PRODUCTION OF CONJUGATED LINOLEIC ACID

BY *LACTOBACILLUS REUTERI*

By

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CHAPTER I

INTRODUCTION

Conjugated linoleic acid (CLA) refers to a group of positional and geometric isomers of linoleic acid (LA; c9c12-C18:2) (MacDonald 2000). The major isomer is the c9t11 octadecadienoic acid (Kepler and Tove 1967), which has been suggested to be the most biologically active (Ha and others 1990). It has shown to be antioxidative (Ip and others 1991), anticarcinogenic (Pariza and Hargraves 1985; Pariza 1997), and antiatherosclerotic (Lee and others 1994; Wilson and others 2000)

Formation of CLA can be achieved by chemical synthesis (Christie and others 1997), by rumen bacteria (Bauman and others 1999) or by lactic acid bacteria (LAB) (Ogawa and others 2001). Among LAB the genus *Lactobacillus* has gained recent attention because of their other potentially beneficial properties. For instance, they can control cancer (Kasper 1998) and lower serum cholesterol (Gilliland 1990).

Lactobacillus reuteri, a probiotic that highly effectively enzymatically transforms LA into CLA (Pariza and Yang 1998), is also bile resistant (De Boever and others 2000). This is an important property for its survival and establishment in the intestinal tract (Gilliland 1990).

Studies on *L. reuteri* showed that exposure to bile acids produced modifications in cell permeability (Taranto and others 2003). Thus since linoleate isomerase, the enzyme

responsible for CLA production is intracellular (Pariza and Yang 1998), it may be that the presence of glycocholic acid, a major bile acid (Brashears and others 1998) could improve its performance. The purpose of this study was to investigate the influence of glycocholic acid on CLA production by *L. reuteri* 55739.

CHAPTER II

REVIEW OF LITERATURE

Conjugated Linoleic Acid and its Benefits

Chemistry of Conjugated Linoleic Acid

Conjugated Linoleic Acid (CLA) is a group of positional and geometric isomers of linoleic acid, c9c12-C18:2 (MacDonald 2000). The major isomers found in CLA are c9t11-, t9c11-, t9t11-, t10t12-, and t10c12-octadecadienoic acids, however CLA also includes, in less amount, c9c11-, t9c11-, c10c12-, c10t12-, and c11c13- octadecadienoic acids (Ha and others 1989).

Formation of Conjugated Linoleic Acid by Chemical Synthesis

The synthesis of CLA can be done commercially by alkaline isomerization of linoleic acid resulting in a mixture of four (8,10-, 9,11-, 10,12-, and 11,13 – C18:2) cis/trans positional isomers (Christie and others 1997). It also can be synthesized chemically via dehydration of castor oil (Padley and others 1994) or by using methyl ricinoleate purified from castor oil to produce methyl c9t11-C18:2 instead of just c9t11-C18:2 (Berdeaux and others 1997). Production is also possible through oxidation of linoleic acid free radicals in the presence of sulfur-rich proteins (Dormandy and Wickens 1987). Jung and others (2001) demonstrated that it is possible to produce soybean oil with a high CLA content by hydrogenation. This procedure involves the use of a

selective Ni catalyst under high temperatures (210 ºC), with agitation (300 rpm) for a short period of time (10 min).

Formation of Conjugated Linoleic Acid in the Rumen

The unsaturated fatty acids linolenic (C18:3) and linoleic (c9c12-C18:2) acid found in forages as well as triglycerides containing linoleic and oleic acid (c9-C18:1) found in seed oils undergo an initial hydrolysis of the ester linkages by microbial lipases prior to the biohydrogenation process in the rumen (Dawson and Kemp 1970). *Butyrivibrio fibrisolvens* as well as a diverse range of other rumen bacteria have the capacity to biohydrogenate unsaturated fatty acids (Bauman and others 1999). Several biochemical steps and different species of rumen bacteria are involved in the complete biohydrogenation of unsaturated lipids (Kemp and Lander 1984). Biohydrogenation of linoleic acid starts with the isomerization of the cis-12 double bond by the linoleate isomerase to yield the major isomer of CLA, c9t11 (Kepler and Tove 1967). A second reaction takes place in which c9t11-C18:2 is rapidly transformed to vaccenic acid (t11-C18:1). Finally, t11-C18:1 is slowly hydrogenated into stearic acid (C18:0) (Tanaka and Shigeno 1976). Because of the slow reduction, t11-C18:1 accumulates in the rumen making it more available for absorption (Bauman and others 1999). Modifications in the biohydrogenation pathways and end products formed can occur as result of changes in populations of rumen bacteria (Leat and others 1977) due to decreased pH (Bauman and others 1999).

Formation of Conjugated Linoleic Acid in Animal Tissue and Milk

There are two sources for the CLA found in meat and milk products of ruminants. The CLA is formed during ruminal biohydrogenation of linoleic acid as well as being synthesized in the animal's tissues from t11-C18:1, an intermediate in the biohydrogenation of unsaturated fatty acids (Bauman and others 1999). Several studies have shown that the concentration of t11-C18:1 isomer is positively correlated to c9t11-C18:2 concentrations in milk fat (Jiang and others 1996; Bauman and others 1999). This close relationship has been explained by the presence of high concentrations of Δ^9desaturase in adipose tissue of growing ruminants (Corl and others 1998, 1999), which seems to be the major site of endogenous synthesis of c9t11-C18:2 from t11-C18:1. The mammary gland contains adipose tissue, which displaces the apparent site of endogenous synthesis of c9t11-C18:2 for lactating ruminants (Bickerstaffe and Annison 1970). The content of CLA in fat from ruminant-derived food products depends on the dietary intake by the ruminant of linoleic acid (Bartlet and Chapman 1961; Parodi 1977), the ruminal production of both CLA and t11-C18:1 by microbial metabolism and the tissue activity of Δ^9desaturase (Bauman and others 1999). Similarly, the content of CLA found in the tissue of nonruminants is synthesized by microorganisms in the gastrointestinal tract that use linoleic acid as a substrate (Chin and others 1992).

Formation of Conjugated Linoleic Acid by Lactic Acid Bacteria

The c9t11-C18:2 isomer has been shown to be the most important isomer in terms of biological activity. Apparently, it is the only isomer incorporated into the phospholipid fraction of tissue of animals fed a mixture of CLA isomers (Ha and others

1990). This has triggered an interest in the development of methods, including the use of bacteria for the selective production of CLA isomers (Ogawa and others 2001). Jiang and others (1998) reported that a dairy starter culture, *Propinibacterium freudenreichii*, had the ability to produce CLA using free linoleic acid as a substrate. This study suggested that, CLA is derived from linoleic acid. Furthermore, they suggested that among CLA producing strains, there is positive correlation between CLA production and tolerance to free linoleic acid. However, Jenkins and Courtney (2003) found that, in MRS broth, the strains capable of producing CLA are the most inhibited by linoleic acid while those that cannot produce CLA are the least affected. Interestingly, in both previous studies, the authors had almost the same conclusion that linoleic acid isomerization to CLA is a detoxification mechanism for survival purposes, respectively for Jiang and others (1998), and Jenkins and Courtney (2003).

The CLA produced by cultures of lactobacilli is mainly present in the extracellular phase (Jiang and others 1998), but is also incorporated into the cellular membrane when grown in the presence of exogenous linoleic acid. It is important to mention that there is no association between the amount of fatty acid incorporated into the membrane and the inhibition of growth by that fatty acid (Jenkins and Courtney 2003). The metabolic pathway by which lactic acid bacteria generally transform free linoleic acid into CLA may be explained as follows: lactic acid bacteria under microaerobic conditions produce hydroxy fatty acids, 10-hydroxy-cis-12-octadecaenoic acid and 10-hydroxy-trans-12-octadecaenoic acid, which were found as possible precursors of the CLA isomers c9t11- or t9c11- C18:2 (Ogawa and others 2001). The accumulation of c9-C18:1, in the extracellular phase, during production of c9t11- or

t9c11 –C18:2 by lactic acid bacteria from medium containing free linoleic acid indicates that some c9t11- or t9c11 –C18:2 are further hydrogenated to c9-C18:1 instead of t11-C18:1, which is similar to what takes place in the bovine rumen (Jiang and others 1998). The conversion of free linoleic acid into CLA by lactobacilli is catalyzed by linoleate isomerase, a membrane bound enzyme (Pariza and Yang 1998). Studies by Pariza and Yang (1998), show detailed biochemical characteristics of *Lactobacillus reuteri.* First, the production of CLA is directly proportional to the cell biomass. Second, there is an enzyme conversion of linoleic acid mainly to the c9t11-C18:2 isomer without producing the t10c12 or the c10c12 isomers. These findings suggest that linoleate isomerase is an enzyme that accumulates, is not required for cell growth, and has a degree of specificity on cis-12, instead of cis-9 double bond of the fatty acid. The reaction between the linoleate isomerase of *L. reuteri* and the free fatty acids can be accomplished in the early stationary phase (Pariza and Yang 1998). Studies on *Lactobacillus acidophilus* found that cells previously subcultured in medium containing linoleic acid had a higher CLA productivity than cells grown in medium without linoleic acid. This suggests that the enzyme system for CLA production is induced by linoleic acid (Ogawa and others 2001). The presence of various medium components such as albumin, starch, cholesterol and lecithin help protect bacteria from free fatty acids during their growth (Nieman 1954). Tween-80 or proteins have an important function in the recovery of the inhibitory effects of free linoleic acid aiding in the production of CLA in MRS broth (Jiang and others 1998). However, inhibition of *Lactobacillus* is less in skim milk than in MRS broth with equal concentration of linoleic acid (Jenkins and Courtney 2003). Furthermore, milk proteins or other components may provide a higher protective effect against fatty acids

than MRS broth (Lin and others 1999). However, the complete mechanisms of CLA production by lactic acid bacteria remain unclear.

Occurrence of Conjugated Linoleic Acid in Foods

Conjugated linoleic acid is found in a wide array of biological materials, including plants and animal tissues, normally consumed by humans. The main dietary source of CLA are animal products, of which ruminants are the greatest contributors. Seventy-six percent of the CLA in meat products from ruminants is the c9t11-C18:2 isomer (Chin and others 1992). This is due to the incomplete biohydrogenation of dietary unsaturated fatty acids in the rumen (Bauman and others 1999). Of meat from ruminant animals, lamb has the highest concentration of CLA with 5.6 mg CLA/g fat, followed by beef which ranges from 2.9 to 4.3 mg CLA/g fat, while veal has the lowest with 2.7 mg CLA/g fat. Foods derived from non-ruminants are far lower in CLA content except for turkey, which contains 2.5 mg CLA/g fat. The content in seafood ranges from 0.3 to 0.6 mg CLA/g fat. Dairy products contain considerable amounts of CLA. For example, the CLA levels in natural cheeses are high and comparable to those in milk (5.5 mg CLA/g fat) ranging from 2.9 to 7.1 mg CLA/g fat (Chin and others 1992). The major isomer of CLA, c9t11-C18:2, represents 80-90% of the total CLA in milk fat (Parodi 1977; Chin and others 1992) and more than 83% in natural cheeses. On the other hand, natural cheeses that have been aged or ripened have a lower CLA content than unripened ones. During ripening of cheese, bacterial enzymes split neutral fat to release free fatty acid (including CLA) (Chin and others 1992), which are very vulnerable to further oxidation. This fact might indirectly cause decline in the CLA concentration in ripened cheeses.

Plant oils contain from 0.1 to 0.7 mg CLA/g fat which is far less CLA than animal products. The two major CLA isomers in plant oils are the c9t11-C18:2 and t10c12-C18:2, accounting for 43 and 40% respectively. Processed foods, except for cooked meats which tend to be lower in the c9t11-C18:2 isomer, are comparable in CLA content to similar unprocessed foods indicating that heat treatment during processing does not change total CLA content or c9t11-C18:2 concentration (Chin and others 1992). In fact some processed cheeses present an increased level of total CLA caused by the combination of both processing temperatures and the presence of whey components, primarily low molecular weight compounds. Processing temperatures could increase the formation of linoleic acid radicals that in the presence of oxygen could lead to either conjugated isomers or lipid peroxides. Lactalbumin and lactoglobulin, the main proteins in whey are sources of hydrogen donors (McDermott 1987), thus aiding in the formation of conjugated double bonds (Shantha and others 1992). These proteins may also protect against isomerization and oxidation, helping to the stability of CLA during processing (Chin and others 1992).

Ocurrence of Conjugated Linoleic Acid in humans

In humans, CLA has been detected mainly in blood, bile, adipose tissue and milk (Fogerty and others 1988). However, CLA also has been found in human cervix because of bacterial colonization and activity (Fairbank and others 1989). The c9t11-C18:2 isomer represents more than 95% of the conjugated dienes in human tissues (Dormandy and Wickens 1987). In human blood serum, c9t11-C18:2 is associated more with triglycerides (58 to 78 %) than with phospholipids (16 to 34 %) or cholesteryl esters (2 to

8 %) (Fogerty and others 1988). By contrast, Harrison and others (1985) reported a similar distribution of CLA among the serum lipids.

In general, CLA in humans can result from two possible sources:

a) Dietary sources such as ruminant meat and dairy fats contribute to CLA levels in human tissues (Jiang and others 1999; MacDonald 2000), human serum and milk (Fogerty and others 1988; Britton and others 1992). Thus fatty acid composition of dietary fat can markedly influence the fatty acid composition of blood, lipids and adipose tissue (Glatz and others 1989). The increased CLA levels in plasma and serum in human subjects after being supplied with a diet high in CLA support this fact. (Huang and others 1994; Britton and others 1992). Studies in rats demonstrate that the incorporation of dietary CLA is tissue-dependent, where adipose tissue and lung tissue contain the most and brain tissue the least (Sugano and others 1997). Studies in humans presented a similar behavior where a higher average concentration of CLA was found in adipose tissue than in serum (Jiang and others 1999).

b) Endogenous synthesis of CLA in humans may be formed by synthesis from linoleic acid in the human organism through anaerobic microbial activity in the large bowel (Fogerty and others 1988). CLA could also be produced in vivo by oxidation of linoleic acid to produce free radicals with subsequent diene conjugation. The conjugated free radicals could then react with protein instead of molecular oxygen to form a CLA molecule and a protein radical (Cawood and others 1983). The desaturation of trans-vaccenic acid by the Δ^9-desaturase as found in rat liver microsomal preparations (Pollard and others 1980; Salminen and others 1998) may also lead to endogenous synthesis of

CLA. Conversion of trans vaccenic acid by the action of intestinal bacteria where CLA formed in the colon would be poorly absorbed (Salminen and others 1998).

Conjugated Linoleic Acid as Antioxidant

In several studies free radicals and radical-mediated oxidation processes have been implicated to play an important role in the development of cancer and atherosclerosis (Ip 1996). Antioxidant activity of CLA has been offered as a possible explanation for its anticarcinogenic effect (Ha and others 1990) and its role in the reduction of atherosclerosis (Lee and others 1994). However, c9t11-C18:2 was initially interpreted as an indicator of free radical attack on polyunsaturated fatty acids (Dormandy and Wickens 1987). Furthermore, in vivo experiments using model membranes showed that CLA did not function as an effective antioxidant or antioxidant precursor and did not act as an efficient radical scavenger in a manner comparable to α-tocopherol (vitamin E) or butylated hydroxytoluene (BHT) (Van den Berg and others 1995). By contrast, previous studies in vitro have revealed that CLA is more resistant to oxidation than LA, a more potent antioxidant than vitamin E, and is almost as effective as BHT (Ha and others 1990). Other studies on animals showed that CLA is also able to reduce thiobarbituric acid that is a reactive lipid peroxidation product (Ip and others 1985). The potent antioxidant activity of CLA (Ha and others 1990; Ip and others 1991) may be explained by oxidative modification of CLA into a metal chelator that stops radical generation (Ha and others 1990). However, the mechanisms of potential CLA antioxidant activity remains unclear and depends on the conditions under which the

experiments are produced and the reliability of the type of assays that are applied to measure lipid oxidation.

Conjugated Linoleic Acid as Anticarcinogenic.

In general, substances that exhibit anticarcinogenic activity in experimental models are mainly from plant origin (Fiala and others 1985; Wattenberg 1992). The only exception found in foods of animal origin is CLA (Fogerty and others 1988; Ha and others 1989; Shantha and others 1992). Although CLA constitutes a relatively minor part of the total fatty acid composition of foods, its protective properties against oxidation have been related to very low levels in the diet (0.05 % by weight). Thus, CLA can be ingested either as free or as a component fatty acid of triglycerides (Ip and others 1995).

Protective properties against human malignant melanoma, colorectal and human breast cancer cells have been shown. It seems to be more effective than beta-carotene, the precursor of vitamin A in this regard (Shultz and others 1992). However, the anticarcinogenic effect of CLA has been somewhat controversial. Since CLA isomers are classified as fatty acids, several studies have revealed that these animal lipids (Erickson and Hubbard 1990; Welsch 1992; Welsch 1994; Kondo and others 1994; Carrol and Hopkins 1979), especially linoleic acid (Ip and others 1985; Roebuck and others 1985), stimulate tumorigenesis in animals. This is possibly due to fatty acid oxidation and lipid peroxidation (Welsch 1987; Fischer and others 1988; Bull and others 1988). Hydroxyperoxy and hydroxyl derivatives of linoleic and arachidonic acids stimulate DNA synthesis and induce ornithine decarboxylase (ODC) activity. Both, DNA synthesis and ODC activity stimulations are markers of tumor promotion (Bull and

others 1988). But, mechanism studies indicate that given the structural similarities between the two (Pariza and others 2000), CLA may compete with linoleic acid in the biosynthesis of arachidonic acid and eicosanoids (Ha and others 1987; Rose and Connolly 1990). Sebedio and others (1997), indicated that, both CLA isomers (c9t11- and t10c12-C18:2) can be elongated and desaturated in similar ways to that of linoleic acid, providing precursors for CLA-derived eicosanoids. Altered eicosanoid signaling and CLA-derived eicosanoid signaling, in particular prostaglandin E_2 (PGE_2) (Lewis 1983), could effect a range of biological activities including lipid metabolism and cytokine synthesis and function (Pariza 1997). Cytokines are hormone-like mediators of immunity and inflammation that are released by macrophages and other immune cells when they are stimulated (Pariza 2000). Tumor necrosis factor-α (TNF-α) and interleukin-1 (IL-1) are cytokines (Lewis 1983) where TNF-α seems to be an important mediator in many chronic pathological processes including carcinogenesis (Okahara and others 1994; Suganuma and others 1996) and atherosclerosis (Ross 1993). Thus, the inhibition of carcinogenesis by CLA isomers or metabolites could result from the combination of different CLA activities such as the effects on eicosanoids (Rose and Connolly 1990). Conjugated linoleic acid can affect prostaglandin metabolism (Liew and others 1995; Pariza and others 1999), in particular that of prostaglandin E_2 (PGE_2) (Li and Watkins 1998). Furthermore, CLA reduces cell proliferation (Belury 2002) by blocking DNA synthesis components and cell cycle proteins (Futakuchi and others 2002) such as bromodeoxyuridine (BrdU) and cyclins A and D, respectively (Ip and others 2001). This increases the tumor suppressor, p53 that induces cell death (apoptosis) (Belury 2002). However, elevated cell death is supported by CLA primarily due to the

reduction of Bc1-2 protein that suppresses apoptosis (Belury, 2002). Conjugated linoleic acid affects cell differentiation in animals by reducing the activity of the structures and sites where carcinogenic transformation takes place from weaning to adult stage (Ip and others 1994). The direct effect of CLA on vitamin A metabolism may influence cell differentiation as well (Banni and others 1999). The antioxidant property of CLA offers significant protection against mammary tumor development by reducing lipid peroxidation products found in mammary tissue such as thiobarbituric acid (Ip and others 1985). The anticarcinogenic effect of CLA continues to be supported by several different studies in vitro and in vivo (Decker 1995; Ip and others 1994; Parodi 1997; Pariza and Hargraves 1985).

Conjugated Linoleic Acid as Antiatherosclerotic

Atherosclerosis is the principal cause of heart attack, stroke and gangrene of the extremities, being a strong cause of mortality in the USA, Europe and Japan (Ross 1993). For these reasons, efforts have been made to find a cure. Research on animals has shown that a diet based on linoleic acid resulted in dramatic decreases in plasma low-density lipoprotein cholesterol (LDL-C) when compared to animals fed with high saturated fat diets. These lipoprotein are greatly associated with reductions in atherosclerosis (Hennessy and others 1992). But, later studies showed that LDL particles enriched with linoleic acid are more susceptible to oxidation and presumably more atherogenic than oleate-rich diets (Reaven and others 1993; Steinberg and others 1989). However, several studies have shown significant reductions in plasma cholesterol concentrations and atherosclerosis in animals fed with CLA when compared with linoleic acid and controls

(Lee and others 1994; Nicolosi and others 1997; Wilson and others 2000). Nicolosi and Laitinen (1996) also reported reduced levels of non-high-density lipoprotein cholesterol (combined very-low- and low-density lipoprotein) and triglycerides with no effect on high-density lipoprotein cholesterol when hamsters were fed a CLA diet. Subsequently, they also observed that blood lipoproteins do not play an important role in CLA's mechanism of action at least against stroke formation. Other authors infer that the anti-atherogenic properties of CLA may be due to an increased plasma tocopherol/total cholesterol ratio where CLA may act in place of tocopherol, suggesting a direct or indirect antioxidant activity in vivo (Wilson and others 2000). However, Lee and coworkers (1994) suggested that the reduction in aortic atherosclerosis with CLA treatment was probably due to alterations in lipoprotein metabolism since oxidative status was not significantly altered. Other studies speculate that CLA might act by altering LDL particle size by producing a larger, less atherogenic particle, since small dense LDL particles are more susceptible to oxidation (De Graaf and others 1991; Hubel and others 1998). Recent studies suggest that the anti-atherosclerotic effect of CLA treatment is not due to direct changes in lipoprotein metabolism or LDL oxidation, but possibly through other mechanisms such as LDL particle size or LDL lipid composition. In summary, CLA may function through decreased oxidative susceptibility of the LDL via lipid composition and not by directly acting as antioxidant on the protection of LDL (Wilson and others 2000).

Bile Salts

Biosynthesis of Bile Salts

Bile acids are synthesized in the liver, stored and concentrated in the gallbladder, and then released as salts into the small intestine. They are the major constituents of bile. The precursor of bile salts is cholesterol, which is converted into trihydroxycoprostanoate and then into cholyl CoA. The activated carboxyl carbon of cholyl CoA reacts with the amino group of glycine to form the conjugated product named glycocholate, or with the amino group of taurine, derived from cysteine, to form taurocholate (Stryer 1995). Glycine conjugated bile acids are the major type in human bile due to the minimal amount of taurine in the normal diet (Dowling 1972). However, there are many different bile salts that vary characteristically with the species (Lehninger 1972). Synthesis of cholic acid is homeostatically regulated by the amount of bile acids that return to the liver through the enterohepatic circulation (Danielsson and Sjovall 1975).

Enterohepatic Circulation of Bile Salts

In animals, the major pathway of degradation of cholesterol is its conversion to bile salts that takes place in the liver. Bile salts are secreted into the small intestine and are largely reabsorbed during lipid absorption. Thus, the circulation of the bile salts from the liver to the small intestine and then to the blood system that goes back to the liver is called the enterohepatic circulation (Lehninger 1972). However bile salts can be deconjugated in the intestine due to an intracellular enzyme named bile salt hydrolase (BSH) which is found commonly in intestinal species bacteria such as *Lactobacillus*

(Gilliland and Speck 1977; Lundeen and Savage 1990). Thus, conjugated bile salts may be hydrolyzed to produce the free bile salt (cholate) and the corresponding amino acid (Corzo and Gilliland 1999). The bile salts sodium glycocholate and sodium taurocholate give rise to sodium cholate and glycine, and sodium cholate and taurine, respectively.

Absorption of bile salts depends highly on the location in the intestine and the type of bile acid being absorbed. Passive diffusion occurs in all segments of the small intestines but in the ileum active transport occurs against an electrochemical gradient (Schiff and others 1972). Molecular charge is important in the absorption of bile salts. The pKa of the acids indicate the net charge at a given pH. The pH of the small intestine ranges from 4.5-6.0. At this pH the deconjugated bile salts (pKa around 6.0) easily diffuse in a non-ionic passive diffusion process while the conjugated acids (pKa 2.0-4.0) are negatively charged and less well reabsorbed by ionic passive diffusion or much more quickly by active transport mechanisms (Dietschy 1968). As result of these transport system, conjugated bile salts are primarily re-absorbed, while most of deconjugated or free bile salts are excreted in the feces.

The Role of Bile Salts

Bile salts promote mainly the absorption of lipids (Lehninger 1972). First, they are excreted into the bile and eventually into the intestinal lumen. Bile salts are highly effective emulsifiers because they contain both polar and non-polar regions (Stryer 1995). They form micelles with fat-soluble materials that are able to pass through the mucosal membranes being absorbed into the body. Bile salts facilitate digestion and absorption of triglycerides, fatty acids, monoglycerides, fat-soluble vitamins, cholesterol, sterols and

other fat soluble compounds (Dietschy 1968). Bile salts that are not absorbed are excreted from the body. Therefore, to maintain the equilibrium in the system, lost bile salts are replaced through synthesis from cholesterol (Shimada and others 1969).

Effect of Bile Salts on Lactobacilli

Studies on behavior of lactobacilli in the presence of bile have been narrowed down to two aspects, bile resistance and cellular permeability. Bile resistance is an important characteristic of some strains of lactobacilli. It enables the bacteria to survive, to grow and to manifest their action in the small intestines (Walker and Gilliland, 1993). At a certain concentration, bile increases cellular permeability, which allows more substrate to enter bacteria cells to be transformed by their intracellular enzymes (Noh and Gilliland 1993).

Effects of Probiotic Cultures on Health

The beneficial effects of probiotic cultures are many, but only those of major relevance will be described in this section.

Probiotic Cultures

Bacteria that provide specific health benefits when consumed as a food component or supplement are called probiotics (Guaner and Malagelada 2003). In other words, consumption of probiotics can improve human or animal health. Among the more popular probiotic cultures used are those that mimic the beneficial bacteria already present in the human system. Examples are *Lactobacillus acidophilus,* followed by

*Lactobacillus reute*ri, different species of *Bifidobacterium*, *Lactobacillus casei*, *Lactobacillus rhamnosis*, among others. However, the level of probiotic bacteria needed in food to provide a benefit to the host is still under discussion (Turner 2003).

Lactic Acid Bacteria

Lactic acid bacteria (LAB) refer to a large group of Gram-positive bacteria with similar properties that obtain their energy from the production of lactic acid as result of carbohydrate fermentation. These organisms are heterotrophic and frequently have complex nutritional requirements because they lack many biosynthetic capabilities. As result, lactic acid bacteria are generally abundant only in communities where these requirements can be provided such as animal oral cavities and intestines, plant leaves as well as decomposing plant or animal matter. Lactic acid bacteria of commercial interest in the food industry are the genera *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Pediococcus* and *Streptococcus* (Daly and others 1998). Lactic acid bacteria are resistant to lysozyme, an enzyme capable of lysing Gram-positive bacteria in the saliva. They reach the intestines to form a layer that adheres to the epithelium, which acts as a defense mechanism against invasion of enteropathogenic bacteria (Kasper 1998). This property among others confers to some of them the attribute of being probiotics. However, it is important to mention that not all probiotics are lactic acid bacteria just as all lactic acid bacteria are not probiotics.

Improved Lactose Utilization by Probiotics

Some people experience cramps, flatulence, and diarrhea after consumption of milk products. This is mainly due to insufficient amounts of the enzyme lactase in the small intestines. This inability to adequately digest lactose can be improved by consuming fermented milk with *L. acidophilus*, a bile resistant bacteria with intracellular β-galactosidase (an enzyme that breaks down lactose). Because of its bile resistance *Lactobacillus. acidophilus* colonizes the small intestines and at the same time produces additional amounts of the enzyme. This is a clear advantage over traditional yogurt starter cultures, which usually die before reaching the small intestines (Gilliland 1990).

Control of Intestinal Infections by Probiotics

Gastrointestinal infections and their consequences are a major clinical problem (Kasper 1998) due to frequent colonization of the gastrointestinal tract by pathogens such as *Clostridium difficile*, *Salmonella typhimurium*, *Escherichia coli* and *Shigella sonnei* (Cross 2002). As a consequence, a wide range of antibiotics has been used over the years developing resistant strains (Kasper 1998). In search of therapeutic improvements, probiotics have taken the challenge due to their properties. Among them, *L. acidophilus* has been successful in the treatment of diarrhea, mucous colitis, and antibiotic colitis (Gililland 1990). The modes of action by which probiotics exert relief are not well defined. However these microorganisms exhibit some inhibitory activity against pathogen growth by producing localized organic acids (acetic and lactic acid), hydrogen peroxide and several antibiotic-like compounds (Kasper 1998) such as acidolin,

acidophilin, lactocidin and bacteriocins (Gilliland 1990). Probiotics also can limit pathogen attachment by means of competitive exclusion because they seed the gut mucosa reducing the space for pathogens to grow (Cross 2002). These friendly bacteria may even reduce more pathogen adhesion to surfaces by producing biosurfactants (Reid and others 1998). Immunomodulatory signals produced by probiotics influence the immune system at the local and systemic level. Some strains of lactobacilli can directly stimulate the immune system by increasing lymphocyte populations and cell surface receptor expression in the gut tissue (Cross 2002). Some strains of lactobacilli can translocate across the intestinal mucosa (without infection) to enter circulation via lymphatic drainage and thoracic duct channeling and interact with leucocytes (white blood cells). Once in contact with leucocytes of host, Gram-positive bacteria transduce signals given by the pathogens. As response, the host releases pro-inflammatory cytokines called neutrophiles and macrophages to fight against infection (Cross 2002).

Effects of Probiotics on Cancer

Environmental factors such as diet have a major role in the development of sporadic colon cancer. High intake of red meat is associated with high risk of colon cancer while fruits and vegetables are associated with reduced risk (Guarner and Malagelada 2003). The effect of diet could be mediated by modifying the metabolic activity and the composition of the gastrointestinal microflora. The ingestion of Lactobacillus acidophilus has proven to bind mutagenic heterocyclic amines formed by cooking protein-rich food (Orrhage and others 1994). Lactic acid bacteria such as Lactobacillus casei can significantly decrease the specific activity of β-glucuronidase,

nitroreductase and azoreductase which are enzymes that transform procarcinogens to the active form (Kasper 1998). In a human study, daily intake of lyophilized L. acidophilus for three months significantly reduced cell division in the upper part of the colonic crypts (Biasco and others 1991). Increased cell division activity in the upper part of the crypts is considered a pre-neoplastic stage (Kasper 1998). Usually, the evidence for the anti-tumorigenic activities of lactic acid bacteria in humans is indirect (Daly and others 1998). However, Aso and others (1992, 1995) showed that oral administration of L. casei Shirota strain reduces the recurrence of a superficial bladder carcinoma in humans. Although the mechanism whereby lactic acid bacteria produce anti-tumor activity is not clear (Gilliland 1990), many of these organisms produce butyrate or butyric acid as a metabolic end product (Daly and others 1998). This fatty acid has been shown to inhibit tumorigenic growth in vitro (Young 1996). Other reports suggest that the antitumor activity extracted from cells of Lactobacillus delbrueckii ssp. bulgaricus was due to glycopeptides (Bogdanov and others 1975). Production of the anticarcinogenic CLA has been claimed by some strains of lactobacilli such as Lactobacillus reuteri (Pariza and Yang 1998). This topic is described in a previous section of this literature review (CLA and its benefits). In general, lactobacilli capable of growing in the human intestinal tract may provide a greater chance for producing antitumor activities than those organisms unable to survive and grow in this environment (Gilliland 1990).

Effects of Probiotics on Serum Cholesterol

The hypocholesterolemic action of *Lactobacillus* species was first noticed when the consumption of cultured or culture containing dairy products supplemented with

certain strains of *L. acidophilus* reduced concentrations of serum cholesterol (Gilliland 1990). These lactobacilli were grown under laboratory conditions such as anaerobiosis and in the presence of bile salts, both of which occur in the small intestines, to see if they could assimilate cholesterol (Noh and Gilliland 1993). Since lactobacilli had success in vitro, studies were prepared in vivo with pigs fed a diet high in cholesterol and supplemented with a strain of *L. acidophilus* that efficiently assimilated cholesterol from the laboratory study. Successfully, the strain of *L. acidophilus* was able to lower serum cholesterol of pigs (Gilliland and others 1985). A similar study conducted by another group of researchers supported the fact that *L. acidophilus* may lower cholesterol levels in vivo (Danielson and others 1989). The hypocholesterolemic action of the lactobacilli might be explained by their ability to deconjugate the bile salts (Gilliland and Speck 1977) and to incorporate some cholesterol into the cellular membrane (Noh and others 1997). However, a later study on cholesterol removal from broth by Brashears and others (1998) indicated that most of the cholesterol removal by strains of *L. acidophilus* was due to assimilation, perhaps by incorporating cholesterol into the cellular membrane, while strains of *L. casei* most likely remove cholesterol by destabilization of cholesterol micelles and coprecipitation of cholesterol with deconjugated bile salts at pH less than 6.0. Nevertheless, how could bile acid deconjugation and cholesterol incorporation decrease cholesterol levels in the human body? Deconjugated bile acids do not function as well as the conjugated ones in the intestinal absorption of cholesterol (Eyssen 1973). Deconjugated bile salts are less likely to be reabsorbed from the intestines (Chikai and others 1987). Increased deconjugation of bile salts could result in greater excretion of free bile salts from the intestinal tract (Chikai and others 1987), stimulating the synthesis

of their replacement from cholesterol (De Rodas and others 1996). Furthermore, free bile salts do not support the absorption of lipids, including cholesterol, from the intestines (Dietschy and Wilson 1970; Playoust and Isselbacher 1964). Thus, both effects, the use of cholesterol to produce de novo bile salts and the interrupted absorption of cholesterol from the intestines may reduce serum cholesterol levels in the body (Brashears and others 1998; De Rodas and others 1996). Strains of lactobacilli that prefer to deconjugate sodium glycocholate, the main bile salt in the human intestinal tract, better than sodium taurocholate may be more relevant to lower serum cholesterol (Brashears and others 1998). *Lactobacillus acidophilus, L. casei* (Corzo and Gilliland, 1999) *and L. reuteri* (Taranto and others 1999) deconjugate bile acids during growth by action of the enzyme bile salt hydrolase. Lactobacilli deconjugate bile salts the best in the late exponential phase (Walker and Gilliland, 1993) and at pH of approximately 6.0 (Gilliland and Speck, 1977). Cholesterol incorporated into or adhered to the bacterial cells would be less available for absorption from the intestine into the blood (Brashears and others 1998). The maximum assimilation of cholesterol by lactobacilli occurs in the late exponential phase of growth (Walker and Gilliland 1993). The assimilation of cholesterol by *L. acidophilus* requires growth under anaerobic conditions and the presence of bile salts. However the most bile-tolerant lactobacilli strains may not necessarily assimilate the most cholesterol, furthermore their ability to assimilate cholesterol varies among strains (Gilliland et al., 1985). Studies on the antiatherosclerotic effect of CLA, a compound produced by some lactobacilli has caught the attention of researchers. How the compound is produced and its beneficial effects are better described in a previous section of this literature review (CLA and its benefits). In brief, any mechanism, bile salt

deconjugation, cholesterol incorporation or CLA production is important in controlling serum cholesterol concentrations in humans.

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CHAPTER III

INFLUENCE OF GLYCOCHOLIC ACID ON PRODUCTION OF CONJUGATED LINOLEIC ACID BY CELLS OF *LACTOBACILLUS REUTERI* 55739

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Abstract

Cells of *L. reuteri* 55739 grown in MRS broth with and without linoleic acid (LA, 0.2%) were harvested and washed. The washed cells were added to buffer containing LA (0.2%) and incubated 18 h at 37 °C. The cells, which had been grown without LA, transformed LA into conjugated linoleic acid (CLA, mainly c9t11-C18:2) better than did those cells grown with it. When glycocholic acid (0.3%) was added to the washed cell suspension about the same levels of CLA was formed regardless of whether or not the cells had been grown in the presence of glycocholic acid.

Introduction

Conjugated linoleic acid (CLA) refers to a group of positional and geometric isomers of linoleic acid (LA; c9c12-C18:2) (MacDonald 2000). The major isomer, c9t11 octadecadienoic acid (Kepler and Tove 1967), has been suggested to be antioxidative (Pariza 1991), anticarcinogenic (Pariza and Hargraves 1985; Pariza 1991), and antiatherosclerotic (Lee and others 1994; Wilson and others 2000)

Conjugated linoleic acid is produced by chemical synthesis (Christie and others 1997), by rumen bacteria (Bauman and others 1999) or by lactic acid bacteria (LAB) (Ogawa and others 2001). Among LAB the genus *Lactobacillus* has gained recent attention because they can control cancer (Kasper 1998) and lower serum cholesterol (Gilliland 1990).

Lactobacillus reuteri, a probiotic that highly effectively enzymatically transforms LA into CLA (Pariza and Yang 1998), is also bile resistant (De Boever and others 2000).

This is an important property for its survival and establishment in the intestinal tract (Gilliland 1990).

Exposure to bile acids has shown to modify cell permeability of *L. reuteri* (Taranto and others 2003). Therefore since linoleate isomerase, the enzyme responsible for CLA production is intracellular (Pariza and Yang 1996), it may be that the presence of glycocholic acid, a major bile acid (Brashears and others 1998) could improve its performance. The purpose of this study was to investigate the influence of glycocholic acid on CLA production by *L. reuteri* 55739.

Material and Methods

Source and Maintenance of Cultures

The culture used in this study, *Lactobacillus reuteri* 55739, was purchased from the American Type Culture Collection (Manassas, VA., U.S.A.).

The culture was activated before experimentation by subculturing three times just prior to use in lactobacilli Man Rogosa and Sharpe (MRS) broth made according to the manufacturer's directions (Difco Laboratories, Detroit, MI., U.S.A.). The MRS broth was inoculated (1%) and incubated for 18 h at 37 °C. The culture was kept at 4 °C between transfers.

Enumeration of Lactobacilli

The number of lactobacilli in the samples was determined using the pour plate method on MRS agar (Vanderzant and Splittstoesser 1992) and expressed as colony

forming units (CFU) per milliliter. Dilution blanks containing 99 mL of sterilized solution of 0.1% peptone (Difco Laboratories, Detroit, MI., U.S.A.) were used. After pouring plates, they were overlayed with about 5 mL MRS agar and incubated at 37 °C. At 48 h all visible colonies were counted and recorded.

Conjugated Linoleic Acid Production and Linoleic acid/Conjugated Linoleic Acid Incorporation by *L.reuteri* 55739

Cells of *L. reuteri* (1% inoculation) were grown at 37 °C in 10 mL tubes containing MRS supplemented with 0.2% linoleic acid (Matreya, State College, PA., U.S.A) added as aqueous solution in 1% v/v Tween 80 (Sigma Chemical Co., St. Louis, MO., U.S.A.). A control tube was prepared with MRS broth containing 0.2% linoleic acid without cells. The cells were harvested by centrifugation (12000 x g for 10 min at 2 to 4 °C) in a Sorvall RC-5 centrifuge (DuPont Co., Newton, CT., U.S.A.) at 0 h and 24 h of incubation time. The cell pellet was resuspended in 1 mL saline solution (0.137 mol/L NaCl, 7.0 mmol/L K_2HPO_4 and 2.5 mmol/L KH_2PO_4). Lipid extraction was performed on the control, the cell free supernatants as well as the pellets.

Influence of Concentrations of Linoleic Acid on Conjugated Linoleic Acid Production by Washed Cells

Cells of *L. reuteri* 55739 (1% inoculation) were grown at 37 °C in 50 mL MRS broth. After 18 h the cells were harvested by centrifugation (12000 x g for 10 min at 2 to 4 °C), washed with 50 mL of cold 0.05 M sodium phosphate buffer (pH 7.0) and resuspended in 45 mL of the same buffer. The washed cells were dispensed in 9 mL

volumes into 5 test tubes. A control set of 5 tubes was prepared with 9 mL volumes of cold 0.05 M sodium phosphate buffer (pH 7.0) without cells. A 1 mL aliquot of linoleic acid stock solution 0.10%, 0.15%, 0.20%, 0.25% and 0.30% in 1% v/v Tween 80 was added to tube 1 thru 5 of each set to obtain a final concentration of linoleic acid from 0.10 thru 0.30 mg/mL respectively. All tubes were incubated at 37 °C for 18 h. After incubation the contents of each tube were centrifuged (12000 x g for 10 min at 2 to 4 °C) and clear supernatants were collected for analysis.

Effect of Sonication on Conjugated Linoleic Acid Production by Washed Cells

Lactobacillus reuteri 55739 (1% inoculation) was grown at 37 °C in 40 mL MRS. After 18 h the cells were harvested and washed as described above and resuspended in 40 mL of the same buffer containing linoleic acid (0.2%). They were dispensed in 10 mL volumes into test tubes. The contents of two tubes were sonicated at power 4 for 30 min using Branson sonicator (Branson Ultrasonics Corp., Danbury, CT., U.S.A.), while 2 other tubes were not. Once sonicated, viable cell count was determined in one tube of each set using the pour plate method. The remaining tubes were incubated at 37 °C for 18 h after which the contents of each tube were centrifuged (12000 x g for 10 min at 2 to 4 °C) and clear supernatants were collected for analysis.

Influence of Glycocholic Acid on Conjugated Linoleic Acid Production by Washed Cells

Cells of *L. reuteri* 55739 (1% inoculation) were grown at 37 °C in 20 mL MRS broth with and without 0.2% linoleic acid added as aqueous solution in 1% v/v Tween 80. After 18 h the cells were harvested by centrifugation (12000 x g for 10 min at 2 to 4 °C).

The cells from each media were washed with 20 mL of cold 0.05 M sodium phosphate buffer (pH 7.0), resuspended in 20 mL of the same buffer containing linoleic acid (0.2%) and dispensed in 10 mL volumes into test tubes. A control set of 2 tubes was prepared by dispensing 10 mL volumes of 0.05 M sodium phosphate buffer (pH 7.0) with linoleic acid (0.2%) without washed cells. To investigate the effect of glycocholic acid on CLA production, 1 mL of 3.0% aqueous glycocholic acid (Sigma Chemical Co., St. Louis, MO., U.S.A.) was added to each tube. All tubes were incubated at 37 °C for 18 h. After incubation the contents of each tube were centrifuged (12000 x g for 10 min at 2 to 4 °C) and clear supernatants collected for lipid extraction procedure.

Lipid Extraction from Cell Free Supernatants

This method is based on that of Alonso and others (2003). First, a 6 mL volume of spent broth or uninoculated broth or supernatant from washed cells was mixed with 60 µL of heptadecanoic acid solution (Sigma Chemical Co., St. Louis, MO., U.S.A.), used as internal standard (64.4 mg of heptadecanoic in 10 mL of hexane). Then 12 mL of isopropanol (Pharmaco Products Inc., Brookfield, CT., U.S.A.) were added and shaken vigorously, followed by 9 mL of hexane (Pharmaco Products Inc., Brookfield, CT., U.S.A.). The mixture was shaken 3 min before centrifugation at 1910 x g for 5 min at 2 to 4 °C. The supernatant was collected and filtered through approximately 5.0 g dry sodium sulphate (Spectrum Quality Products Inc., Gardena, CA., U.S.A.); 7 mL of hexane were used to wash the sodium sulphate. The lipid fraction of the sample thus obtained was placed into a 25-mL pear-shaped flask, and dried under nitrogen gas at 70 °C in a Zymark Turbo Vap LV evaporator (Zymark Corp., Hopkinton, MA., U.S.A.).

The dried sample was redisolved with 500 µL hexane and transferred to a microtube. The extracted lipid fraction was mixed with 100 µL 1 N methanolic sodium hydroxide (Spectrum Quality Products Inc., Gardena, CA., U.S.A), then vortexed for 1 min and held at 70 °C in a water bath for 15 min. At this point the samples are ready to prepare fatty acid methyl esters.

Lipid Extraction from Bacterial Pellet

The CLA analysis for bacterial pellet samples was modified from Coakley and others (2003) where 60 µL of internal standard (6.49 mg/mL heptadecanoic acid in hexane) were added to the pellet suspended in 1 mL saline solution (0.137 mol/L NaCl, 7.0 mmol/L K_2HPO_4 and 2.5 mmol/L KH_2PO_4). Then 2 mL of isopropanol were added and vortexed 60 seconds followed by the incorporation of 1.5 mL of hexane. The mixture was shaken vigorously for 3 min and centrifuged 5 min at 1910 x g at 2 to 4 °C. The upper layer was recovered and dried under nitrogen gas at 70°C for 10 min in the Zymark evaporator. The sample was dissolved in 500 µL hexane and transferred to a microtube where a 100 µL 1N methanolic sodium hydroxide were added and vortexed 1 min. The mixture was hold 15 min in a 70 °C bath. Then fatty acid methyl esters were prepared.

Preparation of Fatty Acid Methyl Ester

Methylation of free fatty acids in the mixture from extracted lipids from cell free supernatants as well as cell pellets was achieved by adding 200 µL of 14% boron trifluoride in methanol (Sigma Chemical Co. St. Lous, MO., U.S.A.) and holding it at

room temperature for 30 min. Then 200 µL of hexane and 100 µL of deionized water were added, the samples were vortexed thoroughly, and centrifuged in a refrigerated microcentrifuge SFR13K-120 (Savant Instruments Inc., Farmingdale, N.Y., U.S.A.) at 2000 x g for 3 min. The upper layer was recovered and stored in a vial at –20 °C, until further analyses by gas chromatography.

Gas Chromatography Analyses

All gas chromatography analyses were performed on a HP6890 gas liquid chromatograph (Hewlett-Packard, Wilmington, DE., U.S.A.), equipped with a split/ splitless injector and flame ionization detector (FID). The fatty acid methyl esters were separated on a CP Sil 88 column (100 m x 0.25 mm i.d) containing 100% cyanopropyl siloxane stationary phase, 0.20 µm film thickness (Chrompack, Varian, Inc., CA., U.S.A). Helium (28 psi, 1.97 kg/cm^2) was used as the carrier gas with a split ratio of 1:50. The conditions used were 190 °C isothermal temperature, 250 °C injector temperature, and 250 °C detector temperature. The amount injected was 2 µL, and the peak of the c9t11 -C18:2 isomer was identified by comparison with the retention times of the reference standard (Matreya, Inc., State College, PA., U.S.A.). The amounts of c9t11 -C18:2 isomer were calculated as µg CLA/mL culture based on the areas of individual CLA and the internal standard peaks.

Statistical Analyses

Experiments were conducted in triplicate or quadruplicate. Each value was the mean of three or four independent trials. Data were analyzed using SAS (SAS Inst.

2001) as a randomized block design. Least significant difference analyses and Tukey's test were used to compare means. Significant differences were determined at $P<0.05$ (Steel and others 1997).

Results and Discussion

Confirmation of Identity of *L. reuteri* 55739

The identity of *L. reuteri* 55739 in this study was confirmed (Appendix A). The identity characteristics of the strain were similar to those listed for this organism in Bergey's Manual of Systemic Bacteriology (Sneath and others 1986). The strain of lactobacilli used in this study matched the published reactions except for arabinose, gluconate and ribose.

Conjugated Linoleic Acid Production and Linoleic acid/Conjugated Linoleic Acid Incorporation by *L.reuteri* 55739

The CLA produced by cultures of lactobacilli is mainly found in the extracellular phase (Jiang and others 1998), however it can also occur in the cellular membrane when the organism is grown in the presence of exogenous linoleic acid (Jenkins and Courtney 2003). In this study the ability to produce CLA by *L. reuteri* 55739 was confirmed, it was primarily exogenous CLA (mainly c9t11-C18:2) as mention by Pariza and Yang (1998). After 24 h incubation the mean cell concentration was $1.9x10^9$ CFU/mL. However, when the incorporation of linoleic acid or CLA into cell membrane was

checked, the results showed no significant incorporation of either compound. Linoleic acid that was not transformed into CLA appeared to remain in the broth (Table 1).

Table 1–Conjugated linoleic acid production and LA/CLA incorporation by *L. reuteri* 55739

		Fatty Acids (µg/mL)[1]	
	Incubation (h)	**LA[2]**	**CLA[3]**
Supernatant	0	205.49[a]	0.00[b]
Supernatant	24	88.29[b]	118.75[a]
Cell pellet	0	0.00[c]	0.00[c]
Cell pellet	24	0.00[c]	3.06[c]

[abc]Means in the same column for the supernatant and for the cell pellet with different superscripts differ (P<0.05)
[1]Data are the means from 3 experiments
[2]LA, linoleic acid; supernatant values compared against control 224.18[a] µg/mL
[3]CLA, conjugated linoleic acid (c9t11-C18:2); supernatant values compared against control 0.00[b] µg/mL

Influence of Concentrations of Linoleic Acid on Conjugated Linoleic Acid Production by Washed Cells

The amounts of CLA (c9t11-C18:2) formed by washed cells previously grown in MRS without linoleic acid was nearly constant when the added amount of free linoleic acid in the buffer was between 0.15 and 0.30%. However, there was a significant increase between buffer containing 0.10 and 0.15% linoleic acid (Table 2). Studies by Jiang and others (1998) found that the formation of CLA during growth by

propionibacteria was related to the amount of linoleic acid in the medium up to 0.20%, between 0.20 and 0.60% the production remained almost constant. In another report 0.20% linoleic acid added to MRS broth resulted in production of more CLA after 24 h incubation time than in broth containing 0.4% by active strains of lactobacilli (Alonso and others 2003). Thus, based on the observations found in this experiment and the literature review, it was recommended to add 0.20% linoleic acid to media in future experiments with washed cells.

Table 2–Influence of concentrations of linoleic acid on CLA production by washed cells of *L. retueri* 55739 at 18 h

		Fatty Acids (µg/mL)[1]		Conversion (%)
Growth media[2]	**LA[3] (%)**	**LA[4]**	**CLA[5]**	**LA into CLA[6]**
MRS	0.10	17.27[e]	48.74[b]	70
MRS	0.15	52.76[d]	65.51[a]	70
MRS	0.20	75.34[c]	66.65[a]	60
MRS	0.25	105.88[b]	60.18[ab]	50
MRS	0.30	150.89[a]	73.69[a]	40

[abcde]Means in the same column with same superscripts do not differ (P>0.05)
[1]Data are the means from 3 experiments
[2]MRS, Man Rogosa and Sharpe broth
[3]Added amount of linoleic acid
[4]LA, linoleic acid detected in supernatant; values compared against control 68.67, 92.44, 121.32, 127.52 and 211.28 µg/mL for 0.1 thru 0.3% LA respectively
[5]CLA, conjugated linoleic acid (c9t11-C18:2) detected in supernatant; values compared against control 0.00 µg/mL for 0.1 thru 0.3% linoleic acid
[6]Conversion values based on those of control

Effect of Sonication on Conjugated Linoleic Acid Production by Washed Cells

Cell viability of washed cells of *L. reuteri* 55739 was compromised when sonication was applied. Washed cells that were sonicated showed a much lower plate count (mean $1.2x10^8$ CFU/mL) than those that were not (mean $1.6x10^9$ CFU/mL). A decrease in the viable cell count is related to disruption of cells and release of endoenzymes (Wang and Sakakibara 1997). However, when CLA production was measured non-sonicated cells transformed more free linoleic acid than did the sonicated ones (Table 3). This implies that although cells of *L. reuteri* 55739 were disrupted by sonication, linoleate isomerase (bound membrane enzyme responsible for CLA production) was not effectively released or was inactivated.

Table 3–Effect of sonication on CLA production by washed cells of *L. retueri* 55739 at 18 h

	Fatty Acids (µg/mL)[1]	
	LA[2]	**CLA[3]**
Non-sonication	124.76[b]	23.32[a]
Sonication	153.98[a]	0.58[b]

[ab]Means in the same column with different superscripts differ ($P<0.05$)
[1]Data are the means from 3 experiments
[2]LA, linoleic acid detected in supernatant
[3]CLA, conjugated linoleic acid (c9t11-C18:2) detected in supernatant

Influence of Glycocholic Acid on Conjugated Linoleic Acid Production by Washed Cells

A study on *L. acidophilus* found that cells previously grown in medium containing LA had a higher CLA productivity that those cells grown in medium without LA (Ogawa and others 2001). However, in the present study, washed cells of *L reuteri* 55739 previously grown without LA (mean $1.1x10^9$ CFU/mL) produced more CLA (c9t11-C18:2) than did those cells previously grown with LA ($1.6x10^9$ CFU/mL). An explanation would be that washed cells of *L. reuteri* previously grown on LA are less active than unexposed washed cells in converting LA into CLA (Lee and others 2003). When glycocholic acid was added to washed cells, CLA production was not significantly higher than in its absence (Table 2). Because *L. reuteri* grows in the presence of 0.3% oxgall, a bile salt (Taranto and others 2003), it is not expected to lyse in the presence of 0.3% glycocholic acid. However, 0.3% bile salt can alter the permeability of cells of lactobacilli (Noh and Gilliland 1993).

Bile salts can produce folds and buds in the cell membrane of *L. reuteri*, which normally exhibits a flat and disorganized distribution (Valdez and others 1996). These changes may not only affect cell permeability but also the interactions between the membrane and the surroundings (Taranto and others 2003). As consequence, it is possible that the whole intracellular enzyme system of *L. reuteri* could be affected by the presence of bile. Some compounds such as acidic phenols once inside the cells of *L. plantarum* inhibit some fatty acid-metabolizing enzymes (Rozes and Peres 1998). The presence of bile salts during growth produced an increase in some unsaturated fatty acids, but a decrease in cyclic fatty acids in *L. reuteri* (Taranto and others 2003). However, when the properties of linoleate isomerase were examined, the enzyme did not require

addition of external cofactors or energy (Peng and others 2003). In fact, experiments performed by Rosson and others (1999) indicate that the concentration of Tween 80 seemed to affect linoleate isomerase activity the most among other components of the medium such as yeast extract, peptone, acetate, glucose, salts and vitamins.

Table 4–Effect of glycocholic acid on CLA production by washed cells of *L. reuteri* 55739 resuspended in 0.05 M sodium phosphate buffer (pH 7.0) with LA (0.2%) at 18 h

		Fatty Acids (µg/mL)[1]	
Growth media[2]	**GA[3]**	**LA[4]**	**CLA[5]**
MRS	-	29.84[b]	59.56[a]
MRS	+	33.48[b]	64.28[a]
MRS-LA	-	60.84[a]	28.79[b]
MRS-LA	+	68.70[a]	25.98[b]

[ab]Means in the same column with different superscripts differ (P<0.05)
[1]Data are the means from 4 experiments
[2]MRS, washed cells grown in Man Rogosa and Sharpe broth; MRS-LA, washed cells grown in MRS broth with 0.2% linoleic acid
[3]GA, 0.3% glycocholic acid; (-) no exposure to, (+) exposure to
[4]LA, linoleic acid detected in supernatant from washed cells; values compared against those from control (uninoculated 0.05 M sodium phosphate buffer) with and without GA added, 101.27 µg/mL and 97.01µg/mL respectively
[5]CLA, conjugated linoleic acid (c9t11-C18:2) detected in supernatant from washed cells; values compared against those from control (uninoculated 0.05 M sodium phosphate buffer) with and without GA added, 0.00µg/mL and 0.00µg/mL respectively

Thus, in this study it may be that bile salts simply display no effect on linoleate isomerase, the enzyme responsible for converting LA into CLA. Therefore no noticeable increase in CLA production was observed when washed cells were exposed to GA.

Further studies are needed to elucidate how LA conversion into CLA by *L reuteri* could be improved.

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APPENDIX A

IDENTITY OF *LACTOBACILLUS REUTERI* 55739

Table 5–Identity characteristics of *Lactobacillus reuteri* 55739

Test	*L. reuteri* 55739	*L. reuteri (Bergey's)*[1]
Amygdalin	-	o
Arabinose	-	-
Cellobiose	-	-
Esculin	-	o
Fructose	+	+
Galactose	+	+
Glucose	+	+
Gluconate	-	-
Lactose	+	+
Maltose	+	+
Mannitol	-	-
Mannose	-	-
Melezitose	-	-
Melibiose	+	+
Raffinose	+	+
Rhamnose	-	-
Ribose	-	-
Salicin	-	-
Sorbitol	-	-
Sucrose	+	+
Trehalose	-	-
Xylose	-	-
Catalase	-	-
Gram stain	+	+
Morphology	Rod	Rod
Growth at 45 °C	+	+
Growth at 15 °C	-	-

[1]*Lactobacillus reuteri* reactions as listed in the 9th edition of Bergey's manual and systemic bacteriology; o, reaction not determined

APPENDIX B

RAW DATA

Table 6–Conjugated linoleic acid production and LA/CLA incorporation by *L. retueri* 55739, raw data.

			Fatty Acids (µg/mL)	
		Incubation (h)	LA[1]	CLA[2]
Experiment 1	Supernatant	0	222.03	0.00
	Supernatant	24	88.57	123.44
	Cell pellet	0	0.00	0.00
	Cell pellet	24	0.00	3.07
Experiment 2	Supernatant	0	212.44	0.00
	Supernatant	24	77.45	103.96
	Cell pellet	0	0.00	0.00
	Cell pellet	24	0.00	3.31
Experiment 3	Supernatant	0	182.00	0.00
	Supernatant	24	98.84	128.84
	Cell pellet	0	0.00	0.00
	Cell pellet	24	0.00	2.80

[1]LA, linoleic acid; supernatant values compared against control 225.55, 194.86, and 224.18µg/mL for experiments 1 thru 3 respectively
[2]CLA, conjugated linoleic acid (c9t11-C18:2); supernatant values compared against control 0.00µg/mL for experiments 1thru 3

Table 7–*Lactobacillus reuteri* 55739 growth in Man Rogosa and Sharpe broth with 0.2 mg/mL linoleic acid

	Plate counts (CFU/mL)[1]			
Incubation (h)	Exp 1	Exp 2	Exp 3	Mean
0	$1.7x10^{8}$	$1.5x10^{8}$	$1.5x10^{8}$	$1.6x10^{8b}$
24	$2.0x10^{9}$	$2.2x10^{9}$	$1.6x10^{9}$	$1.9x10^{9a}$

[ab]Means in the same column with different superscripts differ (P<0.05)
[1]Colony formit units per milliliter

Table 8–Influence of concentrations of linoleic acid on CLA production by washed cells of *L. retueri* 55739 at 18h, raw data

			Fatty Acids (µg/mL)	
	Media[1]	**LA[2] (%)**	**LA[3]**	**CLA[4]**
Experiment 1	MRS-C	0.10	79.31	0.00
	MRS-C	0.15	90.26	0.00
	MRS-C	0.20	117.33	0.00
	MRS-C	0.25	*	0.00
	MRS-C	0.30	238.97	0.00
	MRS	0.10	16.73	43.67
	MRS	0.15	51.95	64.98
	MRS	0.20	70.44	70.08
	MRS	0.25	111.39	47.43
	MRS	0.30	152.64	76.36
Experiment 2	MRS-C	0.10	74.44	0.00
	MRS-C	0.15	91.97	0.00
	MRS-C	0.20	109.25	0.00
	MRS-C	0.25	129.51	0.00
	MRS-C	0.30	211.40	0.00
	MRS	0.10	14.86	53.86
	MRS	0.15	53.74	64.53
	MRS	0.20	80.48	63.79
	MRS	0.25	101.20	67.62
	MRS	0.30	159.63	73.10
Experiment 3	MRS-C	0.10	52.27	0.00
	MRS-C	0.15	95.09	0.00
	MRS-C	0.20	137.37	0.00
	MRS-C	0.25	125.53	0.00
	MRS-C	0.30	183.47	0.00
	MRS	0.10	20.23	48.69
	MRS	0.15	52.60	67.01
	MRS	0.20	75.11	66.09
	MRS	0.25	105.04	65.48
	MRS	0.30	140.41	71.60

[1]MRS, Man Rogosa and Sharpe broth; MRS-C, MRS uninoculated control
[2]Added amount of linoleic acid
[3]LA, linoleic acid detected in supernatant
[4]CLA, conjugated linoleic acid (c9t11-C18:2) detected in supernatant
*Loss value

Table 9–*Lactobacillus reuteri* 55739 initial biomass added to MRS medium with different linoleic acid concentrations at 0 h

		Plate counts (CFU/mL)[3]			
Growh media[1]	Content[2] (%)	Exp 1	Exp 2	Exp 3	Mean[4]
MRS	0.10	$1.8x10^{9}$	$1.5x10^{9}$	$8.6x10^{8}$	$1.4x10^{9a}$
MRS	0.15	$1.4x10^{9}$	$1.8x10^{9}$	$6.5x10^{8}$	$1.3x10^{9a}$
MRS	0.20	$1.4x10^{9}$	$2.0x10^{9}$	$9.8x10^{8}$	$1.5x10^{9a}$
MRS	0.25	$1.0x10^{9}$	$2.1x10^{9}$	$1.1x10^{9}$	$1.4x10^{9a}$
MRS	0.30	$9.5x10^{8}$	$1.7x10^{9}$	$7.0x10^{8}$	$1.1x10^{9a}$

[a]Means in the same column with different superscripts differ (P<0.05)
[1]MRS, Man Rogosa and Sharpe broth
[2]Amount of linoleic acid added to media
[3]Colony formit units per milliliter
[4]The mean value for all initial biomass values is $1.3x10^{9}$ CFU/mL

Table 10–Effect of sonication on CLA production by washed cells of *L. retueri* 55739 at 18 h, raw data

		Fatty Acids (µg/mL)	
		LA[1]	CLA[2]
Experiment 1	Non-sonication	133.84	23.18
	Sonication	157.24	1.74
Experiment 2	Non-sonication	123.49	15.20
	Sonication	150.51	0.00
Experiment 3	Non-sonication	116.95	31.57
	Sonication	154.18	0.00

[1]LA, linoleic acid detected in supernatant
[2]CLA, conjugated linoleic acid (c9t11-C18:2) detected in supernatant

Table 11–Effect of sonication on cell viability of *L. retueri* 55739

	Plate counts (CFU/mL)[1]			
Cells	Exp 1	Exp 2	Exp 3	Mean
Non-Sonicated	$1.8x10^{9}$	$1.4x10^{9}$	$1.6x10^{9}$	$1.6x10^{9a}$
Sonicated	$1.1x10^{8}$	$1.4x10^{8}$	$1.0x10^{8}$	$1.2x10^{8b}$

[a]Means in the same column with different superscripts differ (P<0.05)
[1]Colony formit units per milliliter

Table 12–Effect of glycocholic acid on CLA production by washed cells of *L. reuteri* 55739 resuspended in 0.05 M sodium phosphate buffer (pH 7.0) with LA (0.2%) at 18h, raw data

			Fatty Acids (µg/mL)	
	Growth medium[1]	GA[b]	LA[c]	CLA[d]
Experiment 1	MRS	-	29.68	56.93
	MRS	+	27.52	51.94
	MRS-LA	-	55.17	24.41
	MRS-LA	+	56.23	27.67
Experiment 2	MRS	-	35.99	38.23
	MRS	+	41.24	37.49
	MRS-LA	-	64.55	10.82
	MRS-LA	+	76.98	9.27
Experiment 3	MRS	-	28.00	41.67
	MRS	+	29.18	42.83
	MRS-LA	-	42.51	20.53
	MRS-LA	+	63.49	15.27
Experiment 4	MRS	-	25.70	101.40
	MRS	+	35.97	124.88
	MRS-LA	-	81.14	59.40
	MRS-LA	+	78.12	51.71

[1]MRS, washed cells grown in Man Rogosa and Sharpe broth; MRS-LA, washed cells grown in MRS broth with 0.2% linoleic acid

[b]GA, 0.3% glycocholic acid; (-) no exposure to, (+) exposure to

[c]LA, linoleic acid detected in supernatant from washed cells; values compared against those from control (uninoculated 0.05 M sodium phosphate buffer) with (82.20, 83.50, 82.06 and 157.32 µg/mL) and without (84.64, 88.03, 95.53, and 119.63µg/mL) GA added, experiments 1 thru 4 respectively

[d]CLA, conjugated linoleic acid (c9t11-C18:2) detected in supernatant from washed cells; values compared against those from control (uninoculated 0.05 M sodium phosphate buffer) with (0.00µg/mL) and without (0.00µg/mL) GA added, experiments 1 thru 4 respectively

Table 13–Cells of *L. reuteri* 55739 added to 0.05 M sodium phosphate buffer (pH 7.0) with LA (0.2%) at 0 h

		Plate counts (CFU/mL) [d]				
Growth media[b]	**GA[c]**	**Exp 1**	**Exp 2**	**Exp 3**	**Exp 4**	**Mean[e]**
MRS	-	1.1×10^{9}	9.2×10^{8}	9.2×10^{8}	1.3×10^{9}	1.1×10^{9b}
MRS	+	1.2×10^{9}	8.6×10^{8}	8.9×10^{8}	1.6×10^{9}	1.1×10^{9b}
MRS-LA	-	2.3×10^{9}	1.0×10^{9}	2.1×10^{9}	1.5×10^{9}	1.7×10^{9a}
MRS-LA	+	2.5×10^{9}	1.2×10^{9}	9.9×10^{8}	1.7×10^{9}	1.6×10^{9a}

[a]Means in the same column with different superscripts differ ($P<0.05$)
[b]MRS, washed cells grown in Man Rogosa and Sharpe broth without 0.2% linoleic acid; MRS-LA, washed cells grown in Man Rogosa and Sharpe broth with 0.2% linoleic acid
[c]GA, 0.3% glycocholic acid; (-) no exposure to, (+) exposure to
[d]Colony formit units per milliliter
[e]The mean values of washed cells grown in MRS with and without linoleic acid are 1.6×10^{9} and 1.1×10^{9} CFU/mL

VITA

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Candidate for the Degree of

Master of Science

Thesis: PRODUCTION OF CONJUGATED LINOLEIC ACID BY *LACTOBACILLUS REUTERI*

Major Field: Animal Science

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Title of Study: PRODUCTION OF CONJUGATED LINOLEIC ACID BY *LACTOBACILLUS REUTERI*

Pages in Study: 59 Candidate for the Degree of Master of Science

Major Field: Food Science

Scope of Study: Cells of *L. reuteri 55739* grown in MRS broth with and without linoleic acid (LA, 0.2%) were harvested and washed. The washed cells were added to buffer containing LA (0.2%) and exposed to glycocholic acid (0.3%) to determine its influence on CLA production. Experiments also were conducted to confirm CLA production and to study the effect of different linoleic acid concentrations added as well as sonication on CLA production by washed cells of *L. reuteri* 55739.

Findings and Conclusions: In this study the ability to produce CLA by *L. reuteri* 55739 was confirmed, it was primarily exogenous CLA (mainly c9t11-C18:2). The amounts of CLA formed were nearly constant when the amount of free linoleic acid gradually increased from 0.15 to 0.30%. Thus, 0.20% linoleic acid was used to further test washed cells. Experiments on sonication revealed that although cells of *L. reuteri* 55739 were disrupted, linoleate isomerase was not effectively released or it was inactivated since little or no activity was detected in the sonicated cell preparation. Washed cells of *L. reuteri* 55739 previously grown without LA produced more CLA than did those cells previously grown with LA. Possibly because washed cells previously grown on LA are less active than unexposed washed cells in converting LA into CLA. However, when glycocholic acid was added to washed cells no increase in production of CLA was observed regardless of whether of not the cells had been grown in its presence. Thus, it may be that bile salts simply display no effect on linoleate isomerase. Further studies are needed to elucidate how LA conversion into CLA by *L. reuteri* could be improved.

Advisor's Approval: Dr. Stanley E. Gilliland