets were greater than for those fed the high fat diet. There were also a greater number of *Bifidobacterium spp* in cecal contents of mice fed the RS versus the EC diet. There was no effect of diet on bacteria from the *Bacteroides* genus in cecal contents of mice. However, mice fed the high fat diet had greater levels of *Bacteroides* genus in their cecal contents compared to mice fed the low fat diets. The ratio of Fermicutes measured to Bacteriodetes

measured was significantly greater in cecal contents of mice fed the RS versus EC diet and lower for mice fed the high fat versus low fat diet.

3.4 Discussion

The major reason for conducting this current study was to compare effects of high and low fat diets on fermentation. In a preliminary study (unpublished data) effects of fermentation of RS were not observed in rats fed a high fat diet. The results showed the pH was not reduced, the size of the cecum was not increased, SCFA including butyrate were not increased in cecal contents, and blood levels of PYY were not increased in rats fed RS in a high fat diet (41% of energy) compared to rats fed RS in a low fat diet (18% of energy). These results were in agreement with research with high fat diets in ruminant animals, as it is known that adding higher than usual fat to the diet of the ruminant (Ferguson *et al*., 1990, Harvatine and Allen, 2006) or adding high fat to an *in vitro* model reduces rumen fermentation.

Resistant starch is defined as starch that is resistant to amylotic reaction in the small intestine. Once this resistant starch reaches the cecum of the large intestine it is fermented by the bacterial populations. Thus, resistant starch is considered a fermentable dietary fiber (DRI for Macronutrients, 2002/2005). The complex microbial community in the cecum consists of a diverse range of bacteria that are predominantly obligate anaerobes. These bacteria act together to ferment dietary substrates such as RS, producing a range of beneficial products that are favorable to maintain good health. In this study the main observation was that favorable bacterial populations which are involved in production of butyrate, such as *Lactobacillus spp, Bifidobacterium spp,* Clostridial cluster XIV a - b and Clostridial cluster IV populations were significantly higher in cecal contents of RS fed verses EC animals. Our research has demonstrated that RS increase SCFA in the cecum (Keenan et al., 2006), gene expression of

peptide YY (PYY) and proglucagon (GLP-1) in the cecum (Keenan et al., 2006; Zhou et al., 2006) and peptide levels of PYY and GLP-1 in the blood (Keenan et al., 2006; Zhou et al., 2008; Shen et al., 2009).

Production of SCFA reduces gut pH which helps to maintain a healthy colon. For example, a pH of 6.8 is considered the pH of a healthy mammalian gut, and a pH of 7.5 and greater, can be indicative of many diseases, including bowel cancer (Pitcher & Cummings, 1996; Vulevic *et al.*, 2004; Van Nuenen *et al*, 2004). Butyrate is a well known SCFA, which helps to regulate colonic health (Sakata, 1987; Mortensen & Clausen, 1996; Weaver *et al.*, 1997; Sharp & Macfarlane, 2000; Topping & Clifton, 2001; Hijova & Chmelarova, 2007). Because of these benefits we were interested in studying population changes of bacteria involved in fermentation of RS ultimately to butyrate.

The main observation in this study was favorable bacterial populations which involved the production of butyrate such as *Lactobacillus spp Bifidobacterium spp*, Clostridial clusters IV and Clostridial clusters XIVa - b and Bacteroides group population using qRT-PCR. All these populations were significantly high with the RS fed animal's ceca than EC fed animals except *Bacteroides spp*.

In the present study, we have tested total culturable anaerobic bacteria; total culturable LAB; total Coliform and Enterobacteriaceae family using plate count methods. Both total culturable anaerobic bacteria ($p < 0.001$) and total culturable LAB ($p < 0.001$) were significantly increased with the RS fed animals than EC fed group. Both total Coliform and Enterobacteriaceae family bacterial populations were not significantly different from each other with the diet type.

Lactobacillus spp and *Bifidobacterium spp* are two major probiotic groups that are used in the food industry. Some strains from these two genera start the fermentation of prebiotic by

attaching and changing the position (translocating) starch molecules (Duncan *et al.*, 2002; Louis *et al.*, 2007). Total culturable LAB populations ($p < 0.001$) in plate count method and *Lactobacillus spp* ($p < 0.0004$) and *Bifidobacterium spp* ($p < 0.002$) in Rt-PCR were significantly in mice on the RS diets. Both of these strains have beneficial health effect. Several investigators have shown that *Lactobacillus Spp*, such as *L. acidophilus, L. reuteri, L. casei* and *L. gasseri,* are able to remove cholesterol *via* various mechanisms and reduce serum total cholesterol concentration triglycerides in mice (Akalin *et al.*, 1997; Xiao *et al.*, 2003; Liong & Shah, 2005). *Bifidobacterium spp* such as *Bifidobacterium longum, Bifidus Regularis*™ / *Bifidobacterium animalis* also help to reduce serum total cholesterol concentrations in the blood aswell (Beena & Prasad, 1997; Xiao *et al.*, 2003). In addition *Bifidobacterium spp* have beneficial effects on gut health and liver health (O'Sullivan, 2008).

In this research the *Bacteroides thetaiotaomicron* species was not specifically measured, instead the Bacteroides group populations including *Prevotella* and *Porphyromonas spp* targeted primers were used enumerate the Bacteroides group. That may be the reason for not to get significant effect. In other words in this experiment, we expected significantly higher amounts of bacteria from the Bacteroides group in animals fed the RS diet. Because the *Bacteroides thetaiotaomicron* species is one of the bacterial populations reported to be attach to RS molecules to begin the fermentation process (Louis *et al.*, 2007). However, our results demonstrated no effect of diet on the Bacteroides group; this could be because of the reduction in other species within the Bacteroidetes genus. Even though Bacteria from the Bacteroides group were not significantly increased with feeding of RS, increased amounts of these bacteria were associated with feeding a high fat diet.

With the high fat diet there were significant increases of Bacteroides group ($p < 0.02$) while significant reduction of *Lactobacillus spp (*$p < 0.008$), *Bifidobacterium spp* ($p < 0.07$) Clostridial cluster IV ($p < 0.06$) and Bacterial Domain ($p < 0.003$). Similar results for high fat diet were reported by Cani and his group with non-digestible but fermentable fiber. In that research they found feeding high-fat diet reduced number of *Bifidobacterium spp*, Clostridial cluster XIV a while increasing group and Bacteroides-related mouse intestinal bacteria (Cani & Delzenne, 2007; Cani *et al.*, 2007). Furthermore Their results suggest that gram negative bacteria, which include the Bacteroides group, increase plasma lipo- polysaccharide levels which activate the inflammatory response and the onset of obesity as well as type 2 diabetes (Cani & Delzenne, 20 07). Another group of scientist has been reported *Lactobacillus Spp* have ability to remove cholesterol *via* various mechanisms and reduce serum total cholesterol concentration triglycerides in mice (Akalin *et al.*, 1997; Xiao *et al.*, 2003; Liong & Shah, 2005). Our current study animal fed hi fat EC diets have increased Bacteroides group while decreasing total *Lactobacillus spp.* According above two facts by increasing Bacteroides group while reducing *Lactobacillus spp* in the ceca badly affect for serum total cholesterol concentration triglycerides (Cani & Delzenne, 20 07; Lee *et al.*, 2006).

In this study we have used energy as fat 18% for LF diets and energy as fat 41% for HF diets. The energy values were, for LF 3.5 kcal/g and HF 4.2 kcal/g. Our results have shown reduction of total bacteria domain. Usually 98% of fat as a fatty acid is absorbed by the ileum part of small intestine and only 2% of fat enters in to the Large intestine or to the cecum (Saunders & Sillery, 1988). This 2% of fatty acid which enter in to the cecum or large intestine with the RS might affect two ways. One of them is The free fatty acid entering in to the cecum can kill some of the native commensal bacterial populations by acting as antibacterial compounds. For example it has

been studied beneficial effect of antibiotic-like action of essential fatty acids against pathogenic bacteria (Das, 1985). The other one is some starch granule may cover with fatty acid layer and it might prevent the bacterial attachments.

The Firmicutes to Bacteroidetes ratio may be important as an indicator for obesity (Ley *et al.*, 2006; Mariat *et al.*, 2009; Schwiertz *et al.*, 2009). However, the value of this ratio in favor of Fermicutes or Bacteriodites for obesity is uncertain at this time. Ley *et al.* (2006) and Mariat *et al.* (2009) report greater ratios of Fermicutes to Bacteriodetes in obese subjects. The argument for the increased ratio of Fermicutes to Bacteriodetes in overweight and obese subjects is their increased ability to ferment fermentable fibers (Fermicutes include the bacterial populations that ferment the fermentable fibers such as RS and fructans) that allows for greater harvest of energy from the large bowel. But Schwiertz *et al.* (2009) had conflicting results. According to their study overweight (1.1) and obese subjects (1.2) have a significantly lower Firmicutes to Bacteroidetes ratio in fecal material than for non-overwieght subjects (3.3). They concluded that SCFA produced by the bacteria is more important to the obesity compired to number of bacteria in the gut (Schwiertz *et al.*2009).

Our study had a greater ratio of Fermicutes to Bacteriodetes in mice fed RS, a fermentable fiber, this suggest that Fermicute bacteria can increase in numbers because they ferement RS and fructans (Sharp & Macfarlane, 2000; Louis *et al.*, 2007). Our results of a greater Fermicute to Bacteriodetes ratio with feeding of a fermentable fiber are similar to those of Schwiertz *et al.* (2010) for lean subjects. However, our value is much higher because we used a fermentable fiber in our study, which is a prebiotic that is fermented by Fermicute bacteria. Our results agree with Cani and his group's theory that Gram negative gut microbiota such as Bacteriodetes increases

with the high fat diets and that increase the plasma lipopolysaccharide levels which trigger the inflammatory tone and the onset of obesity and type 2 diabetes.

In previous studies, we showed lower abdominal fat with the consumption of low fat RS diets compared to diets with equal energy density (Keenan, et al., 2006, Shen et al. 2009, Zhou et al., 2009). These results indicated that fermentable RS has possible physiological benefits, including lowering abdominal fat, beyond energy dilution alone. The ability to ferment RS appears to be necessary for the effect of lower abdominal fat as lean mice that fermented RS had lower abdominal fat, but polygenic obese mouse models that did not ferment RS did not have reduced abdominal fat (Zhou et al., 2009). Since rodents that are fed and ferment RS are leaner than controls, it would seem reasonable that the leaner rodents would share characteristics, such as Firmicute to Bacteriodetes ratio in the large gut, with lean people. As a result of reports of different values for the ratio of Firmicutes to Bacterioidetes, more research is needed to resolve the bacterial ecology issue. However, our results of the ratio of Firmicutes to Bacterioidetes are in agreement with those of Schwiertz *et al.* (2010).

In this study, we hypothesized that bacterial fermentation of RS would be reduced and reflected by high fat diet. This result was observed for *Lactobacillus spp*., Clostridial cluster IV ($p<0.06$), the bacterial domain, and the Firmicute to Bacteriodetes ratio (qPCR results). Therefore, high fat diets may reduce beneficial results from incorporation of fermentable fibers in the diet. Our results support nutrition and health recommendations to avoid high fat diets (http://www.cnpp.usda.gov/dietaryguidelines.htm).

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CHAPTER 4: ASSESSMENT OF POPULATION VARIATION IN MICE GUT MICROBIOTA WHEN FED DIETARY RESISTANT STARCH VERSUS ENERGY CONTROL DIET WITH LOW OR MEDIUM FAT LEVELS

4.1 Introduction

A high fat western diet with high energy density is a concern in developed western countries because of health problems associated with it such as obesity, diabetes miletus, coronary heart disease and colon cancer (Assmann *et al.*, 1999; Cani & Delzenne, 2007; Cani *et al.*, 2008; Shikany *et al.*, 2009). Obesity is one of the major concerns in western countries including in the USA. According to resent survey childhood obesity was the number 1 health concern for kids in 2008 (Epstein *et al.*, 2008; Wickham *et al.*, 2009). These health issues are associated with enormous economic costs in leading public health issues in the western countries. As a result interest of dietary fiber diet has increased, because it can help to maintain a healthy body weight and reduce obesity related health issues (Burton-Freeman, 2000; Slavin, 2005). Establishment of diverse gut bacterial microbiota is unique for each individual. Consumption of high fiber diet helps to improve gut microbial ecology. For example dietary fiber such as Resistant Starch, Fructo-oligosaccharides and inulin stimulate the growth of beneficial bacteria in the colon, with the potential to increase fat oxidation and reduce body fat while maintaining bowel health (Ferguson *et al.*, 2000; Higgins, 2004; Higgins *et al.*, 2006; Keenan *et al.*, 2006; Zhou *et al.*, 2008; Shen *et al.*, 2009). Hence, the beneficial effects of RS in controlling overweight and obesity has increased interest in the scientific community.

Butyrate, one of the principal colonic SCFA, is produced by fermentation of fiber including RS diet. Studies have shown that RS protects against colorectal cancer by maintaining healthy epithelium cells in the intestinal tract (McIntyre *et al.*, 1993; Gibson *et al.*, 1998). High fat levels can interfere with the fermentation of RS into butyrate by lowering the bacterial populations in the intestinal tract. Hence high fat diets can lower the expected benefits of RS

(chapter 3). Bearing this in mind, this experiment was designed to study the involvement of moderate levels of fat with RS fermentation using C57bl/6J female mice.

4.2 Material and Methods

4.2.1 Animals and Diets

Thirty-six C57bl/6J female mice ranging in age from 10 to 15 weeks were stratified by age and body fat divided into 2 x 2 factorial four groups (n=9). All four mice groups were fed the control diet for the first six weeks prior to dietary treatment to observe energy intake and body weight gain. Then they were fed one of four diets, Low fat (Lo fat) Control, Moderate fat (M fat) Control, Lo fat RS, or M fat RS. Both M fat diets had equal energy density: 26% fat and Lo fat diets had 18% fat to equalize the energy density of the two diets; increased cellulose was added to the EC diet (Table 4.1).Mice were fed the diets for 10 weeks and at the end of the study, mice were sacrificed

Mice ceca were tied with threads, separated from the rest of the GI tract, and aseptically transferred to separate Whirl-pak bags. Whirl-pak bags were placed into a double Zip lock bag with an anaerobic GasPak™ EZ Gas generating Pouch System (BBL GAS PAK, Voiglobal Distribution INC. P.O. Box 1130, Lawrence, Kansas 66044-8130 USA) and immersed in ice.

4.2.2 Microbial Analysis of the Cecal Contents

4.2.2.1 Direct Plating

Total Anaerobic and Lactic acid (LAB) bacterial counts were enumerated using plate count methods. Ceca contents were diluted 1:4 with peptone buffer solutions (PBS) and serial dilutions were made. LAB counts were enumerated by using de Man-Rogosa-Sharpe Agar (MRS agar) (Difco, Laboratories, *Detroit*, *Michigan*, USA). Reinforced Clostridial agar (Oxoid, Basingstoke, UK) plates were used to quantify total anaerobic and Clostridial counts.

Table 4.1 Diet Compositions

Ingredient (g/kg)	**Energy Control**		**RS diet**	
	Moderate fat	**Low Fat**	**Moderate fat**	**Low Fat**
Corn starch	432.5	424.5	0	0
sistant starch	0	0	540.7	530.7
Sucrose	100	100	100	100
Casein	200	200	200	200
Soybean oil	70	70	70	70
lard	40	0	40	0
Cellulose	108.2	156.2	0	50
Mineral mix (AIN-93G)	35	35	35	35
Vitamin mix (AIN-93G)	10	10	10	10
Choline chloride	1.3	1.3	1.3	1.3
L-cystine	3	3	3	3
Metabolizable Energy	1000g/kg (3.7kcal/g)	1000g/kg (3.3kcal/g)	1000g/kg (3.7kcal/g)	1000g/kg (3.3kcal/g)
Fat content	11%(wt/wt) 26% (cal/cal)	7%(wt/wt) or 18% (cal/cal)	11%(wt/wt) 26%(cal/cal)	7%(wt/wt) or 18% (cal/cal)

3. **Amioca®and Hi-Maize® cornstarches were gifts from National Starch and Chemical Company (Bridgewater, NJ). Hi-Maize® cornstarch consists of 56% resistant starch determined by the Englyst method (Englyst et al., 1992) and provided by National Starch and Chemical Company.**
4. **The fat levels in the low and moderate fat diets were 18% and 26% of energy, respectively.**

The MRS agar plates were anaerobically incubated at 30°C for 48 hrs and reinforced Clostridial agar plates were anaerobically incubated at 37°C for 3-4 days in a chemically generated anaerobic system using anaerobic GasPak™ EZ in an anaerobic box (Mitsubishi Gas Chemical America, Inc., New York, N.Y.). Then the total Colony Forming Units (CFU) were determined.

4.2.2.2 DNA Extraction

Bacterial DNA was extracted using the same method explained in Chapter 3**.** The above mentioned 1:4 diluted ceca samples were used for direct plating for DNA extraction with QIAamp DNA Stool Mini kit (QIAGEN, Valencia, CA). All the manufacturer's instructions were followed with slight modifications. Briefly adding stool lysis (ASL) buffer to the diluted cecal contents (200 µl), the samples were subjected to three cycles of freeze-thaw in liquid nitrogen to a 5 min at 95°C water bath to break the thick Gram-positive bacterial cell walls. After the extraction of DNA the purified DNA was quantified using a NanoDrop ectrophotometer and all DNA extract samples were diluted to 1ng/µl. Purified DNA was stored at -80 ^{o}C freezer until used.

4.2.2.3 Quantitative Real-time PCR

The SYBR® Green method of quantitative real–time PCR (qRT-PCR) assay was performed using an ABI Prism 7900HT Sequence Detection System (serial 100151) (Applied Biosystems, Foster City, CA). The information of the targeted bacterial groups, primer sequences, annealing temperatures and literature references are given in Chapter 3, Table 3.2. All reactions were performed in sterile MicroAmp® optical 384-well reaction plates with barcode sealed with MicroAmp® optical adhesive film (Applied Biosystems, Foster City, CA) with Chapter 3 bacterial samples. Hence construction of a standard curve for real-time PCR bacterial

dilutions, Targeted bacteria, Annealing Temperature (^{o}C), Sequence of oligonucleotide and information of the standard curves were exactly same as done in Chapter 3.

4.2.3 Statistical Analysis

This study was analyzed as a completely randomized factorial with two independent variables, diet (two levels, RS or Energy control) and fat (low and Medium) using Two-way ANOVA followed by least significant difference if the ANOVA F value was significant, $p<0.05$. Statistical analyses were done using SAS version 9.13 (SAS Institute Inc., Cary, NC).

Again this study was analyzed by statistical comparisons of all pairs using the Student's t test following 1-way analysis of variance (ANOVA) (JUMP In version 7.0, SAS Institute Inc., Cary, N.C., U.S.A.). Statistical significance occurs for $P<0.05$.

4.3 Results

The results of both Total culturable anaerobic bacteria and Total culturable Lactic Acid Bacteria (LAB) bacteria are given in Figurer 4. 1 and Figurer 4.2. The statistical significant levels $t < 0.05$ were indicated in either gray or red color.

Total culturable anaerobic bacteria and Total culturable LAB were not affected by the dietary fat level in $p<0.05$ significant level. Both of these two types of bacteria were showing significant diet effect, for animals fed RS diets with significantly higher bacterial counts in cecal contents than those fed EC diets. Both of these populations have significantly higher population in RS fed animals ($p<0.0001$). These results emphasize anaerobe populations and facultative anaerobes such as LAB populations increased with the RS diets but these levels were not affected with a moderate amount of fat.

As we discussed in the Chapter 3, the bacterial populations involved in the process of fermenting resistant starch to butyric acid were further analyzed using qRT-PCR (Figure 4.3-

Figure 4.8). Even though culture based bacterial analysis methods to examine cecal samples gives a limited representation, this culturable method gives opportunity to isolate colonies for future studies. It also gives a good overview on total culturable populations.

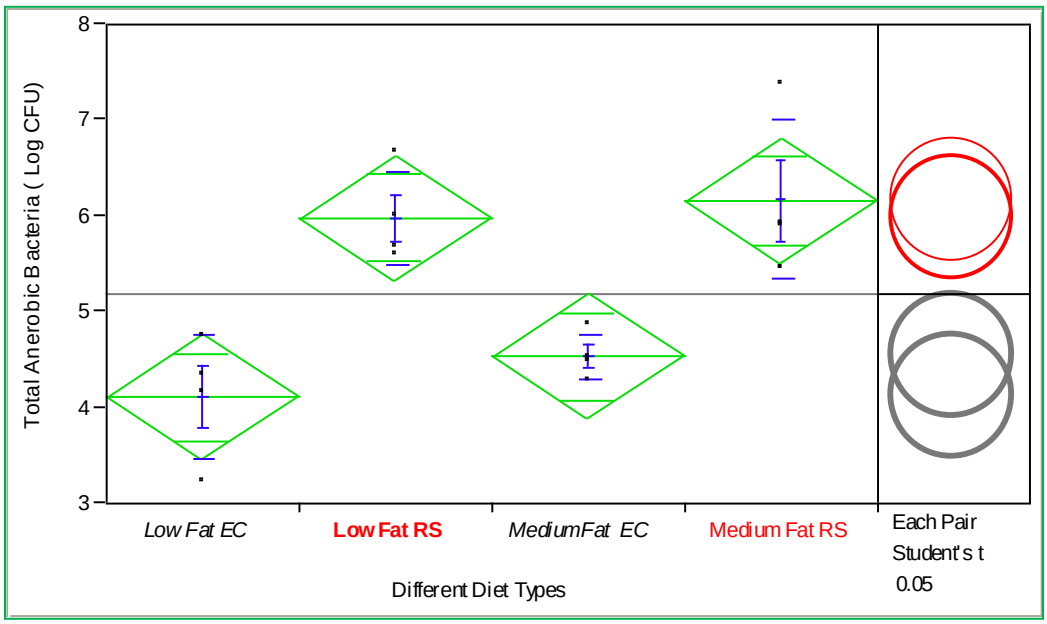

Figure 4.1 Analysis of Total Anaerobic Bacteria Counts: Fat NS, Diet p< 0.001, Fat x Diet NS

All three Total bacterial domain, *Lactobacillus spp*., and *Bifidobacterium spp.* which analized using qRT-PCR were comparable to the Plate count results. Low and Moderate fat levels were not significant and no Fat and Diet interactions were detected for p<0.05 significant levels. RS fed animals had higher levels of Total bacterial domain, *Lactobacillus spp*., and *Bifidobacterium spp.* compared to the EC diet (p< 0.0001). The total bacterial domain Lo EC diet represented significantly lower bacterial population than Lo RS (p< 0.0001) and M RS (p < 0.003) diets. Similarly, bacterial populations in M EC diet fed mice ceca were significantly lower than Lo RS (p<0.0001) and M RS (p< 0.0005) diets. Similar results were found for *Lactobacillus spp*., and *Bifidobacterium spp.*

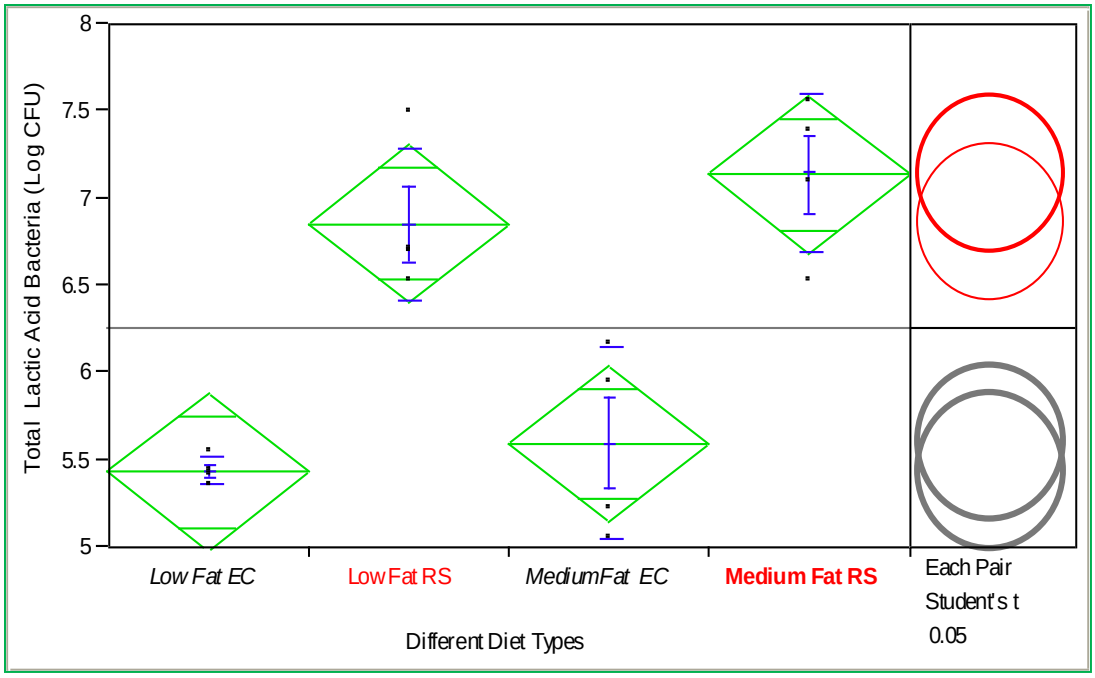

Figure 4.2 Analysis of Culturable *Lactic acid bacteria* Counts: Fat NS, Diet p< 0.0001, Fat x Diet NS RS, resistant starch; EC, energy control

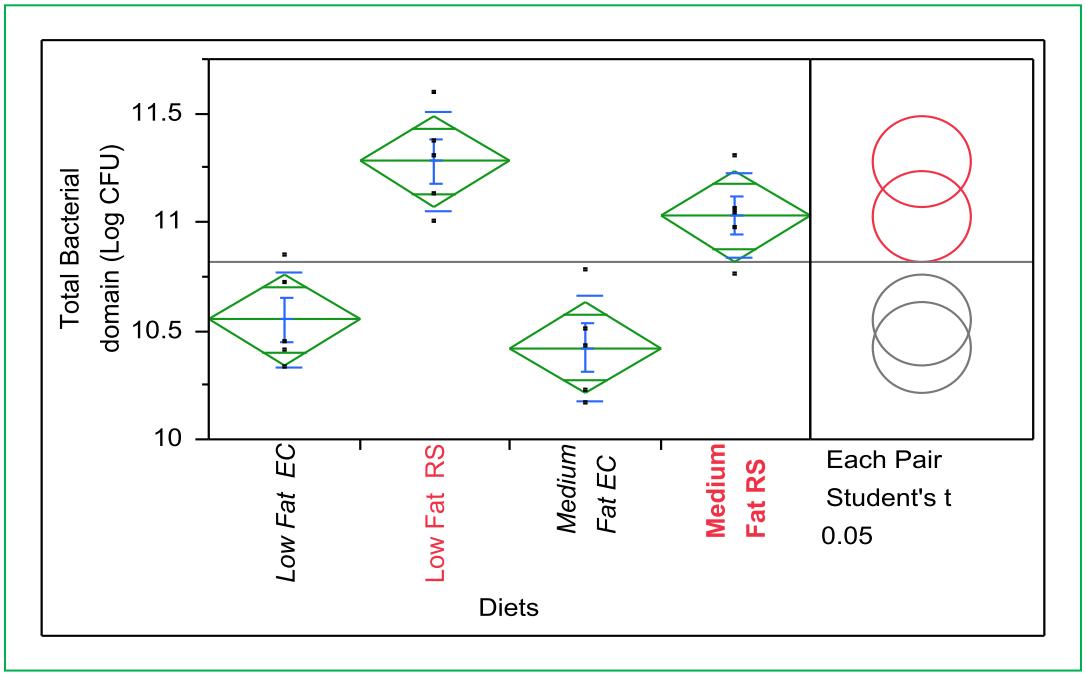

Figure 4.3 Analysis of Total Bacterial Domain Counts: Fat NS, Diet p< 0.0001, Fat x Diet NS

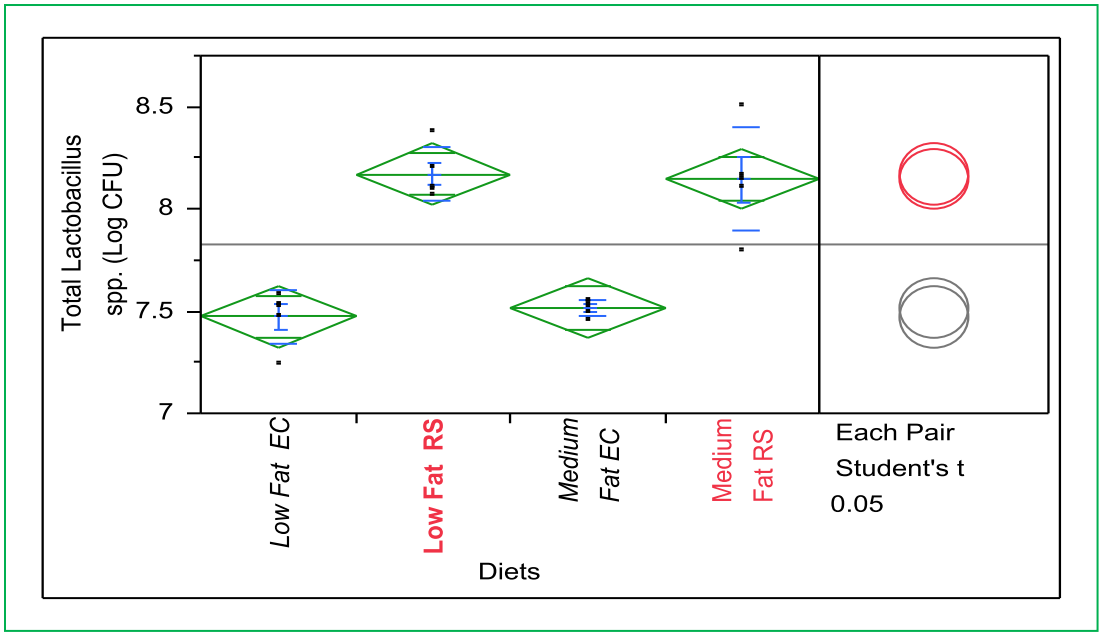

Figure 4. 4 Analysis of *Lactobacillus spp.*Counts: Fat NS, Diet p< 0.0001, Fat x Diet NS

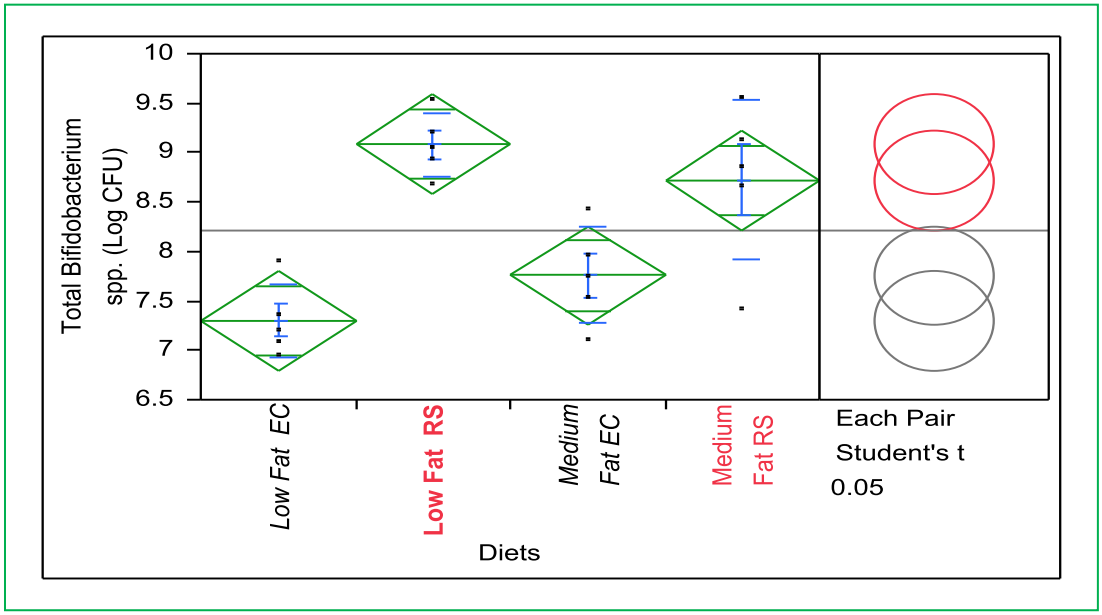

Figure 4.5 Analysis of Culturable *Bifidobacterium Spp.* Counts: Fat NS, Diet p< 0.0001, Fat x Diet NS

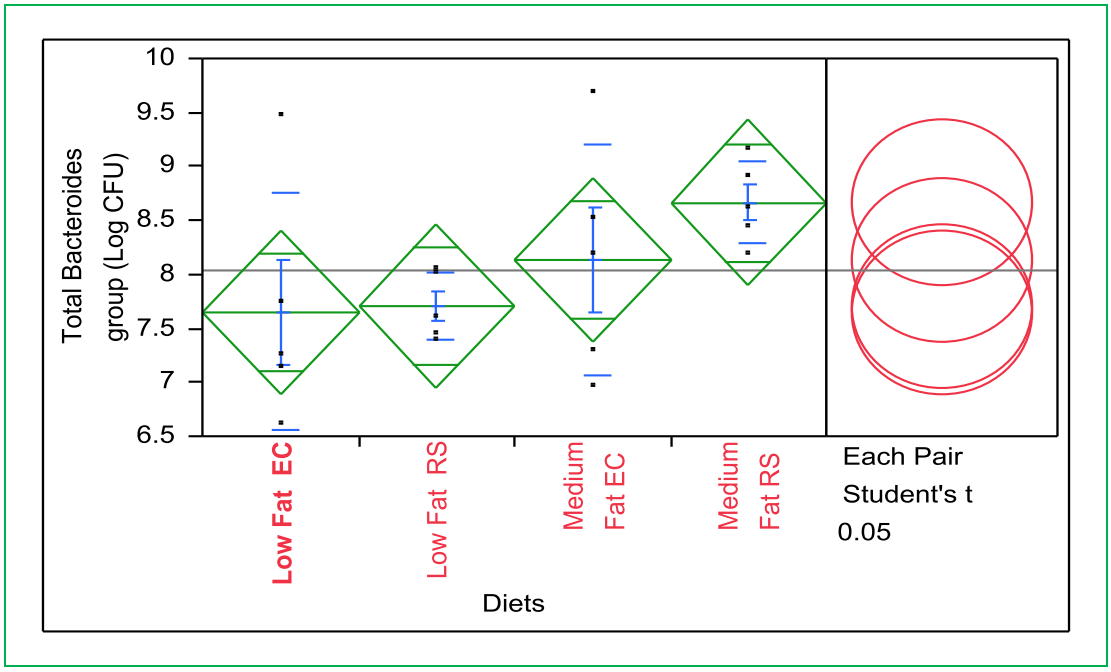

Figure 4. 6 Analysis of Bacteroides group Counts: Fat p< 0.062, Diet NS, Fat x Diet NS

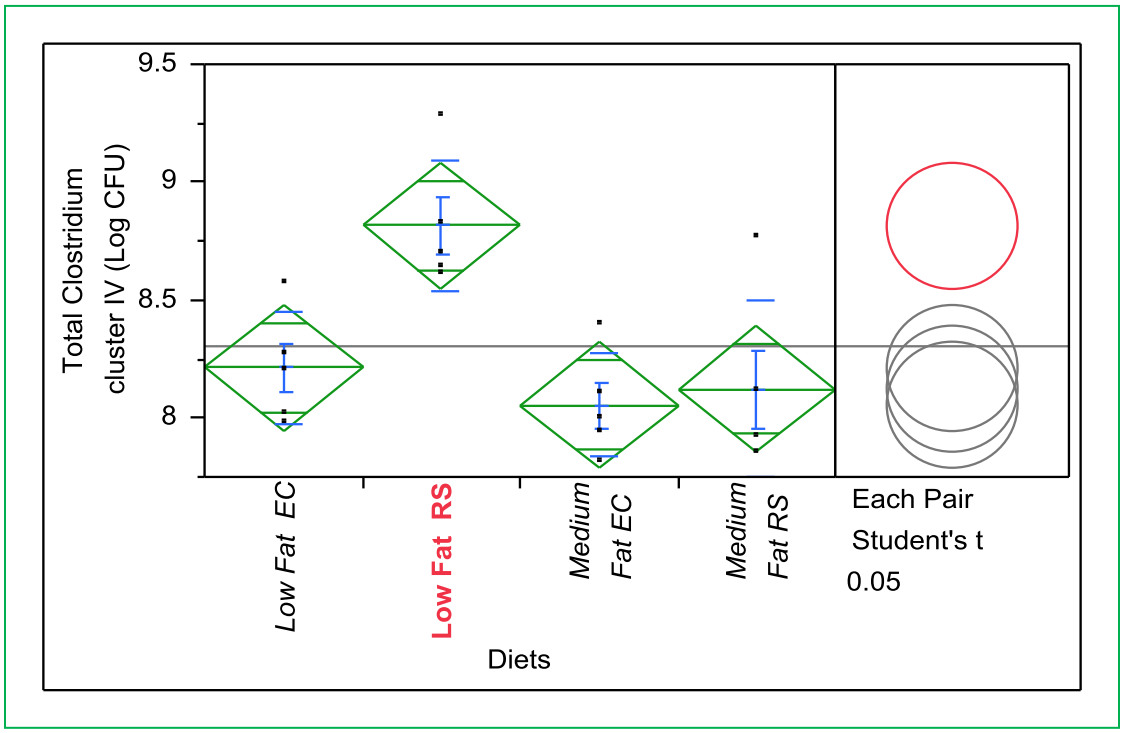

Figure 4.7 Clostridium cluster IV Counts : Fat p< 0.004, Diet p< 0.0175, Fat x Diet p< 0.049

Both Fat levels and Fat and Diet interactions were not significantly detected for Bacteroides group for $p<0.05$ significant level however these fat levels were significant for $p<0.06$. The type of diet the mice were fed had no effect on the bacterial numbers for the Bacteroids group in the cecal contents. However, mice fed the moderate fat diet had higher counts of *Bacteroides* group in their cecal contents compared to mice fed the low fat diets.

The Clostridium cluster group IV the fat levels were significant $p<0.004$ hence low fat diet fed mice ceca had significantly higher Clostridium cluster group IV than the medium fat diet fed mice ceca. Diet levels were also significant ($p<0.01$), in other words RS diet fed animals ceca had significantly higher Clostridium cluster group IV populations. At the same time Fat and diet interactions also significant ($p< 0.05$).

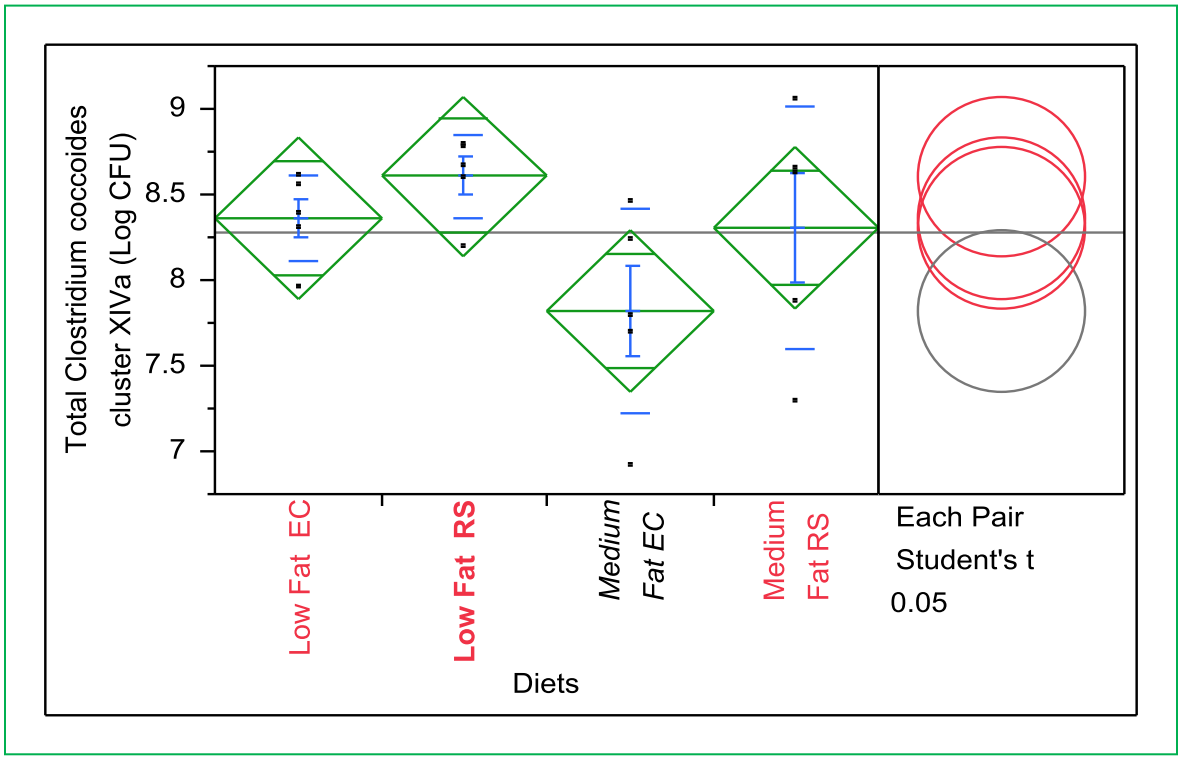

Figure 4.8 Clostridium cluster XIV a-b Counts: Fat NS, Diet NS, Fat x Diet NS

Fat levels, diet type or fat diet interactions were not significant for Clostridium cluster group XIVa counts ($p<0.05$) but for animals fed low fat levels ceca contents had higher population of Clostridium cluster group XIVa counts.

4.4 Discussion

Our previous chapter emphasized that a diet high in fat interfered with the fermentation of RS and reduced the beneficial bacterial population involved in fermenting RS to butyric acid. In this current study our main objective was to identify what effect a moderate level of dietary fat had on fermentation of RS.

Most of the analyzed cecal bacterial population did not significantly changed with moderate fat with compared to low fat diets. Those unchanged bacterial populations were total culturable anaerobic, total culurable LAB, total bacterial domain *Lactobacillus spp*., and *Bifidobacterium spp*. Although fat effect was not significant on these populations the diet levels were significant. In other words these bacterial populations were increased with RS diet with compared to EC diets. This also confirmed that as a prebiotic RS help to increase benificial bacterial populations which involved in fermentation of RS in to butyrate.

Bacteroides group observations in this study (Moderate fat versus Low fat) were similar to observations inchapter 3 (High fat RS diet versus Low fat die). The diet effect was not significant for $p<0.05$.therefor there are no effect RS on Bacteroides group population. Modarate fat fed animals ceca were observed higher population of Bacteroides group than the animal fed low fat in $p<0.06$ significance level.

The bacterial species under Clostridium cluster group IV were sensitive for the medium fat diet and the Clostridium cluster group IV group were reduced by moderate fat in mice ceca

with compared to low fat diets. At the same time the Clostridium cluster group IV group was increased with RS diet. There were fat and Diet interaction also appeared.

According this observation we could argue that it is not essential to have a low fat diet to alter the all the bacteria in gut microbial profile, by consuming a moderate amount of fat with the RS similar benefits could be obtained.

High dietary fat levels (41% of energy) had an impact on Gram positive bacteria which are involved with fermentation of fermentable fiber such as resistant starch (previous chapter). The fatty acids which enter into the cecum or large intestine might act as antibacterial compounds and interfere with bacterial cells by enhancing formation of cytoplasmic lipid droplets and affecting bacterial metabolism. In ruminants, the effect of high fat on fermentation has been well studied and fatty acids have exibited a direct effect on ruminal in-vitro (with pure cultures) bacteria (Jenkins, 1993). Our observation in this study indicated that a medium level of dietary fat (28% of energy) did not have the negative impact that high dietary fat had on fermentation. According to the observations in this chapter and in the previous chapter, the consumption of probiotics or synbiotics can be taken with a low fat or medium fat diet, but not with a high fat diet. Further studies need to be done to determine the timing of the probiotics or synbiotics and consumption of foods contining high fat. Almost all the probiotics (*Lactobacillus spp*., and *Bifidobacterium spp.*) are Gram positive bacteria, and consumption of these organisms with high fat levels might not give the expected result for consumers.

The 2% of presumably digested, but unabsorbed fat from the moderate fat diet that entered the large intestine had a similar effect as the low fat diet. Nevertheless, further investigations are needed to delineate the effect of different types of fat such as saturated, unsaturated and polyunsaturated fat in the fermentation of RS. Furthermore, investigations of

population variation of bacteria involved in RS fermentation to butyric acid in endocrine obesity and aging are needed.

4.5 References

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CHAPTER 5: ASSESSMENT OF POPULATION VARIATION OF GUT MICROFLORA IN AN IN-VITRO FERMENTATION OF PRE-DIGESTED RESISTANT STARCH WITH DIFFERENT PRE-DIGESTED FAT SOURCES BY RAT FECAL INOCULA

5.1 Introduction

Dietary resistant starch (RS) is beneficial to health in numerous ways. These benefits include, a decrease in metabolizable energy, reduction in body fat and obesity and improved bowel health. Short-chain fatty acids (SCFA), such as acetate, propionate, succinate, malate and butyrate are increased in the intestinal tract of animals and humans on a RS diet (Bird *et al.*, 2000). Adding RS to the diet promotes a greater ratio of butyrate to other SCFA and is associated with increased gene and hormone expression for peptide YY (PYY) and glucagon-like peptide-1(GLP-1) and reduced body fat(Keenan *et al.*, 2006; Zhou *et al.*, 2006; Shen *et al.*, 2009). However energy intake is not reduced in RS fed rats. Later studies demonstrated a lower respiratory exchange ratio in RS fed mice, indicating a partitioning of fat to oxidation rather than storage (Zhou *et al.*, 2008). An increase of butyrate in the intestine may be important for reduced body fat and an important component of the mechanism for the control of obesity and for improving the health of the colon. Thus, fermentation of RS in the large intestine, and production of butyrate, continues to attract attention from the scientific community because of its importance to the health.

Our previous work shows that high fat diet reduces cecal and fecal beneficial bacterial populations and fermentation in rats fed RS diets. Hence, effects of fermentation of RS were not observed in rats fed a high fat diet (Chapter 3). The results included: cecal pH was not reduced, the size of the cecum was not increased as expected, SCFA including butyrate and blood levels of PYY were not increased in cecal contents.

Diets high in saturated fat result in poor health including increases in the onset of atherosclerosis **(**fatty material collects along the walls of arteries), obesity, diabetes and cardio

vascular disease. On the otherhand, high consumption of long chain poly unsaturated fatty acid (PUFA) helps to alleviate these conditions and has positive effects on health (Ravnskov, 1998).

Natural fats are complex blends of triglycerides (Carey *et al.*, 1983). For this research we used three types of digested natural fats and oil with RS, to understand the effect of different types of fat and oil on RS fermentation in the large intestine. Three different types of fats used for this study were lard, corn oil, and fish oil from tuna. Average western diet contain high amount of fat. That is about 150 g of triglyceride and 4-8 g of phospholipids mainly from animal origin lecithin (Carey *et al.*, 1983). Hence these foods increase the risk of diseases such as obesity, diabetes and cardiovascular disease

The present study focuses on the inhibitory effects of different types of fat; lard (a major source of saturated fatty acids); corn oil (a major source of unsaturated omega 6 fatty acids) and fish oil (a major source of omega 3 fatty acids) on RS fermentation and butyric acid producing microbiota.

5.2 Materials and Methods

We used an in-vitro anaerobic fermentation system to investigate the effects of different fats on fermentation and growth of bacteria in the following combinations:1) hydrolyzed RS (RS), 2) hydrolyzed RS combined with corn-oil (C/RS), 3) fish-oil (F/RS) and 4) lard (L/RS).

5.2.1 Preparation of Fermentation Substrate

5.2.1.1 Fat Hydrolysis

Corn oil, tuna fish oil and lard were used as the major fat sources. Corn oil is contains mainly triglycerides with proportions of approximately 59% polyunsaturated fatty acids (PUFA), 24% monounsaturated fatty acids (MUFA), and 13% saturated fatty acids (Dupont *et al.*, 1990). Tuna fish oil contains 37.4% saturated fatty acids, 20.2 % MUFA, 5.4% omega 6 (also called ω−6 or n-6) long chain PUFA and 31.9% omega 3 (also called ω−*3* or *n-3*) long chain PUFA

(Jenvanitpanjakul & Laixuthai ,1992: Napier & Sayanova, 2005). The fatty acid composition of corn oil, tuna fish oil and lard pig fat are given in Table 5.1, Table 5.2 and Table 5.3 respectively.

Table 5.1 Fatty Acid Composition of Mazola Corn Oil

Fatty acid Composition	**%**
Myristic	0.2
Palmitic	9.9
Stearic	2.9
Saturated above CL8	0.2
Hexadecenoic	0.5
Oleic	30.1
Linoleic	56.2
Linolenic	0

(Kuksis & Beveridge, 1960)

Table 5.2 Fatty Acid Composition of Tuna Fish oil

Fatty acid Composition	**%**
Myristic	3.8
Palmitic	23
PalmitoleateC16:1 (n-7)	7
Heptadecanoic Acid (C17:0)	1.9
Stearic (C18:0)	8.1
Oleic(C18:0) (n-9 and n-7)	11.9
Linoleic (C18:1)	1.3
Alpha linolenic acid (C18:3)(n-3)	0.6
Therapic Acid(C18:4) (n-3)	0.6
Arachidic acid (C 20:0)	0.4
Gadoleic acid. (20:1)	0.3
Arachidonic acid (20:4)	2
EPA / Eicosapentaenoic acid (C20 :5)	4.8
DTA / Docosatetraenoic acid (C22:4)(n-6)	0.2
DPA/ Docosapentaenoic acid (C22:5) (n-6) and (n-3)	3.5
Tetracosanoic acid	0.2
DHA/docosahexaenoic acid (C22:6) (n-3)	24.3

(Jenvanitpanjakul & Laixuthai, 1992)

Lard is pig fat composed of approximately 40.8% saturated fatty acids, 43.8% MUFA, and 9.6% PUFA (Dupont *et al.*, 1990).

Table 5.3 Fatty acid composition of Lard Fat

Fatty acid Composition	%
Myristic	2
Palmitic	26
Stearic	14
Oleic	44
Linoleic	10
Linolenic	0

All three types of fat (corn oil, fish oil and lard) were separately hydrolyzed using pancreatic lipase as explained in Ferna´ndez-Moya *et al.,* 2000. One gram of fat was hydrolyzed with 100 ml of pancreatic lipase. That is mixture of 2 g of P3292 *Pancreatin* from porcine pancreas powder (Sigma-Aldrich Corp. St. Louis, MO, USA) in 965 mL of 1 M Tris-HCl buffer (pH 8), with 10 mL of CaCl2 (22%) and 25 mL bile sat (0.1%)(Sigma Aldrich) for 5min. After digestion the solution was microwaved 1-2 min to stop the enzyme reaction and cooled over ice. Solidified fat were filtered through No1 watman filter paper (Whatman Int'l. Ltd. Maidstone, England) and washed two times with cooled water. Each digested fat was collected and stored in refrigerator.

5.2.1.2 Carbohydrate Hydrolysis

One hundred gram of RS was hydrolyzed with 240 ml of pancreatic amylase as explained in Al-Rabadi *at el*., (2009). That is mixture of 2 g of P3292 *Pancreatin* from porcine pancreas powder (Sigma-Aldrich Corp. St. Louis, MO, USA) in 965 mL of 1 M sodium acetate buffer (pH 5), with 10 mL of CaCl2 (22%) and 25 mL bile sat (0.1%)(Sigma Aldrich) for 30min. After digestion the solution was microwaved 1-2 min to stop the enzyme reaction and cooled at room temperature. Precipitate included the undigested RS was separated from the solution by

centrifugation and then washed with distilled water twice. Undigested RS was air dried under a sterilized hood and stored in refrigerator.

5.2.1.3 Preparation of Four Different Types of Fermentation Substrates

Fermentation substrates mix were made by using each type of above hydrolyzed fat (2%) separately mixing with hydrolyzed RS (98%) and compared to control RS (100%) to mimic the fermentation substrate entering to the large intestine (Saunders & Sillery, 1988).

5.2.2 *In- vitro* System for Culturing Gut Microbiota

To create complex microbial ecosystem similar to large intestine, four anaerobic fermenters were used. Each of the four reactors contains the microbiota of the rat gastrointestinal tract. The all four vessels were inoculated with pooled rat fecal samples, and the microbial ecosystem was sustained by the addition of a culture medium. A detailed scheme of the reactor setup is provided in Figure 5.1.

5.2.2.1 Experimental Setup

(a) Instrumentation

The Simulator of the rat large intestinal microbial ecosystem consists of one liter media storage bottle with the stainless three steel port assemblies for adding and with drawing media (340 Edrudo Road Vineland, NJ 08360). This setup was maintained at a temperature of 37°C using an incubator. Each vessel simulates a one separate large intestine. One port of each fermenter was used for anaerobic gas supply, next for feeding and the last one for drawing the samples.

The two- way variable-speed compact pump with four-channel pump head was used to fill and draw the vessel (Cole-Parmer, 625 East Bunker Court, Vernon Hills, IL 60061). For the tubing and connections Cole-Parmer Silicone auto analysis tubing, master flex barbed fittings

and connectors were used. After the experiment tubes were cleaned with diluted 2.5% NaOCl (Clorox ® bleach).

(A) **(B)**

Figure 5.1 Large Intestinal Microbial Ecosystem Consists (A)Before adding diets. (B) After adding diets. 1. Anaerobic gas cylinder, 2. LI vessel fermenters [2.1 hydrolyzed RS (RS), 2.2 combination of hydrolyzed RS with hydrolyzed corn-oil (C/RS), 2.3 hydrolyzed fish-oil (F/RS) and 2.4 hydrolyzed lad (L/RS)], 3. Magnetic stirrer, 4. Pump, 5. Pump head

Each vessel was filled with 350 mL of anaerobic solution (Fig 5.1). The anaerobic solution prepared according to Monsma & Marlett, 1995, that was combining nine parts of solution A to one part of solution B. Solution A contained the following (per L of distilled

water): 0.14 mol NaHCO3, 11.1 mL hemin solution (0.78 mmol/L water), 1.1mL menadione solution (0.36 mmol/L water), and 1.1 mL resazurinsolution (3.98mmol/L water) as a redox indicator and autoclaved (121°C, 15 min) after preparation. Solution B was made using autoclaved 1L distilled water and contained the 0.48mol NaCl and 0.02 mmol K2HPO4. Cysteine-HCl (0.63 mmol) was added to the inoculum buffer just before use to reduce the redox potential (Monsma & Marlett, 1995, 1996). After mixing solution A and Solution B pH was adjusted to 7.5pH with 0.1 M NaOH.

After adding the anaerobic solution to the LI vessel fermenters, both anaerobic strip and magnetic string rod were placed in each vessel. All four LI vessels s were set up on top of a magnetic stirrer and anaerobic gas was pumped for 10 min until the color of the strip change from blue to colorless and stable. The composition of anaerobic gas was 80% N2, 10% H2, 10% CO2, supplied by a gas cylinder (Shahin *et al.*, 2003). There was no gas exchange between the different vessels and the headspace of the culture system was flushed after feeding and drawing of samples for 5 min to ensure anaerobic conditions.

(b) Preparation of Fecal Inoculum

Rat feces were used as the inoculums of the LI vessel fermenters. After 12 days of the Resistant starch (RS) diet, within about a 1h period, 50 g of fresh feces were collected immediately upon voiding to Whirl-pak bag (Nasco - Fort Atkinson, 901 Janesville Avenue, P.O. Box 901, Fort Atkinson, WI 53538-0901). Whirl-pak bags were placed in a double Zip lock bag with an anaerobic GasPak™ EZ Gas generating Pouch System (BBL GAS PAK, Voiglobal Distribution INC. P.O. Box 1130, Lawrence, Kansas 66044-8130 USA) and immersed in ice until transfer to the lab. At the beginning of the experiment, the vessels were inoculated with a pooled fecal sample of rats. Aliquots (50 g) of freshly voided fecal samples were diluted and

homogenized with 200 mL anaerobic solution in Whirl-pak filter bag. Filtrate was collected into another sterilized Whirl-pak bag and Incubated in 37°C for1 hr under anaerobic condition using anaerobic Gas Pak™ EZ Gas generating Pouch System.

After one hour, 50 mL of homogenized anaerobic inoculums was added in to the each LI vessels. When the fermenters were stabilized, 10 g of fermentation substrate were introduced separately to each fermenter. Anaerobic contents were aseptically collected into separate 15ml centrifuge tubes at 0, 1/2, 1, 2, 4, 12, 24, 48, and 72h in triplicates. This whole experiment was repeated three times and microbial analysis was done for each time point using plate count methods. qRt-PCR analysis was done for 0-24h samples.

5.2.3 Microbial Analysis of the Anaerobic Contents

5.2.3.1 Direct Plating

Anaerobic and aerobic bacterial counts for each time point were enumerated using plate count methods. Using peptone buffer solutions (PBS), serial dilutions were made in anaerobic contents. MacConkey sorbitol agar was used to detect total *E. coli* (Difco, Laboratories, Detroit, Michigan, USA), and plates were aerobically incubated at 37°C for 24 hrs/ 1day. Lactic acid bacteria (LAB) were enumerated by using de Man-Rogosa-Sharpe Agar (MRS agar) (Difco, Laboratories, Detroit, Michigan, USA). Bifidobacterium agar (Oxoid, Basingstoke, UK) plates were used to quantify total *Bifidobacterium spp.* Counts. Reinforced Clostridial agar (Oxoid, Basingstoke, UK) plates were used to quantitate total anaerobic and Clostridial counts. The MRS agar plates were anaerobically incubated at 30°C for 48 hrs, Bifidobacterium agar plates were anaerobically incubated at 37°C for 2 days and reinforced Clostridial agar plates were anaerobically incubated at 37°C for 3-4 days in a chemically generated anaerobic system using

anaerobic GasPak™ EZ in an *anaerobic box* (Mitsubishi Gas Chemical America, Inc., New York, N.Y.). Then the totals Colony Forming Units (CFU) were determined.

5.2.3.2 DNA Extraction

Collected anaerobic contents from each fermenter in each time points used for DNA extraction using a QIAamp DNA Stool Mini kit (QIAGEN, Valencia, CA) using the manufacturer's instructions with slight modifications. After adding stool lysis (ASL) buffer to samples of the anaerobic contents (200 µl), the samples were subjected to three cycles of freeze-thaw in liquid nitrogen and a 5 min at 95°C water bath to break the thick gram positive bacterial cell walls. Purified DNA was quantified using a NanoDrop ectrophotometer and all DNA sample extracts were diluted to 1ng/µl. Purified DNA was stored in a -80 ^{o}C freezer.

5.2.3.3 Quantitative Real-time PCR

The SYBR® Green method of quantitative real–time PCR (qRT-PCR) assay was performed using an ABI Prism 7900HT Sequence Detection System (serial 100151) (Applied Biosystems, Foster City, CA) as explained in chapter 3.2.3 The information of the targeted bacterial groups, primer sequences, annealing temperatures and literature references were similar to chapter 3 Table 3. 2. Standards curves of DNA quantification disassociation curves are displaying in Appendix A. To determine CFU/ml unknown Ct values were compared to the Ct of the standard curves.

5.2.4 Statistical Analysis

The study was analyzed as a mixed model repeated time followed by Tukey to determine differences among levels of treatment ($p<0.05$). Statistical analyses were done using SAS version 9.13 (SAS Institute Inc., Cary, NC). All data are presented as least square means (ls means) with pooled SE.

5.3 Results

5.3.1 Direct Plate Count Results

Culture-based methods were used to examine culturable bacterial counts in all four different anaerobic LI vessel fermenters and repeated three times. Each of these fermenters represented a single large intestine. Even though plating gives limited representation (10 - 40%) of the microbiota (Sharp & Macfarlane, 2000); plating helps to quantitate populations of culturable (live) bacterial populations. The variability of culturable bacterial population over time is given in the following Figures. Both substrate and time effects could be seen for all bacteria that were analyzed by plating ($p < 0.05$ significance level).

There were significant time and treatment effects (The effects of different fermentation substrate mixtures when means were collapsed over all times, up to and including 72 hrs) observed for all culturable bacterial groups which were analyzed in this study. Only the *Bifidobacterium spp*. ($p < 0.002$) demonstrated a significant interaction between time and the fermentation substrates. The total culturable bacterial counts were increased over the time up to 24 hours and then decreased after that up to 72 hours, which was the total time of the incubation. The reason for the decline after 24 hours appears to be the reduction of substrate and the data after 24 hours are likely not meaningful. Hence the results were further analyzed up to and including 24hrs.

Figure 5.2 indicates the population variation of total anaerobic bacteria including *Clostridium spp* up to 24 hrs. Treatment level means collapsed across all time points up to and including 24hrs for total anaerobic counts were RS=9.57, F/RS=9.01, L/RS=8.75 and C/RS=8.64 Log CFU/ml. Total Anaerobic counts were significantly higher in RS substrate compared to the rest of the substrates. The second largest population was observed for F/RS substrate and that

also was significantly different from the growth of bacteria fed RS and L/RS. The populations that grew in L/RS and C/RS were not significantly different from each other.

When we compared the growth over time for the population changes, all four fermentation substrates; RS, C/RS, L/RS and F/RS; when comparing the means across all time points through the 12 hr time point, RS fed anaerobic fermenters had higher populations of total anaerobic bacteria than F/ RS, C/RS and L/RS. F/RS and L/RS were not significantly different whereas C/RS had the lowest bacterial population. RS and F/RS were not significantly different and C/RS and L/RS were not significantly different.

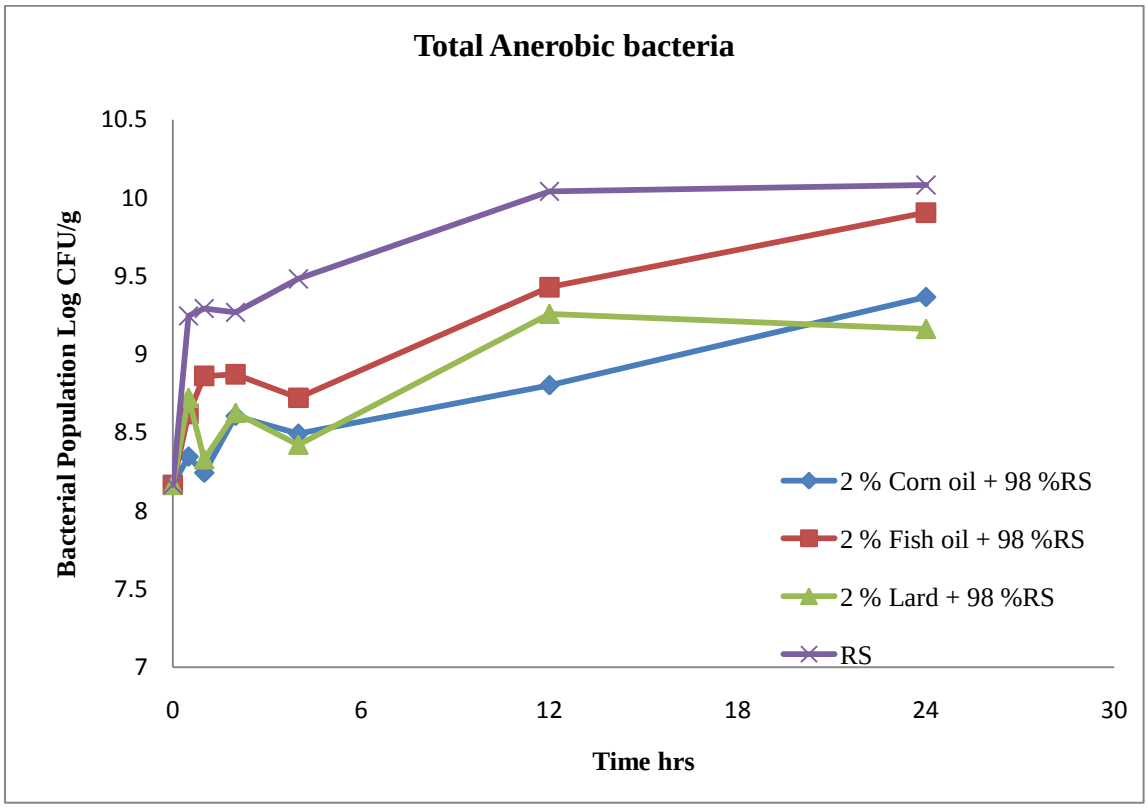

Figure 5.2 Effects of Different Fermentation Substrates on Total Culturable Anaerobic Bacteria Population Variation Analyzed by Direct Plate Count Method (Log CFU/mL): treatment p< 0.001, time p< 0.001, and treatment x time NS. Groups different from other groups are indicated with a, b and c for significant differences of substrate means across all time points for the 72hours: RS^{a}, C/RS$^{b\ c}$, L/RS^{c} and F/RS^{b}.

Figure 5.3 indicates population variation of Total culturable *Bifidobacterium spp*. bacteria. Treatment level means collapsed across all time points up to and including 24hrs for Total *Bifidobacterium spp*. counts were RS=9.40, F/RS=9.17, L/RS=8.55 and C/RS=8.47 Log CFU/ml. Total *Bifidobacterium spp*. counts were significantly higher in the RS substrate than for the rest of the fermentation substrates, and the second largest population was observed for the F/RS substrate, which was also significantly different from the other two fermentation substrates. The L/RS and C/RS substrate samples were not significantly different from each other.

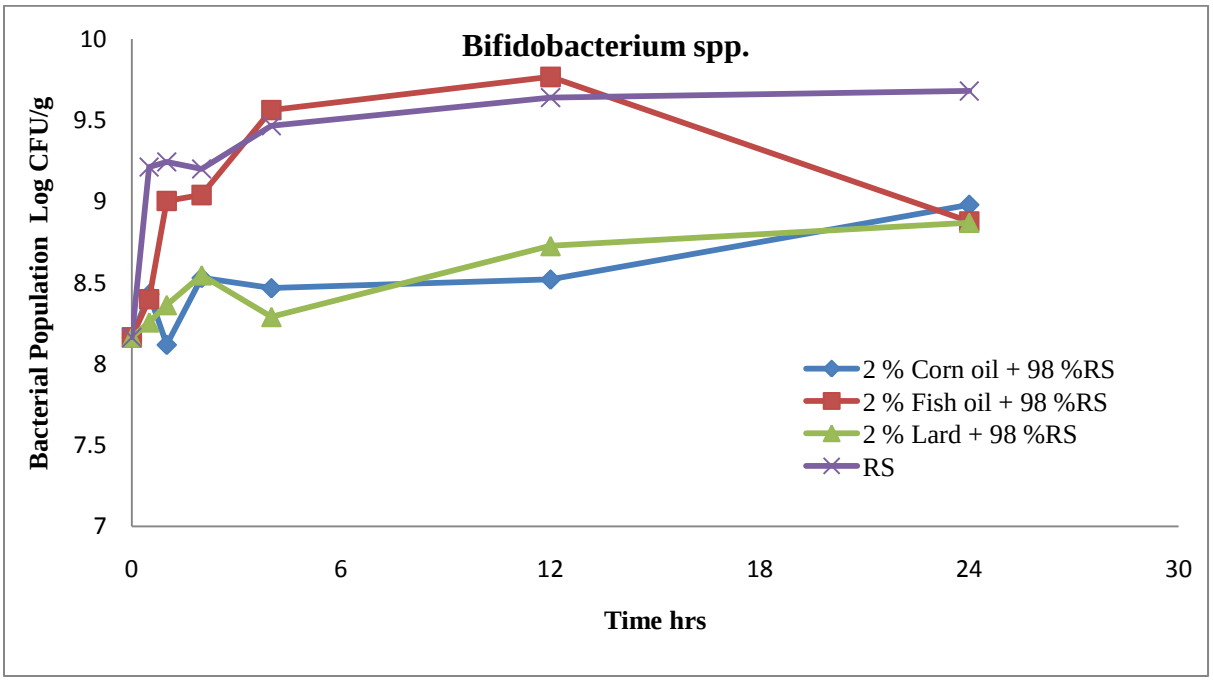

Figure 5.3 Effects of Different Fermentation Substrates on Total Culturable *Bifidobacterium spp.* Population Variation Analyzed by Direct Plate Count Method (Log CFU/mL): treatment p< 0.001, time p< 0.001, and treatment x time p <0.002. Groups different from other groups are indicated with a, b and c for significant differences of substrate means across all time points for the 72hours: RS[a], C/RS [c], L/RS[c] and F/RS[b].

When we compared the trend of time with the growth of the population for all four substrates, RS, C/RS, L/RS and F/RS, the total culturable *Bifidobacterium spp.* were increased over the first 24 hrs. The highest bacterial populations were after 12hrs for F/RS and RS substrates, but for C/RS and L/RS media, the highest bacterial population was at 24hrs. There

also was a substrate by time interaction observed for culturable *Bifidobacterium spp*, likely the result of the F/RS substrate effect. The effect of hydrolised tuna fish oil on fermentation was very strong over the first 12 hours of incubation and then dropped off by 24 hours. This may be due to using up all the fish oil substrate.

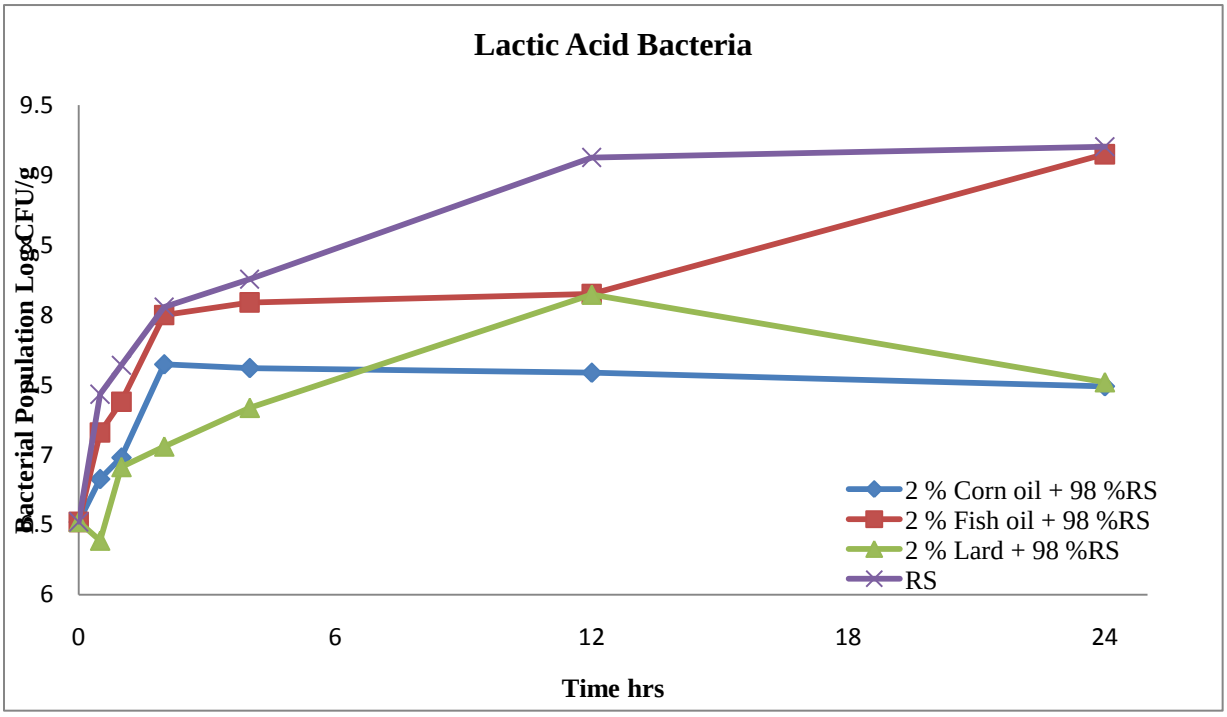

Figure 5.4. Effects of Different Fermentation Substrates on Total Culturable LAB Population Variation Analyzed by Direct Plate Count Method (Log CFU/mL): treatment p< 0.001, time p< 0.001, and treatment x time NS. Groups different from other groups are indicated with a, b and c for significant differences of substrate means across all time points for the 72hours: .RS^{a}, C/RS^{b}, L/RS^{b} and F/RS^{a}.

Treatment level means collapsed across all time points for Total Lactic Acid Bacteria (LAB) populations time points up to and including 24hrs were RS=8.28, F/RS=7.96, L/RS=7.28 and C/RS=7.41 Log CFU/ml (Figure 5.4). LAB counts were significantly higher in both RS substrate and F/RS substrate and bacterial counts for these substrate samples were not significantly different from each other. These substrates were also significantly different from

the other two fermentation substrates. The L/RS and C/RS substrate samples were not significantly different from each other.

The mean population of LAB at 12 hrs in RS fed fermenter was significantly higher than the means for F/ RS, C/RS and L/RS. Bacterial populations of F/RS and L/RS means were not significantly different whereas the C/RS substrate had the lowest mean bacterial population. The analysis of the means at the 24 hour time point indicated that both the RS and F/RS fed anaerobic fermenters had significantly higher populations of total LAB than both C/RS and L/RS. Means for RS and F/RS were not significantly different from one another, and similarly C/RS and L/RS were not significantly different.

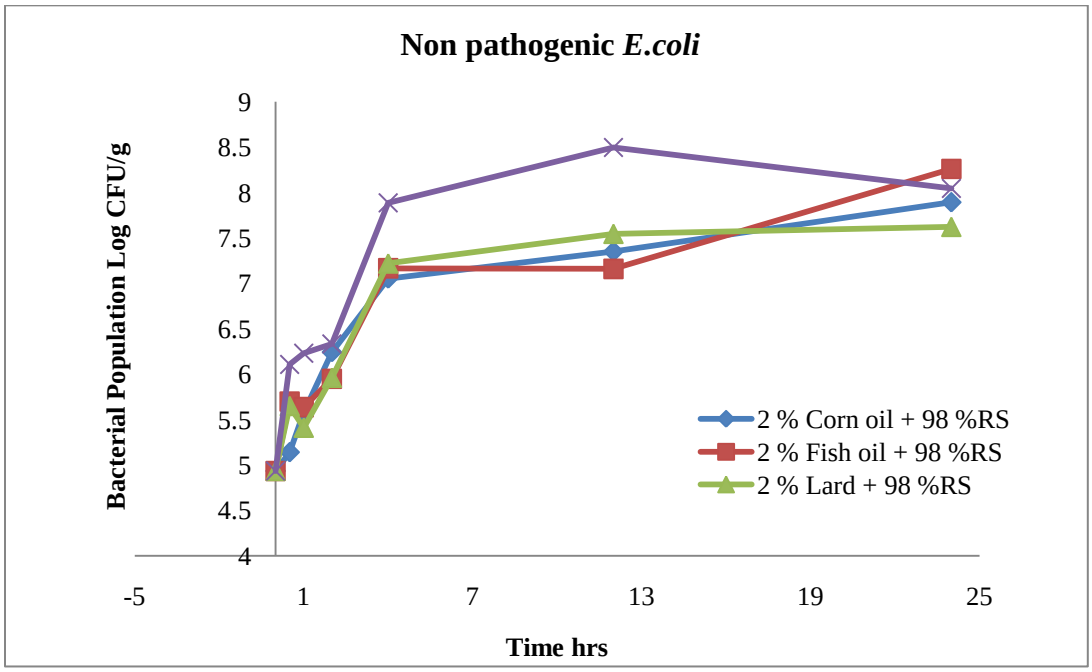

Figure 5.5 Effects of Different Fermentation Substrates on Total Culturable *E.coli* Population Variation Analyzed by Direct Plate Count Method (Log CFU/mL): treatment p< 0.04, time p< 0.001, and treatment x time NS. Groups different from other groups are indicated with a, b and c for significant differences of substrate means across all time points for the 72hours: RS^{a}, C/RS^{b}, L/RS^{b} and F/RS^{b}.

Culturable *E. coli* counts were plated on Sorbitol MacConkey Agar and all the colonies were dark pink in color. Hence we did not see any potentially pathogenic *E. coli* counts on these

plates, which would have appeared as a light pink. The only difference for *E. coli* counts populations time points up to and including 24hrs was significant for RS compared to the other fermenters. Treatment level means collapsed across all time points up to 24hours were RS=7.18, F/RS=6.64, L/RS=6.57 and C/RS=6.54 Log CFU/ml. The analysis of the means culturable *E. coli* counts at the 24 hour time point indicated that all four fermenters (RS, F/RS C/RS and L/RS) were not significantly different.Further detailed information about the analysis of culturable bacterial levels is in the Appendix.

5.3.2 Quantitative RT-PCR Count Results

The plate count results were further confirmed by qRT- PCR analysis. The bacterial populations for the qRT-PCR analyses were not identical to the cultured analyses as the goal was to more specifically target the bacteria known to ferment RS to butyric acid. QRT-PCR is more targeted and allows for better detection of specific bacterial populations than with culture. As with culture, LAB populations and bifidobacteria were included. However, Clostridial clusters IV and Clostridial clusters XIVa and b are major butyrate producers (Louis *et al.*, 2004; Louis *et al.*, 2007). Instead of total anaerobic culture, the total bacterial domain was also included in qRT-PCR analyses. In order to calculate a Bacteriodetes to firmicutes ratio, the Bacteriodes group including Prevotella and Porphyromonas was also included. All the qRT-PCR sample analyses were performed on samples collected up to and including the 24 hour time point as the culture analyses indicated that substrates for total anaerobic bacteria and *Bifidobacteriam spp.* may have become limited after 24 hours.

There was a significant time effect observed for all bacterial groups and strains which were analyzed using qRT-PCR. The effect of different fermentation substrate mixtures when means were collapsed over all times, up to and including 24 hrs was significant for three of the

six bacterial populations measured, *Lactobacillus spp*. (p< 0.005), *and Bifidobacterium spp*. (p< 0.001) and Clostridial cluster IV(p< 0.04) . For Clostridial cluster XIVa and b there was not a significant effect for fermentation substrate mixtures, but the fermentation substrates effect approached significance (p< 0.09). Similar to the results with culturable bacterial populations, only the *Bifidobacterium spp*. demonstrated an interaction between time and the fermentation substrates that only approached significance (p<0.06). Again, the F/RS substrate mixture appears to have a different response in *Bifidobacterium spp.* growth over time compared to the other three media groups (Figure 5. 6).

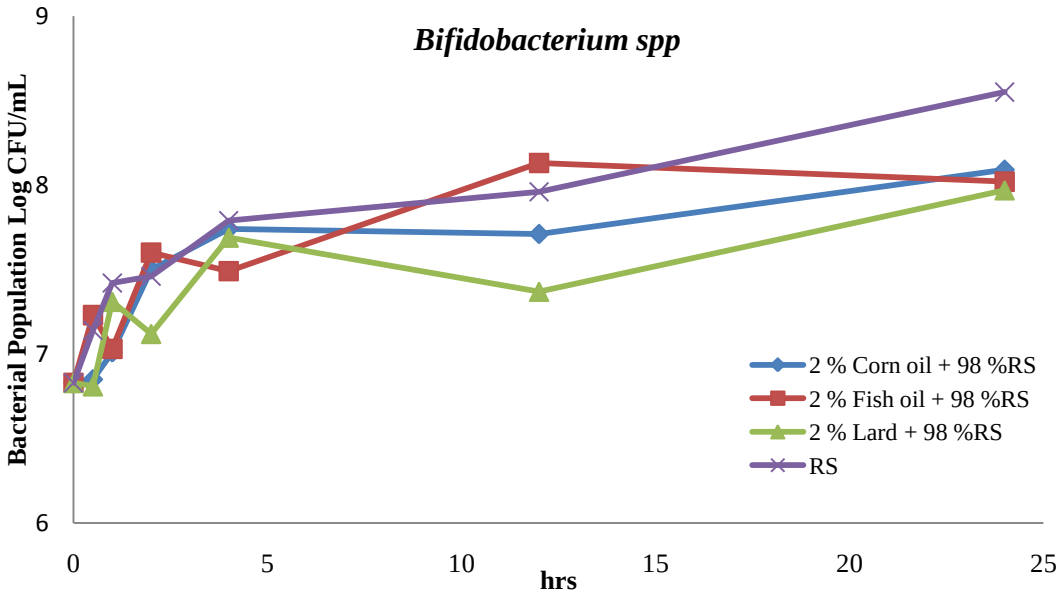

Figure 5.6 Effects of Different Fermentation Substrates on *Bifidobacterium spp.* Population Variation Analyzed by qRT PCR (Log CFU/mL): treatment p< 0.007, time p< 0.006, treatment x time p< 0.06. Groups different from other groups are indicated with a, b and c for significant differences of substrate means across all time points for the 72hours: RS^{a}, C/RS^{bc}, L/RS^{c} and F/RS^{ab}.

The *Bifidobacterium spp*. treatment level substrate means collapsed across all time points up to and including 24 hours were RS=7.72, F/RS=7.59, L/RS=7.38 and C/RS=7.49 Log CFU/ml. *Bifidobacterium spp*. populations were significantly higher for RS compared to C/RS and L/RS substrates. However, levels in RS fermenters only approached a significant difference

with F/RS (P< 0.103). The F/RS fermenter was significantly different from L/RS and F/RS fermenters. *Bifidobacterium spp*. with F/RS increased until 12 hours and then decreased by 24 hours. This appears to be what produced the time by substrate effect for Bifidobacteria spp.that approached significance (p<0.06). At the 12 hr time point L/RS had the lowest count compared to the rest of substrate fermenters.

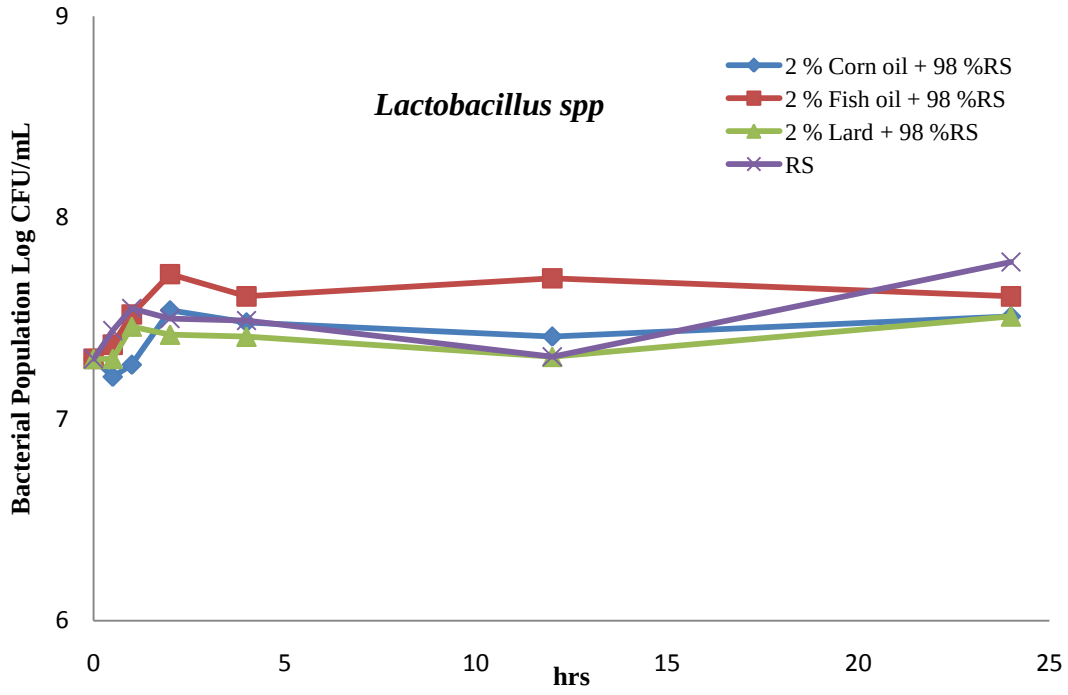

Figure 5.7. Effects of Different Fermentation Substrates on *Lactobacillus spp.* Population Variation Analyzed by qRT PCR (Log CFU/mL): treatment p< 0.007, time p< 0.006, and treatment x time NS. Groups different from other groups are indicated with a, b and c for significant differences of substrate means across all time points for the 72hours: RS^{a}, C/RS^{ab}, L/RS^{b} and F/RS^{a}.

The means for *Lactobacillus spp*. fermentation substrates collapsed across all time points up to and including 24 hours were RS=7.51, F/RS=7.59, L/RS=7.40 and C/RS=7.41 Log CFU/ml. The F/RS substrate had significantly higher levels of *Lactobacillus spp.* compared to both C/RS and L/RS substrate, but the difference between the RS fed fermenters and the L/RS

(p< 0.06) and the C/RS (p<0.10) only approached significance Both F/RS and RS were not significantly different from one another and, both C/RS and L/RS were not significantly different from one another.

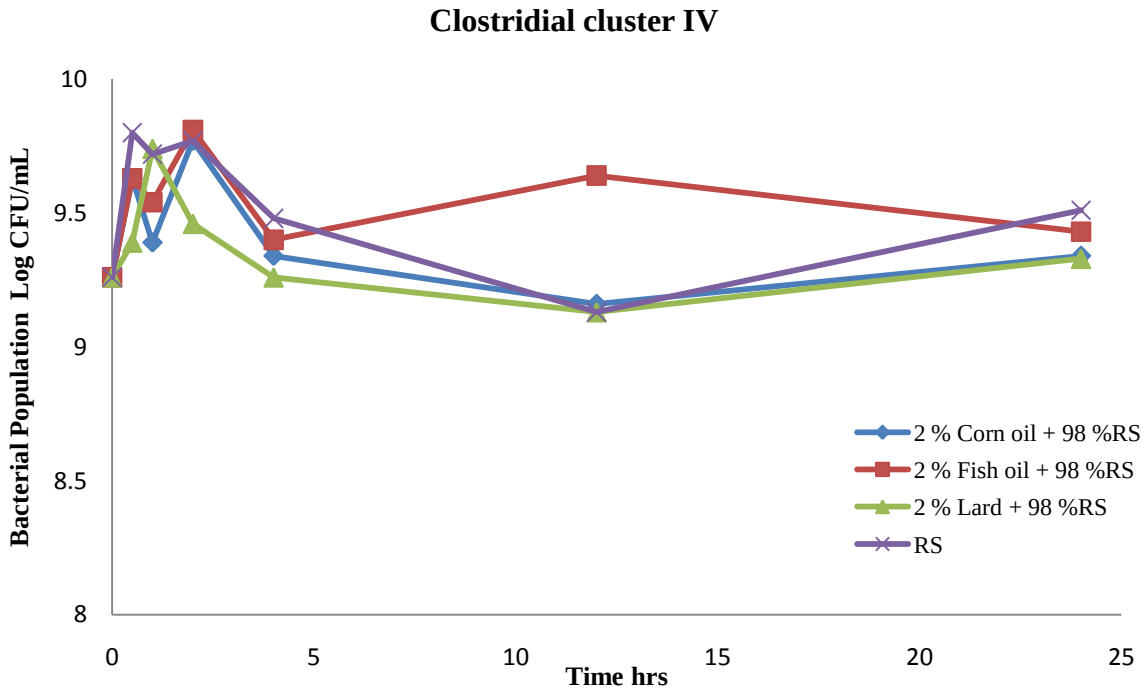

Figure 5. 8. Effects of Different Fermentation Substrates on Clostridial cluster IV Population Variation Analyzed by qRT PCR (Log CFU/mL): treatment p< 0.04, time p< 0.002, and treatment x time NS. Groups different from other groups are indicated with a, b and c for significant differences of substrate means across all time points for the 72hours: RS^{a}, C/RS^{a}, L/RS^{b} and F/RS^{a}.

For the Clostridial cluster IV populations (Figure 5.8), the substrate means collapsed across all means up to and including 24 hours were RS=9.58, F/RS=9.58, L/RS=9.38 and C/RS=9.44 Log CFU/ml. Analyses of these means demonstrated that the RS and F/RS substrate means were higher than for the L/RS substrate mean. However, the difference between the RS and F/RS substrate means compared to the C/RS substrate mean only approached significance (p< 0.1) and RS and F/RS were not significantly different. The mean population of Clostridial cluster IV at the 12 hr time point was significantly higher in the F/ RS fermenter than the means

of RS, C/RS and L/RS, whereas this population was not significantly different when compared to the rest of the substrates at the 24 hr time point.

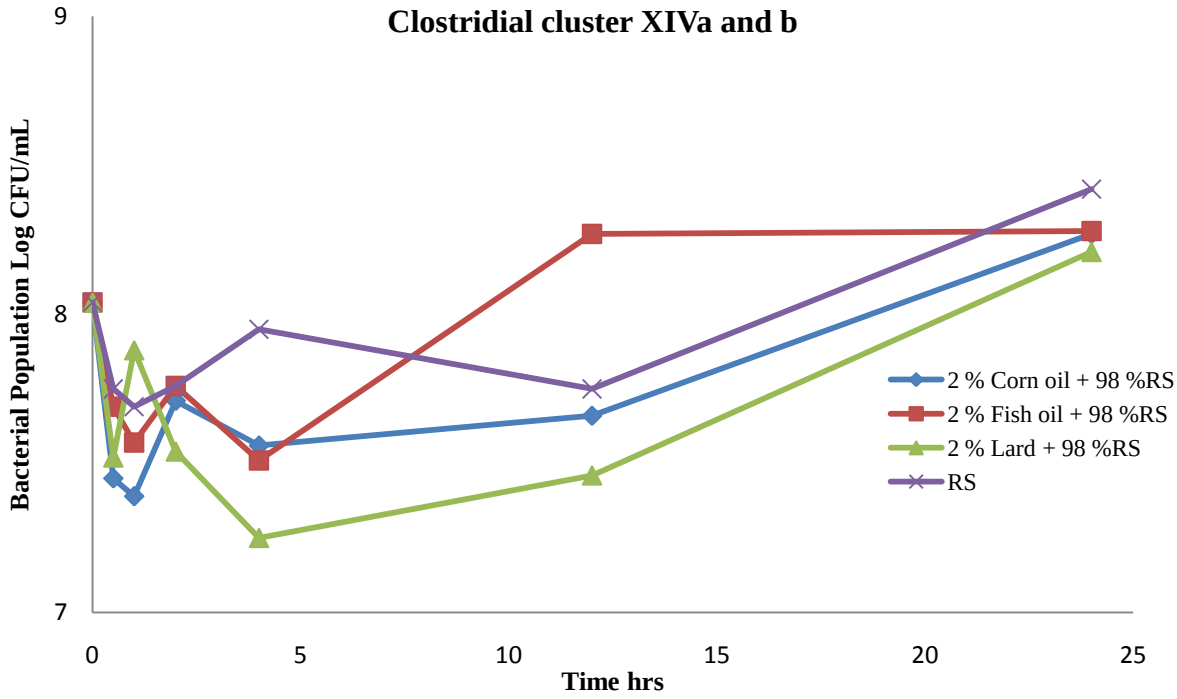

Figure 5.9. Effects of Different Fermentation Substrates on Clostridial cluster XIVa - b Population Variation Analyzed by qRT PCR (Log CFU/mL): treatment p< 0.09, time p< 0.006, and treatment x time NS. Groups different from other groups are indicated with a, b and c for significant differences of substrate means across all time points for the 72hours: RS^{a}, C/RS^{a}, L/RS^{b} and F/RS^{a}.

The substrate means for the Clostridial cluster XIVa and b (Figure 5. 9) collapsed across all time points up to and including 24 hours were RS=7.89, F/RS=7.84, L/RS=7.64 and C/RS=7.67 Log CFU/ml. The Clostridial clusters XIVa-b bacterial means were similar to the Clostridial cluster IV at the 12 hr time point. The mean population of Clostridial cluster XIVa-b at the 12 hr time point was significantly higher for the F/ RS fermenter than the means of RS, C/RS and L/RS Clostridial cluster XIVa-b bacterial population.

The substrate means for both the Bacterial Domain and the Bacteroides group populations collapsed across all times up to and including 24 hours demonstrated no significant differences among substrate means for either bacterial population.

A list of means for the Bacteroides (Figure 5. 10) and the Bacterial Domain (Figure 5. 11) group were: RS=8.99, F/RS=8.94, L/RS=8.91 and C/RS=8.84 and RS=10.69, F/RS=10.67, L/RS=10.55 and C/RS=10.54 Log CFU/ml; Log CFU/ml, respectively.

Summary of Results

All bacterial populations involved in this study regardless of measurement tool, culture or qRT-PCR, demonstrated significant time effects. Hence, the bacterial populations regardless of fermentable substrate were changed with time. Those populations only analyzed by culture all demonstrated treatment effects (effect associated with Fermentable substrate). Those effects were: Total Anaerobic bacteria (treatment $p< 0.001$, treatment x time NS), culturable *Bifidobacterium spp.* (treatment $p< 0.001$, treatment x time $p <0.002$), LAB population variation (treatment $p< 0.001$, treatment x time NS) *E.coli* (treatment $p< 0.04$, treatment x time NS). Populations measured by qRT-PCR demonstrated *Bifidobacterium spp.* population treatment $p< 0.007$, treatment x time $p< 0.06$, *Lactobacillus spp*. population (treatment $p< 0.007$ treatment x time NS), Clostridial cluster IV population (treatment $p< 0.04$, treatment x time NS), Clostridial cluster XIV (treatment $p< 0.09$, treatment x time NS), Bacteroides group (treatment NS, treatment x time NS), Bacterial Domain (treatment NS, treatment x time NS).

5.4 Discussion

Our previous study concluded there were effects of high fat levels on fermentation of RS (Chapter 3 and 4). The main reason for conducting the current study was to compare the direct effects of three major types of hydrolised fat; lard, corn oil, and tuna fish oil on RS fermentation.

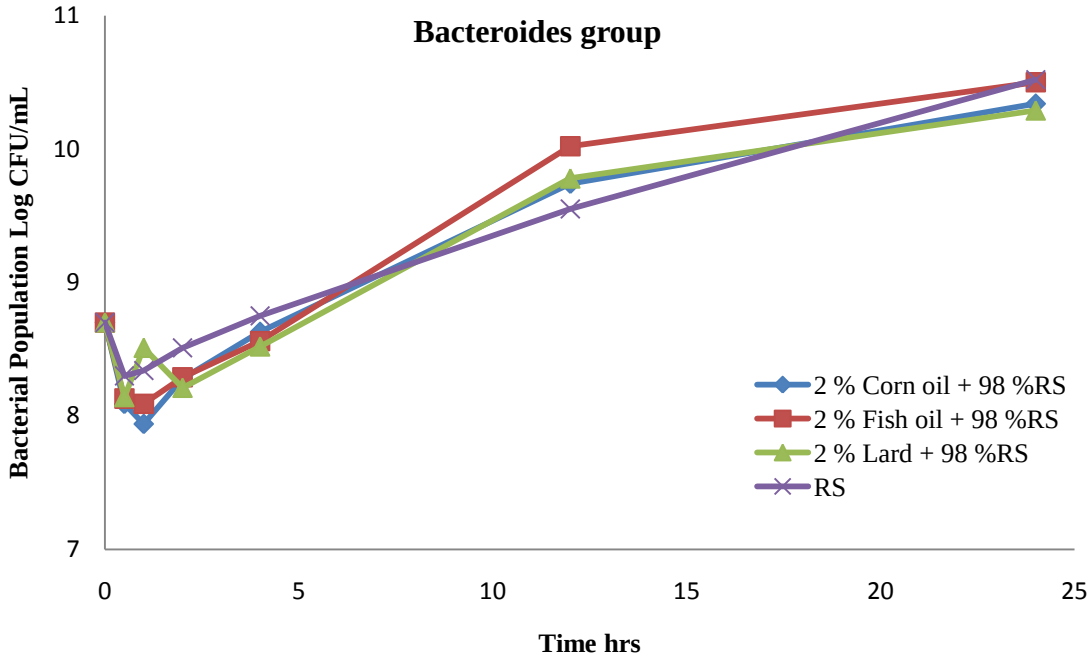

Figure 5.10. Effects of Different Fermentation Substrates on Bacteroides group Population Variation Analyzed by qRT PCR (Log CFU/mL): treatment NS, time p< 0.001, treatment x time NS.

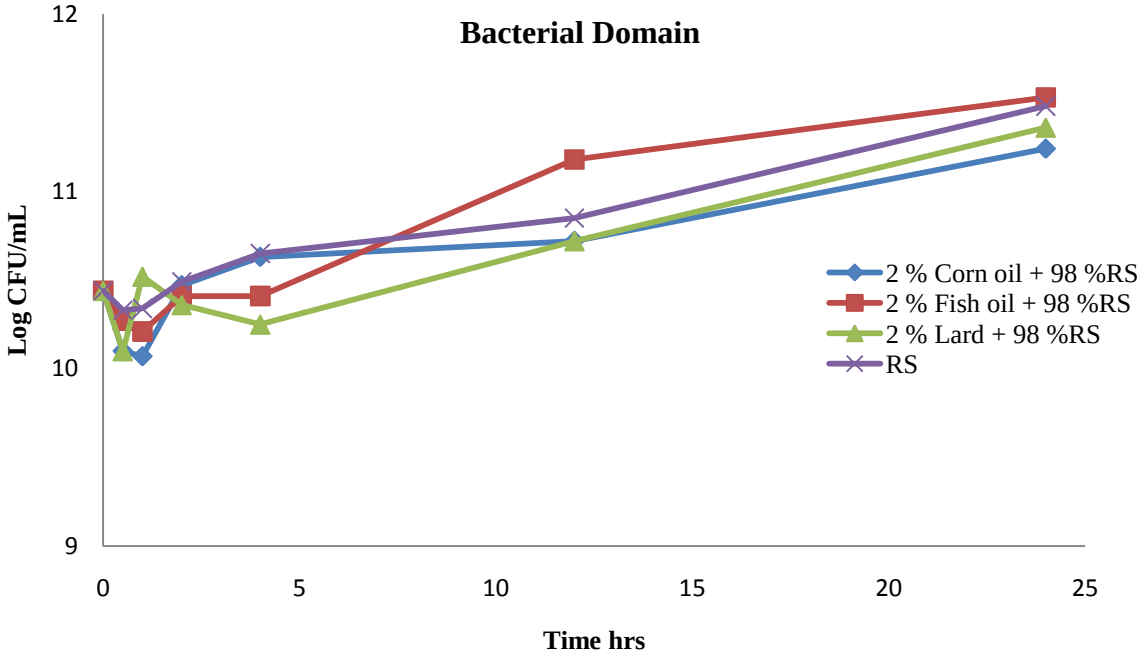

Figure 5.11. Effects of Different Fermentation Substrate on Bacterial Domain Population Variation Analyzed by qRT PCR (Log CFU/mL): treatment NS, time p< 0.001, treatment x time NS

For instance, L/RS added to an in vitro fermenter in the current study indicated reduction of populations of most of the bacteria which are involved with butyric acid production. Namely those populations were total culturable anaerobic bacteria, culturable LAB, and culturable total *Biffidobacterium spp* which were analyzed by direct plating as well as *Lactobacillus spp*. *Bifidobacterium spp,* Clostridial cluster IV and Clostridial cluster XIVa and b population which were analyzed by qRT-PCR. Similar results were observed with the C/RS diet, and during the study period analyzed, bacterial populations were generally not significantly different between L/RS fed fermenter and C/RS fed fermenter. These fermenters were fed with 2% of different sources of fat with 98% of digested RS to simulate composision of large intestine. Because in the small intestine 98% of the fat which digested is absorb and only (2%) carry over into the large intestine (Saunders & Sillery, 1988). Hence low level of 2% of the fatty acids coming from corn oil digestion and lard digestion interfered with RS fermentation. In other words, fat which enter to the large intestine by escaping the absorption from the small intestine also interferes with the fermentation process in the large intestine.

Bacterial suppression by fatty acids was studied by several investigators for decades (Boughton & Pollock, 1953; Jenkins & Jenny, 1989, 1992; Jenkins, 1995; Onetti *et al.*, 2001; Kim *et al.*, 2008; Amorocho *et al.*, 2009). Almost all these studies were on rumen microbes. In this study we targeted the effect of fat interference in the large intestine on a non-ruminant animal. The rat fecal inoculums were used as the inoculums for all fermenters to mimic large intestine bacterial populations because the main target was investigation of different types of fat involvement in large intestine of rodents. The basic fermentative reaction occurred in omnivorous are reported similar fermentative reaction to that in obligate herbivores (Topping & Clifton, 2001). The reason for that is because; the intestinal microbial community structure is

more similar in mammalian species than in between different species (Leser & Molbak, 2009). Different types of Clostridium clusters, some *Bacteroides spp.Bifidobacterium* spp and *Lactobacillus spp* were frequently isolated from rumen contents in early investigations (Trovatelli & Matteuzzi, 1976; Yanke & Cheng, 1998; Cook *et al.*, 2007). Even though some of the bacterial populations which are involved in fermentation of prebiotics in the non-ruminant are similar in the ruminant, fat interference in fermentation of the mono-gastric digestive system is different from complex stomachs of the ruminant. In the ruminant, the whole amount of fat that is consumed interferes with the fermentation, but in the mono-gastric animals 98% of fat is absorbed in small intestine before it reaches the large intestine. Therefore only 2% of the fat can interfere with the fermentation of the large intestine (Saunders & Sillery, 1988). In a high fat diet this may be a substantial amount of fat in the large intestine that can interfere with bacterial fermentation.

The main reason why rumen microbes were observed to have reduced function with increased fatty acids such as unsaturated fatty acids is antimicrobial effect of the unsaturated fatty acid (Ferguson *et al.*, 1990; Jenkins, 1993), was that the fatty acids associate with the bacterial cell wall and disrupts the cell membrane. These disruptions primarily impact Gram-positive bacteria as they are generally more susceptible to fatty acids and other anionic agents than the gram negative bacteria (Maczulak *et al.*, 1981). The Gm - bacteria less susceptible to Fatty acid due bauble cell membrane in their cell wall. Gram-negative bacteria have a restricting sieving mechanism in their outer membranes, which reduces the penetration of fatty acids into bacterial cell. This is probably the main reason for reduction of Gram positive populations such as *Lactobacillus spp*. *Bifidobacterium spp,* Clostridial cluster IV and Clostridial cluster XIVa-b populations in the fermenters containing lard and corn oil.

Soon after a fatty acid adsorbs to a bacterial cell membrane, it will penetrate into the protoplast membrane changing the cell layer orientation and destroying the bacterial activity (Galbraith & Miller, 1973; Jenkins, 1993). On the other hand high fat diets have been shown to increase gram negative bacteria. The Gram negatives such as the family Enterobacteriaceae and the Bacteroides group were reported to increase with high fat diets (Cani & Delzenne, 2007; Cani *et al.*, 2009). The main reason of increasing population of Gram negative bacteria could be due to better opportunity by reducing the competition from native Gram positive bacteria.

The data showed that the substrate F/RS had the opposite effect compared to C/RS and L/RS; F/RS had a significantly greater mean population for most of the favorable bacterial populations. Similar results were observed by our research group with *in vivo* study, in rat fed fish oil combined with RS in the diet (Unpublished data). In that study fish oil fed animals had significantly greater ceca weight and lower pH compared to rats fed the control diet and rats fed the RS diet without fish oil. Hence, fish oil appears to interact synergistically with RS to enhance its fermentation.

A similar phenomenon has been observed by several investigators with fish oil supplemented diets in rumen fermentation (Kim *et al.*, 2008; Amorocho *et al.*, 2009). A recent study by Kim *et al* (2008) showed that the addition of fish oil to the ruminant diet has an effect on bio-hydrogenation. Bio-hydrogenation could be known as biological process which occurred in the rumen with the help of rumen microbes (Mosley *et al.*, 2002). During bio-hydrogenation conjugated linoleic acid was produced by ruman microbes which have antioxidant and anti carcinogenic properties. These effects of fish oil (LCPUFA) on bio-hydrogenation in the rumen have not been clearly explained yet (Kim *et al.*, 2008). But for signal stomach animal does not occur bacteria which were able to break down dietary fat.

In this present study, we observed a significantly greater population of total culturable anaerobic bacteria, culturable LAB, and culturable total *Biffidobacterium spp.* with the F/RS substrate compared to both C/RS and L/RS. With qRT-PCR there was also a significantly higher population of *Lactobacillus spp* observed with the F/RS substrate.

In our previous *in vivo* study in chapter 3, we observed a significant increase in the Bacteroides group populations with a high fat diet. However, in the present study, we haven't seen a significant increase of the Bacteroides group populations with the substrates containing fats ($p > 0.05$). For this study we used 100% digested RS as the control diet while rest of three diets contained 98% of the RS. Hence control diets had extra 2% of RS than the rest of the diets (rest of the diets had 2% of different digested fat).

In this study total mean Bacteroidetes group was not significantly different within each diet. The one reason for this could be because of the high variations of Bacteroidetes group in each individual (Layton *et al.*, 2006). The second reason could be increase of population of *Bacteroides thetaiotaomicron* during RS fermentation (Bird *et al.*, 2000; Louis *et al.*, 2007; Louis, 2007) might have diluted the increase of Gram negative Bacteroides group populations that have been observed with high fat diets not containing RS (Cani & Delzenne, 2007; Cani *et al.*, 2009).

The main conclusion of this study is that hydrolyzed fish-oil which presumably enters the large intestine promoted the growth of some beneficial bacteria during fermentation of RS whereas hydrolyzed unsaturated corn-oil and saturated lard fat reduced the counts of beneficial bacteria. Hence our findings agree with the recent nutritional advice of need to decrease the intake of saturated fatty acid and to increase the intake of omega 3 fatty acids in fish oil in the diet relative to unsaturated omega 6 fatty acids in vegetable oils.

5.5 References

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CHAPTER 6: CHANGES IN POPULATIONS OF BUTYRIC ACID FORMING BACTERIA CAUSED BY RESISTANT STARCH FERMENTATION IN A RAT MODEL OF HUMAN ENDOCRINE OBESITY

6.1 Introduction

Overweight and obesity are a growing epidemic in many developed western countries including the United States. In the United States more than half of the population is overweight and 30% of the population is obese. According to National Institute of Health (NIH) Clinical Guidelines classification, overweight and obesity in adults is defined according to BMI, the ratio of weight in kilograms to the square of height in meters (kg/m2). People with 25-29.9 BMI are classified as overweight and people with 30.0 or above BMI are considered obese. Obesity has been divided into three levels; level1 is the BMI range of 30.0-34.9, level II is a BMI ranging from 35.0-39.9, and level III includes anyone with a BMI greater than or equal to 40.

Obesity has a direct relationship to serious health consequences such as type 2 diabetes mellitus, cardiovascular diseases, pulmonary hypertension, obstructive sleep apnea, gastro-esophageal reflux disease, musculoskeletal disorders, a variety of cancers, and with the relationship with these diseases and disorders that there is an increased risk of mortality. The mortality associated with overweight and obesity is more frequently common among women (Guallar-Castillon *et al.*, 2002). This obesity epidemic could be known as a multi-factorial disorder deriving from genetic and metabolic factors as well as environmental, socioeconomic, and behavioral factors (Marin-Guerrero *et al.*, 2008). Endocrine changes, such as reductions in the hormone estrogen in females at menopause, also increases fat accumulation and this is considered an endocrine cause of obesity. However, it is well known that not all individuals in a group of people subjected to similar conditions are susceptible to the same negative effects, such as diet-induced weight gain and hyperglycemia. Other factors may help trigger the weight gain and hyperglycemia. Gastrointestinal microbial ecology varies from to individual to individual and may also have a direct effect on obesity by influencing energy harvest from dietary

substances as well as affecting genes that regulate how energy is expended and stored (Ley; Ley *et al.*, 2005; Ley *et al.*, 2006; Turnbaugh *et al.*, 2006; DiBaise *et al.*, 2008; Tsukumo *et al.*, 2009).

The endocrine system is composed of tissues and glands. Hormones are the chemical messengers of the body, and are involved with transformation of information and instructions from one set of cells to another. Hormone levels also can be influenced by factors such as stress, infection, and changes in the balance of fluid and minerals in blood. Endocrine glands are responsible for releasing more than 20 major hormones directly into the bloodstream where they can be transported to cells in other parts of the body. The major glands that are involved with the endocrine system are the hypothalamus, pituitary, thyroid, parathyroids, adrenals, pineal body, and the reproductive glands, which include the ovaries and testes. Hormones from these reproductive glands influence energy metabolism related to body fat content and body weight. However, females primarily undergo menopause, either surgically or naturally, and this is often related to increased body fat and weight gain. Thus, the postmenopausal period is related is caused by a loss of functioning ovaries, including the production and release of estrogen (Wing *et al.*, 1991a; Wing *et al.*, 1991b; Arabi *et al.*, 2003). Reduced estrogen production during menopause is believed to be a major cause for the increase in body fat and one of the triggers that induces obesity in the later period of female life (Burger *et al.*, 1995; Shimizu *et al.*, 1997; Danilovich *et al.*, 2000; Gotoh *et al.*, 2009).

Possible modification of gastro-intestinal microbiota may be able to alleviate obesity by controlling energy expenditure and storage. Antibiotics such as norfloxacin and ampicillin have been studied for their modification of gastro-intestinal microbiota and improvement of oral glucose tolerance and reduced hepatic steatosis in *ob/ob* mice. These antibiotic treatments

regulate the metabolic endotoxaemia (ME), and lower plasma lipopolysaccharide (LPS) levels. Other effects of antibiotic treatment include reduced gut permeability for bacteria, a lower occurrence of visceral adipose tissue inflammation and macrophage infiltration in high-fat-fed mice. Finally this tends to reduce glucose intolerance and weight gain (Cani & Delzenne, 2007; Membrez *et al.*, 2008).

Taking antibiotics for modification of gut microbiota is not a good solution for obesity. In fact, we now have antibiotic-resistant diseases that are much more difficult to treat. Those diseases are caused by certain strains of bacteria such as gonorrhea, tuberculosis, and Methicillin resistant *Staphylococcus aureus* (MRSA) (Levy & Marshall, 2004). Another way to control gut microbial ecology is adding prebiotics and probiotics to the diet (Kleessen *et al.*, 1997; Weaver *et al.*, 1997; Sharp & Macfarlane, 2000).

This study was conducted to examine the effects of resistant starch (RS) as a prebiotic on cecal microbial community populations in an endocrine model of obesity, using ovariectomized (OV) or sham-operated (SH) virgin female Sprague-Dawley rats.

6.2 Materials and Methods

6.2.1 Animals and Diets

Twenty OV and 20 sham (SH) 10 week old virgin rats were purchased from Harlan (Indianapolis, IN) and they were fed an energy control (EC) diet for six weeks prior to dietary treatment to observe energy intake and body weight gain during the hyperphagic period after surgery. After six weeks, rats were stratified by body weight within a 2 x 2 factorial (surgery and diet) and fed either the EC or the diet containing resistant starch (RS, Hi-Maize, National Starch, Bridgewater, NJ). Both diets had equal energy density: 3.3 kcal/g and to equalize the energy density of the two diets, increased cellulose was added to the EC diet (Table 6. 1). Each group of

rats was fed the diets for 13 weeks and food intake and body weights were monitored 3 times per week. At the end of the study, rats were killed and ceca were tied with threads, separated from the rest of the GI tract, and aseptically transferred to separate Whirl-pak bags (Figure6.1). Whirl-pak bags were placed in a double Zip lock bag with an anaerobic GasPak™ EZ Gas generating Pouch System (BBL GAS PAK, Voiglobal Distribution INC. P.O. Box 1130, Lawrence, Kansas 66044-8130 USA) and immersed in ice.

Table 6.1 Diet Compositions

	Energy Control		**Resistant Starch**	
Ingredients	grams	Kcal	grams	Kcal
Amioca	424.5	1527	0	0
Hi-Maize	0	0	530.7	1486
Sucrose	100	400	100	400
Casein	200	716	200	716
Soybean Oil	70	591.5	70	591.5
Cellulose	156.2	0	50	0
Mineral Mix	35	30.8	35	30.8
Vitamin Mix	10	38.7	10	38.7
Choline Chloride	1.3	0	1.3	0
L-Cystine	3	12	3	12
	1000 g/kg	3.3 Kcal/g	1000 g/kg	3.3 Kcal/g

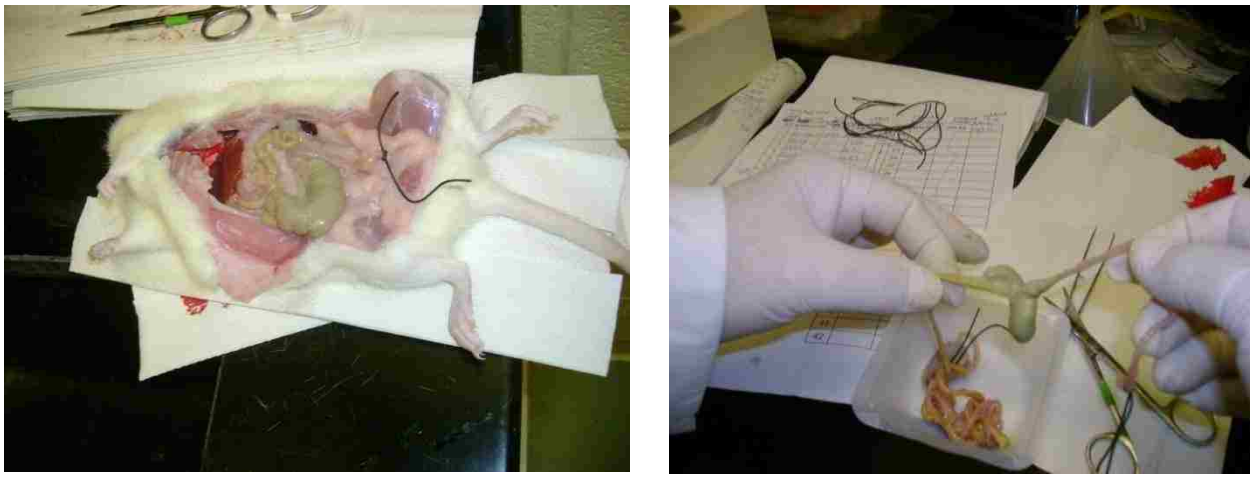
Amioca®and Hi-Maize® cornstarches were gifts from National Starch Food Innovation (Bridgewater, NJ). Hi-Maize® cornstarch consists of 56% resistant starch determined by the Englyst method (Englyst et al., 1992) as measured by National Starch Food Innovation.

6.2.2 Microbial Analysis of the Cecal Contents

6.2.2.1 Direct Plating

Anaerobic bacterial counts were enumerated using plate count methods. Ceca and contents were diluted 1:4 with peptone buffer solutions (PBS) and serial dilutions were made. *Lactic acid bacteria (LAB)* were enumerated by using de *Man-Rogosa*-Sharpe *Agar* (MRS agar)

(Difco, Laboratories, *Detroit*, *Michigan*, USA). Reinforced Clostridial agar (Oxoid, Basingstoke, UK) plates were used to quantitate total anaerobic and Clostridial counts. The MRS agar plates were anaerobically incubated at 30°C for 48 hrs and reinforced Clostridial agar plates were anaerobically incubated at 37°C for 3-4 days in a chemically generated anaerobic system using anaerobic GasPak™ EZ in an *anaerobic box* (Mitsubishi Gas Chemical America, Inc., New York, N.Y.). Then the total Colony Forming Units (CFU) were determined. Colonies were isolated and verified by gram staining.



(a) (b)

Figure 6.1 Rat Dissection (a) Rat Digestive System (b) Rat Cecum and Large Intestine

6.2.2.2 DNA Extraction

Previously mentioned 1:4 diluted ceca samples which were used for direct plating were also used for DNA extraction. DNA was extracted using a QIAamp DNA Stool Mini kit (QIAGEN, Valencia, CA) using the manufacturer's instructions with slight modifications in the third step. The samples were then subjected to three cycles of freeze-thaw in liquid nitrogen and 5 min at 95°C in a water bath to break the thick gram positive bacterial cell walls. Purified DNA

was quantified using a NanoDrop Spectrophotometer and all DNA sample extracts were diluted to 1ng/µl. Purified DNA was stored in a -80 ^{o}C freezer until the quantitative real–time PCR (qRT-PCR) analysis.

6.2.2.3 Quantitative Real-time PCR

The SYBR® Green method of quantitative real–time PCR (qRT-PCR) assay was used to quantify bacteria as explained in chapter 3 using an ABI Prism 7900HT Sequence Detection System (serial 100151-Applied Biosystems, Foster City, CA). The information of the targeted bacterial groups, primer sequences, annealing temperatures and literature references are given in chapter 3, Table 3. 2. All reactions were performed in sterile MicroAmp® optical 384-well reaction plates with barcode sealed with MicroAmp® optical adhesive film (Applied Biosystems, Foster City, CA). All reactions consisted of 5µl of 2X SYBR Green Master Mix (Applied Bio systems, Foster City, CA,USA), 0.5 µl of each primer at 10 µM, 0.5 µl of bovine serum albumin (BSA) (final concentration 250 mg /ml), 0.5 µl of nuclease free water and 3 µl of DNA template in a 10 µl total volume. The cycling conditions were 50°C for 2 min, 95°C for 10 min, then 40 cycles of 95°C for 15 s, followed by the primer-specific annealing temperature (Chapter 3 :Table 3.2) for 1 min, then 78°C for 30 s. Following amplification, a dissociation step was included to analyze the melting curve of the amplified product to determine the specificity of the amplification. Further information of thermal profiles, standerd curves, diassociation curves are in Apendix A.

6.2.2.4 Preparation of PCR Standards and Quantification of Target Bacterial DNA in Pure Cultured for Fecal Samples by Quantitative Real Time-PCR

The standard curve was conducted by using serial dilutions of quantified pure cultures of *Lactobacillus acidophilus* ATCC 4646, *Clostridium leptum* ATCC 29065, *Clostridium coccoides* ATCC 29236, *Bacteroides fragilis* ATCC 25285, *Bifidobacterium longum* ATCC 15708 and

Escherichia coli ATCC 25947. Bacterial cultures were grown overnight and serial dilutions were made. Then for each dilution the Ct (cycles to threshold) value was measured by qRT- PCR and plated onto appropriate media to determine the actual bacterial cells present in the overnight culture (CFU/ml). For qRT- PCR, 200 µl of each dilution tube was used to isolate DNA templates from the bacterial standard species listed above. DNA was isolated by the commercial QIAamp method as explained above. qRT- PCR primers were used to amplify the DNA for the 16S rRNA (Table 3. 2). To determine CFU/ml unknown Ct values were compared to the Ct of the standard curves.

6.2.3 Statistical Analysis

This study was analyzed as a completely randomized factorial with two independent variables with two levels for each variable, diet (RS or Energy Control) and Surgery (OV and SH) using two-way ANOVA followed by least significant difference if the ANOVA F value was significant, $p<0.05$. Statistical analyses were done using SAS version 9.13 (SAS Institute Inc., Cary, NC). All data are presented as least square means (ls means) with pooled SE.

6.3 Results

Total culturable anaerobic bacteria including *Clostridium spp.* (Figure 6.2b) and total culturable Lactic Acid Bacteria (LAB) (Figure 6.2a) were significantly affected by the diet type because animals fed RS diets had greater numbers of these bacterial populations in the cecal contents than those fed EC diets.

These results emphasize anaerobe populations and facultative anaerobes such as LAB populations increased with the RS diets. These diet effects confirmed previous findings in non-endocrine studies (Kleessen *et al.*1997; Louis *et al* 2007).

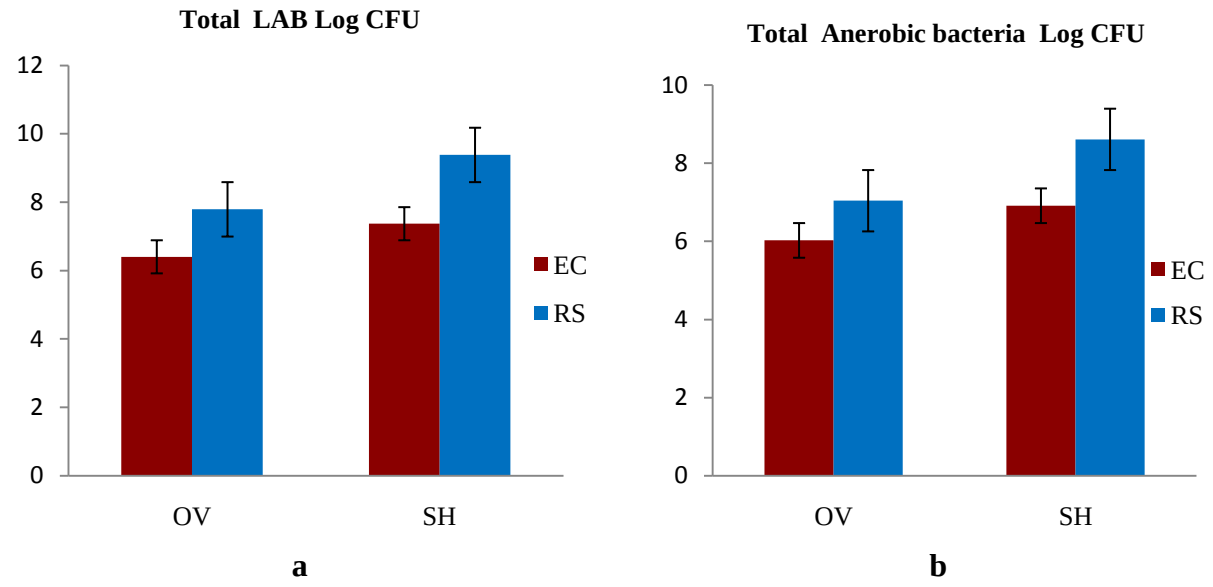


Figure 6.2 (a) Total Culturable Lactic Acid Bacteria Diet, p < 0.004; Surgery,NS; Interaction, NS (b) Total Culturable Anaerobic/Aerobic Bacterial Populations in the Cecum: Diet, p < 0.004; Surgery, p < 0.04 ;Interaction, NS.

In this experiment MRS plates were incubated anaerobically in 30^{o}C for 48 hrs. Hence, MRS plates also indicated the growth of *Bifidobacterium spp.* which is found in another bacterial Order and also produces bacteria under anaerobic conditions.

Lactic Acid Bacteria presence in cecal contents was not significantly affected by surgery. There was a significant increase of total culturable anaerobic bacteria including *Clostridium spp* for the SH groups compared to the OV groups. These results emphasize that total culturable anaerobic bacteria including *Clostridium spp* populations have endocrine effect on their growth.

The size and the weight of the GI tract and ceca in RS fed animals were always greater than those in the EC fed animals. Contrasting results were observed regarding the total abdominal fat pads that included gonadal, perirenal and retroperitoneal fat in the abdominal cavity. There was a greater weight of the abdominal cavity fat pads observed in animals fed EC diet versus RS diets (data not shown**;** Robert *et al.*2008).

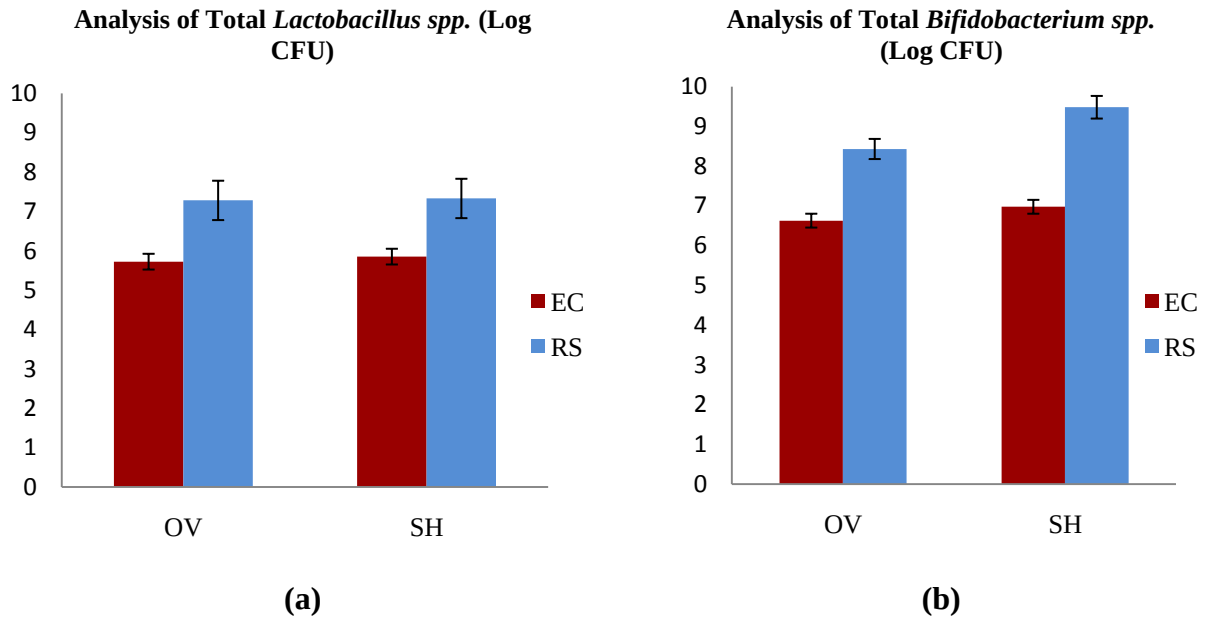


Figure 6. 3 (a) *Lactobacillus spp* Populations: Diet, p < 0.001; Surgery, p < NS ; Interaction, NS, (b) *Bifidobacterium spp*. Diet, p < 0.001; Surgery, p < 0.04; Interaction, NS Analyzed by qRT-PCR.

The effect of the two independent variables on bacteriaint the cecal contents was further analyzed using quantitative qRT-PCR. For this purpose we chose to analyze beneficial bacteria which help to produce butyric acid as an end product. Standard curves, bacterial DNA amplification plots and disassociation graphs were shown in the last chapter.

Results for the, *Bifidobacterium spp* (Figure 6. 3b) Clostridial clusters IV (Figure 6. 4a), Clostridial clusters XIVa, b (Figure 6. 4b), *Bacteroides spp* and Bacterial Domain (Figure 6. 5b) were significantly affected by surgery without any significant interactions. Animals fed the RS diet had greater populations of all bacteria analyzed. Except for *Lactobacillus spp* (Figure 6. 3a), and the entire bacterial domain ($p<0.06$), the rest of the bacterial populations were significantly greater in the SH groups versus the OV groups.

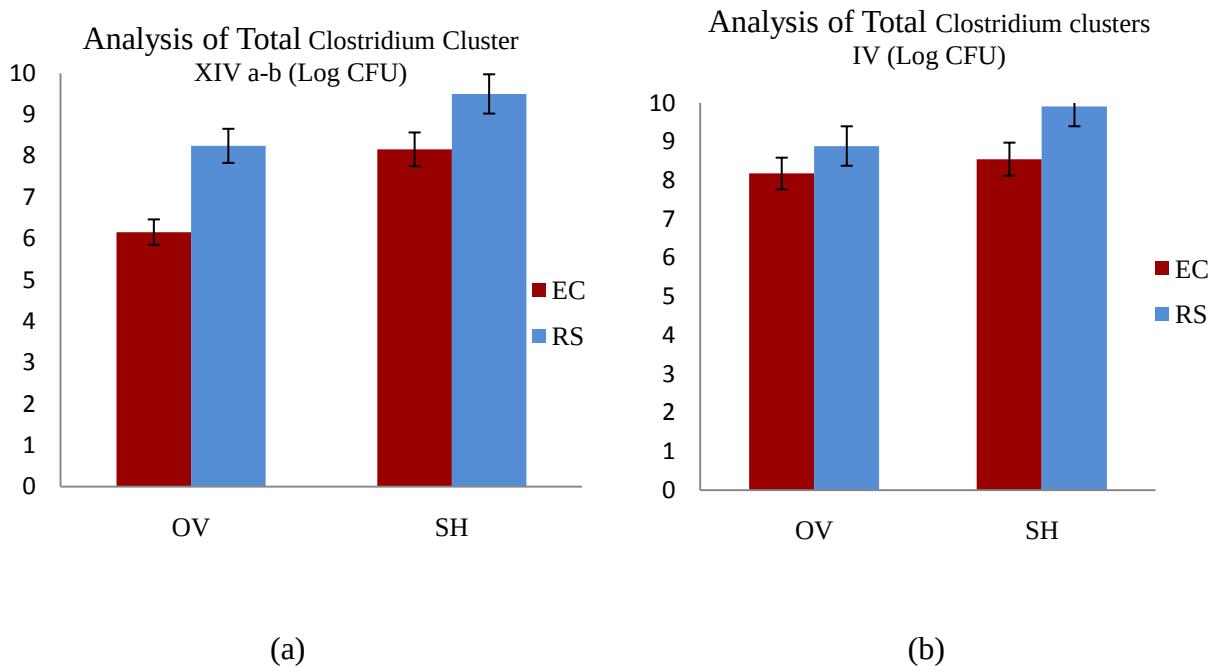


Figure 6. 4 (a) Clostridial clusters XIVa - b Population ;Diet, p < 0.0006; Surgery, p < 0.008; Interaction, NS (b) Clostridial clusters IV Population; Diet, p < 0.004; Surgery, p < 0.02; Interaction, NS Analyzed by qRT-PCR

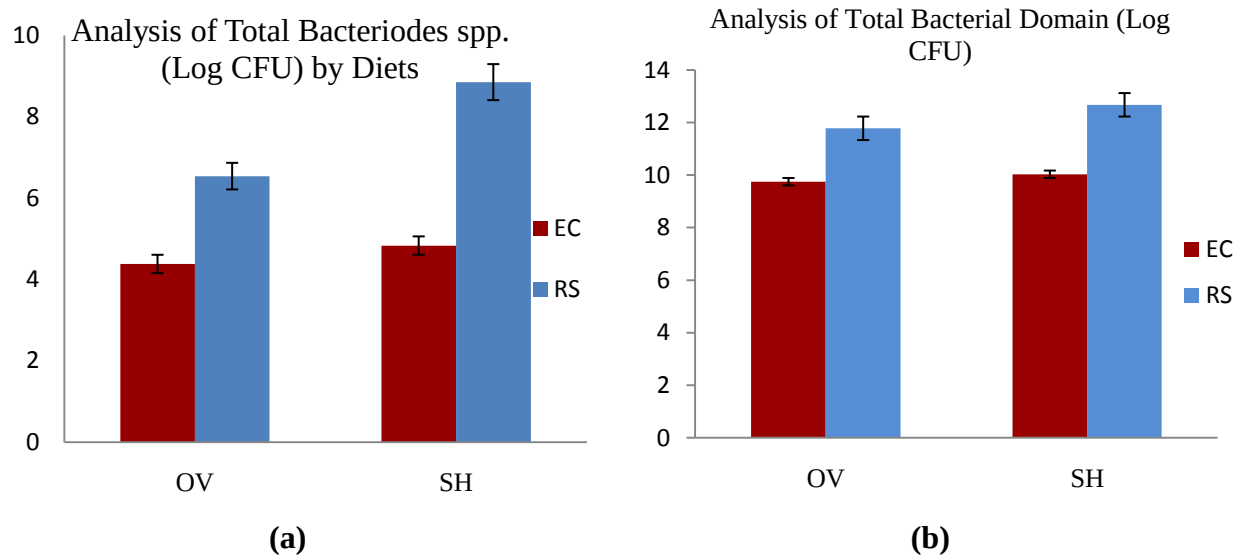


Figure 6. 5 (a) Bacteroides group Population ; Diet, p < 0.003; Surgery, p < 0.004; Interaction, NS (b) Clostridial clusters IV Population; Diet, p < 0.005; Surgery, p < 0.06; Interaction, NS, NS Analyzed by qRT-PCR

All three studies presented in chapters 3, 4 and 6 that involved isolation of bacterial DNA and performance of qRT-PCR analysis were conducted together. Hence Standard curves and melting curves were common to all three studies.

Cycling conditions for bacterial types are given in Apendix Figure I and Figure II. Standard curves for each bacterial type and disassociation curves were given in Figure III-VIII and Figure IX-XIV, respectively. The square regression coefficients in the linear regressions of all bacterial types, determined for experiments in chapters 3, 4 and 5, indicated a good correlation between the amount of template (total bacterial copies) and the amount of product (represented by the Cts) in the standard curves (R^2 = 0.99). The linearity of the standard curves and the fact that the PCR operates with consistent efficiency confirmed that the assay was well suited for quantitative measurements of each bacterial type.

There were no contaminating DNA fragments, such as primers forming dimers and mispriming PCR products, present in the reactions for the Bacterial Domain, *Lactobacillus spp*, Clostridial clusters XIVa-b and Clostridial clusters IV. There were slight shoulders visible in *Bifidobacterium spp.* and the Bacteroides group due the effect of primers forming dimers. The reason for this effect is when primers anneal to themselves and create small templates for PCR amplification. This primer dimmer effect disassociation is show a small fall off in measured concentration at the highest level and peak is less in proportion, since the effect is very subtle primer dimmer effect can be neglected. These results are shown in disassociation curves in Figure IX- XIV.

6.4 Discussion

Dietary resistant starch is a fermentable fiber and a prebiotic, which resists the amylotic reaction in the digestive system because of its granular structure and it also, enhances the

butyrate production (Kleessen *et al.*, 1997; Ferguson *et al.*, 2000; Higgins, 2004; Higgins *et al.*, 2006). This study was conducted to observe the effects of resistant starch (RS) as a prebiotic on beneficial cecal bacteria in the microbial community populations and their relation in the endocrine model of obesity, using ovariectomized (OV) or sham-operated (SH) virgin female Sprague-Dawley rats. Sham-operated rats mimic the stress of surgry of OV rats without removal of ovaries.

The targeted beneficial bacterial populations, which were analyzed in this study, were bacteria involved with the butyrate production in the large intestine. In this study, all analyzed bacterial populations, which support the production of butyric acid in the gut, were significantly increased in RS fed rats compared to rats fed EC diets regardless of type of surgery.

Data on the rats separate from the microflora results were reported previously in the thesis of Julina Robert. In her study, she analyzed body fat, body weight in the beginning and the end of the study, gut weight (Full GI tract, Full small intestine, and Full large intestine and Energy intake). The major result was reduced body fat and increased gut size for rats fed the RS diet versus the EC diet. Similar results were previously demonstrated by several investigators with the RS consumption (de Deckere *et al.*, 1993; Keenan *et al.*, 2006; Shen et al., 2009; Zhou et al., 2009). Thus, increased gut size and decreased body fat in animals fed the RS versus the EC diet, was accompanied by changes in the cecal microflora. Total colony forming units determined by plate counts were assessed for total lactic acid producing bacteria and total culturable anaerobic. Using RT-PCR, bacterial analyses were broadened on the one hand to include the entire bacterial domain and fine-tuned on the other hand to include Bifidobacteria, Clostridial clusters XIV a-b and Clostridial clusters IV, and Basteriodes. Bacterial results with

the use of both methods support the effects of reduced body fat with increased bacterial growth of bacteria known to be associated with fermentation of resistant starch.

Lactobacillus spp have been studied regarding obesity and increased levels are associated with reduced body weight and reduced serum total cholesterol concentrations in the blood. Various proposed mechanisms for these effects have been reported. Specifically, increases in *Lactobacillus Spp, such as L. acidophilus, L. reuteri , L. casei* and *L. gasseri,* are reported to be associated with significantly reduced the serum total cholesterol concentrations and triglyceride concentrations in mice (Akalin *et al.*, 1997; Xiao *et al.*, 2003; Liong & Shah, 2005). Use of probiotics, such as *Lactobacillus rhamnosus* (*L. rhamnosus*) PL60, have been reported to result in a significant reduction of obesity in mice by producing weight loss without reducing energy intake (Lee *et al.*, 2006).

Bifidobacterium spp have been proven to have a beneficial effect on health by improving gut permeability and it also suppresses pathogenic species such as the Enterobacteriaceae family (O'Sullivan, 2008). This same group of bacterial species has also been involved with reduction of serum total cholesterol concentrations in the blood (Beena & Prasad, 1997; Xiao *et al.*, 2003)

Although lactobacilli and bifidobacteria both feed on resistant starch, they do not produce butyrate. Both Clostridial clusters IV and Clostridial clusters XIVa-b are the major bacterial groups which produce butyrate. Butyrate has been identified as a beneficial SCFA which helps to maintain gut health (Cummings & Macfarlane, 1991; Gibson, 1998; Sato *et al.*, 2008).

The ovarian hormone estrogen is known to influence energy metabolism. The major factor which may contribute to the gradual fall in resting energy expenditure with age and menopause is the loss of the luteal phase. This specific effect of menopause may decrease the resting metabolic rate (Poehlman, 1993; Heymsfield *et al.*, 1994; Panotopoulos *et al.*, 1997).

Ovarian hormones are reported to delay the processes of aging (Morrison *et al.*, 2006). Sham operation was done to imitate the ovariectomized operation but they have active ovaries. So that OV animals should age faster than SH animals. This aging process also may affect the ecology of gut microbial populations as well. The results of the current study may be a reflection of the aging process. *Lactobacillus spp* populations are reported to increase with increasing age, while the populations of *Bifidobacterium spp* and *Bacteriodes spp.* are reduced with increasing age. Although, *Bacteriodes spp* population is reported to decrease with increasing age, their bacterial diversity has been reported to be increased (Mitsuoka, 1992). In agreement with the past studies, *Bifidobacterium spp* and *Bacteriodes spp* were significantly lower in OV compared to SH rats in the current study. However, *Lactobacillus spp* analyzed by either method were not change increased with OV surgery. The latter may be the result of measuring a broad range of bacteria with measurement of *Lactobacillus spp,* and specific lactobacilli that increased in previous studies were diluted with numerous other Lactobacilli that are not changed with OV. It is also possible that the aging process was not advanced enough by OV surgery during the study to observe the effect of an increase in *Lactobacillus spp*. It may take a longer study to observe the effects of aging on increased *Lactobacillus spp* in OV rats.

All other bacterial populations analyzed in this study, the entire Bacterial Domain, Clostridial clusters IV, Clostridial clusters XIVa, b and Total anaerobic bacteria including *Clostridium spp,* were higher in cecal contents of SH versus OV rats. However, the values for the bacterial domain only approached significance ($p<0.06$). The populations of Clostridial clusters IV, Clostridial clusters XIVa-b are also reported to be reduced with increasing age (Hayashi *et al.*, 2003; Zwielehner *et al.*, 2009).

6.5 Conclusion

The present study demonstrated that, adding resistant starch to the diet may improve health by increasing beneficial bacteria and reducing body fat. This may mean that prebiotics like resistant starch could be used in the diet to overcome increased fat gaining during the postmenopausal period of women.

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CHAPTER 7: COMPARISON OF DIET RESTRICTION VERSUS A LOW FAT RESISTANT STARCH DIET

7.1 Introduction

One of the major problems each one of us has face in our life time is aging. Aging is often related with health related chronic problems and severe infectious disease. Most of these conditions are often associated with the gastrointestinal tract (Garibaldi & Nurse, 1986). With aging the the gastro intestinal tract pH increases due to lack of acid production (Hurwitz *et al.*, 1997). This increase of pH has negative effects on the health numerous ways, such as increased ability to absorb toxic compounds, and promotion of pathogenic strains such as *Helicobacter pylori* in the gut consequently it also up regulates the ability gastritis, ulcers gastric cancers and colorectal cancers (Thornton, 1981; Sgouros & Bergele, 2006).

Diet /Calorie restriction is a strategy of under-nutrition without malnutrition, which has been shown to improve longevity by improving health in rodents and primates including human (Hursting *et al.*, 2003). Diet restriction has anti-cancer effects, and anti leukemia effect in rats (Hursting *et al.*, 1993). Furthermore, HDL cholesterol increases were observed in monkeys and Muslims who fast during the daylight hours of the holy month of Ramadan cit original article here. Similar results were indicated by inhabitants of Okinawa, Japan, with fewer calories and they indicated lower death rates from cancer and vascular diseases (Hursting *et al.*, 2003). These evidences conclude that practice of calorie restriction will extend the healthy human life span.

With the aging gut microbial diversity and population varies. For example, in elderly populations, the following occurs, a reduction of percentage of *Bifidobacterium spp. Bacteroides spp*. and Clostridium cluster IV (Clostridium *leptum*) and an increase in Fungi, members of family Enterobacteriaceae, *Lactobacillus spp.* and some *Clostridia spp.* such as *C. difficile* group *and C.perfringens* (Hopkins *et al.*, 2001; Mueller *et al.*, 2006; Tuohy, 2007).

As discussed in early chapters RS is a prebiotic and it beneficially affects the host by selectively stimulating the growth of beneficial bacteria while improving host health in numerous ways (Brown *et al.*, 1997; Bird *et al.*, 2000; Higgins, 2004; Keenan *et al.*, 2006; Zhou *et al.*, 2008; Shen *et al.*, 2009).

Hence calorie restriction and RS diets both help to improve host health. The main focus of this study is compare on the gut microbiota of the calorie restricted aged mice with the low fat RS fed mice main beneficial gut microbial populations.

7.2 Material and Methods

7.2.1Animals and Diets

This study was conducted as two separate studies of restricted calorie diet study versus continuous diet and RS versus EC with low fat diet study.

7.2.1.2 Study 1

Two-year old twelve female C57BL/6 mice from the Mutant *Mouse* Aging *Colony* (National Institute of Aging colony) were fed either restricted calorie diet or normal EC diet (n=6) to measure the effect of calorie restricted diet on the variation of beneficial gut microbial populations. Both restricted calorie diet fed animals and normal EC diet fed animals were individually housed. CR was initiated at 14 weeks of age at 10% restriction, increased to 25% restriction at 15 weeks, and to 40% restriction at 16 weeks where it is maintained until 24 months of age.

All 2 groups mice were Mice were fed the control diet for the first six weeks prior to dietary treatment to observe energy intake and body weight gain. Then these animals were fed with either restricted or continuous diet for 3 months. At the end of the study animals were sacrificed and mice ceca were tied with threads, separated from the rest of the GI tract, and

aseptically transferred to separate Whirl-pak bags. Whirl-pak bags were placed in a double Zip lock bag with an anaerobic GasPak™ EZ Gas generating Pouch System (BBL *GAS* PAK, Voiglobal Distribution INC. P.O. Box 1130, Lawrence, Kansas 66044-8130 USA) and immersed in ice.

7.2.1.2 Study 2

Eighteen C57bl/6J female mice were used to compare low fat Energy control (LO EC) diet versus Low fat RS (LO RS) diet (n=9). These 18 animals were similar to animals which we used for Chapter 3. All 2 groups mice were Mice were fed the control diet for the first six weeks prior to dietary treatment to observe energy intake and body weight gain. Then they were fed one of four diets, Low fat (Lo fat) Control, or Lo fat RS. Both Low fat diets had 18% fat to equalize the energy density of the two diets (Table 1 Chapter 3).Mice were fed each one of the diets for 10 weeks and at the end of the study, mice were sacrificed and ceca were collected as explained in study 1.

7.2.2 Microbial Analysis of the Cecal Contents

7.2.2.1 DNA Extraction

Bacterial DNA were extracted using the same method explain in Chapter 3. DNA was extracted from ceca samples with the help of QIAamp DNA Stool Mini kit (QIAGEN, Valencia, CA). All the manufacturer's instructions were followed with slight modifications. The slight modification was after adding stool lysis (ASL) buffer to samples of the diluted cecal contents (200 µl), the samples were subjected to three cycles of freeze-thaw in liquid nitrogen and a 5 min at 95°C water bath to break the thick gram positive bacterial cell walls. After the extraction of DNA the purified DNA was quantified using a NanoDrop ectrophotometer and all DNA sample extracts were diluted to 1ng/µl. Purified DNA was stored in a -80 ^{o}C freezer.

7.2.2.3 Quantitative Real-time PCR

The SYBR® Green method of quantitative real–time PCR (qRT-PCR) assay was performed using an ABI Prism 7900HT Sequence Detection System (serial 100151) (Applied Biosystems, Foster City, CA) as explained in previous chapters. The information of the targeted bacterial groups, primer sequences, annealing temperatures and literature references are given in Chapter 3, Table 2. All reactions were performed in sterile MicroAmp® optical 384-well reaction plates with barcode sealed with MicroAmp® optical adhesive film (Applied Biosystems, Foster City, CA). Hence construction of a standard curve for real-time PCR bacterial dilutions, Targeted bacteria, Annealing Temperature (^{o}C) and Sequence of oligonucleotide were exactly same to the Chapter 3.

7.2.3 Statistical Analysis

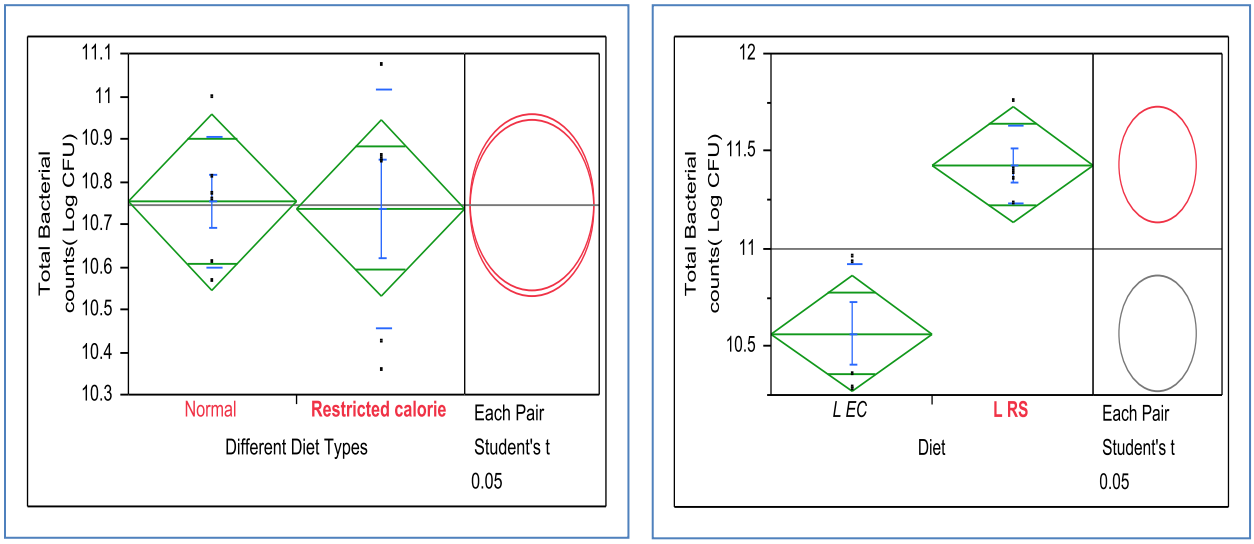
Both the Study 1 (restricted calorie diet study and continuous diet) and Study 2 fat (Energy control diet and Low fat RS) were analyzed by statistical comparisons of all pairs using the Student's t test following 1-way analysis of variance (ANOVA) (JUMP In version 7.0, SAS Institute Inc., Cary, N.C., U.S.A.). Statistical significance occurs for $P<0.05$.

7.3 Results

The bacterial populations involved in the process of fermenting resistant starch to butyric acid were further analyzed using qRT-PCR (Figure 7.1-Figure7. 6).The statistical significant levels $t < 0.05$ were indicated in either gray or red color.

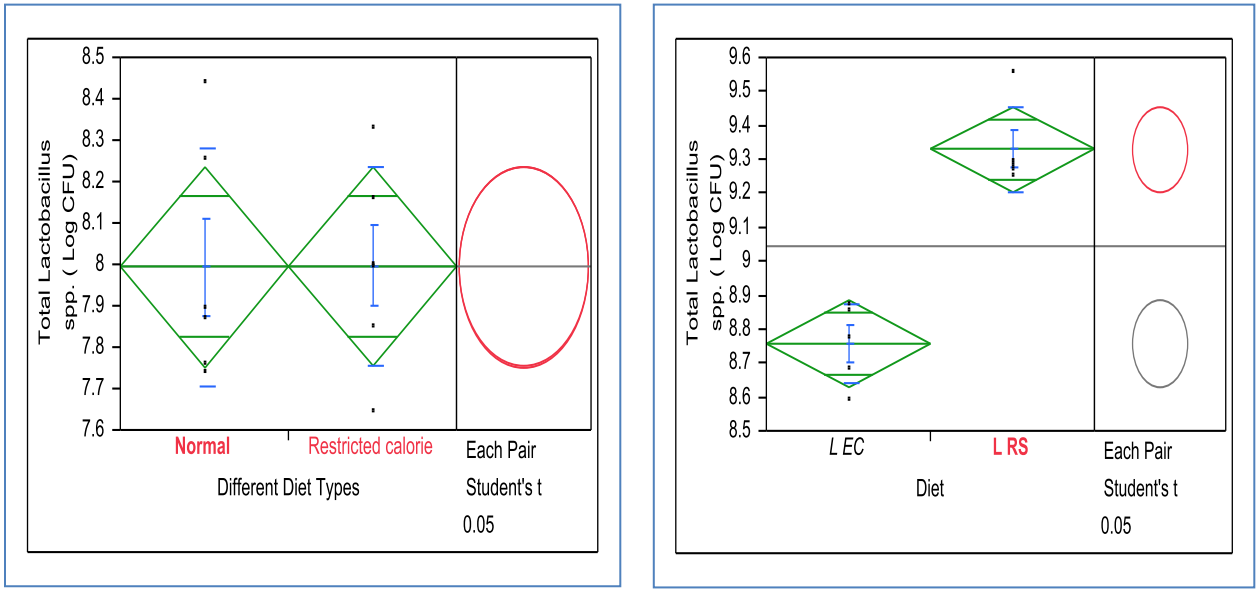
The Total bacterial domain (Figure 7.1) and *Lactobacillus spp* (Figure 7.2) were comparable for each other. Both total bacterial domain and *Lactobacillus spp* were not significantly different for caloric restriction diet fed animal and control diet fed animals.

Whereas, low fat RS fed animals had significantly high Total bacterial domain ($P < 0.0014$) and *Lactobacillus spp* ($P < 0.0001$).



(a) **(b)**

Figure 7.1 Analysis of Bacterial Domain (a) Restricted Calorie Diet Study Versus Continuous Diet (b) RS Versus EC with Low Fat Diet Study: Diet p<0.0014



(a) **(b)**

Figure 7.2 Analysis of *Lactobacillus spp.* (a) Restricted Calorie Diet Study Versus Continuous Diet (b) RS Versus EC with Low Fat Diet Study: Diet p<0.0001

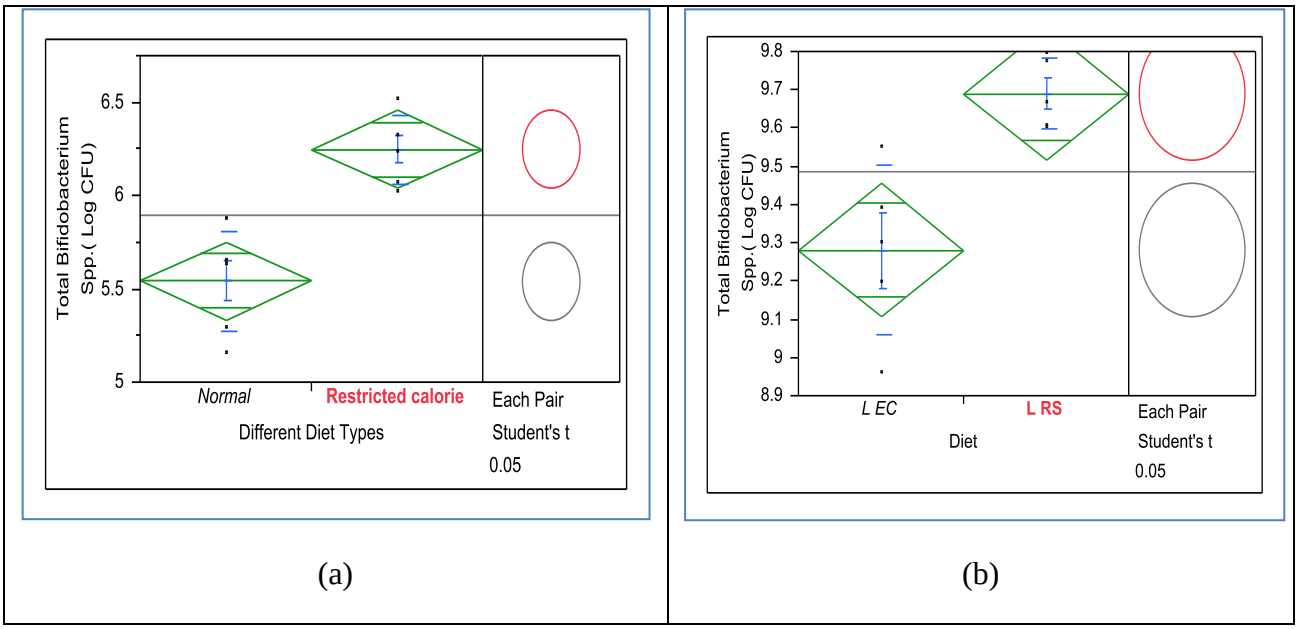


Figure 7.3 Analysis of Total *Bifidobacterium Spp.* (a) Restricted Calorie Diet Study Versus Continuous Diet :Diet p<0.0003. (b) RS versus EC with Low Fat Diet Study: Diet p<0.005

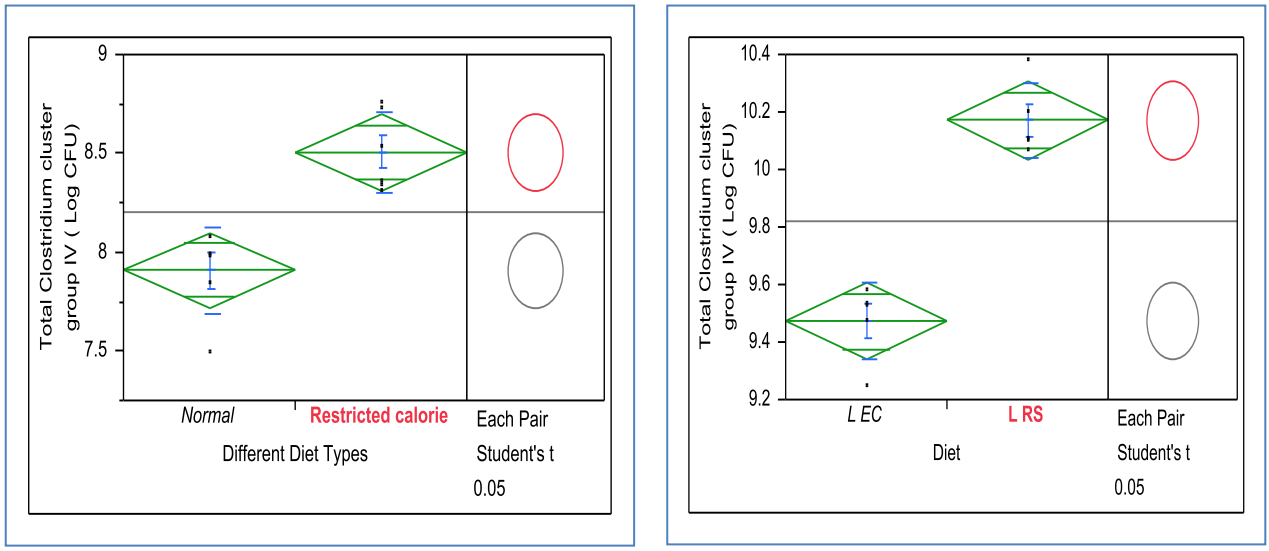
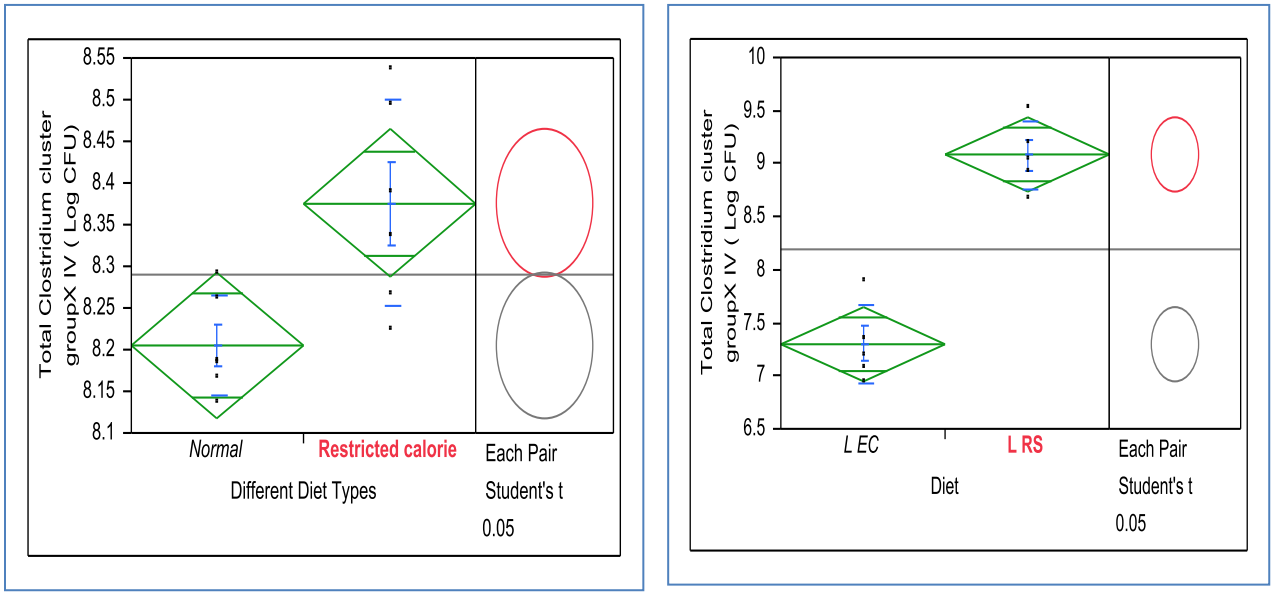


Figure 7.4 Analysis of Total Clostridium Cluster IV (a) Restricted Calorie Diet Study Versus Continuous Diet :Diet p<0.0006. (b) RS versus EC with Low Fat Diet Study: Diet p<0.0001

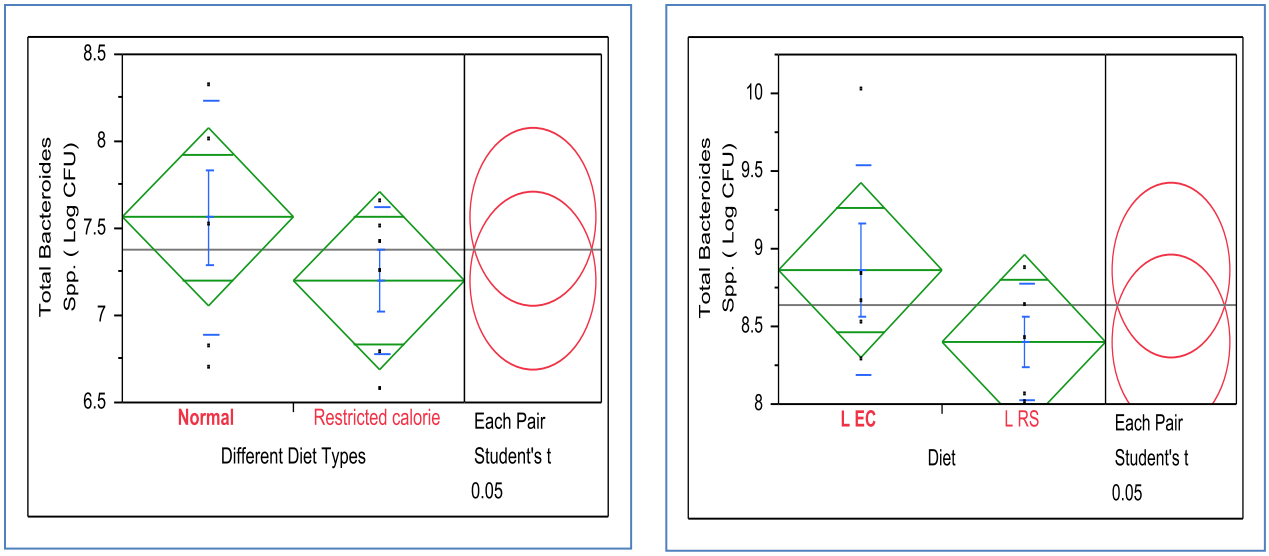
The Total *Bifidobacterium spp.* (Figure 7.3) Clostridium cluster group IV (Figure 7.4) and Clostridium cluster group XIVa-b (Figure 7.5) were comparable for Study 1 and study 2. Ceca from mice fed Restricted calorie diet had higher levels of Total *Bifidobacterium spp.* ($P < 0.0003$), Clostridium cluster group IV ($P < 0.0006$) and Clostridium cluster group XIVa-b ($P < 0.0125$) populations than ceca from mice fed normally. Similarly, ceca from mice fed the low fat RS diet had significantly higher levels of total *Bifidobacterium spp.* ($P < 0.005$), Clostridium cluster group IV ($P < 0.0001$), and Clostridium cluster group XIVa-b ($P < 0.0001$) populations than in ceca from mice fed a low fat diet without RS.

There was no effect of diet treatment on bacteria from the Bacteroides group in cecal contents of mice with both studies.



(a) **(b)**

Figure 7.5 Analysis of Clostridum Cluster XIVa-b(a) Restricted Calorie Diet Study Versus Continuous Diet: Diet p<0.01. (b) RS Versus EC with Low Fat Diet Study: Diet p<0.0001



(a) **(b)**

Figure 7.6 Analysis of Bacteroides group Counts (a) Restricted Calorie Diet Study Versus Continuous Diet (b) RS Versus EC with Low Fat Diet Study

7.4 Discussion

Several investigators support the possibility of restricted calorie diet that can exert life span by reducing aging related diseases such as cardiovascular disease, cancer, leukemia and digestive tract related problems (Ma *et al.*, 1992; Hursting *et al.*, 2003; Hursting *et al.*, 2007; Heilbronn *et al.*, 2006; Sun *et al.*, 2009). In present investigation we observed significant increase of beneficial bacterial populations such as populations of *Bifidobacterium spp.* Clostridium cluster group IV and Clostridium cluster group XIVa-b with restricted calorie diet in aged mice than the normal diet fed aged mice (Figure 7.3, 7.4 and 7.5).

The relationship between aging and the *Bifidobacterium spp.* were studied by several investigators and according to all of their finding; the *Bifidobacterium spp.* populations were reducedwith the age (Gorbach *et al.*, 1967; Benno *et al.*, 1992; Mitsuoka, 1992). Similar results were observed for Clostridium cluster group IV group. Clostridium cluster group IV group were decreased in terms of percentage microbiota composition in the elderly (Zwielehner *et al.*, 2009).

Hayashi *et al*, 2003 have indicated cecal microbial variation in six elderly individuals using 16S rDNA. They found that the proportion of Clostridium cluster XIVa was lower than in healthy adults (Hayashi *et al.*, 2003).

In our present study animals fed restricted calorie diet were able to elevate *Bifidobacterium spp.*, Clostridium cluster group IV group and Clostridium cluster group XIVa group in aged mice. Hence restricted diet fed animals experienced an increase in beneficial bacteria. For example *Bifidobacterium spp* helps to improves the digestion absorption and immune system while decreasing the side-effects of antibiotic therapy . Mean while it also provides protection against enteric pathogens, putrefactive substances, and believed to be involved with mechanisms of reduction of cholesterol levels and anti-tumoral activity (Leahy *et al.*, 2005). The Clostridium cluster group IV and Clostridium cluster group XIVa-b are the major two phylogenic groups involve with production of butyric acid. Butyric acid is well established for improving health (Scheppach *et al.*, 2001; Hijova & Chmelarova, 2007; Sato *et al.*, 2008). Some of these benefits are butyric acid act as a major energy source for epithelial cells of colonic mucosa which stimulates colorectal cell proliferation, while maintaining healthy epithelium by blocking the absorption of cancer-causing substances (Scheppach *et al.*, 2001; Sato *et al.*, 2008 Hagopien *et al.*, 1977 ,Cummings & Macfarlane, 1991, Gibson *et al.*, 1998).

It is well known that as a prebiotic RS diet stimulates beneficial bacterial populations which involve fermenting RS in to Butyric acid. In this study low fat RS diet fed animals ceca were shown to significantly increase populations of *Lactobacillus spp, Bifidobacterium spp.* Clostridium cluster group IV and Clostridium cluster group XIVa-b and the total bacterial domain than the low fat EC fed Animals. In both studies (Restricted calorie diet versus nomal diet and RS diet versus EC diet) were not significant for Bacteroidetes group in $p<0.05$ level

because Bacteroidetes group have been reported in high variations between individuals (Layton *et al.,* 2006).

Both Restricted calorie diet and RS diets comparable for four out of six investigated bacterial populations. It is well established that diet restriction leads to improved health and increased longevity in many species (Ma *et al.,* 1992; Hursting *et al.,* 2003; Heilbronn *et al.,* 2006; Sun *et al.,* 2009). Most of the prebiotics have identified as a healthy aging diets (Tuohy, 2007; Guigoz *et al.,* 2008; Vulevic *et al.,* 2008). In addition, resistant starch diets improve gut function and metabolic status. Hence we could argue that low fat RS diet as healthy diet which helps to increase life span.

7.5 References

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CHAPTER 8: CONCLUSION

This study demonstrated that, Resistant starch (RS) increases beneficial gastro-intestinal bacterial populations which involved in fermentation RS in to butyrate. Those beneficial bacterial populations which increased by RS diets were Total culturabale anerobic bacteria, Lactic Acid Bacteria, Culturable *Bifidobacterium spp* which analysed by plate count metod; *Lactobacillus spp, Bifidobacterium spp,* Clostridium cluster IV and Clostridium cluster XIVa-XIVb which analysed by qRT-PCR.

The High fat levels (41% dietary energy) were interfered with this process and prevented fermentation of RS and reduced bacterial populations in the ceca compared to a low fat diet (18% dietary energy). But Modarate fat levels (22% dietary energy) had relatively less effect on fermentation of RS and reduced bacterial populations in the ceca compared to a low fat diet. The type of diet the mice were fed had no effect on the bacterial numbers for the Bacteroids group in the cecal contents. However, high fat diet fed micehad higher counts of *Bacteroides* group in their cecal contents compared to mice fed the low fat diets. As a diferant type of fat both Corn oil and Lard reduced bacterial populations which are involved in fermentation of RS. However, if the fat used was fish oil there were no negative effects on the fermentation of RS or the bacterial population. These studies were highlighted importance of controling the type and level of fat when consuming resistant starch as a prebiotics in diet.

With the ovariectomy and aging process simulate and with the age *Bifidobacterium spp, Bacteroides spp,* Clostridium cluster IV and Clostridium cluster XIV decreased. RS diet was able to reduced body fat in an endocrine model of obesity caused by ovariectomy (OV) while increasing the beneficial bacterial populations. In Aged mice both calorie restricted diet and RS diets were able to improve *Bifidobacterium spp,* Clostridium cluster IV and Clostridium cluster XIV populations.

APPENDIX A: RT-PCR PROTOCOL AND STANDERD CURVES

(a)Thermal Cycler Protocol of Rt-PCR

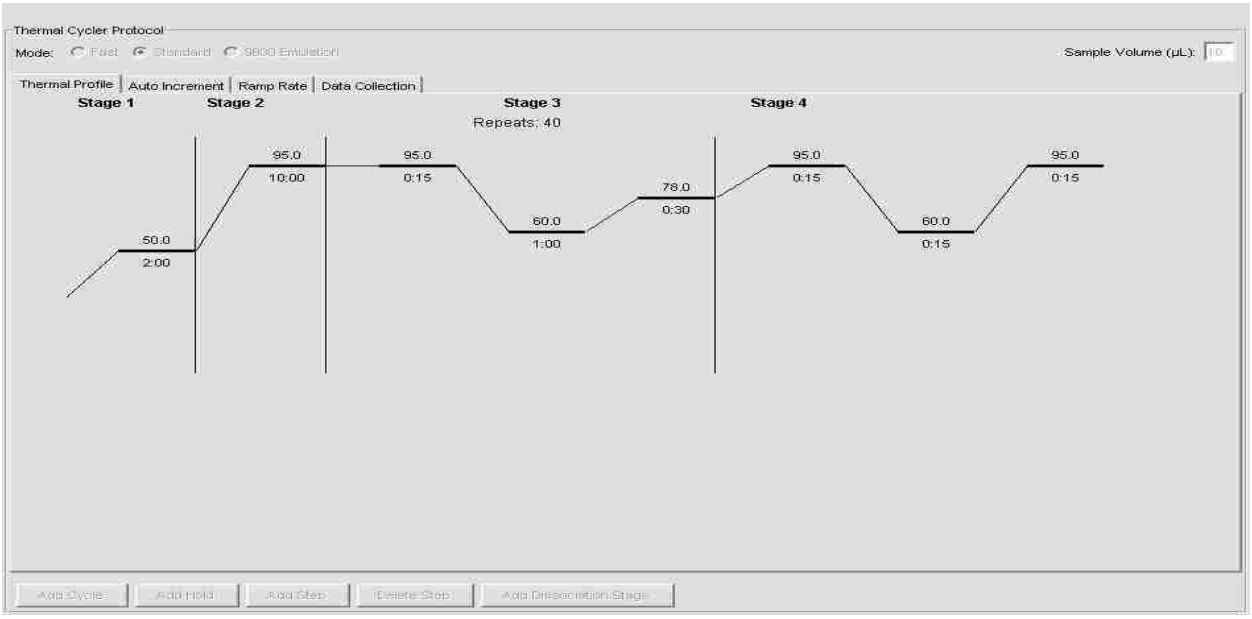

Figure I Thermal Cycler protocol for Bacterial Domain, *Lactobacillus spp*, Clostridial clusters IV, Bacteroides group and *Bifidobacterium spp.*

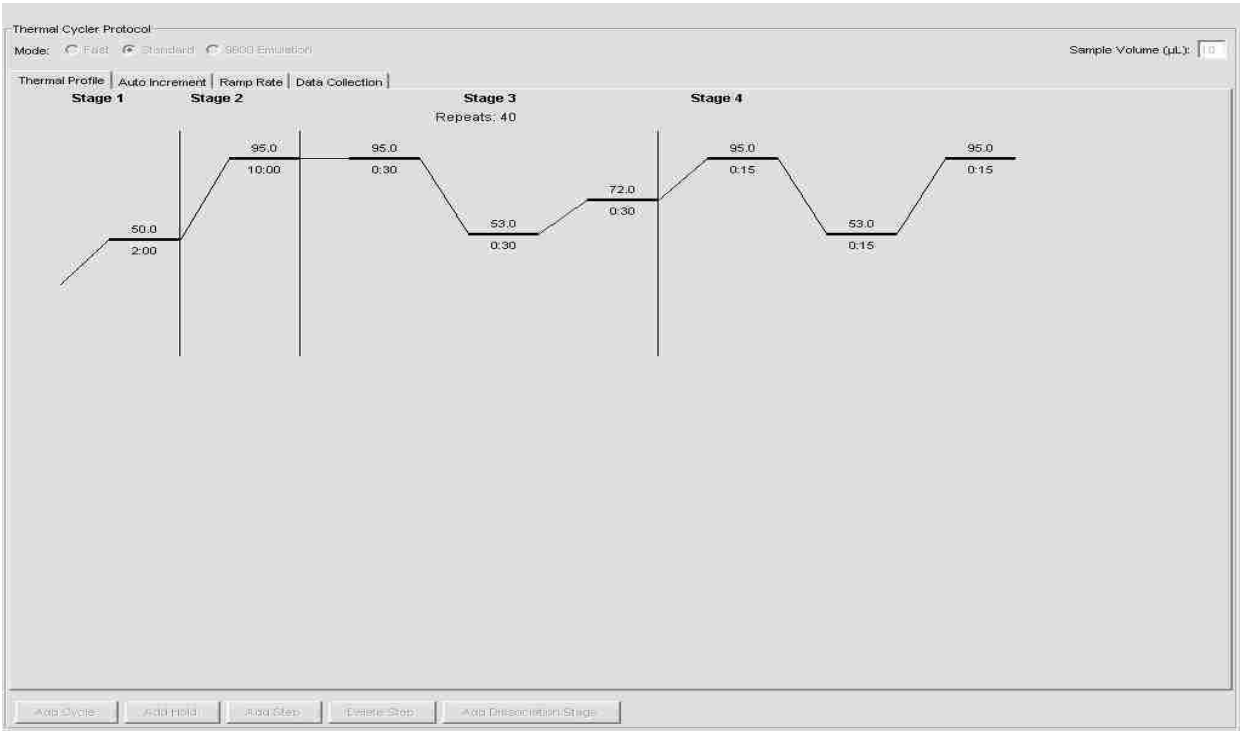

Figure II Thrmal Cycler Protocol for Clostridial clusters XIVa-b

(b)Bacterial Standerd Curves Which Analysed by Rt-PCR

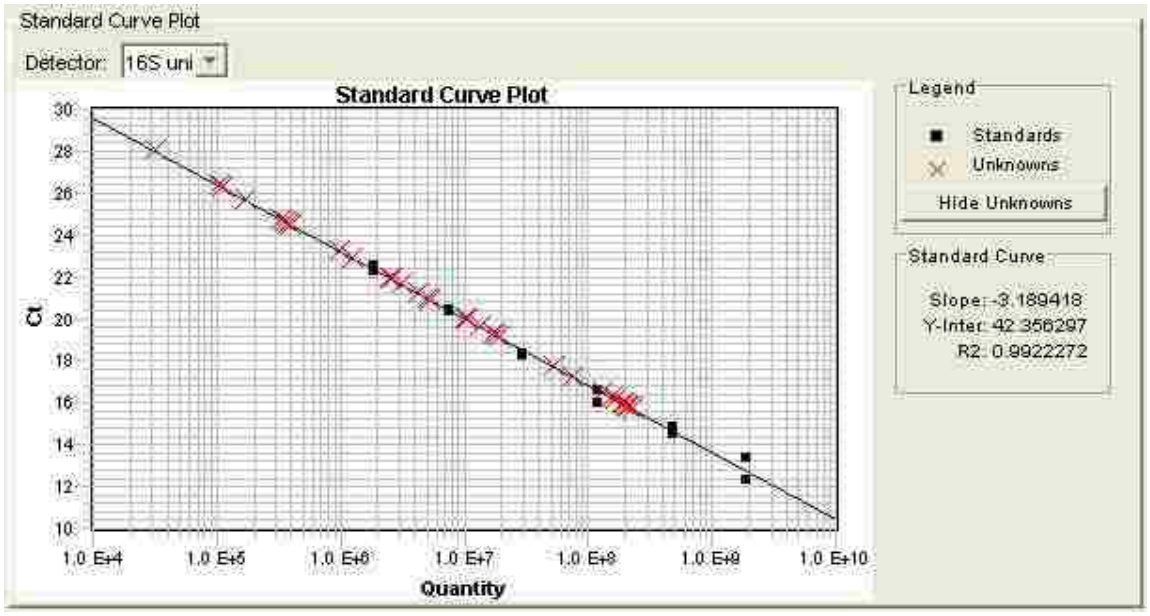

Figure III Standard Curve for Bacterial Domain

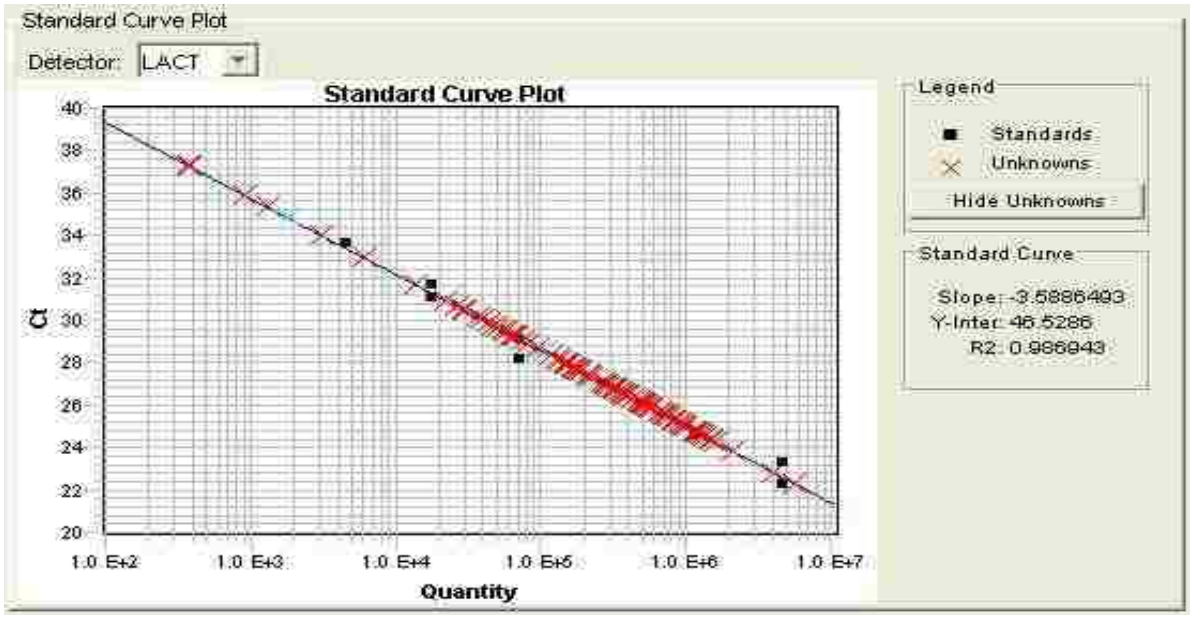

Figure IV Standard Curve for *Lactobacillus spp*

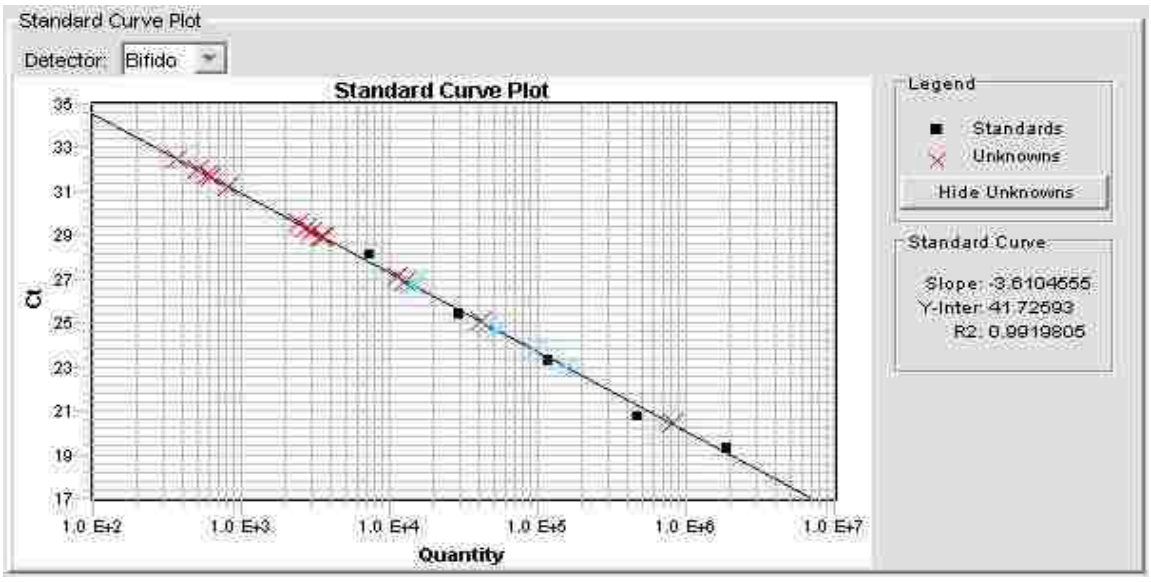

Figure V Standard Curve for *Bifidobacterium spp.*

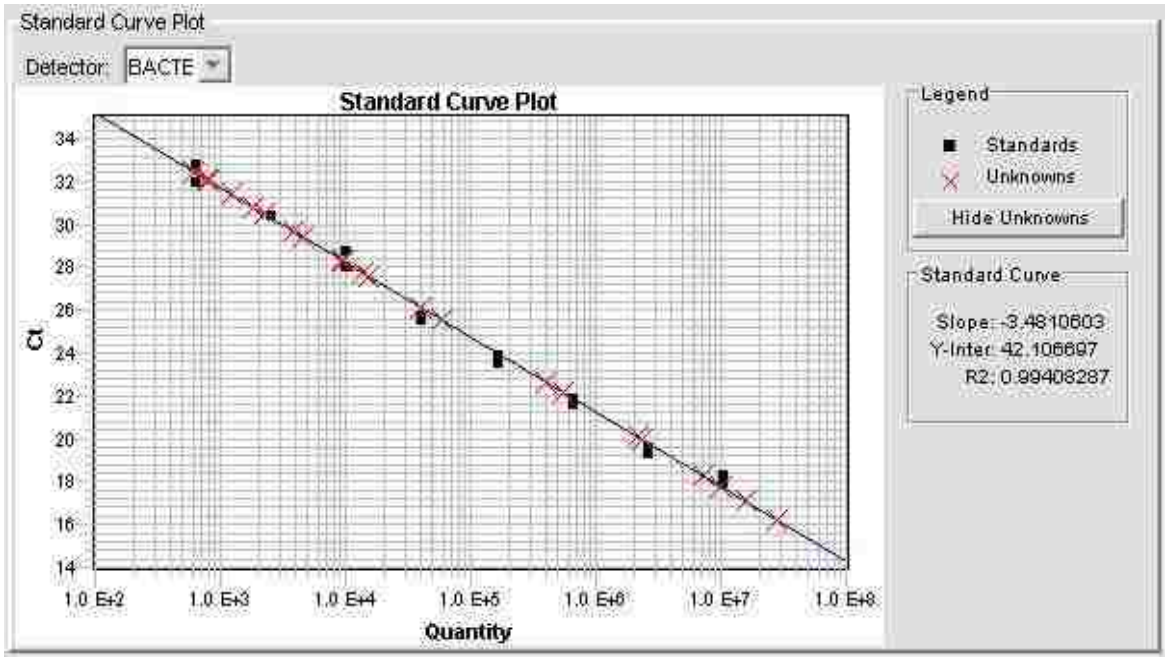

Figure VI Standard Curve for Bacteroides group

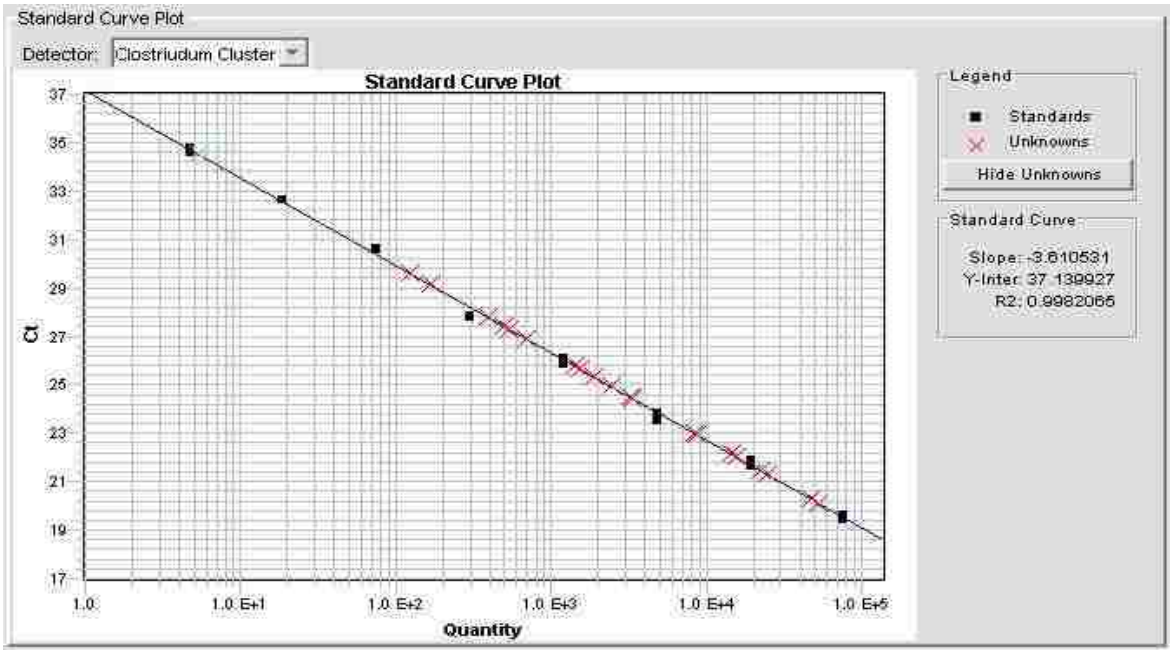

Figure VII Standard Curve for Clostridial clusters XIVa-b

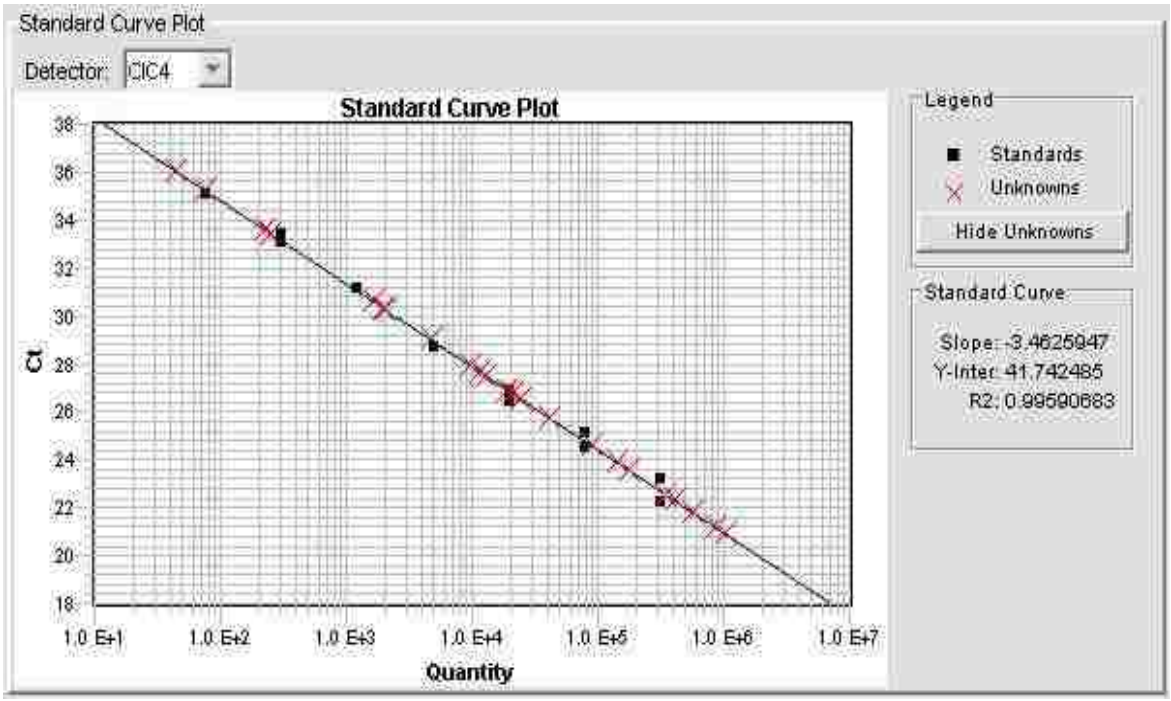

Figure VIII Standard Curve for Clostridial clusters IV

(c)Bacterial Dissociation Curves Which Analysed by Rt-PCR

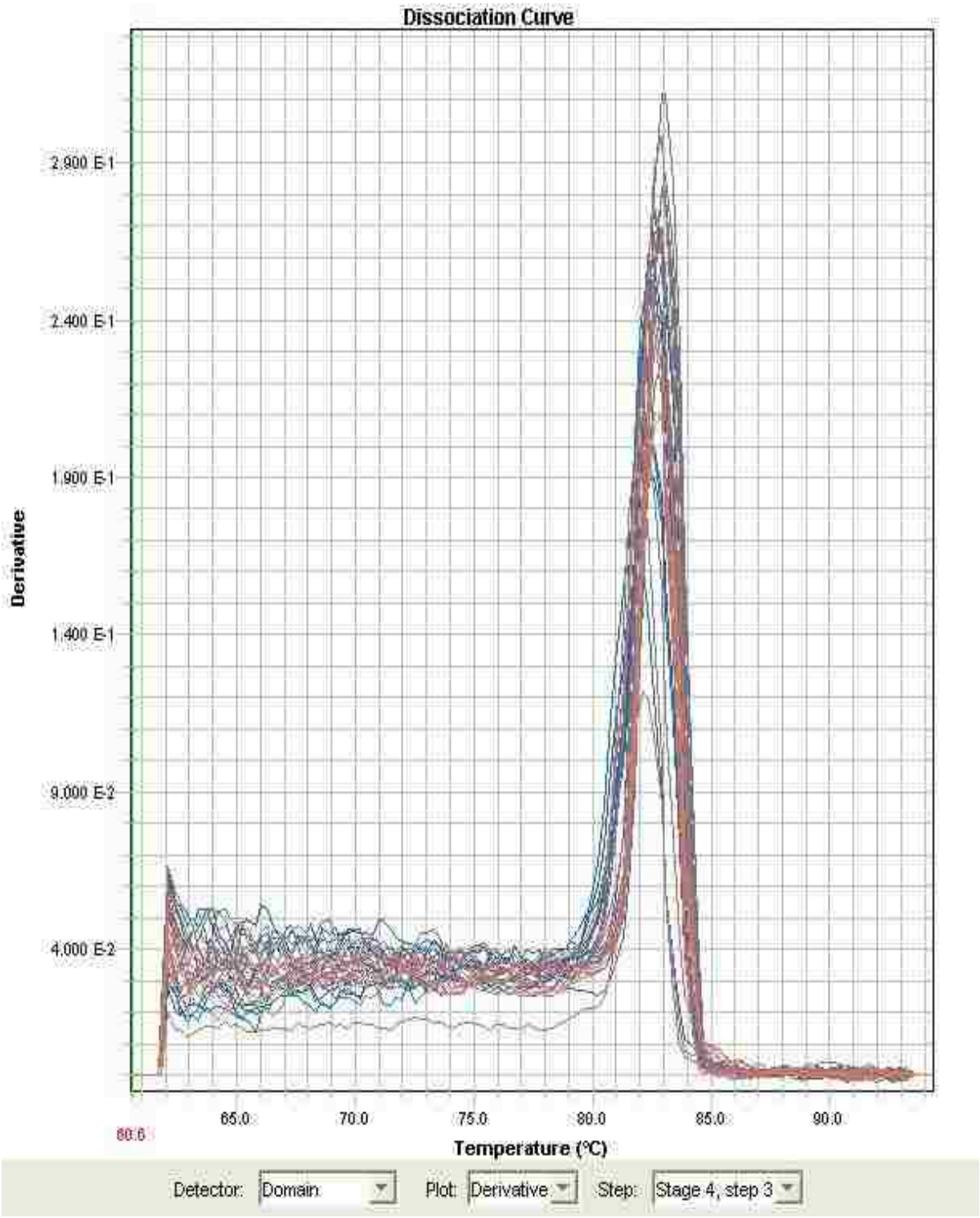

Figure IX Bacterial Domain (Melting T 82.5 oC)

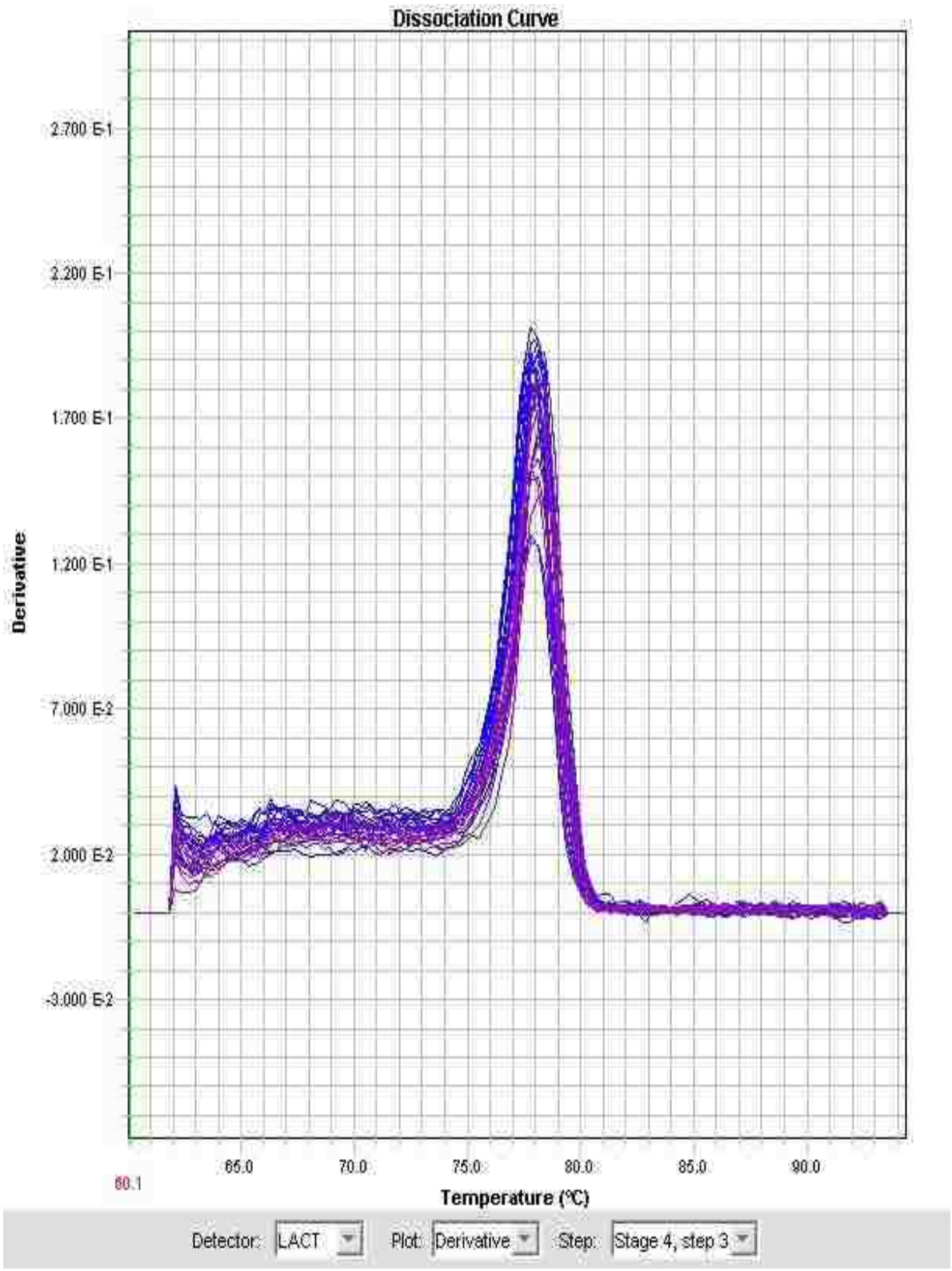

Figure X *Lactobacillus spp* (Melting T 78 oC)

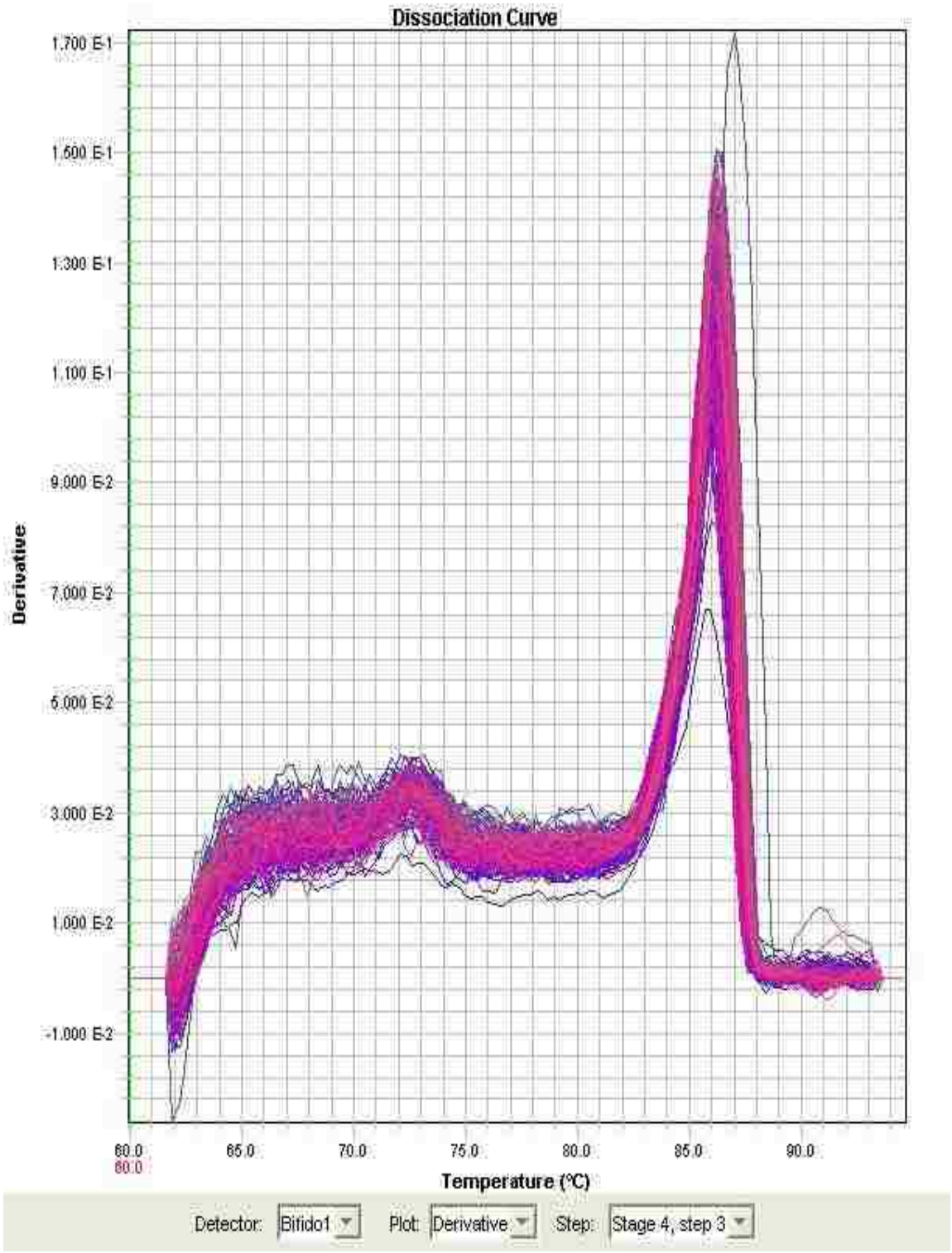

Figure XI *Bifidobacterium spp.* (Melting T 86 oC)

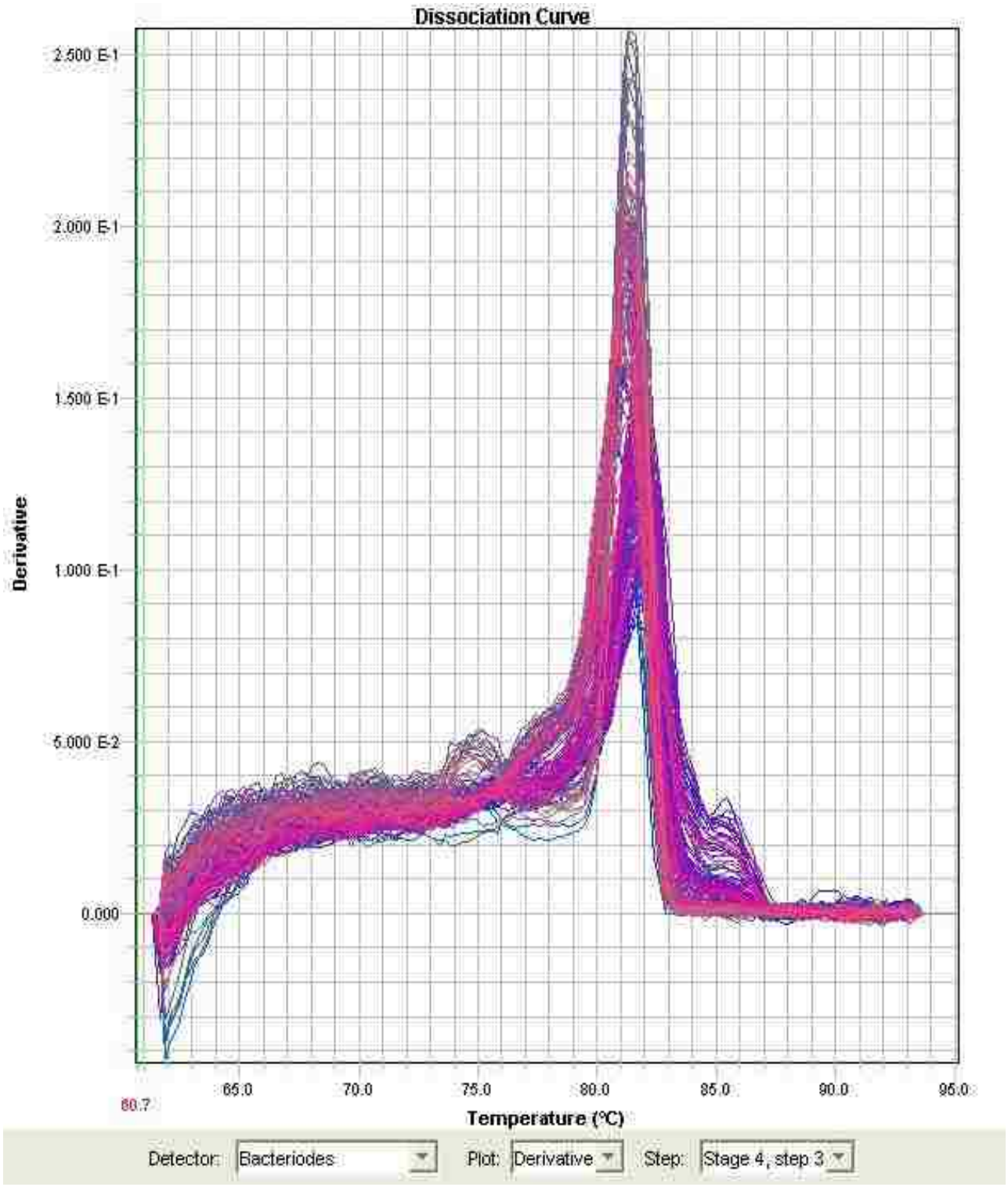

Figure XII Bacteroides group (Melting T 81 oC)

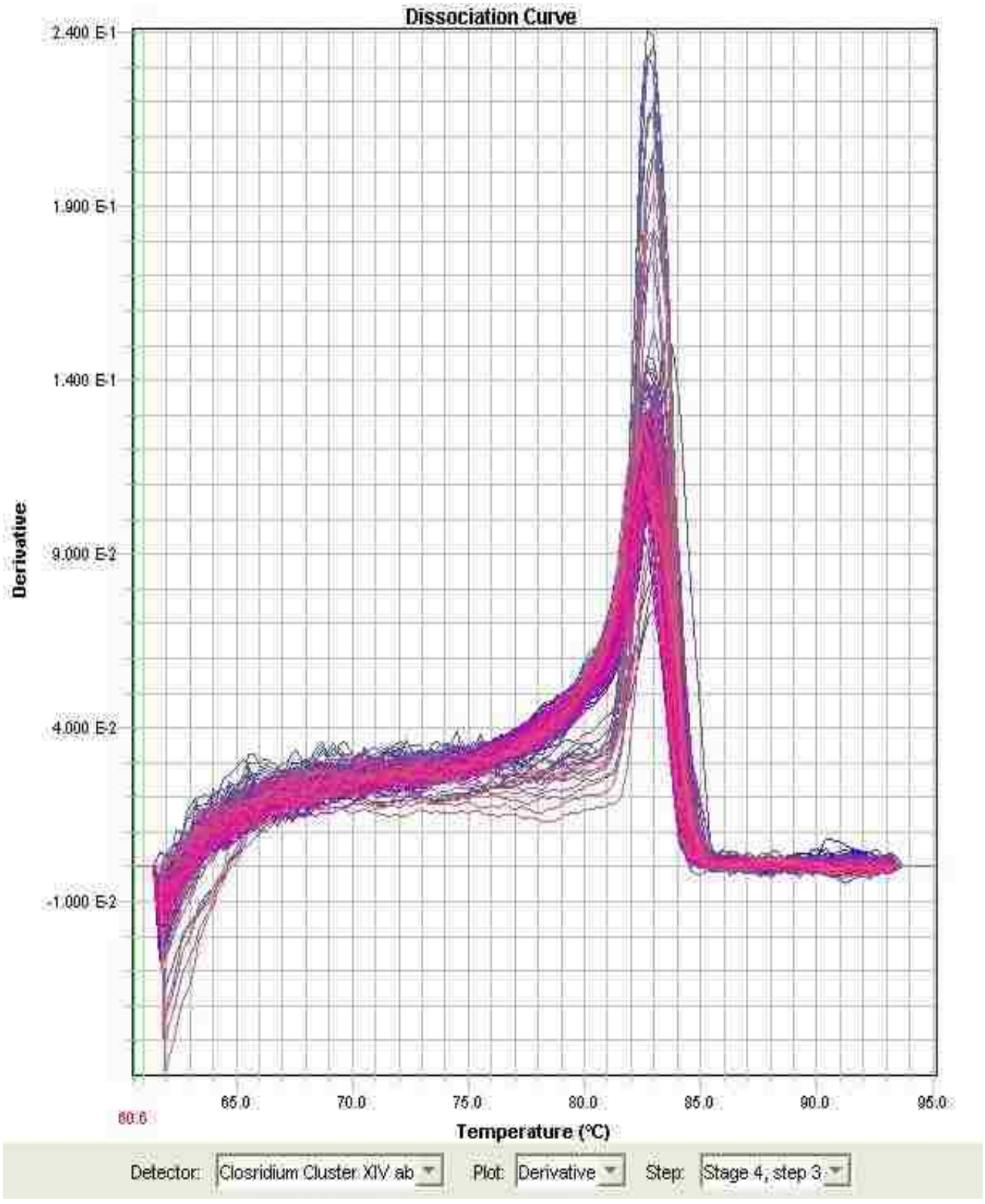

Figure XIIIClostridial clusters XIVa-b (Melting T 83 ^{o}C)

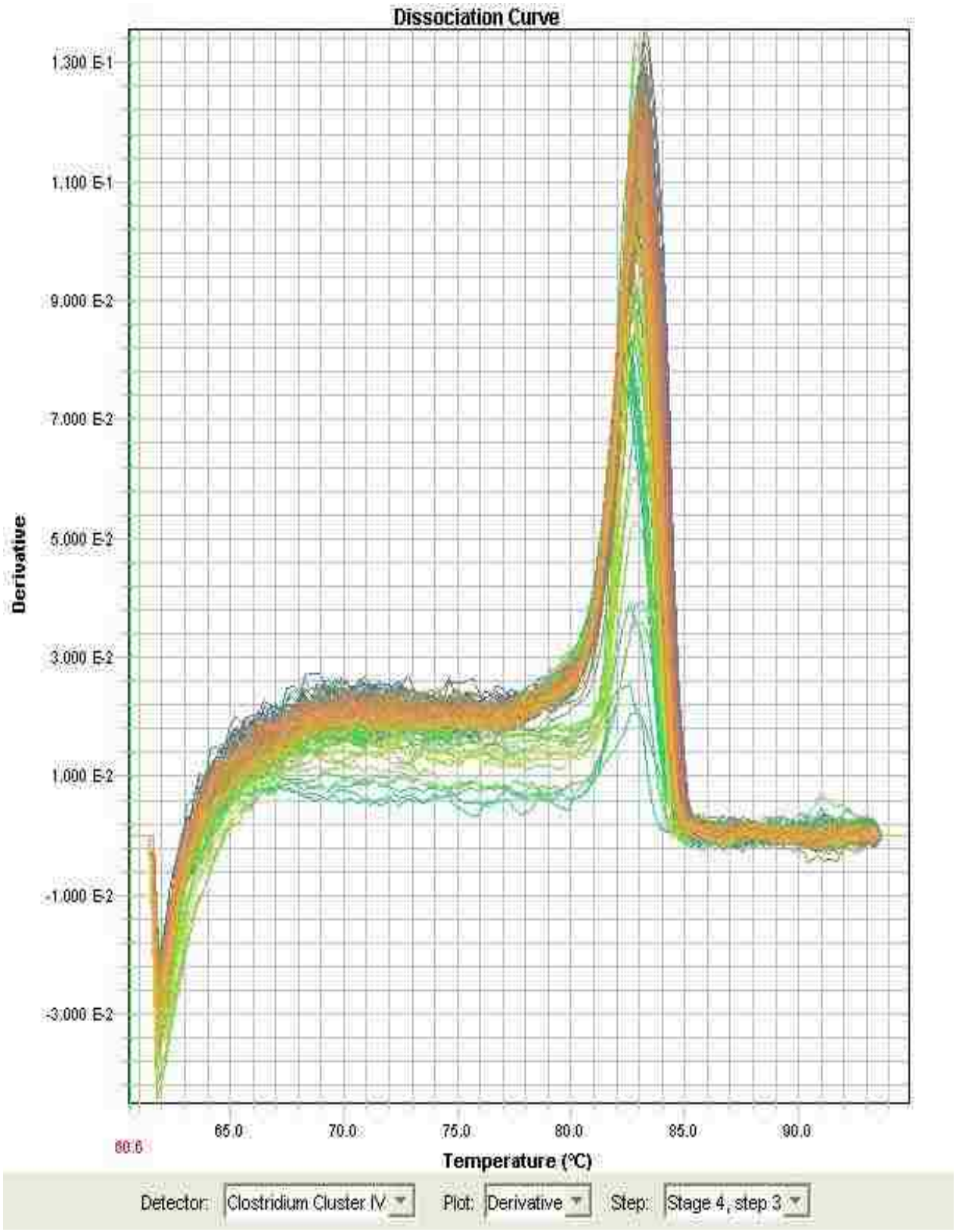

Figure XIVClostridial clusters IV (Melting T 82.5 oC)

APPENDIX B: CHAPTER 5 SAS PROGRAM

(a) SAS Programme for Chapter 5

(a.1)SAS Cocde for programme

```
proc options option = macro; run;
dm'log;clear;output;clear';
options nodate nocenter nonumber ps=512 ls=132 nolabel;
ODS HTML style=minimal body='C:\Documents and Settings\rsenev1\Desktop\ClosXiv.html' ;
Title1 'Invitro Study';
data invitro;
input  Diet $ Time Bacte1      Bacte2 Closiv  Bif      Lac Dom ClosXiv ;
datalines;

 C          0.5  6.181871  6.003654  9.506346  6.755419   7.25122  9.873611  7.415339  7.755419
 C          0.5  7.073775  6.853974  10.21879  7.098233  7.256507  10.88366  8.231394  8.098233
 C          0.5  5.564315  5.414847  9.178546  6.698154  7.142024  9.565257   6.71726  7.698154
 C            1  6.366887  6.180006  9.462617  7.432922  7.387964  10.17754  7.708172  8.432922
-
-
-
-
-
-

/*Proc print data = invitro ; run;*/

PROC mixed DATA=invitro cl covtest;
  class Diet Time;
  MODEL ClosXiv= Diet|Time /DDFM = KR outp=resids;
  repeated Time/ type=AR(1) ;
lsmeans Diet|Time / adjust=tukey cl;
run;
```

(b)SAS Results

Table (a1) Results of Type 3 Tests of Fixed Effects of Total Anaerobic Bacteria Counts

Effect	Num DF	Den DF	F Value	Pr > F
Diet	3	64	27.85	<.0001
Time	7	64	15.86	<.0001
Diet*Time	21	64	1.1	0.3678

Table (a2) Results of Least Squares Means of Total Anaerobic Bacteria Counts

Effect	Diet	Time	Estimate	t Value	Pr > |t|
Diet	**C**		8.6879	125.65	<.0001
Diet	**F**		9.0317	130.62	<.0001
Diet	**L**		8.7533	126.59	<.0001
Diet	**R**		9.4904	137.25	<.0001
Time		**0.5**	8.6592	88.55	<.0001
Time		**1**	8.6825	88.79	<.0001
Time		**2**	8.8442	90.44	<.0001
Time		**4**	8.7808	89.79	<.0001
Time		**12**	9.3842	95.96	<.0001
Time		**24**	9.63	98.48	<.0001
Time		**48**	9.31	95.21	<.0001
Time		**72**	8.6358	88.31	<.0001
Diet*Time	**C**	**0.5**	8.3467	42.68	<.0001
Diet*Time	**C**	**1**	8.2433	42.15	<.0001
Diet*Time	**C**	**2**	8.6067	44.01	<.0001
Diet*Time	**C**	**4**	8.4933	43.43	<.0001
Diet*Time	**C**	**12**	8.8033	45.01	<.0001
Diet*Time	**C**	**24**	9.3667	47.89	<.0001
Diet*Time	**C**	**48**	9.1167	46.61	<.0001
Diet*Time	**C**	**72**	8.5267	43.6	<.0001
Diet*Time	**F**	**0.5**	8.32	42.54	<.0001
Diet*Time	**F**	**1**	8.8633	45.32	<.0001
Diet*Time	**F**	**2**	8.8733	45.37	<.0001
Diet*Time	**F**	**4**	8.7233	44.6	<.0001
Diet*Time	**F**	**12**	9.43	48.22	<.0001
Diet*Time	**F**	**24**	9.9067	50.65	<.0001
Diet*Time	**F**	**48**	9.57	48.93	<.0001
Diet*Time	**F**	**72**	8.5667	43.8	<.0001
Diet*Time	**L**	**0.5**	8.7233	44.6	<.0001
Diet*Time	**L**	**1**	8.33	42.59	<.0001
Diet*Time	**L**	**2**	8.6267	44.11	<.0001
Diet*Time	**L**	**4**	8.4233	43.07	<.0001
Diet*Time	**L**	**12**	9.26	47.35	<.0001
Diet*Time	**L**	**24**	9.1633	46.85	<.0001
Diet*Time	**L**	**48**	8.91	45.56	<.0001
Diet*Time	**L**	**72**	8.59	43.92	<.0001

Table (a2) Cont'd

Effect	Diet	Time	Estimate	t Value	Pr > |t|
Diet*Time	**R**	**0.5**	9.2467	47.28	<.0001
Diet*Time	**R**	**1**	9.2933	47.52	<.0001
Diet*Time	**R**	**2**	9.27	47.4	<.0001
Diet*Time	**R**	**4**	9.4833	48.49	<.0001
Diet*Time	**R**	**12**	10.0433	51.35	<.0001
Diet*Time	**R**	**24**	10.0833	51.56	<.0001
Diet*Time	**R**	**48**	9.6433	49.31	<.0001
Diet*Time	**R**	**72**	8.86	45.3	<.0001

Table (a3) Results of p Values of Total Anaerobic Bacteria Counts

Effect	Diet	_Diet	t Value	Pr > |t|
Diet	**C**	**F**	-3.52	0.0008
Diet	**C**	**L**	-0.67	0.5059
Diet	**C**	**R**	-8.21	<.0001
Diet	**F**	**L**	2.85	0.0059
Diet	**F**	**R**	-4.69	<.0001
Diet	**L**	**R**	-7.54	<.0001

Table (b1) Results of Type 3 Tests of Fixed Effects of Lactic Acid Bacteria Counts

Effect	Num DF	Den DF	F Value	Pr > F
Diet	3	64	15.7	<.0001
Time	7	64	18.74	<.0001
Diet*Time	21	64	0.99	0.487

Table (b2) Results of Least Squares Means of Lactic Acid Bacteria Counts

Effect	Diet	Time	Estimate	t Value	Pr > |t|
Diet	**C**		7.6596	74.35	<.0001
Diet	**F**		8.1167	78.79	<.0001
Diet	**L**		7.5704	73.49	<.0001
Diet	**R**		8.4438	81.96	<.0001
Time		**0.5**	6.9517	47.72	<.0001
Time		**1**	7.2283	49.61	<.0001

Table(b2) Cont'd

Effect	Diet	Time	Estimate	t Value	Pr > |t|
Time		**4**	7.8258	53.72	<.0001
Time		**12**	8.205	56.32	<.0001
Time		**24**	8.5108	58.42	<.0001
Time		**48**	8.575	58.86	<.0001
Time		**72**	8.5933	58.98	<.0001
Diet*Time	**C**	**0.5**	6.8267	23.43	<.0001
Diet*Time	**C**	**1**	6.98	23.96	<.0001
Diet*Time	**C**	**4**	7.62	26.15	<.0001
Diet*Time	**C**	**12**	7.5867	26.04	<.0001
Diet*Time	**C**	**24**	7.83	26.87	<.0001
Diet*Time	**C**	**48**	8.2867	28.44	<.0001
Diet*Time	**C**	**72**	8.5	29.17	<.0001
Diet*Time	**F**	**0.5**	7.16	24.57	<.0001
Diet*Time	**F**	**1**	7.38	25.33	<.0001
Diet*Time	**F**	**2**	8	27.46	<.0001
Diet*Time	**F**	**4**	8.09	27.76	<.0001
Diet*Time	**F**	**12**	7.96	27.32	<.0001
Diet*Time	**F**	**24**	9.1533	31.41	<.0001
Diet*Time	**F**	**48**	8.86	30.41	<.0001
Diet*Time	**F**	**72**	8.33	28.59	<.0001
Diet*Time	**L**	**0.5**	6.3867	21.92	<.0001
Diet*Time	**L**	**1**	6.9133	23.73	<.0001
Diet*Time	**L**	**2**	7.06	24.23	<.0001
Diet*Time	**L**	**4**	7.3367	25.18	<.0001
Diet*Time	**L**	**12**	8.1467	27.96	<.0001
Diet*Time	**L**	**24**	7.8567	26.96	<.0001
Diet*Time	**L**	**48**	8.2733	28.39	<.0001
Diet*Time	**L**	**72**	8.59	29.48	<.0001
Diet*Time	**R**	**0.5**	7.4333	25.51	<.0001
Diet*Time	**R**	**1**	7.64	26.22	<.0001
Diet*Time	**R**	**2**	8.0567	27.65	<.0001
Diet*Time	**R**	**4**	8.2567	28.34	<.0001
Diet*Time	**R**	**12**	9.1267	31.32	<.0001
Diet*Time	**R**	**24**	9.2033	31.59	<.0001
Diet*Time	**R**	**48**	8.88	30.48	<.0001
Diet*Time	**R**	**72**	8.9533	30.73	<.0001

Table (b3) Results of p Values of Lactic Acid Bacteria Counts

Effect	Diet	_Diet	t Value	Pr > |t|
Diet	**C**	**F**	-3.14	0.0026
Diet	**C**	**L**	0.61	0.5427
Diet	**C**	**R**	-5.38	<.0001
Diet	**F**	**L**	3.75	0.0004
Diet	**F**	**R**	-2.25	0.0282
Diet	**L**	**R**	-5.99	<.0001

Table (c1) Results of Type 3 Tests of Fixed Effects of Culturable *Bifidobacterium spp.* Counts

Effect	Num DF	Den DF	F Value	Pr > F
Diet	3	64	32.73	<.0001
Time	7	64	7.75	<.0001
Diet*Time	21	64	2.52	0.0024

Table (c2) Results of Least Squares Means of Culturable *Bifidobacterium spp.* Counts

Effect	Diet	Time	Estimate	t Value	Pr > |t|
Diet	**C**		8.5127	129.74	<.0001
Diet	**F**		9.0081	137.29	<.0001
Diet	**L**		8.5458	130.25	<.0001
Diet	**R**		9.2881	141.56	<.0001
Time		**0.5**	8.6742	93.48	<.0001
Time		**1**	8.6821	93.57	<.0001
Time		**2**	8.8292	95.15	<.0001
Time		**4**	8.9142	96.07	<.0001
Time		**12**	9.1633	98.75	<.0001
Time		**24**	9.185	98.99	<.0001
Time		**48**	8.8525	95.4	<.0001
Time		**72**	8.4092	90.63	<.0001
Diet*Time	**C**	**0.5**	8.43	45.42	<.0001

Table (c2) Cont'd

Effect	Diet	Time	Estimate	t Value	Pr > |t|
Diet*Time	**C**	**1**	8.1183	43.75	<.0001
Diet*Time	**C**	**2**	8.53	45.96	<.0001
Diet*Time	**C**	**4**	8.2667	44.54	<.0001
Diet*Time	**C**	**12**	8.52	45.91	<.0001
Diet*Time	**C**	**24**	8.98	48.39	<.0001
Diet*Time	**C**	**48**	8.79	47.36	<.0001
Diet*Time	**C**	**72**	8.4667	45.62	<.0001
Diet*Time	**F**	**0.5**	8.3983	45.25	<.0001
Diet*Time	**F**	**1**	9.0033	48.51	<.0001
Diet*Time	**F**	**2**	9.04	48.71	<.0001
Diet*Time	**F**	**4**	9.6333	51.91	<.0001
Diet*Time	**F**	**12**	9.7667	52.63	<.0001
Diet*Time	**F**	**24**	9.21	49.63	<.0001
Diet*Time	**F**	**48**	8.7067	46.92	<.0001
Diet*Time	**F**	**72**	8.3067	44.76	<.0001
Diet*Time	**L**	**0.5**	8.6567	46.65	<.0001
Diet*Time	**L**	**1**	8.3633	45.07	<.0001
Diet*Time	**L**	**2**	8.5467	46.05	<.0001
Diet*Time	**L**	**4**	8.29	44.67	<.0001
Diet*Time	**L**	**12**	8.7267	47.02	<.0001
Diet*Time	**L**	**24**	8.87	47.8	<.0001
Diet*Time	**L**	**48**	8.57	46.18	<.0001
Diet*Time	**L**	**72**	8.3433	44.96	<.0001
Diet*Time	**R**	**0.5**	9.2117	49.64	<.0001
Diet*Time	**R**	**1**	9.2433	49.81	<.0001
Diet*Time	**R**	**2**	9.2	49.57	<.0001
Diet*Time	**R**	**4**	9.4667	51.01	<.0001
Diet*Time	**R**	**12**	9.64	51.94	<.0001
Diet*Time	**R**	**24**	9.68	52.16	<.0001
Diet*Time	**R**	**48**	9.3433	50.35	<.0001
Diet*Time	**R**	**72**	8.52	45.91	<.0001

Table (c3) Results of p Values of Culturable *Bifidobacterium spp.* Counts

Effect	Diet	_Diet	t Value	Pr > |t|
Diet	**C**	**F**	-5.34	<.0001
Diet	**C**	**L**	-0.36	0.7223
Diet	**C**	**R**	-8.36	<.0001
Diet	**F**	**L**	4.98	<.0001
Diet	**F**	**R**	-3.02	0.0037
Diet	**L**	**R**	-8	<.0001

Table (d1) Results of Type 3 Tests of Fixed Effects of Culturable *E.coli.* Counts

Effect	Num DF	Den DF	F Value	Pr > F
Diet	3	64	2.96	0.0388
Time	7	64	33.99	<.0001
Diet*Time	21	64	0.68	0.8381

Table (d2) Results of Least Squares Means of Culturable *E.coli* Counts

Effect	Diet	Time	Estimate	t Value	Pr > |t|
Diet	**C**		6.9883	54.59	<.0001
Diet	**F**		6.9733	54.47	<.0001
Diet	**L**		6.8942	53.86	<.0001
Diet	**R**		7.3846	57.69	<.0001
Time		**0.5**	5.6492	31.2	<.0001
Time		**1**	5.7142	31.56	<.0001
Time		**2**	6.1217	33.81	<.0001
Time		**4**	7.3317	40.5	<.0001
Time		**12**	7.6383	42.19	<.0001
Time		**24**	7.9558	43.95	<.0001
Time		**48**	7.9858	44.11	<.0001
Time		**72**	8.0842	44.65	<.0001
Diet*Time	**C**	**0.5**	5.14	14.2	<.0001
Diet*Time	**C**	**1**	5.58	15.41	<.0001
Diet*Time	**C**	**2**	6.2433	17.24	<.0001
Diet*Time	**C**	**4**	7.0533	19.48	<.0001
Diet*Time	**C**	**12**	7.35	20.3	<.0001
Diet*Time	**C**	**24**	7.8933	21.8	<.0001

Table (d2) Cont'd

Effect	Diet	Time	Estimate	t Value	Pr > |t|
Diet*Time	**C**	**48**	8.3567	23.08	<.0001
Diet*Time	**C**	**72**	8.29	22.9	<.0001
Diet*Time	**F**	**0.5**	5.7	15.74	<.0001
Diet*Time	**F**	**1**	5.6367	15.57	<.0001
Diet*Time	**F**	**2**	5.95	16.43	<.0001
Diet*Time	**F**	**4**	7.1667	19.79	<.0001
Diet*Time	**F**	**12**	7.16	19.77	<.0001
Diet*Time	**F**	**24**	8.26	22.81	<.0001
Diet*Time	**F**	**48**	7.7233	21.33	<.0001
Diet*Time	**F**	**72**	8.19	22.62	<.0001
Diet*Time	**L**	**0.5**	5.65	15.6	<.0001
Diet*Time	**L**	**1**	5.41	14.94	<.0001
Diet*Time	**L**	**2**	5.96	16.46	<.0001
Diet*Time	**L**	**4**	7.22	19.94	<.0001
Diet*Time	**L**	**12**	7.5467	20.84	<.0001
Diet*Time	**L**	**24**	7.6233	21.05	<.0001
Diet*Time	**L**	**48**	7.97	22.01	<.0001
Diet*Time	**R**	**0.5**	6.1067	16.87	<.0001
Diet*Time	**R**	**1**	6.23	17.21	<.0001
Diet*Time	**R**	**2**	6.3333	17.49	<.0001
Diet*Time	**R**	**4**	7.8867	21.78	<.0001
Diet*Time	**R**	**12**	8.4967	23.47	<.0001
Diet*Time	**R**	**24**	8.0467	22.22	<.0001
Diet*Time	**R**	**48**	7.8933	21.8	<.0001
Diet*Time	**R**	**72**	8.0833	22.32	<.0001

Table (d3) Results of p Values of Culturable *E.coli* Counts

Effect	Diet	_Diet	Estimate	Standard Error	DF	t Value	Pr > |t|
Diet	**C**	**F**	0.015	0.181	64	0.08	0.9342
Diet	**C**	**L**	0.09417	0.181	64	0.52	0.6048
Diet	**C**	**R**	-0.3962	0.181	64	-2.19	0.0323
Diet	**F**	**L**	0.07917	0.181	64	0.44	0.6634
Diet	**F**	**R**	-0.4112	0.181	64	-2.27	0.0265
Diet	**L**	**R**	-0.4904	0.181	64	-2.71	0.0087

Table (e1) Results of Type 3 Tests of Fixed Effects of Bacterial Domain Analyzed by qRT-PCR

Effect	Num DF	Den DF	F Value	Pr > F
Diet	3	48	1.54	0.2164
Time	5	48	34.25	<.0001
Diet*Time	15	48	1.01	0.4649

Table (e2) Results of Least Squares Means of Bacterial Domain Analyzed by qRT-PCR

Effect	Diet	Time	Estimate	Pr > |t|
Diet	**C**		10.5431	<.0001
Diet	**F**		10.6724	<.0001
Diet	**L**		10.5573	<.0001
Diet	**R**		10.6951	<.0001
Time		**0.5**	10.2064	<.0001
Time		**1**	10.291	<.0001
Time		**2**	10.437	<.0001
Time		**4**	10.4894	<.0001
Time		**12**	10.8713	<.0001
Time		**24**	11.4067	<.0001
Diet*Time	**C**	**0.5**	10.1075	<.0001
Diet*Time	**C**	**1**	10.0758	<.0001
Diet*Time	**C**	**2**	10.4756	<.0001
Diet*Time	**C**	**4**	10.6361	<.0001
Diet*Time	**C**	**12**	10.7209	<.0001
Diet*Time	**C**	**24**	11.2426	<.0001
Diet*Time	**F**	**0.5**	10.2772	<.0001
Diet*Time	**F**	**1**	10.21	<.0001
Diet*Time	**F**	**2**	10.4199	<.0001
Diet*Time	**F**	**4**	10.4101	<.0001
Diet*Time	**F**	**12**	11.185	<.0001
Diet*Time	**F**	**24**	11.5322	<.0001
Diet*Time	**L**	**0.5**	10.104	<.0001
Diet*Time	**L**	**1**	10.5281	<.0001
Diet*Time	**L**	**2**	10.362	<.0001
Diet*Time	**L**	**4**	10.2593	<.0001

Table (e2) Cont'd

Effect	Diet	Time	Estimate	Pr > |t|
Diet*Time	**L**	**12**	10.7251	<.0001
Diet*Time	**L**	**24**	11.3652	<.0001
Diet*Time	**R**	**0.5**	10.337	<.0001
Diet*Time	**R**	**1**	10.3499	<.0001
Diet*Time	**R**	**2**	10.4905	<.0001
Diet*Time	**R**	**4**	10.652	<.0001
Diet*Time	**R**	**12**	10.8541	<.0001
Diet*Time	**R**	**24**	11.4868	<.0001

Table (e3) Results of p Values of Bacterial Domain Analyzed by qRT-PCR

Effect	Diet	_Diet	t Value	Pr > |t|
Diet	**C**	**F**	-1.46	0.1517
Diet	**C**	**L**	-0.16	0.8737
Diet	**C**	**R**	-1.71	0.0933
Diet	**F**	**L**	1.3	0.2009
Diet	**F**	**R**	-0.26	0.7993
Diet	**L**	**R**	-1.55	0.1271

Table (f1) Results of Type 3 Tests of Fixed Effects of *Lactobacillus spp.* Analyzed by qRT-PCR

Effect	Num DF	Den DF	F Value	Pr > F
Diet	3	48	5.97	0.0015
Time	5	48	33.11	<.0001
Diet*Time	15	48	1.84	0.0567

Table (f2) Results of Least Squares Means of *Lactobacillus spp.* Analyzed by qRT-PCR

Effect	Diet	Time	Estimate	t Value	Pr > |t|
Diet	**C**		7.4969	126.51	<.0001
Diet	**F**		7.5866	128.02	<.0001
Diet	**L**		7.3831	124.59	<.0001
Diet	**R**		7.7258	130.37	<.0001
Time		**0.5**	7.0121	96.61	<.0001
Time		**1**	7.2136	99.39	<.0001
Time		**2**	7.4239	102.29	<.0001
Time		**4**	7.679	105.8	<.0001

Table (f2) Cont'd

Effect	Diet	Time	Estimate	t Value	Pr > |t|
Time		**12**	7.7968	107.43	<.0001
Time		**24**	8.1633	112.48	<.0001
Diet*Time	**C**	**0.5**	6.8506	47.19	<.0001
Diet*Time	**C**	**1**	7.0892	48.84	<.0001
Diet*Time	**C**	**2**	7.497	51.65	<.0001
Diet*Time	**C**	**4**	7.7415	53.33	<.0001
Diet*Time	**C**	**12**	7.7154	53.15	<.0001
Diet*Time	**C**	**24**	8.0877	55.72	<.0001
Diet*Time	**F**	**0.5**	7.2324	49.82	<.0001

Table (f3) Results of p Values of *Lactobacillus spp.* Analyzed by qRT-PCR

Effect	Diet	_Diet	t Value	Pr > |t|
Diet	**C**	**F**	-1.07	0.2896
Diet	**C**	**L**	1.36	0.1811
Diet	**C**	**R**	-2.73	0.0088
Diet	**F**	**L**	2.43	0.019
Diet	**F**	**R**	-1.66	0.1032
Diet	**L**	**R**	-4.09	0.0002

Table (g1) Results of Type 3 Tests of Fixed Effects of *Bifidobacterium spp.* Analyzed by qRT-PCR

Effect	Num DF	Den DF	F Value	Pr > F
Diet	3	48	5.97	0.0015
Time	5	48	33.11	<.0001
Diet*Time	15	48	1.84	0.0567

Table (g2) Results of Least Squares Means of *Bifidobacterium spp.* Analyzed by qRT-PCR

Effect	Diet	Time	Estimate	t Value	Pr > |t|
Diet	**C**		7.4969	126.51	<.0001
Diet	**F**		7.5866	128.02	<.0001
Diet	**L**		7.3831	124.59	<.0001
Diet	**R**		7.7258	130.37	<.0001
Time		**0.5**	7.0121	96.61	<.0001
Time		**1**	7.2136	99.39	<.0001
Time		**2**	7.4239	102.29	<.0001
Time		**4**	7.679	105.8	<.0001

Table (g2) Cont'd

Effect	Diet	Time	Estimate	t Value	Pr > |t|
Time		**12**	7.7968	107.43	<.0001
Time		**24**	8.1633	112.48	<.0001
Diet*Time	**C**	**0.5**	6.8506	47.19	<.0001
Diet*Time	**C**	**1**	7.0892	48.84	<.0001
Diet*Time	**C**	**2**	7.497	51.65	<.0001
Diet*Time	**C**	**4**	7.7415	53.33	<.0001
Diet*Time	**C**	**12**	7.7154	53.15	<.0001
Diet*Time	**C**	**24**	8.0877	55.72	<.0001
Diet*Time	**F**	**0.5**	7.2324	49.82	<.0001
Diet*Time	**F**	**1**	7.0288	48.42	<.0001
Diet*Time	**F**	**2**	7.6019	52.37	<.0001
Diet*Time	**F**	**4**	7.4929	51.62	<.0001
Diet*Time	**F**	**12**	8.1362	56.05	<.0001
Diet*Time	**F**	**24**	8.0276	55.3	<.0001
Diet*Time	**L**	**0.5**	6.8172	46.96	<.0001
Diet*Time	**L**	**1**	7.3123	50.38	<.0001
Diet*Time	**L**	**2**	7.1289	49.11	<.0001
Diet*Time	**L**	**4**	7.6901	52.98	<.0001
Diet*Time	**L**	**12**	7.3718	50.79	<.0001
Diet*Time	**L**	**24**	7.9785	54.96	<.0001
Diet*Time	**R**	**0.5**	7.1482	49.24	<.0001
Diet*Time	**R**	**1**	7.4241	51.15	<.0001
Diet*Time	**R**	**2**	7.4678	51.45	<.0001
Diet*Time	**R**	**4**	7.7916	53.68	<.0001
Diet*Time	**R**	**12**	7.9637	54.86	<.0001
Diet*Time	**R**	**24**	8.5595	58.97	<.0001

Table (g3) Results of p Values of *Bifidobacterium spp.* Analyzed by qRT-PCR

Effect	Diet	_Diet	Estimate	t Value	Pr > |t|
Diet	**C**	**F**	-0.08974	-1.07	0.2896
Diet	**C**	**L**	0.1137	1.36	0.1811
Diet	**C**	**R**	-0.2289	-2.73	0.0088
Diet	**F**	**L**	0.2035	2.43	0.019
Diet	**F**	**R**	-0.1392	-1.66	0.1032
Diet	**L**	**R**	-0.3427	-4.09	0.0002

Table (h1) Results of Type 3 Tests of Clostridium cluster IV Analyzed by qRT-PCR

Effect	Num DF	Den DF	F Value	Pr > F
Diet	3	48	2.92	0.0432
Time	5	48	6.14	0.0002
Diet*Time	15	48	1.17	0.323

Table (h2) Results of Least Squares Means of Clostridium cluster IV Analyzed by qRT-PCR

Effect	Diet	Time	Estimate	t Value	Pr > |t|
Diet	**C**		9.442	169.44	<.0001
Diet	**F**		9.5791	171.9	<.0001
Diet	**L**		9.3887	168.49	<.0001
Diet	**R**		9.5729	171.79	<.0001
Time		**0.5**	9.615	140.89	<.0001
Time		**1**	9.6021	140.7	<.0001
Time		**2**	9.705	142.2	<.0001
Time		**4**	9.3747	137.36	<.0001
Time		**12**	9.2689	135.81	<.0001
Time		**24**	9.4083	137.86	<.0001
Diet*Time	**C**	**0.5**	9.6346	70.59	<.0001
Diet*Time	**C**	**1**	9.3917	68.81	<.0001
Diet*Time	**C**	**2**	9.7726	71.6	<.0001
Diet*Time	**C**	**4**	9.3436	68.45	<.0001
Diet*Time	**C**	**12**	9.1663	67.16	<.0001
Diet*Time	**C**	**24**	9.3433	68.45	<.0001
Diet*Time	**F**	**0.5**	9.6296	70.55	<.0001
Diet*Time	**F**	**1**	9.5429	69.91	<.0001
Diet*Time	**F**	**2**	9.8147	71.91	<.0001
Diet*Time	**F**	**4**	9.4056	68.91	<.0001
Diet*Time	**F**	**12**	9.6465	70.67	<.0001
Diet*Time	**F**	**24**	9.4354	69.13	<.0001
Diet*Time	**L**	**0.5**	9.3901	68.79	<.0001
Diet*Time	**L**	**1**	9.7488	71.42	<.0001
Diet*Time	**L**	**2**	9.4624	69.32	<.0001
Diet*Time	**L**	**4**	9.2628	67.86	<.0001
Diet*Time	**L**	**12**	9.1311	66.9	<.0001
Diet*Time	**L**	**24**	9.3369	68.41	<.0001
Diet*Time	**R**	**0.5**	9.8058	71.84	<.0001

Table (h2) Cont'd

Effect	Diet	Time	Estimate	t Value	Pr > |t|
Diet*Time	**R**	**2**	9.7701	71.58	<.0001
Diet*Time	**R**	**4**	9.4869	69.5	<.0001
Diet*Time	**R**	**12**	9.1317	66.9	<.0001
Diet*Time	**R**	**24**	9.5175	69.73	<.0001

Table (h3) Results of p Values of Clostridium cluster IV Analyzed by qRT-PCR

Effect	Diet	_Diet	t Value	Pr > |t|
Diet	**C**	**F**	-1.74	0.0884
Diet	**C**	**L**	0.68	0.5018
Diet	**C**	**R**	-1.66	0.1034
Diet	**F**	**L**	2.42	0.0195
Diet	**F**	**R**	0.08	0.9373
Diet	**L**	**R**	-2.34	0.0237

Table (i1) Results of Type 3 Tests of Clostridium cluster XIVa-b Analyzed by qRT-PCR

Effect	Num DF	Den DF	F Value	Pr > F
Diet	3	48	2.21	0.0987
Time	5	48	7.38	<.0001
Diet*Time	15	48	0.97	0.5033

Table (i2) Results of Least Squares Means of Clostridium cluster XIVa-b Analyzed by qRT-PCR

Effect	Diet	Time	Estimate	t Value	Pr > |t|
Diet	**C**		7.6775	93.76	<.0001
Diet	**F**		7.8472	95.84	<.0001
Diet	**L**		7.6463	93.38	<.0001
Diet	**R**		7.8915	96.38	<.0001
Time		**0.5**	7.6092	75.88	<.0001
Time		**1**	7.635	76.13	<.0001
Time		**2**	7.6942	76.72	<.0001
Time		**4**	7.5681	75.47	<.0001
Time		**12**	7.7879	77.66	<.0001

Table (i2) Cont'd

Effect	Diet	Time	Estimate	t Value	Pr > |t|
Time		**24**	8.2995	82.76	<.0001
Diet*Time	**C**	**0.5**	7.4547	37.17	<.0001
Diet*Time	**C**	**1**	7.3929	36.86	<.0001
Diet*Time	**C**	**2**	7.7104	38.44	<.0001
Diet*Time	**C**	**4**	7.5678	37.73	<.0001
Diet*Time	**C**	**12**	7.6679	38.23	<.0001
Diet*Time	**C**	**24**	8.2714	41.24	<.0001
Diet*Time	**F**	**0.5**	7.6966	38.37	<.0001
Diet*Time	**F**	**1**	7.5674	37.73	<.0001
Diet*Time	**F**	**2**	7.7583	38.68	<.0001
Diet*Time	**F**	**4**	7.5057	37.42	<.0001
Diet*Time	**F**	**12**	8.2656	41.21	<.0001
Diet*Time	**F**	**24**	8.2899	41.33	<.0001
Diet*Time	**L**	**0.5**	7.5267	37.53	<.0001
Diet*Time	**L**	**1**	7.8852	39.31	<.0001
Diet*Time	**L**	**2**	7.5436	37.61	<.0001
Diet*Time	**L**	**4**	7.2467	36.13	<.0001
Diet*Time	**L**	**12**	7.4617	37.2	<.0001
Diet*Time	**L**	**24**	8.2142	40.95	<.0001
Diet*Time	**R**	**0.5**	7.7588	38.68	<.0001
Diet*Time	**R**	**1**	7.6947	38.36	<.0001
Diet*Time	**R**	**2**	7.7644	38.71	<.0001
Diet*Time	**R**	**4**	7.9521	39.65	<.0001
Diet*Time	**R**	**12**	7.7565	38.67	<.0001
Diet*Time	**R**	**24**	8.4226	41.99	<.0001

Table (i3) Results of p Values of Clostridium cluster XIVa-b Analyzed by qRT-PCR

Effect	Diet	_Diet	t Value	Pr > |t|
Diet	**C**	**F**	-1.47	0.1492
Diet	**C**	**L**	0.27	0.7891
Diet	**C**	**R**	-1.85	0.0707
Diet	**F**	**L**	1.73	0.0892
Diet	**F**	**R**	-0.38	0.7039
Diet	**L**	**R**	-2.12	0.0394

Table (j1) Results of Type 3 Tests of Bacteroidetes Group Analyzed by qRT-PCR

Effect	Num DF	Den DF	F Value	Pr > F
Diet	3	48	0.43	0.7348
Time	5	48	60.46	<.0001
Diet*Time	15	48	0.42	0.9642

Table (j2) Results of Least Squares Means of Bacteroidetes Group Analyzed by qRT-PCR

Effect	Diet	Time	Estimate	t Value	Pr > |t|
Diet	**C**		8.8407	68.65	<.0001
Diet	**F**		8.9442	69.69	<.0001
Diet	**L**		8.9126	69.38	<.0001
Diet	**R**		8.9963	70.22	<.0001
Time		**0.5**	8.1627	50.5	<.0001
Time		**1**	8.2294	51.05	<.0001
Time		**2**	8.3247	51.83	<.0001
Time		**4**	8.6191	54.24	<.0001
Time		**12**	9.7778	63.74	<.0001
Time		**24**	10.427	69.06	<.0001
Diet*Time	**C**	**0.5**	8.0908	24.96	<.0001
Diet*Time	**C**	**1**	7.9448	24.36	<.0001
Diet*Time	**C**	**2**	8.2836	25.75	<.0001
Diet*Time	**C**	**4**	8.6313	27.17	<.0001
Diet*Time	**C**	**12**	9.7462	31.74	<.0001
Diet*Time	**C**	**24**	10.3473	34.2	<.0001
Diet*Time	**F**	**0.5**	8.1377	25.15	<.0001
Diet*Time	**F**	**1**	8.0969	24.98	<.0001
Diet*Time	**F**	**2**	8.2942	25.79	<.0001
Diet*Time	**F**	**4**	8.5684	26.91	<.0001
Diet*Time	**F**	**12**	10.0218	32.87	<.0001
Diet*Time	**F**	**24**	10.5461	35.02	<.0001
Diet*Time	**L**	**0.5**	8.1432	25.17	<.0001
Diet*Time	**L**	**1**	8.5171	26.7	<.0001
Diet*Time	**L**	**2**	8.2138	25.46	<.0001
Diet*Time	**L**	**4**	8.5187	26.71	<.0001
Diet*Time	**L**	**12**	9.7881	31.91	<.0001
Diet*Time	**L**	**24**	10.2945	33.98	<.0001
Diet*Time	**R**	**0.5**	8.279	25.73	<.0001
Diet*Time	**R**	**1**	8.3587	26.05	<.0001
Diet*Time	**R**	**2**	8.5071	26.66	<.0001
Diet*Time	**R**	**4**	8.758	27.69	<.0001
Diet*Time	**R**	**12**	9.5549	30.95	<.0001
Diet*Time	**R**	**24**	10.5201	34.91	<.0001

Table (j3) Results of p Values of Bacteroidetes Group Analyzed by qRT-PCR

Effect	Diet	Diet	t Value	Pr > |t|
Diet	**C**	**F**	-0.73	0.4662
Diet	**C**	**L**	-0.51	0.6123
Diet	**C**	**R**	-1.1	0.2749
Diet	**F**	**L**	0.22	0.8235
Diet	**F**	**R**	-0.37	0.7131
Diet	**L**	**R**	-0.59	0.5552

VITA

Reshani Nisansala Senevirathne was born in Western province of Sri Lanka. She received her primary education from Holy Family Convent Kaluthara and St Joseph Balika Girls School in Colombo 14, Sri Lanka. She received her secondary education from St. Paul's girls school Milagiriya in Colombo 4, entered Wayamba University of Sri Lanka in 1999. She received her Bachelor of Science in Agricultural Science degree majoring in food technology in 2004, and she earned another Bachelor of Science Degree majoring in chemistry in 2000 from Institute of Chemistry, Ceylon. She entered Louisiana State University in 2005 for her Graduate studies and graduated in May 2007 with her master in degree from Department of Food Science.

She actively participated in both research and extracurricular activities since joining the Department of Food Science at LSU in the spring of 2005 as graduate student in food microbiology. During her graduate career, she was an active member of the Food Science Club and elected President of Food Science Club of Louisiana State University in 2007 – 2008 and elected Vice President of Food Science Club in 2006 – 2007. She was awarded the Barkate Scholarship for Outstanding Graduate Student in 2006 by the Department of Food Science. Reshani has received IFT – Gulf Coast Section 2006 Tom Quinn Student Scholarship, based on her abstract, "Direct Colony Immunoblot for Enumeration of *Vibrio vulnificus*". She was a team member of a multi-state project titled "Ecology and control of pathogenic strains of *Vibrio vulnificus* and *Vibrio parahaemolyticus* in U.S. Gulf Coast oysters" and Food Product Development competition team of LSU in IFT 2007.

During the 2008 – 2009 academic years she has servered as Director of the Undergraduate Research Paper Competition in Institute of Food Technologist Student Association (IFTSA).

During 2009 IFT annual conference (Anaheim, California) she organized the Undergraduate Research Paper Competition symposia.

Reshani has received Graduate School Dissertation Fellowship by LSU Graduate School in 2009. She was awarded the Graduate Student Merit Honor Roll awarded in 2009 by Gamma Sigma Delta LSU Chapter Honor Society. Reshani also received Graduate Scholarship 2nd place from Carbohydrate Division of the Institute of Food Technologists (IFT) in 2009 and 2nd place of IFT – Gulf Coast Section poster Competition in 2009.

Reshani also actively mentored many students on their research from high school students to graduate students. Upon her graduation she plan to serve as a Post Doctoral Researcher and try to use both the academic and leadership abilities she have gained from LSU for the betterment of the society.