PRODUCTION OF CONJUGATED LINOLEIC ACID AND CONJUGATED LINOLENIC ACID BY *BIFIDOBACTERIUM BREVE* JKL03 AND ITS APPLICATION

By

Yun-Kyoung Jung

Department of Food Science and Agricultural Chemistry
Macdonald Campus of McGill University
Montreal, Québec, Canada

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ABSTRACT

Conjugated linoleic acid (CLA) is predominantly found in foods of ruminant origin such as milk and processed cheese, and has gained much interest recently due to its beneficial health and biological effects on animals and humans.

The bioconversion of linoleic acid (LA) and linolenic acid (LNA) by a selected *Bifidobacterium* from healthy infant feces was studied. *Bifidobacterium breve* JKL03 had the ability to convert linolenic acid (0.2 mg/ml) to CLNA in fermentation of skim milk medium for 24 h up to a yield of 72.0% (up to 74.7% under aerobic conditions) and linoleic acid (0.2 mg/ml) into CLA by fermentation in skim milk medium for 24 h up to a yield of 23.9% (up to 28.0% under aerobic conditions).

B. breve JKL03 was also co-fermented with *Lactobacillus acidophilus* (NCFM® strain), a commonly added starter culture, to observe the resulting effects on growth during fermentation for yogurt production. Fermentation of LNA in skim milk with *B. breve* JKL03 and *L. acidophilus* (NCFM) maintained high CLNA production level. On the other hand, CLA production in the same media with both strains did not exhibit as high level as with the single *B. breve*.

These results are important for the advancement of knowledge on the production of CLA and CLNA in dairy products and for knowledge on the basic metabolic mechanisms for such conversion.

RÉSUMÉ

L'acide linoléique conjugué (ALC) se retrouve principalement dans les aliments de source laitière tels le lait et les fromages. Ce composé a récemment attiré l'attention à cause de ses effets biologiques salutaires sur la santé de l'humain et de l'animal.

La bio-conversion des acides linoléique (AL) et linolénique (ALN) par une souche spécifique de *Bifidobacterium* isolée des matières fécales de bébés sains est l'objet de la présente recherche. *Bifidobacterium* isolée possède la capacité de convertir l'acide linolénique (0.2 mg/ml) en ALNC par bioréaction fermentaire de 24 heures dans un médium à base de lait écrémé, et ce avec un rendement de 72.0 % (jusqu'à 74.7 % en conditions aérobiques); quant à l'acide linoléique (0.2 mg/ml), la conversion en ALC dans un médium à base de lait écrémé a atteint 23.9 % sur 24 heures (jusqu'à 28.0 % en conditions aérobiques).

B. breve JKL03 a également été co-fermentée avec *Lactobacillus acidophilus* (souche NCFM®), une souche d'amorcage populaire, afin d'en observer les effets sur la croissance durant la production fermentation de yogourt. La fermentation de l'ALN en medium de lait écrémé avec *B. breve* JKL03 et *L. acidophilus* (NCFM) a maintenu le haut rendement en ALNC. Toutefois, la production d'ALC dans le même médium avec les deux souches en simultané n'a pas permis de maintenir une haute productivité comparativement à *B. breve* seule.

Ces résultats sont importants pour l'avancement des connaissances quant à la production d'ALC et de ALNC dans les produits laitiers et dans l'élucidation des mécanismes métaboliques de base pour une telle conversion.

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FORWARD

This thesis is submitted in the form of original papers suitable for journal publication. A general introduction states the rationale and the objectives of this study, and the first chapter is literature review that presents the theory and background information on this topic. The next two chapters represent the body of the thesis, each being a complete manuscript. The last section is a summary of the major conclusions. The format of this thesis has been approved by the Faculty of Graduate and Postdoctoral Studies at McGill University and follows the conditions outlined in the Guidelines concerning Thesis Presentation, which are as follows:

"Candidates have the option of including, as part of the thesis, the text of one or more papers submitted, or to be submitted, or the clearly-duplicated text of one or more published papers. These texts must conform to the "Guidelines for thesis Preparation" with respect to font size, line spacing and margin sizes and must be bound together as an integral part of the thesis.

The thesis must be more than a collection of manuscripts. All components must be integrated into a cohesive unit with a logical progression from one chapter to the next. In order to ensure that the thesis has continuity, connecting texts that provide logical bridges between the different papers are mandatory.

The thesis must conform to all other requirements of the "Guidelines for Thesis Preparation" in addition to the manuscripts. The thesis must include: a table of contents, an abstract in English and French, an introduction which clearly states the rational and objectives of the research, a comprehensive review of the literature, a final conclusion and summary, a final all-inclusive bibliography.

As manuscripts for publication are frequently very concise documents, where appropriate, additional materials (experimental and design data as well as descriptions of equipment) must be provided where appropriate and sufficient detail to allow a clear and precise judgment to be made of the importance and originality of the research reported in the thesis.

In general, when co-authored papers are included in a thesis the candidate must have made a substantial contribution to all papers included in the thesis. In addition, the candidate is required to make an explicit statement in the thesis as to who contributed to such work and to what extent. This statement should appear in a single section entitled "Contributions of Authors" as a preface to the thesis.

CONTRIBUTION OF CO-AUTHORS FOR PUBLICATIONS

Dr. Byong H. Lee, my supervisor, is the co-author on the publications presented in this thesis and contributed in a supervisory role in this research, and he fully reviewed the manuscripts.

Dr. Geun B. Kim, a former graduate student at McGill University under Dr. Byong H. Lee, contributed to part of this work and he is the co-author of the manuscripts.

Dr. Mun Y. Jung from Department of Food Science at Woosuk University in Korea provided technical support and reviewed the manuscripts. He is the co-author on the manuscripts.

A. Part of this work has been prepared for publication:

1. Jung, M. Y., G. B. Kim., E. S. Jang., Y. K. Jung. and B. H. Lee. 2005. Improved Extraction Method with Hexane for Gas Chromatographic Analysis of Conjugated Linoleic Acids from Culture Media as Application in Probiotic Studies. *J.Dairy Sci.* (In preparation).

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Permission to use part of this material has been obtained from the co-authors of the manuscripts listed above.

GENERAL INTRODUCTION

Conjugated linoleic acid is positional and geometric isomers of linoleic acid. It occurs naturally as a mixture of isomers in dairy foods such as milk and meats derived from ruminant animals and could be converted from linoleic acid by linoleic acid isomerase of rumen bacteria.

The double bonds of conjugated linoleic acid could exist at any location from the 2 to 18 positions along a carbon chain and the most common positional isomers include those with double bonds located at positions 9- 11- and 10- 12- along the carbon chain.

Numerous researches on CLA have been carried out and CLAs have been found to be anticarcinogenic, atherosclerotic, body fat reductant, enhancing immune system, and antidiabetic on diabetes type II. The increasing demand for biologically active polyunsaturated fatty acids (PUFA) has attracted commercial attention, especially on conjugated linoleic acid (CLA) - based nutraceuticals and therapeutics. From a human health viewpoint, it appears desirable to increase CLA levels in foods to protect against disease and enhance general health.

Conjugated linolenic acid (CLNA) is also known to be anticarcinogenic and is present in plant seed oils (Kishino *et al.*, 2003), such as Kerela and flax oil. The conjugated linoleic acids and conjugated linolenic acids produced by a strain of bifidobacteria can be good sources for nutritional and health benefits, as functional foods or fermented dairy products, for human and animal intake. However, the quantities of CLA and CLNA in natural foods are considerably low. The application of bifidobacteria capable of producing high levels of CLA and CLNA could be useful in fermented dairy foods.

In this study, many human and rumen origins of *Bifidobacterium* and *Lactobacillus* species were assessed for microbial conversion of linoleic acid to conjugated linoleic acid and linolenic acid to conjugated linolenic acid. Among the strains investigated, we have selected *Bifidobacterium breve* JKL03 and assessed the conversion rate in MRS and NFDM media. Furthermore, the application studies were carried out with *Bifidobacterium breve* JKL03 and *Lactobacillus acidophilus* NCFM to enrich CLA and CLNA in yogurt.

The main objectives of this research were:

1) To screen human and animal origins of *Bifidobacterium* and *Lactobacillus* species, and select a strain with the highest CLA converting ability.

2) To optimize the growth condition of *Bifidobacterium breve* JKL03 for the production of conjugated linoleic acid and conjugated linolenic acid in MRS broth.

3) To optimize the growth condition of *Bifidobacterium breve* JKL03 for the production of conjugated linoleic acid and conjugated linolenic acid in skim milk.

4) To apply *Bifidobacterium breve* JKL03 to produce enhanced CLA and CLNA in yogurt with *Lactobacillus acidophilus* NCFM.

CHAPTER 1

LITERATURE REVIEW: MICROBIAL CONVERSION OF LINOLEIC ACID TO CONJUGATED LINOLEIC ACID

In this chapter, structure and sources of conjugated linoleic acid and the biochemical and molecular characteristics of CLA are reviewed in Part 1.2.

Several health benefits for human and animal on CLA are in Part 1.3.

Part 1.4 continued about CLA synthesis by chemical methods, and biological methods using various microorganisms, and previous works on CLA production by strains of bacteria.

This chapter also included the recent information on the pros and cons of trans fatty acid of CLA.

1.1 INTRODUCTION

The term, conjugated linoleic acid (CLA), refers to a group of positional and geometric isomers of octadecadienoic acid (C18: 2) (Scimeca and Miller, 2000). CLA is a nutritional product that has well documented benefits for the prevention of cancer, atherosclerosis, weight gain, immune system, and diabetes. Linoleic acid is converted to CLA by linoleic acid isomerase enzyme of rumen bacteria. For these reasons, CLA occurs naturally as a mixture of isomers in dairy foods such as milk and meats derived from ruminant animals (Pariza *et al.*, 1999).

Pariza *et al.* (2001) showed evidence on CLA's multifunction and implications regarding the possible biochemical mechanisms. Emerging evidence indicates that the *cis*-9, *trans*-11 and *trans*-10, *cis*-12 CLA isomers have different effects. There are structural differences between these isomers, and there is more than one biochemical mechanism involved. In fact, there is evidence indicating that more than one biochemical mechanism is involved in the specific effects of the *trans*-10, *cis*-12 CLA isomer.

1.2 Conjugated Linoleic Acid (CLA)

1.2.1 Structure and characters

The general structure of conjugated linoleic acid is similar to that of other fatty acids containing varying lengths of hydrocarbon chains linked to a carboxyl group; however, the conjugated nature of the double bonds present along this eighteen-carbon chain molecule makes this group unique. Specifically, the double bonds within the fatty acid molecules are separated from each other by single bonds, rather than the methylene group ($-CH_2-$) present in fatty acids such as linoleic acid (Figure 1.1).

The double bonds of conjugated linoleic acid could exist at any location from the Δ2 to Δ18 position along a carbon chain (Watkins *et al.*, 2001a and 2001b). Fritsche *et al.* (2001) reported conjugated linoleic acid as having a combined total of 56 possible positional and geometric isomers. Not all isomers appear in appreciable quantities in mixtures of conjugated linoleic acid. The most common positional isomers include those with double bonds located at positions 9- 11- and 10- 12- along the carbon chain (Sehat *et al.*, 1998; Watkins *et al.*, 2001a).

1.2.2 Sources of CLA

Conjugated linoleic acid may be found in a variety of food products including those of animal and plant origin. The actual content of conjugated linoleic acid and the distribution of particular isomers vary widely according to the particular product in question.

1.2.2.1 Ruminants

Animal sources may be further subdivided into ruminant and non-ruminant sources. Typically, meats and dairy products from ruminants including cows and lamb contain elevated amounts of conjugated linoleic acid compared with non-ruminant animals and their by-products. This trend results largely from the process of bacterial biohydrogenation of linoleic acid in the rumen, which is absent in non-ruminant species.

Conjugated linoleic acid may be found in milligram quantities per gram of fat in dairy products and meats from lamb, veal and cattle (Watkins *et al.*, 2001b). Moreover, rumenic acid or *cis*-9, *trans*-11 octadecadienoic acid has been identified as one of the major isomers of conjugated linoleic acid in milk fat (Baumgard *et al.*, 2001) and meats from ruminants (Fritsche *et al.*, 1999).

The principle mechanism by which naturally occurring conjugated linoleic acid and conjugated linolenic acid in these ruminants is through the biohydrogenation of polyunsaturated fatty acids illustrated in Figure 1.2 (Bauman *et al.*, 2003), this pathway uses isomerase and reductase of microorganisms. Moseley *et al.* (2002) also concluded that the biohydrogenation of oleic acid by mixed ruminal microbes involves the formation of several positional isomers of *trans* monoenes rather than only the direct biohydrogenation to form stearic acid.

According to Dhiman *et al.* (2000), conjugated linoleic acid may escape the rumen and become available for absorption in the lower digestive tract, thus providing a source of conjugated linoleic acid to the body. While it was first thought that all conjugated linoleic acid in animal tissues were derived from ruminal escape of the biohydrogenation

intermediate, it was later concluded that much of the conjugated linoleic acid was biohydrogenated to vaccenic, and stearic acid (Palmquist, 2001). In 1994, Parodi proposed that Δ9 desaturation could prove an important mechanism for humans to utilize the vaccenic acid in milk fat for endogenous synthesis of conjugated linoleic acid. Palmquist (2001) also noted the endogenous synthesis of rumenic acid in animal tissue and recognized its significance in relation to the fat content of milk. In an experiment whereby pure vaccenic acid was fed to mice, it was found that 50 % of the vaccenic acid stored in tissues was desaturated to conjugated linoleic acid (Palmquist, 2001). Griinari *et al.* (2000) also demonstrated that vaccenic acid was desaturated to rumenic acid in lactating cows with endogenous synthesis of conjugated linoleic acid accounting for approximately 64 % of that present in milk fat. Other researchers also confirm that *trans*-vaccenic acid can be converted further in mammalian tissues to the same *cis*-9, *trans*-11 isomer of conjugated linoleic acid via Δ9 desaturase (Fritsche *et al.*, 1999).

1.2.2.2 Non-ruminants

Although present in lower quantities due to the absence of microbial biohydrogenation, conjugated linoleic acid may also be found naturally in non-ruminant animals such as turkey. Turkey meat was reported to have some content of conjugated linoleic acid among non-ruminant species with 2.5 mg of CLA per gram of fat (Ha *et al.*, 1987; Watkins *et al.*, 2001b). Amounts found in chicken samples were much less than this number. Non-ruminant byproducts such as egg yolk were also found to contain minimal amounts of conjugated linoleic acid (Watkins *et al.*, 2001b).

The predominant isomer in all non-ruminant samples was also found to be that of the *cis*-9, *trans*-11 isomer of conjugated linoleic acid. Almost exclusive to animal products, conjugated linoleic acid may be detected at very low levels in certain plant oils such as safflower, sunflower, canola, and olive oil. Among these oil types, safflower oil was found to have the highest concentration of conjugated linoleic acid with a content of 0.7 mg of CLA per gram of oil, while corn oil was among the lowest with only 0.2 mg of CLA per gram of oil. Furthermore, the *cis*-9, *trans*-11 isomer of conjugated linoleic acid accounted for only 39 % of the conjugated linoleic acid mixture. Differing significantly from the distribution of isomers in animal products (Watkins *et al.*, 2001b), Gnädig *et al.* (2001) suggest that while plant oils are limited in their CLA content, oils rich in non-conjugated linoleic acid including sunflower, soybean and safflower oils, provide ideal starting materials for the production of synthetic conjugated linoleic acid (CLA) mixtures.

1.2.2.3 CLA in food products

Fats and meats from ruminant sources are rich in conjugated linoleic acid; however, dairy products from these same sources have been known to contain even higher amounts of CLA. For instance, fats and meats from ruminants such as lamb, and beef were assessed as having CLA content between 2.7 and 5.6 mg of CLA per gram of fat. Analysis of dairy products including milk, cheese and yogurt from ruminants resulted in higher CLA contents, ranging between 3.6 and 6.2 mg of CLA per gram of fat. (Jiang *et al.*, 1996; Fritsche and Steinhart, 1998)

A variety of products and their conjugated linoleic acid contents are outlined briefly in Table 1.1. Dairy products originating from ruminants contained high levels of conjugated linoleic acid, as did meats derived from these same ruminants. According to Fritsche *et al.* (1999), the wide range of values obtained when analyzing the CLA content in dairy products reflects the differences in the CLA contents of the milk fat. Furthermore, the CLA content in milk fat is influenced heavily by the cows' feed consumption (Fritsche *et al.*, 1999), the content of polyunsaturated fatty acids in the feed (Park *et al.*, 1999b; Fritsche *et al.*, 1999) and the presence of ionophores (Fritsche *et al.*, 1999). Hurst *et al.* (2001) analyzed a series of milk-chocolate-based confectionery samples for the presence of conjugated linoleic acid. It was found that the *cis*-9, *trans*-11 isomer of conjugated linoleic acid was present in quantities up to 0.3 mg/g milk chocolate.

1.2.2.4 Trans Fatty Acid

Trans fatty acids have been found to increase total cholesterol levels and LDL cholesterol levels, and to reduce HDL cholesterol levels. In other words, trans fatty acids have negative effects on cholesterol levels. Food products that contain large amounts of trans fatty acids are tropical oils (palm and coconut), stick margarines, high fat baked and fried goods and any product for which the label says, "partially hydrogenated vegetable oils". Therefore the US FDA has made it mandatory that as of 2006 nutritional labels reveal the content of trans fat in foods and dietary supplements (FRRR, 2003). However, trans fatty acids in ruminant meat and milk, such as vaccenic acid and CLA, have positive effects on human health.

Cesano *et al.* (1998) studied animals fed a standard diet or diets supplemented with 1% linoleic acid (LA) or 1% conjugated linoleic acid (CLA) for 2 weeks prior (total of 14 weeks). Mice receiving LA-supplemented diet displayed significantly higher body weight, lower food intake and increased local tumor load as compared to the other two groups of mice. Mice fed the CLA-supplemented diet displayed not only smaller local tumors than the regular diet-fed group, but also a drastic reduction in lung metastases. Ledoux *et al.* (2000) indicated that trans fatty acids in milk fat products have their origin in the hydrogenation of polyunsaturated fatty acids by rumen microorganisms and the effect of the diet of mammals on the trans fatty acid production in milk is preponderant. The bio-hydrogenation results mainly in 18:1 *trans* isomers, especially the 11-*trans*-vaccenic acids, and produces also noticeable amounts of 9-*cis*, 11-*trans* 18:2 rumenic acids, a conjugated linoleic acid (CLA) having anticarcinogenic properties. Fatty acid isomerization during heating treatments affects linoleic (18:2) and alpha-linolenic (18:3) acids, two essential fatty acids.

For these reasons, conjugated linoleic acid would be excluded from US FDA's definition of "trans fat" for the purposes of nutritional labeling (US FDA, Labeling and Dietary Supplements, 2003; *Bauman et al.*, 2004).

1.3 Health Benefits

1.3.1 Anticarcinogenic

Throughout the literature, there is much evidence to demonstrate the effectiveness of CLA as an antioxidative (Ha *et al.*, 1990) and anticancer agent in animal models. Although the protective properties (Belury, 1995; O'Shea *et al.*, 1998; Stanton *et al.*, 1998). Cancer chemoprevention by CLA has been known to extend over a number of cancer sites. Ip (2000) reported that tumor development in the mammary gland appeared to be particularly sensitive to CLA intervention. Preferential accumulation of conjugated linoleic acid in neutral lipid of adipocytes, the dominant cell type in mammary tissue was suggested as a possible explanation for this. Experiments on rat mammary epithelium offered some insight by revealing that CLA was able to inhibit the formation of premalignant injuries from terminal end buds, the primary sites for chemical induction of mammary carcinomas, after exposure to a carcinogen. Overall results from experiments suggest a decrease in proliferation and an increase in apoptosis. Banni *et al.* (2001) also noted that CLA had the ability to modulate mammary stromal cell differentiation resulting in inhibition of mammary carcinogenesis.

Further study on the inhibition of mammary tumor growths induced by N-methylnitrosourea in the rat was done by Bougnoux *et al.* (2000), and Guthrie and Carroll (1999) observed the specific vs. nonspecific effects of dietary fat on carcinogenesis. Corl *et al.* (2001 and 2003) studied the role of 9-desaturase in the production of *cis*-9, *trans*-11 and also *cis*-9, *trans*-11 CLA reduces cancer risk in rats. Specifically they compared the effects

of a typical CLA mixture of isomers and the single predominant *cis*-9, *trans*-11 isomer of CLA on the incidence and growth of mammary tumors and concluded that similar inhibition of mammary tumor growth was observed using both the mixture and the single isomer. It is also interesting to note that Fritsche *et al.* (1997) identified CLA isomers in human subcutaneous adipose tissue using gas chromatography-direct deposition-Fourier transform infrared spectroscopy (GC-DD-FTIR) and gas chromatography electron ionization mass spectrometry (GC-EIMS). The most abundant adipose tissue isomers present were *cis*-9, *trans*-11 and *trans*-9, *trans*-11 octadecadienoic acids (C18: 2). Minor isomers identified in adipose tissue included both *trans*-9, *cis*-11 and *cis*-9, *cis*-11 octadecadienoic acids.

In addition, Schønberg and Krokan (1995) investigated the influence of linoleic acid and conjugated linoleic acid on the growth of different cancer cell lines in culture. They found that CLA exerted a moderate growth inhibitory effect in a dose- and time- dependent manner, while linoleic acid had no effect, or for one cell line, a slight stimulatory effect. The biochemical mechanism by which CLA exerted its anticancer activity was thought to include the formation of cytotoxic lipid peroxidation products. Wahle and Heys (2002), and Chujo *et al.* (2004) studied a similar research about investigating the effects of CLA on cultures of sensitive and insensitive cell lines of human breast and prostate cancer. Significant effects on cell signal mechanisms associated with inhibition of tumorigenesis and cell proliferation were induction of redox enzymes, attenuation of cytokine-induced transcription factor activation, activation or inhibition of specific protein kinase C isoforms involved in proliferate or apoptotic events and modulation of eicosanoid production from gene expression to product formation.

In humans, researchers such as Chajes *et al.* (2003) also investigated the role of conjugated linoleic acid on breast cancer evolution by measuring CLA in adipose breast tissue removed during cancer surgery and by monitoring for subsequent secondary growths. No relationship was found between the concentration of CLA and the prognostic features of breast cancer such as tumor size or vascular invasion. Furthermore, the potential of CLA to decrease the risk of secondary growths subsequent to treatment could not be documented (Bougnoux *et al.*, 2000). Thus, it should be noted that while CLA administered in animal models and cell culture systems demonstrates anticarcinogenic activity, there is no direct evidence that CLA protects against cancer in humans.

1.3.2 Cardiovascular disease

Cardiovascular disease is a general term used to describe all diseases of the heart and blood vessels. Atherosclerosis is the main form of this disease, characterized by mounds of lipid material mixed with smooth muscle cells and calcium, also called plaques, along the inner walls of the arteries.

Conjugated linoleic acid has already been shown to reduce atherogenesis in animal studies (Nicolosi and Wilson, 1997; Watkins *et al.*, 2001a). Moreover, Kritchevsky (2000) reported that conjugated linoleic acid inhibited cholesterol-induced atherogenesis in hamsters when fed as 1 % of the diet. Diets rich in *trans*-10, *cis*-12 CLA were also reported by Deckere *et al.* (1999) to decrease values of LDL- and HDL-cholesterol, increase VLDL and decrease epididymal fat in hamsters. As well, the severity of atherosclerosis was reduced by 30 % in an experiment monitoring rabbits with pre-established atherosclerosis fed a diet containing 1 % CLA over 90 days.

Atherosclerosis disturbs the body's ability to effectively regulate blood clotting due to the fact that platelets respond to plaques in the same way as they would respond when encountering injured blood vessels. Platelets form blood clots and remain attached to plaques inside of arteries, gradually increasing in size until the blood supply is finally blocked off. Clots may also break loose and lodge themselves along smaller arteries with the same end result. The action of platelets is controlled by eicosanoids made from omega-6 and omega-3 fatty acids and an imbalance among these compounds may actually contribute to the formation of clots.

1.3.3 Body fat and lipid metabolism

Many authors conducting animal studies have already noted that CLA-fed animals showed equal or greater growth rate gains compared with controls while consuming smaller quantities of food. Specifically, improved feed efficiency was confirmed in mice, rats (Chin *et al.*, 1994) and pigs (Cook and Pariza, 1998). Researchers reasoned that increased feed efficiency was a result of changes in body composition following the consumption of conjugated linoleic acid. Later studies on CLA-fed mice validated this observation as these mice experienced over a 50 % reduction in whole body fat and a significant increase in body protein, water and ash. Further measurements also showed that conjugated linoleic acid inhibited post-heparin lipoprotein lipase and increased glycerol release from adipocytes during fat metabolism, suggesting an increase in lipolysis (Cook and Pariza, 1998).

Park *et al.* (1999a) investigated the effects of conjugated linoleic acid (CLA) preparations, which were enriched for the *cis*-9, *trans*-11 CLA isomer or the *trans*-10, *cis*-12

CLA isomer, on body composition in mice. They had found that body composition changes were associated with feeding the *trans*-10, *cis*-12 CLA isomer. By contrast, the *cis*-9, *trans*-11 and *trans*-9, trans-11 CLA isomers did not affect these biochemical activities. Yang *et al.* (2003) observed that supplementation of CLA in the diet of layer hens decreased the concentration of docosahexaenoic acid in all of the tissue lipids and concluded that dietary CLA can transfer to the tissue but that incorporation of CLA isomers into the tissue is selective in hens.

Examining the reduction in body fat accumulation (Bouthegound *et al.*, 2002) in animal models further, DeLany and West (2000) and West *et al.* (2000) fed mice both high and low fat diets supplemented with conjugated linoleic acid. From this, they determined that 0.5 % and 1.0 % CLA in the diet reduced body fat regardless of food intake. As well, they concluded that one mechanism by which CLA reduced fat was by increasing the body's energy expenditure and that fat oxidation was also implicated in reduced fat accumulation in the body. Miner *et al.* (2001) also established apoptosis of adipocytes in white adipose tissue within five days of CLA supplementation in the diet, confirming that CLA selectively reduces fat deposits in the body. Further research carried out on the adipocyte 3T3-L1 cell culture system showed that the *trans*-10, *cis*-12 isomer of CLA was able to reduce lipoprotein lipase activity, intracellular triacylglycerol and glycerol levels while enhancing release of glycerol into the medium. As a result, the physiological mechanism of body fat reduction in mice was attributed to the inhibition of fat storage in adipocytes coupled with both β-oxidation in skeletal muscle and an increase in skeletal muscle mass (Park *et al.*, 1999a). Demaree *et al.* (2002) and Evans *et al.* (2002) also studied about CLA isomers on adiposity and lipid metabolism. Other authors such as de Deckere *et al.* (1999) noted the

activity of the *trans*-10, *cis*-12 isomers while the predominant *cis*-9, *trans*-11 isomer had little or no effect on lipid metabolism in hamsters, and Berven *et al.* (2000) studied about the safety of CLA in overweight or obese human volunteers.

Conjugated linoleic acid has been shown to modulate the maturation and differentiation of adipocytes in addition to affecting lipid metabolism through the regulation of lipid mediators, namely the prostaglandins and leukotrienes (Pariza *et al.*, 1999). Since adipose tissue is also an important site for the regulation of glucose metabolism, the ability of CLA to affect glucose metabolism was investigated. Human studies reported by Risérus (2001) pointed to the *cis*-10, *trans*-12 conjugated linoleic acid isomer rather than a commercial mixture of CLA isomers as a potent regulator of glucose metabolism, causing several metabolic disturbances related to insulin resistance in pre-diabetic men.

1.3.4 Enhancing immune system

According to Cook and Pariza (1998), sources of immune stimulation can range from vaccination to exposure to environmental lipopolysaccharides. Throughout stimulation, cells of the immune system interact with antigens and release cytokine communication signals including interleukin (IL-1) and tumor necrosis factor α (TNF) to direct the immune response. While both are critical in the defense process, they redirect nutrient flow to immune related products and induce skeletal muscle degradation.

It was determined through successive experiments in CLA-fed chicks (Cook and Pariza, 1998), mice (Miller and Gipson, 1994; Cook and Pariza, 1998) and rats that conjugated linoleic acid prevented immune-induced weight loss. At first, it was

hypothesized that CLA was protective against immune-induced catabolism by prompting immune suppression (Marha *et al.*, 2003), however further animal studies suggested enhanced immune function in addition to protection against immune-induced wasting. Cook *et al.* (1993) explained this phenomenon by the ability of CLA to affect eicosanoid production, reasoning that CLA could prevent the down-regulation of the immune response on one hand and prevent muscle wasting on the other.

The effects of conjugated linoleic acid on autoimmune diseases and the asthmatic response were also examined (Cook *et al.*, 1993). Results indicated that while CLA did not worsen responses to allergens, it actually reduced type I hypersensitivity in guinea pigs.

1.3.5 Antidiabetic

The possible effects of the CLA isomers on blood insulin are confused by marked differences in species response. Mice fed CLA-supplemented diets developed mild insulin resistance (DeLany and West, 2000), which may be related to a shift toward enhanced use of fatty acids as fuel and the regulation of insulin sensitivity in humans on CLA were studied by Brown and McIntosh (2003).

Moya-Camarena and Belury (1999) studied that conjugated linoleic acid inhibits carcinogenesis and atherosclerotic plaque formation and delays the onset of diabetes in experimental animals. Whereas numerous data has demonstrated beneficial effects in rodent models, but little work has been done to determine the role of dietary CLA in human health.

CLA isomers have a number of beneficial health effects, as shown in biomedical studies with animal models. Ryder *et al.* (2001) reported that a mixture of CLA isomers

improved glucose tolerance and CLA (47% c9, t11; 47.9% c10, t12) diet reduced adiposity and improved glucose tolerance. Insulin-stimulated glucose transport and glycogen synthesis activity in skeletal muscle were improved with the 50:50 diet compared with all other treatments.

1.4 CLA Synthesis

Synthetic CLA mixtures differ somewhat in isomeric composition from which occurs naturally in cows as a result of microbial conversion in the rumen and Δ9-desaturation in the mammary gland (Griinari *et al.*, 2000; Gnädig *et al.*, 2001).

1.4.1 Chemical method

Actual isomeric composition of synthetic CLA depends to a great extent on the exact synthetic procedure used and can vary within a rather wide range (Gnädig *et al.*, 2001). Typically, oils rich in linoleic acid (C18: 2) are converted into their conjugated forms by treatment with strong alkali (Gnädig *et al.*, 2001). Predictably, such treatment results in a mixture of many isomers of linoleic acid. Fritsche *et al.* (1999) reported the major isomers resulting from alkali isomerization as *trans*-8 *cis*-10, *cis*-9 *trans*-11, *trans*-10 *cis*-12 and *cis*-11 *trans*-13 isomers. Varying parameters including solvent type (i.e. ethylene glycol, glycerol, *tert*-butanol or water), catalysts present (i.e. lithium-, sodium- or potassium

hydroxide) or reaction vessels allow for the formation of certain isomer mixtures predominantly over others (Reaney *et al.*, 1999).

Berdeaux *et al.* (1998) and Chen *et al.* (1999) presented methods for the synthesis of *trans*-10, *cis*-12 and *cis*-9, *trans*-11 isomers of linoleic acid (C18: 2). The exception is *cis*-9, *trans*-11 octadecadienoic acid, which may be produced either chemically from ricinoleic acid or microbial from linoleic acid using cultures of *Lactobacillus* sp. (Pariza *et al.*, 1999). Chemically synthesized CLA used in anticarcinogenic studies was composed of approximately 48 % each of *cis*-9, *trans*-11 CLA and *trans*-10, *cis*-12 CLA (Kim *et al.*, 2000a).

1.4.2 Biological method

Several biotechnological methods to produce significant quantities of CLA for the enrichment of foods were approached. One method performed previously by Martinez *et al.* (1999) involved the use of lipase from *Candida Antarctica* to catalyze the acidolysis reaction of CLA with corn oil. This reaction resulted in a 20-fold enrichment of CLA content. McNeill (1999) and Gnädig *et al.* (2001) also described the enzymatic enrichment and incorporation of CLA into triacylglycerols using lipases from *Geotrichum candidum* and *Rhizomucor miehei.* This procedure generated one triacylglycerol enriched with the *cis*-9, *trans*-11 isomer of linoleic acid (C18: 2) and another enriched with the *trans*-10, *cis*-12 isomer. Another biotechnological approach for the actual synthesis of CLA includes the use of genetic engineering for transferring the genetic information of the isomerase of

Butyrivibrio fibrisolvens into microorganisms like *E. coli* (Gnädig *et al.*, 2001), and then the lipid extraction and purification method, which was developed, by Bligh and Dyer (1959).

Here are some valuable results about CLA formation through microorganisms and the summary of conversion studies on linoleic acid by various bacteria strains is shown in Table 1.2.

1.4.2.1 CLA Formation by *Lactobacillus*, and *Lactococcus* species

Kim and Liu (2000) identified the factors and procedures responsible for increasing the conjugated linoleic acid content in fermented milk. Fourteen lactic acid bacteria were screened for CLA-producing ability using sunflower oil (containing 70% linoleic acid) as a substrate. Among the screened strains, they found that *Lactococcus lactis* I-01 showed the highest CLA-producing ability. The optimal concentration of sunflower oil for CLA production was 0.1 g/L in whole milk, which accounted for 0.25% of total milk fat. The results demonstrated that CLA formation in fermented milk could be affected by numerous factors such as bacterial strain, cell number, optimal substrate concentration, and the period of incubation at neutral pH.

Lin *et al.*, (2003) researched on the production of conjugated linoleic acid by enzyme extract of *Lactobacillus acidophilus* CCRC 14079 and Kishino *et al.* (2002) screened 14 strains of lactic acid bacteria, *Lactobacillus plantarum* AKU 1009a was selected as a potential strain for CLA production from linoleic acid. Washed cells of *L. plantarum* with high levels of CLA production were obtained by cultivation in a nutrient medium with 0.06% (w/v) linoleic acid. Under the optimal reaction conditions with the free form of linoleic acid as the substrate, washed cells of *L. plantarum* produced 40 mg CLA/mL

reaction mixture (33% molar yield) from 12% (w/v) linoleic acid in 108 h. The resulting CLA was a mixture of two CLA isomers, *cis*-9, *trans*-11 and *trans*-9, *trans*-11-octadecadienoic acid.

Jiang *et al.* (1998) incubated different lactobacilli in MRS medium with a linoleic acid concentration of 25 mg/ml. None of the used lactobacilli, lactococci or streptococci formed CLA but the growth of some of these strains was inhibited by linoleic acid (25 mg/ml) in the medium. Strains from *Lactobacillus, Lactococcus* and *Pediococcus* were found to lack this ability while strains from *Bifidobacterium* and *Propionibacterium* were among the most efficient producers.

1.4.2.2 CLA Formation by propionibacteria

Rainio *et al.* (2002) studied the production of conjugated linoleic acid by *Propionibacterium freudenreichii* ssp. *shermanii*, and Jiang *et al.* (1998) had found that three *Propionibacterium* strains were able to form CLA in the MRS medium, and with the three former strains, the CLA formation was studied at different concentrations of free linoleic acid and in two additional culture media.

Lactobacillus reuteri and *Clostridium sporogenes* strains were formed to possess membrane-bound isomerases that produced the *cis*-9, *trans*-11 isomer of CLA while strains from *Propionibacterium acnes* were found to possess soluble isomerases capable of producing the *trans*-10, *cis*-12 isomer. The isomerase genes were cloned using peptide sequences of enzymes purified from *Lactobacillus reuteri* and *Propionibacterium acnes.* Analysis of linoleic acid isomerase sequences showed that these genes shared little

homology to genes of known enzymes. Furthermore, the isomerases contained an NAD/FAD binding domain in the region close to the N-terminus and further works on the expression of the isomerases, in particular the hosts are underway.

1.4.2.3 CLA Formation by bifidobacteria

Although production by the genus *Bifidobacterium* exhibited considerable interspecies variation, *B. breve* and *B. dentium* converted 65% of linoleic acid to *cis*-9, *trans*11-CLA at linoleic acid concentrations of 200-1000 μg/ml in MRS media. Coakley *et al.* (2003) used strains of *Lactobacillus, Lactococcus, Pediococcus* and *Bifidobacterium* to investigate their ability of producing the health-promoting fatty acid, conjugated linoleic acid from free linoleic acid. Nine strains of *Bifidobacterium* produced the c9, t11 CLA isomer from free linoleic acid. The t9, t11 CLA isomer was also produced by some strains, but at much lower concentrations. Coakley *et al.* (2003) found that *Bifidobacterium breve* and *B. dentium* were the most efficient CLA producers among the range of strains tested, with *B. breve* converting up to 65% linoleic acid to c9, t11 CLA when grown in 0.55 mg/ml linoleic acid. Strains also varied considerably with respect to their sensitivity to linoleic acid. The production of CLA by probiotic bifidobacteria offers a possible mechanism for some health-enhancing properties of bifidobacteria and provides novel opportunities for the development of functional foods.

1.4.3 Effects of processing on CLA levels

Fritsche and Steinhart (1998) noted that conjugated linoleic acid could be formed during the process of partial hydrogenation of vegetable oils. Lavillonniere *et al.* (1998) and Juanéda *et al.* (2001) further reported that CLA isomers were also detected in oils subjected to severe heating in the laboratory. Comparison of CLA mixtures produced by processes such as frying with both naturally present and chemically synthesized CLA mixtures revealed differences in composition (Sébédio *et al.*, 1999).

According to Juanéda *et al.* (2001), CLA mixtures in unsaturated used frying oils may be characterized by a high level of *trans-trans* isomers (i.e. approximately 50%) and the presence of 9, 11- and 10, 12- octadecadienoic acid in each different geometrical configuration. Because of the huge differences in conjugated linoleic acid content, Garcia-Lopez *et al.* (1994) investigated whether processing conditions influenced CLA levels in cheese. CLA content in cheese was found to increase from 9.5 mg/g of fat in the raw ingredients to 10.7 mg/g fat in the finished product without any apparent changes in isomeric distribution. It was suggested that the increase in CLA contents resulted mainly from the heating of the raw ingredients opposed to any other step in the cheese processing. However, the mechanism behind CLA formation is still not clearly understood.

1.5 CONCLUSION

This review discussed the published data and new findings that relate to underlying biochemical mechanisms of action. The *cis*-9, *trans*-11 and *trans*-10, *cis*-12 CLA isomers occur naturally in food. The *cis*-9, *trans*-11 isomer is the principal dietary forms of CLA, but both the *cis*-9, *trans*-11 and *trans*-10, *cis*-12 CLA isomers appear to be active in inhibiting carcinogenesis and reducing body fat in human and animal models.

For human nutrition, CLA-rich sources other than milk and dairy products are ruminant meats and some non-ruminant meats, while vegetable products contain only small amounts of CLA. As CLA has many nutritionally important biological properties, CLA intake is highly desirable for humans. The CLA levels of milk and dairy products can be enriched by manipulation of the diet of ruminants and by manufacturing fermented dairy products or cheese with starter cultures selected for a high CLA-producing potential. Use of lactic acid bacteria or bifidobacteria with high CLA-producing abilities could further increase the opportunity to increase CLA level in common fermented dairy foods.

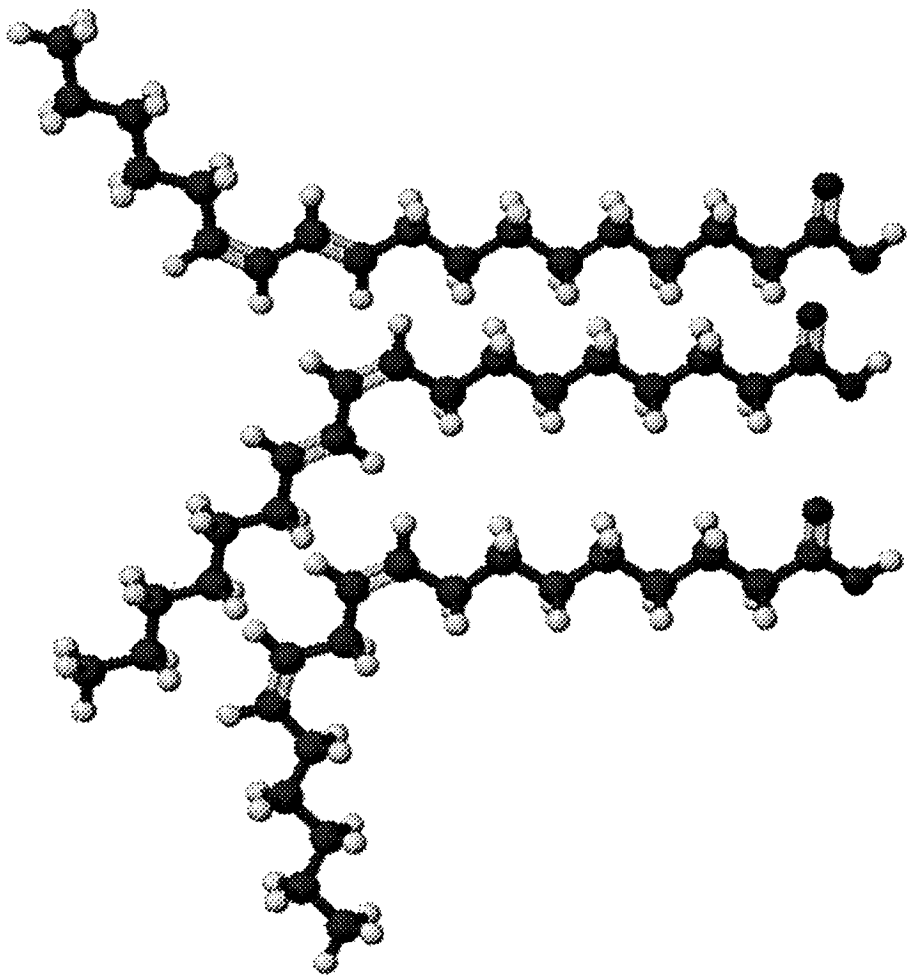

Figure 1.1 Structures of t-10, c-12 octadecadienoic acid (top), c-9, t-11 octadecadienoic acid (center), and ordinary linoleic acid, c-9, c-12 octadecadienoic acid: linoleic acid. (bottom). (Pariza *et al.*, 2001)

Figure 1.2 Pathway of biohydrogenation of linoleic acid and linolenic acid to stearic acid by rumen microorganisms (Bauman *et al.*, 2003).

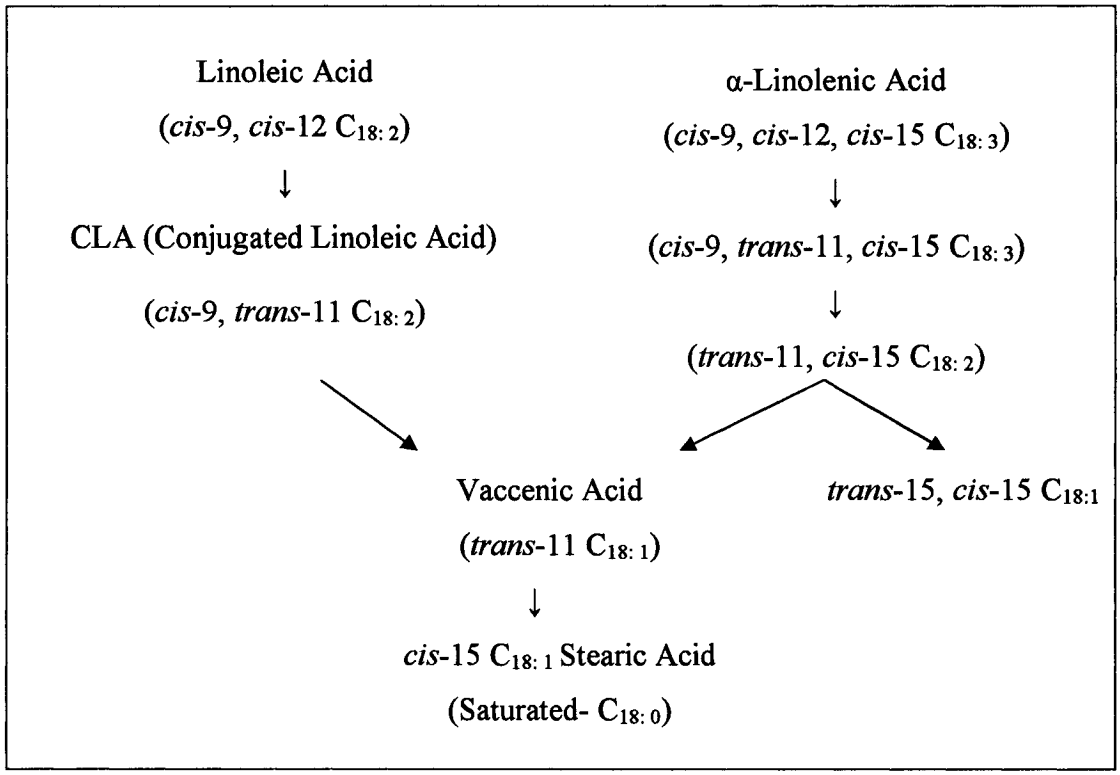

Table 1.1 Concentration of CLA in representative foods. (Chin *et al.*, 1992)

Food	Total CLA (mg/g fat)	c-9, t-11 isomer (%)
Meat		
Fresh ground beef	4.3	85
Beef round	2.9	79
Beef smoked sausage	3.8	84
Veal	2.7	84
Lamb	5.6	92
Pork	0.6	82
Poultry		
Chicken	0.9	84
Fresh ground turkey	2.5	76
Dairy Products		
Homogenized milk	5.5	92
Butter	4.7	88
Sour cream	4.6	90
Plain yoghurt	4.8	84
Ice cream	3.6	86
Processed cheese	6.7	93
Sharp cheddar cheese	3.6	93
Colby cheese	4.9	95
Reduced fat Swiss	4.5	83
Cottage cheese	6.1	92
Seafood		
Salmon	0.3	n.d.
Lake trout	0.5	n.d.
Shrimp	0.6	n.d.
Vegetable Oils		
Safflower	0.7	44
Sunflower	0.4	38
Canola	0.5	44
Corn	0.2	39

n.d* : Not detectable.

Table 1.2 Summary of conversion studies for CLA with various microorganisms.

Author (Year)	Strains*	Media	Reaction Condition	LA (mg/ml)	CLA (mg/ml)
Jiang *et al.* (1998)	*P. fr.* ssp. *shermanii*	MRS	72 h, 20°C	0.2 0.5	0.11 0.175
	P. fr. ssp. *shermanii*	SLM [1]	72 h, 20°C	0.1	0.001
Lin *et al.* (1999 and 2000)	*L. acidophilus*	Skim milk	24 h, 37°C	1.0	0.1
Rainio *et al.* (2001)	*P. fr.* ssp. *shermanii*	Whey permeate	96 h, 30°C	1.0	0.57
Ogawa *et al.* (2001)	*L. acidophilus*	Washed cells with KPB [2]	Anaerobic 72 h, 28°C	5.0	3.76
Kim *et al.* (2002)	*L. lactis*	MRS	Anaerobic 12 h, 37°C	0.1	2 mg CLA/g fat
	L. lactis	Whole milk	Anaerobic 12 h, 37°C	0.1	4 mg CLA/g fat
Kishino *et al.* (2002)	*L. acidophilus* *L. brevis* *L. plantarum* *L. rhamnosus*	KPB [2] (pH 6.5)	Anaerobic 24 h, 37°C	4.0 4.0 4.0 4.0	1.5 0.55 3.41 1.41
Alonso *et al.* (2003)	*L. acidophilus* *L. casei*	MRS	24 h, 37°C	0.2	0.132 0.111
	L. acidophilus *L. casei*	Skim milk	24 h, 37°C	0.2	0.116 0.996
Coakley *et al.* (2003)	*B. breve* *B. lactis* *B. dentium*	MRS	Aerobic 24 h 48 h	0.55	0.364 0.1569 0.1266
Lee *et al.* (2003)	*L.reuteri*	Washed cells with HCl buffer	37 °C, 1 h	0.5	0.175
Ando *et al.* (2004)	*L. plantarum*	Washed cells with buffer	Anaerobic	5.0 30.0	2.7 7.5

*P. : *Propionibacterium*
*L. : *Lactobacillus*
*B. : *Bifidobacterium*
SLM [1]: Sodium lactate medium
KPB [2]: Potassium phosphate buffer (pH 6.5)

CHAPTER 2

PRODUCTION OF CONJUGATED LINOLEIC ACID (CLA) AND CONJUGATED LINOLENIC ACID (CLNA) BY *BIFIDOBACTERIUM BREVE* JKL03

After screening many human and animal origins of *Bifidobacterium* and *Lactobacillus* species for microbial conversion of linoleic acid to conjugated linoleic acid, *Bifidobacterium breve* JKL03 had the highest converting ability, and was selected for further studies.

The growth studies were carried out in MRS media and the optimal conditions for *B. breve* to convert free linoleic acid to CLA and free linolenic acid to CLNA were established.

The results of this study were summarized as a manuscript suitable for journal publication. The manuscript entitled "Production of conjugated linoleic acid and conjugated linolenic acid by *Bifidobacterium breve* JKL03" was co-authored by Yun-Kyoung Jung, Geun-Bae Kim, Mun-Yhung Jung, and Byong H. Lee, and was written by Yun-Kyoung Jung and edited by Byong H. Lee.

2.1 ABSTRACT

Among many different species that we have studied for CLA converting ability, *Bifidobacterium breve* JKL03 showed the highest CLA converting activity.

Bifidobacterium breve JKL03 was grown in MRS media under different conditions to observe its ability for CLA conversion. The strain was cultured in MRS medium with linoleic acid under anaerobic and aerobic condition for 0 to 72 h. The productions were also carried out at various concentrations of linoleic acid. The microbial conversion rate of linoleic acid (0.5 mg/ml in 2% Tween 80) to conjugated linoleic acid in MRS medium for 24 h was 68.46%, respectively.

Linolenic acid, which is also C_{18} but has 3 double bonds, was used as the substrate. The conversion rate of linolenic acid into conjugated linolenic acid in MRS medium for 24 h was up to 83.02%.

KEYWORDS: Conjugated linoleic acid (CLA); Conjugated linolenic acid (CLNA); *Bifidobacterium breve* JKL03; MRS medium

2.2 INTRODUCTION

The increasing demand for biologically active polyunsaturated fatty acids (PUFA) has attracted commercial attention, especially on conjugated linoleic acid (CLA) - based nutraceuticals and therapeutics. CLA has gained considerable attention in recent years because of many beneficial effects, including anticarcinogenic activity (Chin *et al.*, 1992; Liew *et al.*, 1995), antiatherogenic activity (Lee *et al.*, 1994), immune stimulation (Cook *et al.*, 1993), as well as the ability to reduce body fat in animals (Park *et al.*, 1999a) and humans (Smedman and Vessby, 2001; Miner *et al.*, 2001). Evidence also exists to suggest that CLA may be effective in the prevention and treatment of non-insulin dependant diabetes mellitus (Houseknecht *et al.*, 1998). From a human health viewpoint, it appears desirable to increase CLA levels in foods to protect against disease and enhance general health.

Conjugated linoleic acids (CLAs) are geometrical or positional isomers of the fatty acid known as linoleic acid (*cis*-9, *cis*-12 C $_{18:2}$). This acid is converted to CLA by the linoleic acid isomerase of rumen bacteria, and CLAs are predominantly found in dairy products and meats from ruminants in levels up to 30 mg/g fat. There are several possibilities for CLA structures, all containing conjugated double bonds. Often several isomers are found simultaneously in foods, with *cis*-9, *trans*-11 C $_{18:2}$ (rumenic acid) being one of the most abundant isomers. Thus, this isomer represents 91%, 82%, and about 60% of the total CLA content of butter, yogurt, and T-bone steaks, respectively.

Conjugated linolenic acid (CLNA) is also known to be anticarcinogenic (Igarashi and Miyazawa, 2000; Suzuki *et al.*, 2001) and is present in plant seed oils (Kishino *et al.*, 2003), such as Kerela and flax oil. The conjugated linoleic acids and conjugated linolenic

acids produced by a strain of bifidobacteria can be good sources for nutritional and health benefits as functional foods or fermented dairy products itself for human and animal intake.

In this study, many human and animal origins of *Bifidobacterium* and *Lactobacillus* species were assessed for microbial conversion of linoleic acid to CLA and linolenic acid to CLNA. Among the strains that we have screened, *Bifidobacterium breve* JKL03 was found to have the highest converting ability from LA to CLA.

2.3 MATERIALS AND METHODS

2.3.1 Bacterial strains and Media

Among the strains (Table 2.1) tested for their ability to convert LA to CLA, *Bifidobacterium breve* JKL03 showed the highest converting ability. This strain was initially isolated from baby faces and was verified for genus and species by 16s rRNA gene sequence typing according to the method of Kaufman (2000).

This strain was anaerobically pre-cultured for 24 h in MRS medium (Difco, Sparks, MD) and then inoculated again into MRS broth, which contained 0.05% (w/v) of L-cysteine-hydrochloride. MRS medium was pasteurized at 121 °C for 15 min, and portions (5 ml) of MRS medium were added into screw capped-glass tubes (20 ml volume) for the conversion studies.

2.3.2 Chemicals (LA, LNA stock preparation)

Unless otherwise specified, all reagents of analytical grade were purchased from Sigma (St. Louis, MO), Fisher (Fair Lawn, NJ), and Aldrich (Milwaukee, WI).

Linoleic acid was filter sterilized by 0.2 μm minisart filter (Fisher) and used as 0.5 mg/ml stock solution supplemented to 2 % (v/v) Tween 80 (Sigma) which is also previously filter-sterilized by 0.2 μm minisart filter. Tween 80 (Polyoxyethylene sorbitan monooleate) improves the solubility of free LA in aqueous media. They were stored in the dark at –10 °C for further use.

2.3.3 Fermentation conditions

This strain was cultured with either linoleic acid (0.5 mg/ml; w/v) solution or different concentrations of LA solutions under anaerobic (GasPak Plus, BBL, Sparks, MD) or aerobic conditions in MRS broth (Difco) supplemented with 0.05 %(w/v) L-cysteine-hydrochloride. The cultures were then incubated at 37 °C for 24 h. The CLA production was also monitored in MRS broth containing 0.5 mg/ml (w/v) linoleic acid at different times for 0-72 h. The experiments were repeated in triplicates.

2.3.4 Extraction of lipids

After the cells were grown for 24 h, and neutralized to pH 7.0 by adding diluted NaOH solution, the lipids from the cultures were extracted and prepared for the gas

chromatography analysis. The internal standard (2.5 mg/ml w/v), $C_{17:0}$ heptadecadianoic acid in hexane was added to 0.9 ml of the fermented culture and vortexed for 3 min. For the lipid extraction, hexane (0.9 ml) was added and mixed for 5 min and then centrifuged at 13000 g for 20 min. The lipids were extracted with 0.9 ml of hexane 4 times, and the hexane layers were collected in a screw capped-glass tube and dried off under nitrogen at room temperature.

The extracted and dried lipids were methylated with 1 ml of 4 % HCl in methanol at 60 °C for 20 min. After methylation, 2 ml of saturated NaCl and 1 ml of hexane were added and gently shaken. The esters were extracted twice with 1 ml of hexane and the hexane layer, which contains methylated fatty acids, was collected in 1.5 ml tube and dried off by nitrogen flushing at room temperature.

150 µl of 2, 2, 4- trimethylpentane was added into the 1.5 ml tubes to solubilize methylated fatty acids, then 2 µl was injected for the analysis.

2.3.5 Spectrophotometric assay

Bifidobacterium breve cells were cultured with various concentrations of free linoleic acid or free linolenic acid in MRS medium for 24 h at 37 °C. The strain was cultured in different concentrations of free linoleic acid or free linolenic acid.

After fermentation, portions (0.5 ml) of the culture were mixed with 0.5 ml of MRS broth for the dilution in a glass cuvette, inserted into the spectrophotometer (UltrospecII, Biochrom, Cambridge, England) and absorbance readings were taken at 600 nm. The graph (Figure 2.4) was created based on these OD readings.

2.3.6 Gas chromatography

For the GC analysis, the Gas Chromatography from Perkin Elmer (Autosystem, Connecticut, USA) was used. Fatty acid methyl esters (2 µl) were injected and separated on a cyanopropyl type fused silica capillary column (100 m x 0.25 mm; Supelco, Bellefonte, PA). Helium (Matheson, Whitby, ON) was used as the carrier gas and FID (flame ionization detector), split mode for the injection were used. The oven temperature was initially set at 120 °C for 1 min, and then increased at the rate of 7 °C /min to 200 °C. The temperatures of injector and detector were 200 °C and 210 °C, respectively.

CLA contents were calculated by using the Formula (Jung and Jung, 2002), CLA (µg/mg) = (peak area of CLA)(weight of internal std)(theoretical correction factor) / (peak area of internal standard)(sample weight), and the theoretical correction factor (0.978) was calculated by using the theoretical detector response of internal standard (hexadecanoic acid) compared to that of conjugated linoleic acids (Ackman and Sipos, 1964).

2.3.7 Silver ion high performance liquid chromatography

Silver ion HPLC separation of CLA and CLNA methyl esters was carried out with HPLC (Shimadzu, Tokyo, Japan) equipped with a 20 µl injection loop (Waters) and UV detector operated at 233 nm (Sehat *et al.*, 1999). Three ChromSpher 5 Lipids analytical silver-impregnated columns (Chrompack, Bridgewater, NJ) were used in series. The mobile phase was 0.1% acetonitrile in hexane, and the flow rate was 1.0 ml/min.

2.3.8 Preparation of 4,4-Dimethyloxazoline (DMOX) derivatives

Individual isomers of CLA and CLNA methyl esters were collected from the silver ion impregnated HPLC and were converted into DMOX derivatives (Fay and Richli, 1991) and analyzed by a GC/MS to identify the double bonds location in CLA and CLNA isomers (Shantha *et al.*, 1993).

Small portions (5 mg) of FAMEs were placed in microscale reaction vials, an amount of 2-amino-2-methyl-1-propanol equal to ca. 5 times the weight of the FAMEs was added, and the vial purged with argon and kept in a 170 °C dry oven for 6 h. On cooling diethyl ether/hexane (1:1, vol/vol; 5 ml) was added to the tube. The organic layer was washed with distilled water (3 ml) and dried carefully over anhydrous sodium sulfate. Then the organic solvents were taken to dryness by a gentle stream of nitrogen. The obtained DMOX derivatives of fatty acids were dissolved in 2,2,4-trimethylpentane for GC/MS analysis.

2.3.9 Gas chromatography-mass spectrometry

The gas chromatography-mass spectrometry was carried out with a Perkin Elmer Autosystem XL to identify the chemical structures of CLA and CLNA. The same column was used in GC/MS as in GC. However, the oven temperature program was slightly different due to the slightly lower volatility of DMOX derivatives than the methyl esters of CLA and CLNA.

2.4 RESULTS AND DISCUSSION

Bifidobacterium breve JKL03 was studied for its CLA and CLNA converting ability. The c-9, t-11 CLA, which is known as rumenic acid, and c-9, t-11, c-15 - CLNA were mainly produced by the strain with the high conversion efficiency.

When Jiang *et al.* (1998) and Ogawa *et al.* (2001) had studied CLA production by dairy starter cultures, they did not remove the bacteria cells, but Alonso *et al.* (2003) removed cells to assay only free CLA in the media. However, in our study, we did not remove the cells because the results were not stable when the cells were removed.

Throughout this study, 92% of CLA and remaining LA were recovered that is higher than 89.4% reported by Jiang *et al.* (1996). This high recovery was probably due to the neutralization of the supernatants before the lipid extraction.

2.4.1 CLA/CLNA production and Inhibitory effects

To observe whether the presence of linoleic acid could increase CLA conversion rate, the strain was pre-cultured in MRS media containing 0.2 and 0.5 mg/ml of linoleic acid not as the substrate. However, the addition of linoleic acid during pre-culturing did not increase CLA production.

CLA production was increased when the concentration of free linoleic acid increased up to 2.5 mg/ml (0.25% w/v). However, LA could not be converted at a concentration of 5.0 mg/ml. LNA could not be converted at a concentration higher than 0.5 mg/ml (0.05% w/v) (Figures 2.1 and 2.2).

The conversion of LNA was drastically inhibited by aerobic conditions when the concentration is higher than 0.1 mg/ml (Figure 2.3). The conversion of LNA was more reduced than that of LA by high concentration of free linoleic acid.

Jiang *et al.* (1998) suggested that production of CLA from free linoleic acid might function as a detoxification mechanism. The results of CLA/CLNA production by different concentrations of substrates indicated that free LNA was more toxic than free LA to the strain.

When the concentration was higher than 1.0 mg/ml under aerobic conditions, CLNA could not be formed when compared with anaerobic conditions. Higher production rates of CLA and CLNA were observed under anaerobic conditions. The presence of oxygen partially inhibited the conversion of linoleic acid to CLA and linolenic acid to CLNA.

2.4.2 Optimum growth conditions

The growth rates of the strain decreased as the concentration of LA and LNA were increased (Figure 2.4). Also at a concentration level between 0.1 and 0.25 mg/ml, the cell growth was drastically decreased. Boyaval *et al.* (1995) and Jiang *et al.* (1998) reported that free linoleic acid inhibited the growth of *Propionic thoenii* ssp. *shermanii,* but the growth of *P. jensenii* and *P. thoenii* were not affected by free linoleic acid. In this study, free LNA had a stronger negative effect than LA on the growth of *Bifidobacterium breve* JKL03.

CLA and CLNA productions with 0.05% (w/v) linoleic acid or linolenic acid, under anaerobic condition, at different fermentation times, were monitored. The results shown in Figures 2.5 and 2.6 showed that other isomers, which are not normally produced by *B. breve*

JKL03, such as *trans*-9, *trans*-11 CLA and *trans*-9, *trans*-11, *cis*-15 CLNA, increased under longer fermentation time.

B. breve JKL03 rapidly converted LA into CLA after 6 h of fermentation. After 12 h the conversion was almost complete. However, for the LNA, the strain rapidly converted LNA into CLNA after 12 h of fermentation. The CLA conversion into CLNA was complete after 24 h. The amount of CLA produced in 72 h did not increase significantly as compared with those of 12 h. The amount of CLNA produced during 72 h, also did not significantly increase, compared with that of 24 h. The conversion rates of CLA and CLNA during 24 h fermentation in cys-MRS media were summarized in Table 2.2. It appears that LNA can be converted to CLNA by *B. breve* JKL03 with a higher productivity (82.5%) than LA (63.2%) under anaerobic condition.

We also monitored CLA and CLNA productions with 0.05% (w/v) linoleic acid and linolenic acid under aerobic condition, at different fermentation times (Figure 2.7). The results showed that CLA and CLNA productions significantly decreased under aerobic condition.

The amount of LA produced was the highest when *Bifidobacterium breve* JKL03 was fermented with 1.0 mg/ml (w/v) linoleic acid as a substrate for 12 h under anaerobic condition. The greatest amount of CLNA production was achieved when 0.5 mg/ml (w/v) of linolenic acid was fermented for 24 h under anaerobic condition.

2.4.3 Identification of CLA and CLNA isomers

Identification of the isomers was carried out by GC/MS and silver ion impregnated HPLC method. (Jung and Jung, 2002)

Figure 2.8 shows the partial Ag^+-HPLC chromatogram of authentic conjugated linoleic acids. The conjugated linoleic acid isomers were separated into three isomer groups (*trans, trans*; *cis, trans* and *trans, cis*; and *cis, cis* conjugated linoleic acids), and the elution patterns of chromatographic peaks for the authentic CLA methylesters were exactly the same as those reported previously (Sehat *et al.*, 1998).

Even though c10, t12 or t10, c12, and t9, c11 or c9, t11 isomers were appearing at the same time in HPLC chromatogram, these peaks can be recognized as c10, t12 and c9, t11 by gas chromatograph with flame ionization detector due to their different retention times. It has been reported that the DMOX derivative of *cis, trans*-isomer eluted consistently earlier than that of *trans, cis*-isomer for all geometric pairs on cyanopropylsiloxane-phase column gas chromatograph (Sehat *et al.*, 1998).

Figure 2.9 shows partial Ag^+-HPLC chromatogram of CLA isomers that we have obtained from the fermentation of LA with *B. breve* JKL03.

In the chromatogram of co-injection (Figure 2.10), which was mixed with 50% of the authentic conjugated linoleic acid and 50% of my sample, the peaks were noticeably increased due to my sample.

The fractions of the highest peaks of CLA (Figure 2.9) and CLNA (chromatogram not shown) were collected by Ag^+-HPLC runs and converted into their DMOX derivatives.

The DMOX derivatives of the individual fractions were injected into a GC/MS for further analysis to confirm their conjugated double bonds locations.

For CLA isomer, the differences of 12 amu, instead of the usual 14, between *m/z* 196 and 208 and between 222 and 234 were the indication of double bond presence at 9 and 11 carbons. For CLNA isomer, the differences of 12 amu between *m/z* 196 and 208, between 222 and 234, and between 276 and 288 were the indication of double bond presence at 9, 11 and 15 carbons.

The isomers of Ag^+-HPLC peaks in Figure 2.9 were identified as *trans*-9, *trans*-11 CLA, *cis*-9, *trans*-11 CLA, and *cis*-9, *cis*-11 CLA isomers.

2.5 CONCLUSIONS

Fermentation of linoleic acid (LA) and linolenic acid (LNA) with *Bifidobacterium* species was found to be useful for the production of CLA and CLNA that have health benefits for human and animals.

Bifidobacterium and *Lactobacillus* strains derived from human and animal origin were screened and *Bifidobacterium breve* JKL03 was selected as the highest CLA producer.

The production of CLA increased by increasing free LA up to 2.5 mg/ml, but LNA could not be converted at a concentration of more than 0.5 mg/ml. Under aerobic conditions, CLA and CLNA conversions were significantly reduced.

CLA was most efficiently produced when *Bifidobacterium breve* JKL03 was grown in MRS medium containing 1.0 mg/ml (w/v) of LA for 12 h under anaerobic condition. To produce CLNA, a 0.5 mg/ml (w/v) of LNA and a 24-h fermentation under anaerobic condition was essential. The maximum conversion rate of LA to CLA under anaerobic condition was 63.2%, and that of LNA to CLNA was 82.5%.

This study indicates that the production of CLA or CLNA by *Bifidobacterium breve* JKL03 could be applied to enrich the level of CLA and CLNA in dairy products.

Table 2.1 Bioconversion of linoleic acid to conjugated linoleic acid by selected human or rumen origin *Bifidobacterium* and *Lactobacillus* species during 24 h fermentation in MRS broth.

Species	CLA (μg/mL) converted from 500 μg/mL linoleic acid[1]				
	Strain	c9, t11-	t9, t11-	Total	Conversion %
Human origin					
B. breve	NCIBM 2258	96.69 ± 11.81	1.09 ± 0.43	97.78 ± 12.05	19.56 ± 2.41
B. breve	NCIBM 2257	20.08 ± 2.45	ND	20.08 ± 2.45	4.02 ± 0.49
B. breve	DSM 20091	8.22 ± 1.88	ND	8.22 ± 1.88	1.64 ± 0.38
B. breve	KCTC 3220	9.30 ± 2.73	ND	9.30 ± 2.73	1.86 ± 0.55
B. breve	ATCC 15698	184.35 ± 22.31	3.21 +0.27	187.56 ± 22.17	37.51 ± 4.43
B. breve	**JKL03**	321.46 ± 32.26	4.30 + 0.25	372.48 ± 21.37	63.62 ± 11.99
B. catenulatum	ATCC 27539	9.85 ± 1.03	n.d.	9.85 ± 1.03	1.97 ± 0.21
B. catenulatum	ATCC 27675	n.d.	n.d.	-	-
B. catenulatum	ATCC 27676	51.61 ±3.64	2.89 ± 0.91	54.50 ± 4.85	10.90 ± 0.97
B. dentium	ATCC 27534	8.17 ± 0.68	ND	8.17 ± 0.68	1.63 ± 0.14
B. dentium	ATCC 27678	25.96 ± 2.75	2.49 + 0.26	28.45 ± 3.01	5.69 ± 0.60
B. dentium	ATCC 27679	17.41 ± 3.29	n.d.	17.41 ± 3.29	3.48 ± 0.66
B. dentium	ATCC 27680	n.d.	n.d.	-	-
B. dentium	ATCC 15424	n.d.	n.d.	-	-
Rumen origin					
B. merycicum	KCTC 3369	n.d.	n.d.	-	-
B. boum	ATCC 27917	n.d.	n.d.	-	-
B. pseudolongum ssp. *globosum*	ATCC 25865	n.d.	n.d.	-	-
B. pseudolongum ssp. *globosum*	ATCC 25864	n.d.	n.d.	-	-
B. merycicum	ATCC 49391	n.d.	n.d.	-	-
B. ruminantium	ATCC 49390	n.d.	n.d.	-	-
B. adolescentis	DSM 20087	n.d.	n.d.	-	-
B. theromphilum	ATCC 25867	n.d.	n.d.	-	-
B. theromphilum	ATCC 25866	n.d.	n.d.	-	-
B. thermophilum	KCTC 3471	n.d.	n.d.	-	-
Bifidobacterium sp.	KCTC 3373	2.30 ± 0.84	n.d.	2.30 ± 0.84	0.46 ± 0.17
Lb. plantarum	ATCC 10241	n.d.	n.d.	-	-
Lb. vitulinus	ATCC 27783	n.d.	n.d.	-	-
Lb. Ruminis	ATCC 27780	n.d.	n.d.	-	-

[1]Mean value assessed from the duplicate or triplicate fermentation in cys-MRS with 0.5 mg/mL linoleic acid added. ATCC:American Type Culture Collection NCIMB:National Collection of Industrial & Marine Bacteria DSM: Deutsche Sammlung von Mikroorganismen und Zellkulturen

Table 2.2 Bioconversion of LA to CLA and LNA to CLNA by *Bifidobacterium breve* JKL03 during 24 h fermentation in cys-MRS media*.

	c9, t11 - CLA	**t9, t11 - CLA**	**Total**	**Conversion (%)**
Linoleic acid (Anaerobic)	309.185 ± 0.474	9.426 ± 0.028	318.611	63.72
Linoleic acid (Aerobic)	147.836 ± 7.009	9.128 ± 7.075	156.964	31.39
	c9, t11, c15 - CLNA	**t9, t11, c15 - CLNA**		
Linolenic acid (Anaerobic)	406.296 ± 20.208	6.421 ± 0.120	412.717	82.54
Linolenic acid (Aerobic)	n.d	n.d	n.d	n.d

*CLA and CLNA (μg/mL) were converted from 500 μg/mL of free linoleic acid and linolenic acid (Mean value and standard deviation assessed from the triplicate)

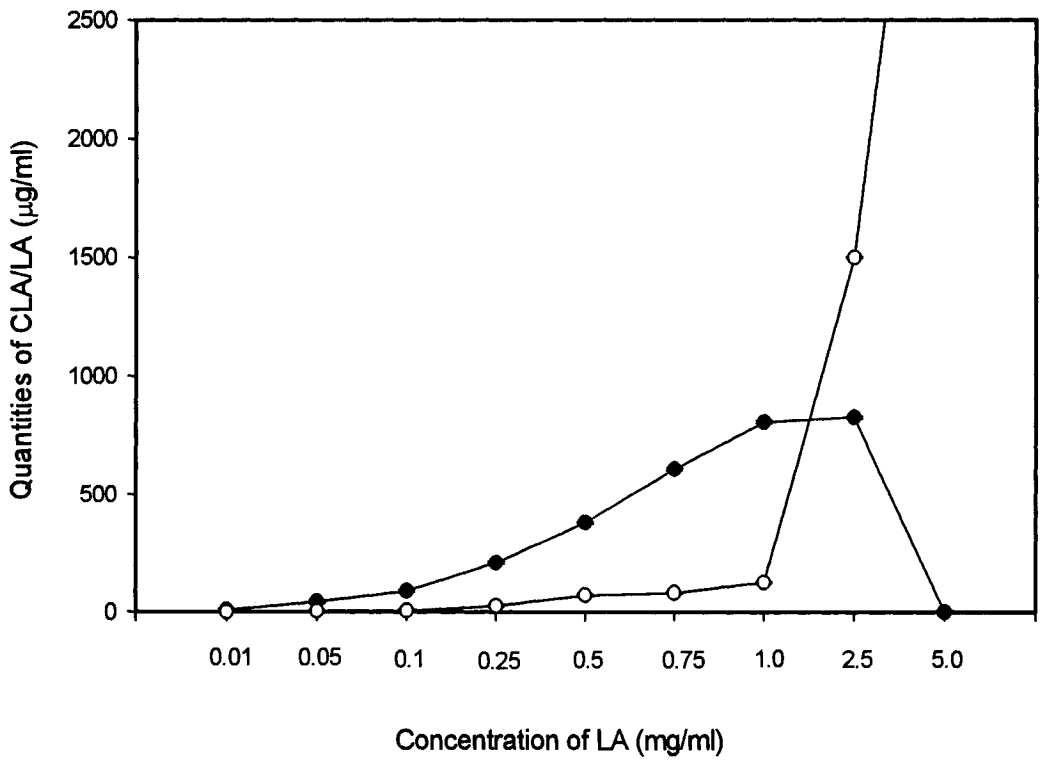

Figure 2.1 CLA (●) production by *Bifidobacterium breve* JKL03 under anaerobic condition at different concentrations of free linoleic acid (○) for 24 h.

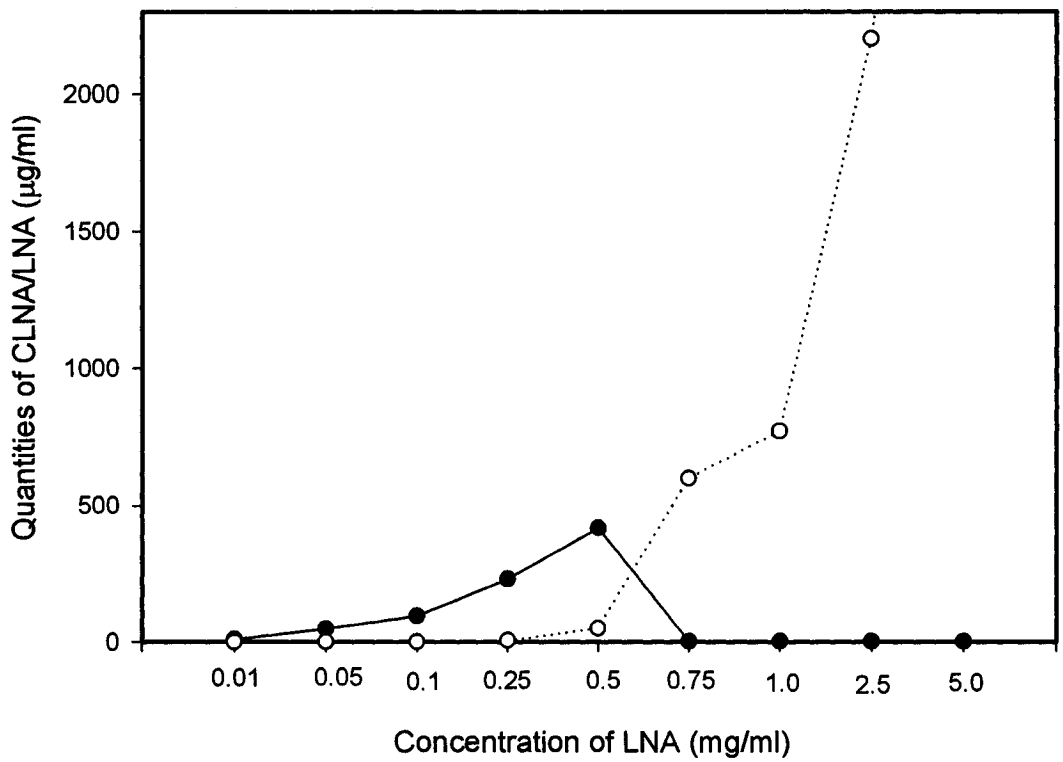

Figure 2.2 CLNA (●) production by *Bifidobacterium breve* JKL03 under anaerobic condition at different concentrations of free linolenic acid (○).

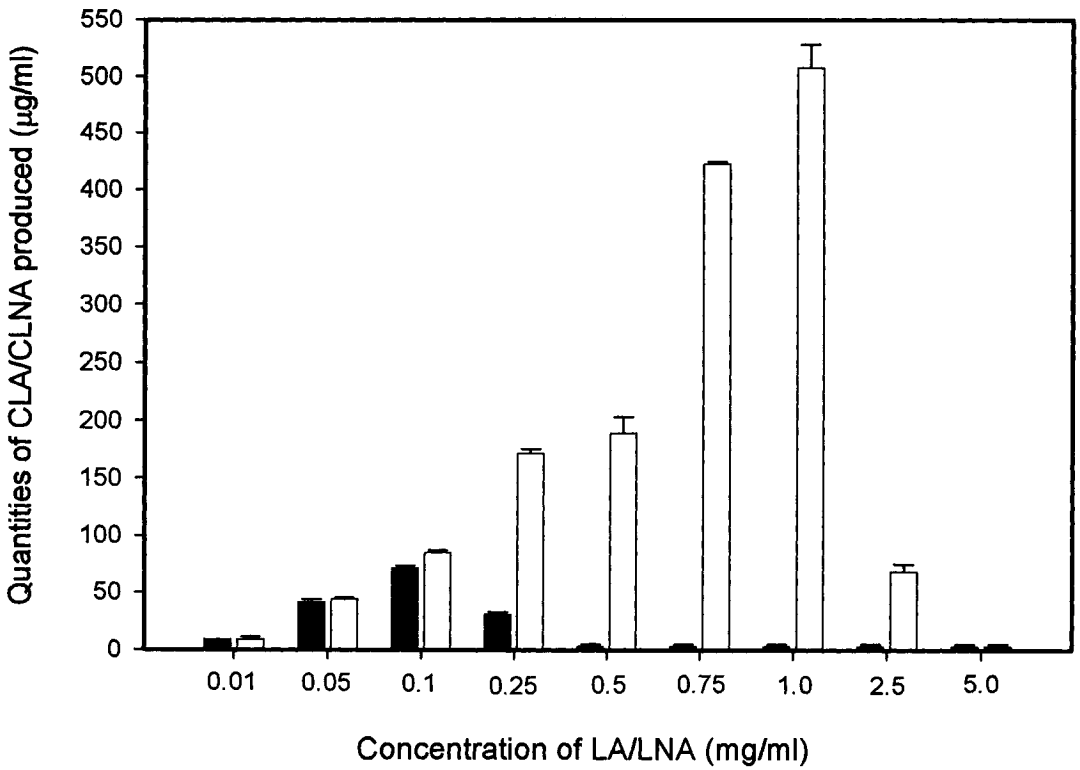

Figure 2.3 Productions of CLA and CLNA by *Bifidobacterium breve* JKL03 under aerobic condition at different concentrations of linoleic acid (□)and linolenic acid (■).

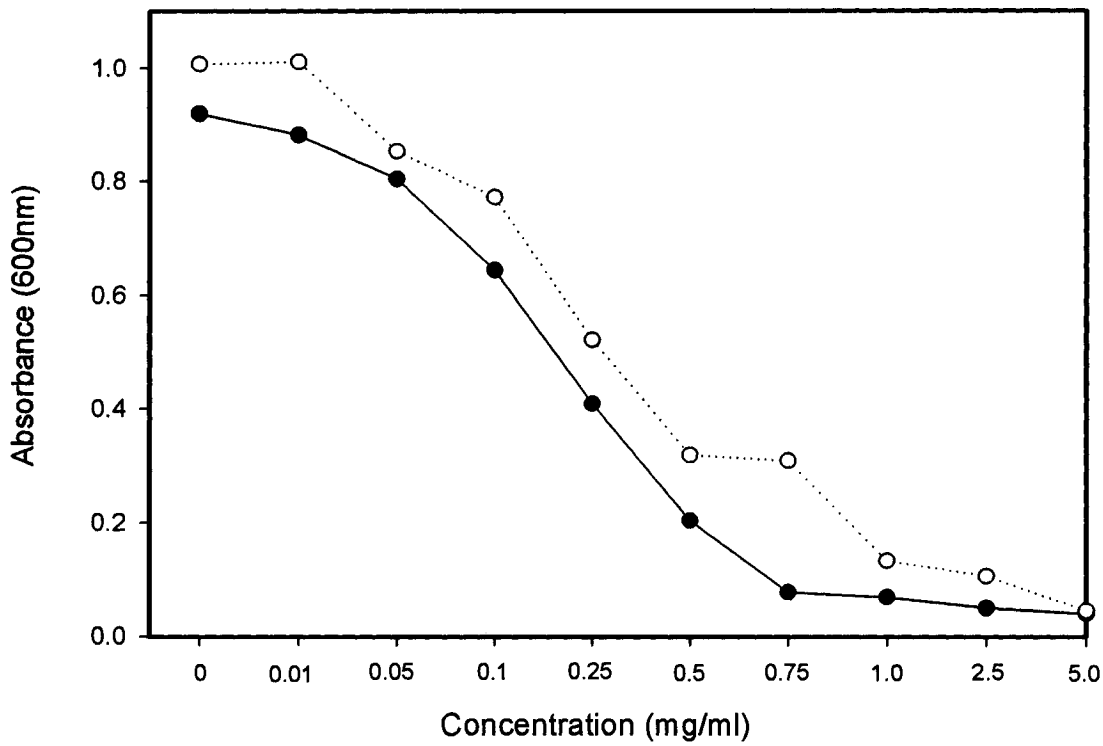

Figure 2.4 The growth rates of *Bifidobacterium breve* JKL03 under anaerobic condition at different concentrations of linoleic acid (○) and linolenic acid (●) after 24 h.

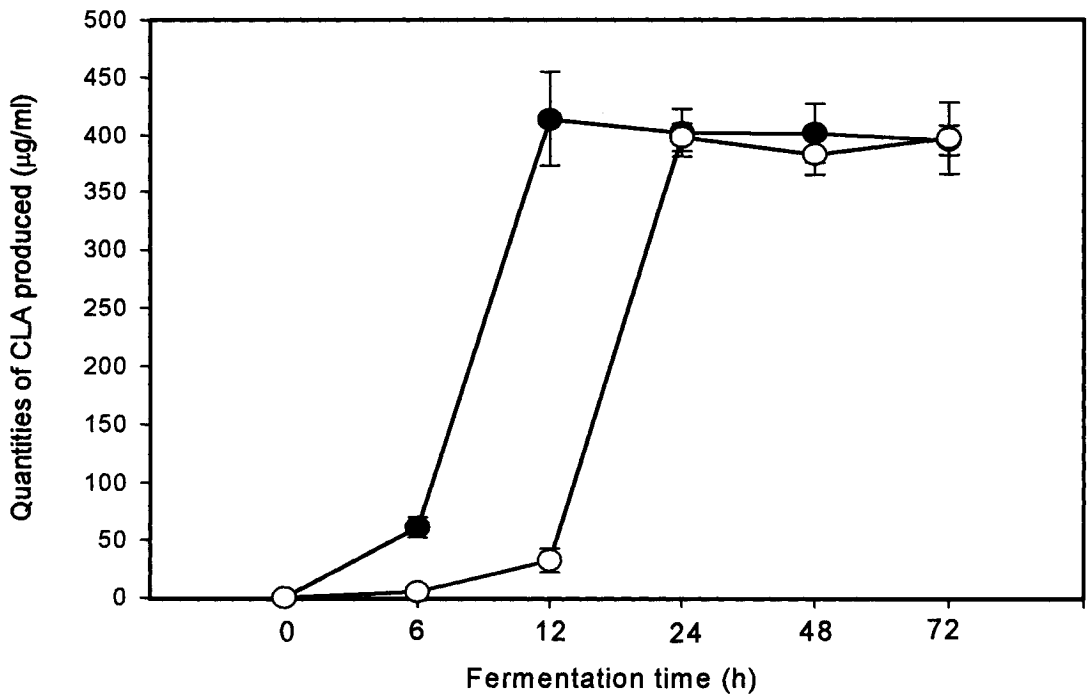

Figure 2.5 Productions of conjugated linoleic acid (●) and conjugated linolenic acid (○) by *Bifidobacterium breve* JKL03 under anaerobic condition at different times.

* CLA and CLNA (μg/mL) were produced from 500 μg/mL of free linoleic acid and linolenic acid

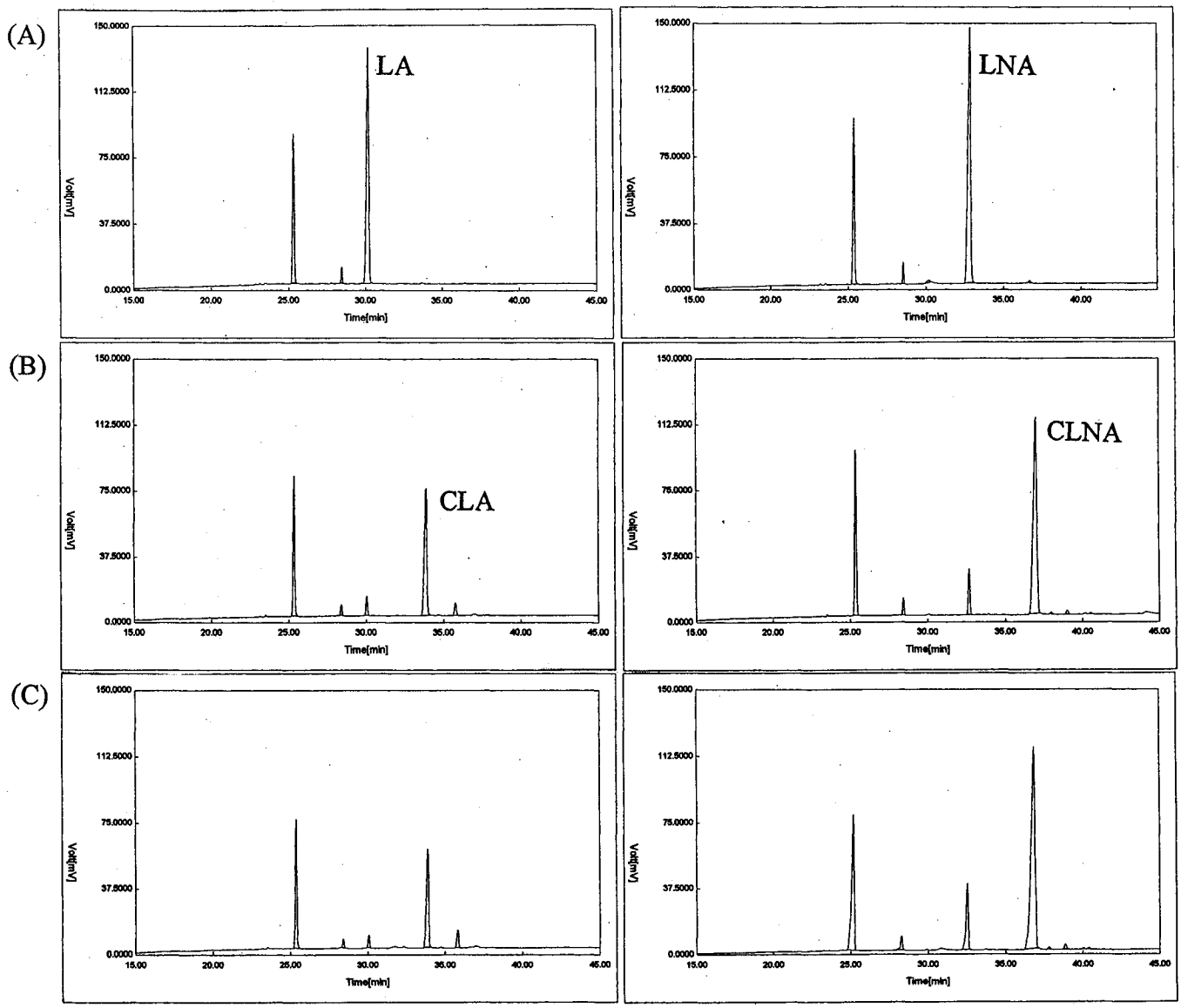

Figure 2.6 Chromatograms on CLA and CLNA productions by *Bifidobacterium breve* JKL03 at 0 (A), 24 (B), and 72 (C) h.

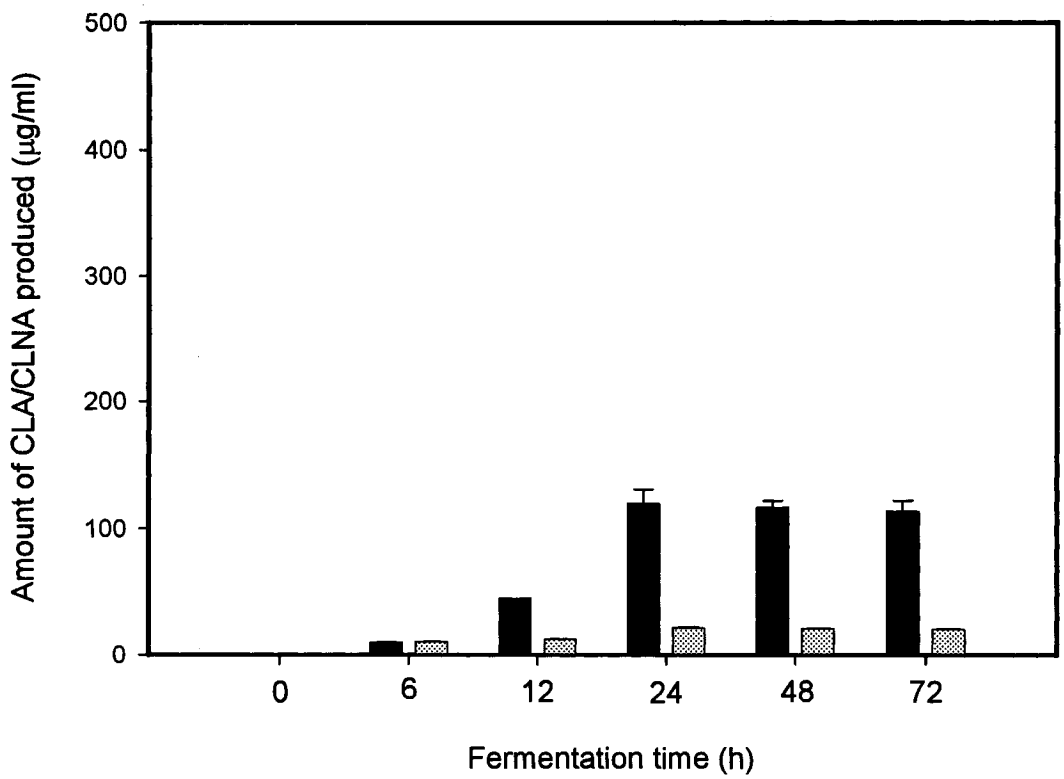

Figure 2.7 Conjugated linoleic acid (■)and conjugated linolenic acid (□) productions by *Bifidobacterium breve* JKL03 under aerobic condition at different fermentation hours.

* CLA and CLNA (μg/mL) were converted from 500 μg/mL of free linoleic acid and linolenic acid (Mean value and standard deviation assessed from the triplicate)

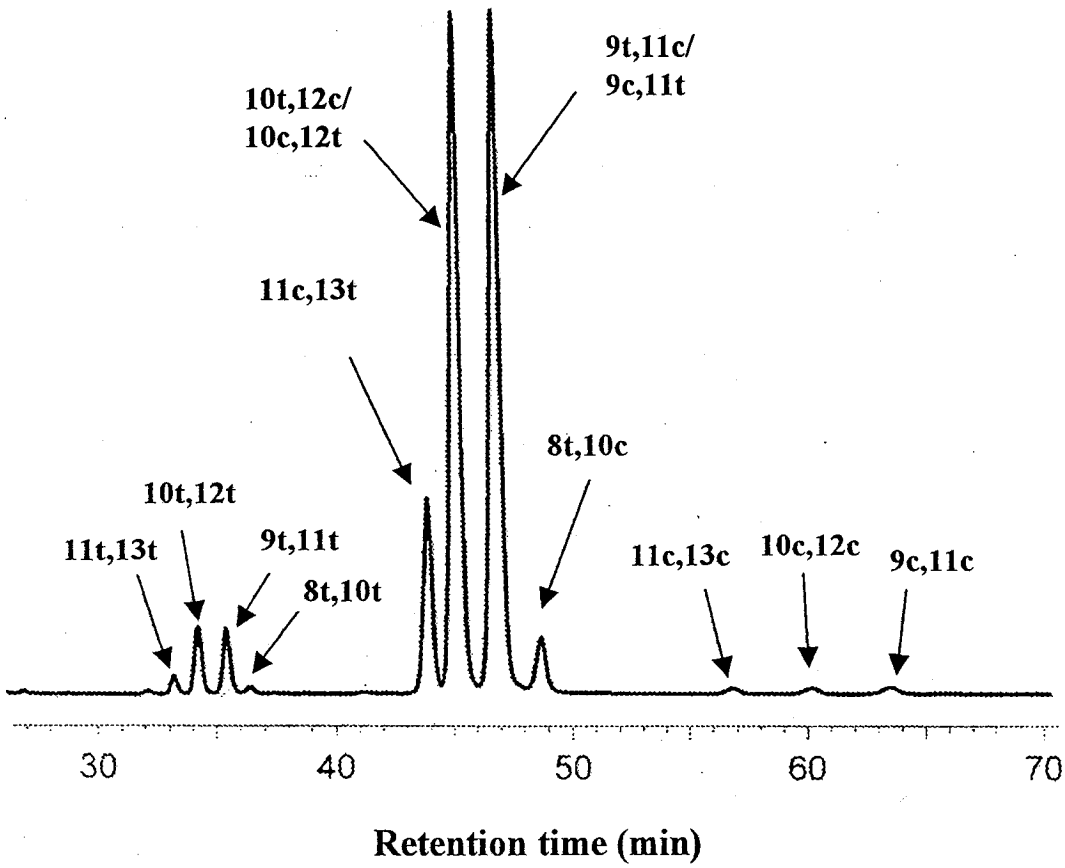

Figure 2.8 Partial Ag^{+}-HPLC chromatogram of authentic CLA methylesters.

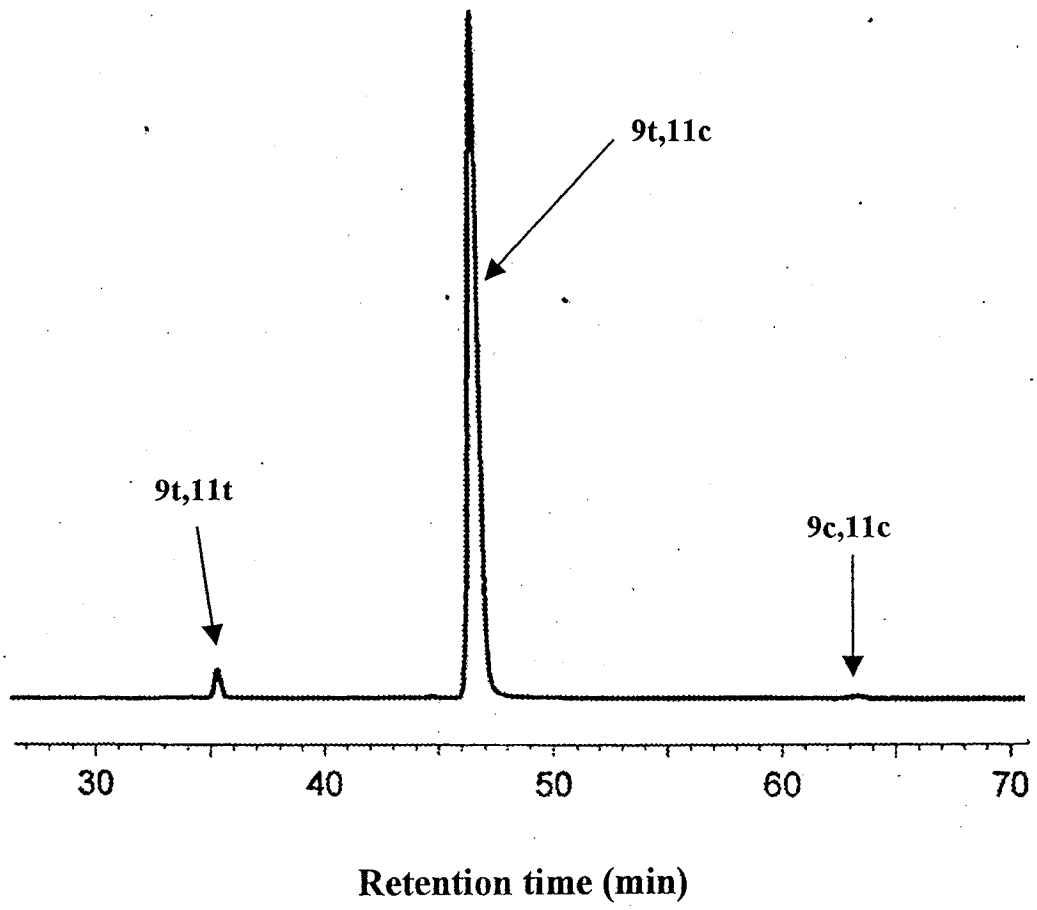

Figure 2.9 Partial Ag^{+}-HPLC chromatogram of CLA methylesters produced by *Bifidobacterium breve* JKL03.

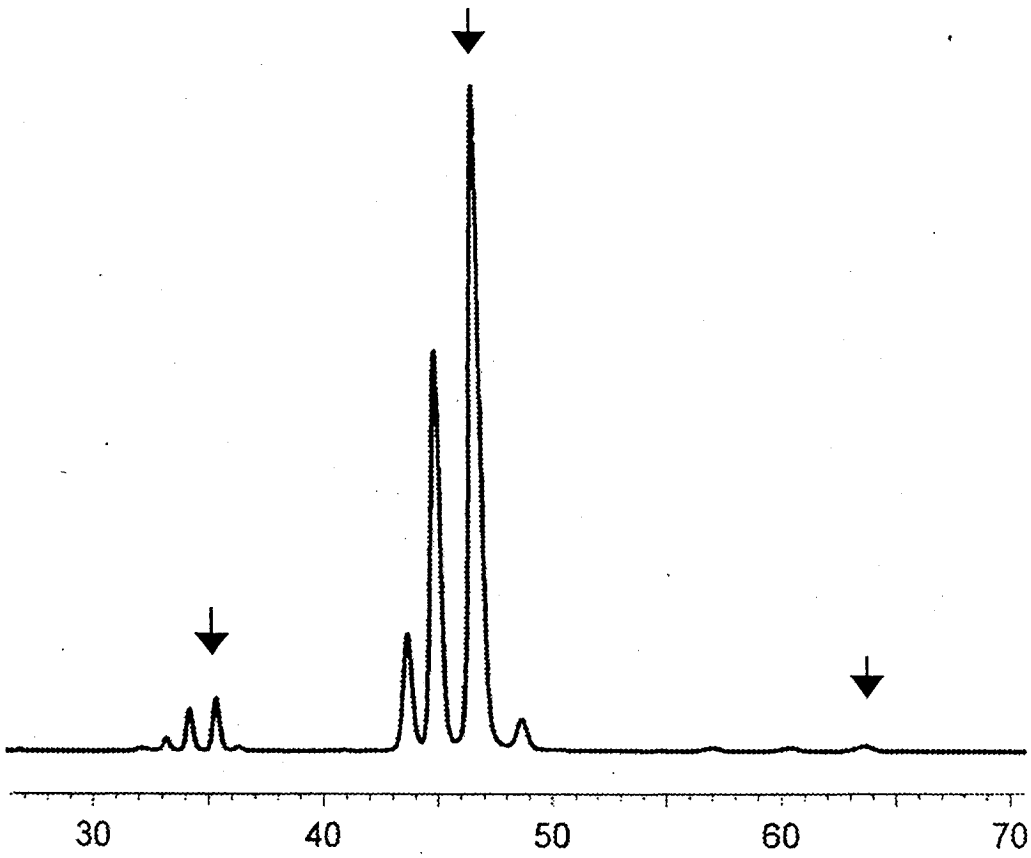

Figure 2.10 Partial Ag^{+}-HPLC chromatogram of coinjection with authentic CLA methylesters, and CLA methylesters produced by *Bifidobacterium breve* JKL03.

CHAPTER 3

ENRICHMENT STUDIES OF CLA AND CLNA IN DAIRY PRODUCTION BY *BIFIDOBACTERIUM BREVE* JKL03

Bifidobacterium breve JKL03 had been selected for its CLA and CLNA producing ability was evaluated in MRS and skim milk media.

Yogurt production by *B. breve* JKL03 and *Lactobacillus acidophilus* were further applied to enrich CLA and CLNA.

The results of this study were summarized as a manuscript suitable for journal publication. The manuscript, "Production of conjugated linoleic acid and conjugated linolenic acid by *Bifidobacterium breve* JKL03" was co-authored by Yun-Kyoung Jung, Geun-Bae Kim, Mun-Yhung Jung, and Byong H. Lee, and was written by Yun-Kyoung Jung and edited by Byong H. Lee.

3.1 ABSTRACT

To optimize the production of CLA using *Bifidobacterium breve* JKL03, the growth studies of this strain were carried out in skim milk and yogurt condition with LA and LNA.

This strain, which was grown for 24 h, showed that the ability to convert linolenic acid (0.2 mg/ml) to CLNA in skim milk up to 72.00% (up to 74.74% under aerobic condition) and linoleic acid (0.2 mg/ml) to CLA up to 23.87% (up to 27.97% under aerobic condition).

When *B. breve* JKL03 was co-cultured with *Lactobacillus acidophilus* (NCFM® strain), the most commonly added starter culture significantly inhibited the growth of *B. breve* JKL03, which resulted in the decrease of CLA and CLNA productions during yogurt process.

KEYWORDS: Conjugated linoleic acid (CLA); Conjugated linolenic acid (CLNA); *Bifidobacterium breve* JKL03; *Lactobacillus acidophilus*; NFDM (skim milk); yogurt

3.2 INTRODUCTION

CLA has gained considerable attention in recent years because of its many beneficial effects, including anticarcinogenic activity (Chin *et al.*, 1992; Liew *et al.*, 1995), antiatherogenic activity (Lee *et al.*, 1994), immune stimulation (Cook *et al.*, 1993), as well as the ability to reduce body fat in animals (Park *et al.*, 1999a) and humans (Smedman and Vessby, 2001; Kelley and Erickson, 2003). CLA also appeared to be effective in the prevention and treatment of non-insulin dependant diabetes mellitus (Houseknecht *et al.*, 1998).

The increasing demand for biologically active polyunsaturated fatty acids (PUFA) has attracted commercial attention, especially on conjugated linoleic acid (CLA)-based nutraceuticals and therapeutics. From a human health viewpoint, it appears desirable to increase CLA levels in foods to help protect against disease and to enhance general health.

Lactobacillus acidophilus NCFM, a probiotic strain available in conventional foods (milk, yogurt, and toddler formula) and dietary supplements, is also important to human probiotic functionality for its biochemical and physiological attributes (Sanders and Klaenhammer, 2001). It is known to decrease the risk of colon cancer, the incidence of pediatric diarrhea, and levels of toxic amines in the blood of dialysis patients.

Bifidobacterium breve JKL03 was previously evaluated in NFDM media for microbial conversion of linoleic acid to CLA and linolenic acid to CLNA. Furthermore, *B. breve* JKL03 and *Lactobacillus acidophilus* (NCFM® strain) were used to investigate the possibility of enrichment of CLA and CLNA in yogurt.

3.3 MATERIALS AND METHODS

3.3.1 Bacterial strains and Media

Among 28 strains (Table 2.1) tested for their ability to convert LA to CLA, *Bifidobacterium breve* JKL03 showed the highest converting ability. This strain was initially isolated from baby faces and was verified for genus and species by 16s rRNA gene sequence typing according to the method of Kaufman (2000).

This strain was anaerobically pre-cultured for 24 h in MRS medium (Difco, Sparks, MD) and then inoculated again into MRS broth, which contained 0.05% (w/v) of L-cysteine-hydrochloride. *Lactobacillus acidophilus* was used as yogurt starter strain and it was pre-cultured for 12 h aerobically in MRS broth.

MRS medium was pasteurized at 121 °C for 15 min, and NFDM medium, which contained 10 % of (w/v) skim milk powder, was pasteurized at 80 °C for 30 min. Portions (5 ml) of NFDM medium were added into screw capped-glass tubes (20 ml volume) that were prepared for the conversion studies. For the yogurt production, 15ml of NFDM media was prepared in screw capped-glass tubes (50 ml volume).

TOS (Transgalacto-oligosaccharides; Yakult co, Japan), Rogosa (Difco), and MRS (Difco) agar media were used to count the colony forming units per milliliter (CFU/ml). Each medium, which contains agar, was poured into the sterile dishes, then set and dried. Peptone water for the dilutions was also prepared.

3.3.2 Chemicals (LA, LNA stock preparation)

Unless otherwise specified, all reagents of analytical grade were purchased from Sigma (St. Louis, MO), Fisher (Fair Lawn, NJ), and Aldrich (Milwaukee, WI).

Linoleic acid was filter sterilized by a 0.2 μm minisart filter (Fisher) and used as 0.5 mg/ml stock solution supplemented to 2 % (v/v) Tween 80 (Sigma) which is also previously filter-sterilized by 0.2 μm minisart filter. Tween 80 (Polyoxyethylene sorbitan monooleate) improves the solubility of free LA in aqueous media. They were stored in the dark at -10 °C for further use.

3.3.3 Fermentation conditions

Bifidobacterium breve JKL03 was cultured in MRS medium which containing linoleic acid (0.2 or 0.5 mg/ml; w/v) under anaerobic (GasPak Plus, BBL, Sparks, MD) and aerobic conditions in NFDM media (Difco, Sparks, MD) supplemented with or without 0.05 %(w/v) L-cysteine-hydrochloride (98% pure; Fisher, Fair Lawn, NJ) and incubated at 37 °C for 24 h and 48 h. The experiments were repeated in triplicates.

3.3.4 Yogurt production

For free linoleic acid (LA) as substrate, two-step fermentation was performed to eliminate the growth inhibition of *Bifidobacterium breve* JKL03. *B. breve* JKL03 was inoculated (1 %) into 15 ml of NFDM media and incubated aerobically for 24 h for the

initial fermentation. *Lactobacillus acidophilus* was then added at the rate of 1 % inoculation and incubated for 24 h (Total 48 h).

Portions of *B. breve* JKL03 (5%) and *Lactobacillus acidophilus* (1%) cultures were inoculated into NFDM media supplemented with 0.2 mg/ml (w/v) of free linolenic acid (LNA) and grown for 8 h and then 16 h more (total 24 hours), respectively.

pH measurement and colony forming unit count (CFU/ml) were carried out at different times during the fermentation.

3.3.5 Microbiological analysis

Yogurt samples (1 ml) were serial diluted in 9 ml peptone water containing 0.05% (w/v) L-cysteine-hydrochloride and mixed by vortex. Several series of dilutions were prepared for the colony counting before plating in different agar plates.

TOS-agar plates were used for counting colonies of *Bifidobacterium breve* JKL03, Rogosa-agar plates (pH 5.4) for *Lactobacillus acidophilus,* and MRS agar plates were used for total colonies.

3.3.6 Extraction of lipids

The lipids from the cultures were extracted and prepared for the gas chromatography analysis. The internal standard (2.5 mg/ml w/v), $C_{17:0}$ heptadecadianoic acid in hexane was added to 0.9 ml of the fermented culture and vortexed for 3 min. For the lipid extraction, hexane (0.9 ml) was added and mixed for 5 min and then centrifuged at 13000 g for 20 min.

The lipids were extracted with 0.9 ml of hexane 4 times, and the hexane layers were collected in a screw capped-glass tube and dried off under nitrogen at room temperature.

The extracted and dried lipids were methylated with 1 ml of 4% HCl in methanol at 60°C for 20 min. After methylation, 2 ml of saturated NaCl and 1 ml of hexane were added and gently shaken. The esters were extracted twice with 1 ml of hexane and the hexane layer, which contains methylated fatty acids, was collected in 1.5 ml tube and dried off by nitrogen flushing at room temperature.

150 µl of 2, 2, 4- trimethylpentane was added into the 1.5 ml tubes to solubilize methylated fatty acids, then 2 µl was injected for the analysis.

3.3.7 Gas chromatography

For the GC analysis, the Gas Chromatography from Perkin Elmer (Autosystem, Connecticut, USA) was used. Fatty acid methyl esters (2 µl) were injected and separated on a cyanopropyl type fused silica capillary column (100 m x 0.25 mm; Supelco, Bellefonte, PA). Helium (Matheson, Whitby, ON) was used as the carrier gas and FID (flame ionization detector), split mode for the injection were used. The oven temperature was initially set at 120 °C for 1 min, and then increased at the rate of 7 °C /min to 200 °C. The injector and detector temperatures were 200 °C and 210 °C, respectively.

3.4 RESULTS AND DISCUSSION

3.4.1 The production of CLA or CLNA in skim milk

To investigate the possibility of enrichment of CLA or CLNA in dairy products, *Bifidobacterium breve* JKL03 was grown in skim milk supplemented with LA or LNA. The c-9, t-11 CLA, which is known as rumenic acid, was mainly produced using *B. breve* JKL03 with the high conversion efficiency. The cells of *B. breve* in growth media were not removed because the recovery of remaining LA or LNA and CLA or CLNA formed were stable without the removal, and 89.10% was recovered throughout this study.

When the selected bacteria strain was fermented in skim milk supplemented with 0.2 mg/ml (w/v) and 0.5 mg/ml of linoleic acid for 24 h and 48 h (Figures 3.1 and 3.2), the amount of CLA produced was significantly increased by increasing fermentation time and adding 0.05% of cysteine. The effects of fermentation time and substrate concentration were significant and the differences were even larger when 0.05 % of cysteine was added.

The reactions of different quantities of linolenic acid as substrate were also carried out. When the selected strain was fermented with 0.2 mg/ml or 0.5 mg/ml (w/v) of LNA in skim milk for 24 h and 48 h (Figures 3.3 and 3.4), the amount of CLNA was less than that of CLA. However, cysteine in media has increased the amount of CLNA significantly.

When the fermentation of LNA in skim milk was carried out under various conditions, the amount of CLNA produced was less when the concentration of LNA was increased.

3.4.2 Optimum conditions

Conversions of linoleic acid to CLA and linolenic acid to CLNA by *B. breve* JKL03 were not complete after growth in skim milk for 24 h, unlike the results of MRS media. After 48 h fermentation, the amount of CLA or CLNA produced was less than that of skim milk supplemented with 0.05% of cysteine.

As cysteine is not added in the yogurt production, *B. breve* JKL03 was grown in cysteine free skim milk under aerobic condition, and also, 0.2 mg/ml (w/v) of LA and LNA was chosen due to the higher conversion rates for the application study.

3.4.3 Yogurt production with *Bifidobacterium breve* JKL03 and *Lactobacillus acidophilus*

When *Bifidobacterium breve* JKL03 and *Lactobacillus acidophilus* were grown simultaneously in skim milk supplemented with linoleic acid (0.2 mg/ml), conjugated linoleic acid was not formed after 24 h of fermentation, not even with 5 times more inoculation of *B. breve* JKL03 under aerobic condition. *B. breve* JKL03 could not be grown due to the rapid growth of *L. acidophilus* and drop of pH of the media.

Several studies have demonstrated that commercial yogurts contain much less numbers of bifidobacteria than *L. acidophilus* (Iwana *et al.*, 1993; Shah *et al.*, 1995). The viability of probiotic bacteria could be affected by many factors and the main factors for loss of viability of probiotic organisms have been referred to the decrease in the pH of the medium by organic acids during fermentation (Hood and Zottola, 1988; Shah and Jelen,

1990; Gueimonde *et al.*, 2004). Two-step fermentation (Lankaputhra and Shah, 1997) was one of the methods used to improve viability of probiotic bacteria in yogurt or other fermented dairy foods made with commercial starter cultures (Shah and Lankaputhra, 1997).

For this reason, LA (0.2 mg/ml) had been fermented initially with *B. breve* JKL03 for 24 h in skim milk, and then *L. acidophilus* was inoculated for further fermentation (Table 3.1). However, the production of CLA in skim milk media under aerobic condition was still low.

Figure 3.5 illustrates the growth of *B. breve* JKL03 and *Lactobacillus acidophilus* in NFDM fermentation containing LA. A gradual pH change was observed during yogurt fermentation with free linoleic acid (Table 3.1). Though CLA formation was almost stopped after 24 h and the pH was 4.89, *L. acidophilus* could start growing rapidly (Figure 3.5).

On the contrary, an almost similar amount of CLNA was produced by fermentation with *L. acidophilus*, when *B. breve* JKL03 at the rate of 5% inoculation and *Lactobacillus acidophilus* at the rate of 1% were grown simultaneously in skim milk containing 0.2 mg/ml of LNA (Table 3.2). Rapid growth of *L. acidophilus* and lowering media pH by *L. acidophilus* did not affect the conversion of LNA to CLNA during the fermentation.

Figure 3.6 showed that after 8 h of fermentation, low pH (4.69) caused the protein coagulation and thus the conversion rate was not increased after 24 h of fermentation. (Table 3.2)

3.5 CONCLUSION

The production conditions of CLA or CLNA using *B. breve* JKL03 grown in skim milk were optimized and applied to enrich CLA or CLNA in dairy products.

After 24 h of growth, this strain alone could convert linolenic acid (0.2 mg/ml w/v) to CLNA up to 72.00% (up to 74.74% under aerobic condition) and linoleic acid (0.2 mg/ml w/v) into CLA up to 23.87% (up to 27.97% under aerobic condition).

As *Lactobacillus acidophilus* NCFM inhibited the growth of *B. breve* JKL03 resulting in the reduction of CLA yields, two-step fermentation will likely be the solution to enrich CLA in fermented dairy products.

This study, in response to the increasing demand for biologically active polyunsaturated fatty acids, has proven that it is possible to have higher CLA or CLNA levels in fermented dairy foods.

However, further studies on other yogurt strain that would not effect CLA conversion or even increase the production with *B. breve* JKL03 are needed to increase the yields of CLA in dairy products.

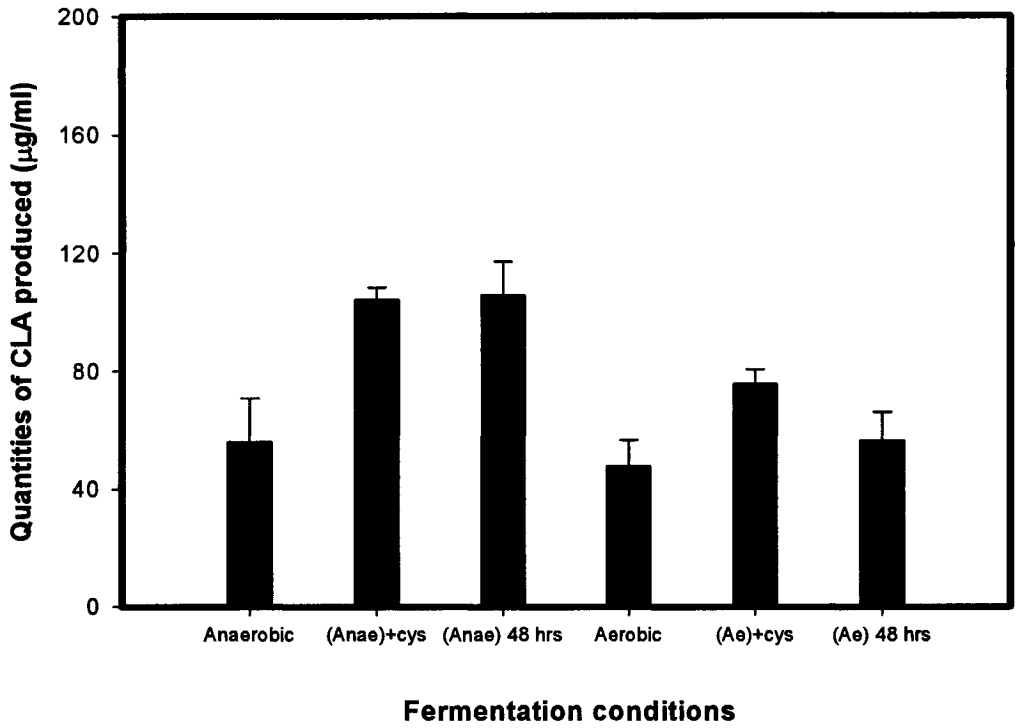

Figure 3.1 CLA production by *Bifidobacterium breve* JKL03 in skim milk.

* *cis*-9, *trans*-11 CLA and *trans*-9, *trans*-11 CLA were calculated as total CLA.
** Error bar indicate standard deviation from the triplicate.
*** CLA (μgmL^{-1}) converted from 200 μgmL^{-1} free linoleic acid and linolenic acid

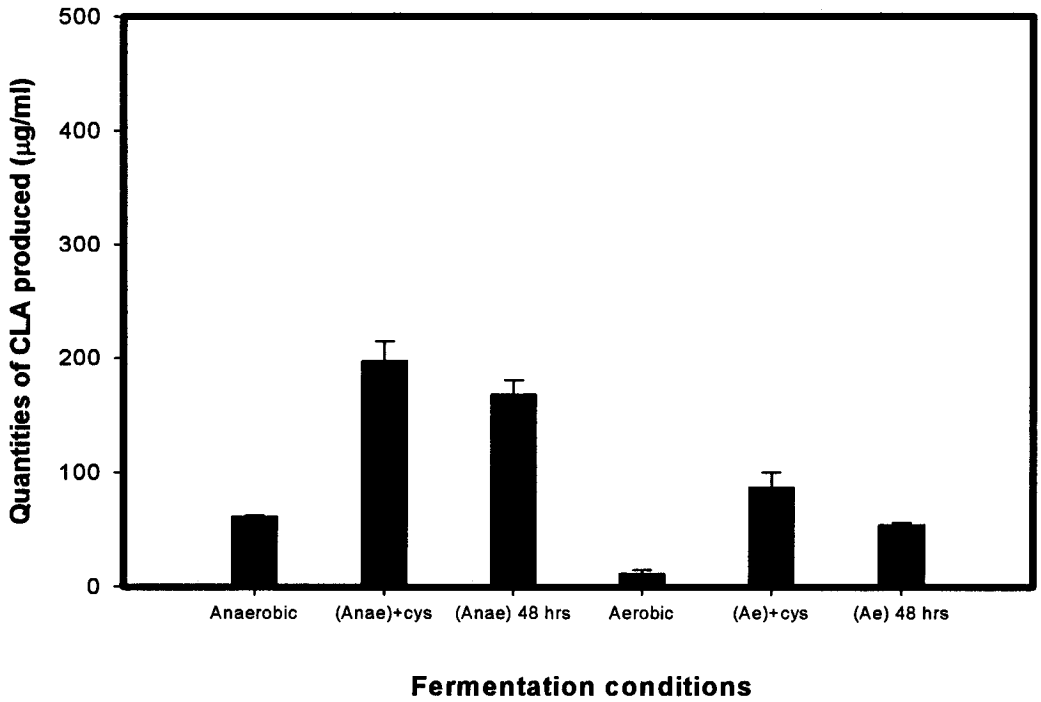

Figure 3.2 CLA production by *Bifidobacterium breve* JKL03 in skim milk.

* CLA (μgmL^{-1}) converted from 500 μgmL^{-1} free linoleic acid

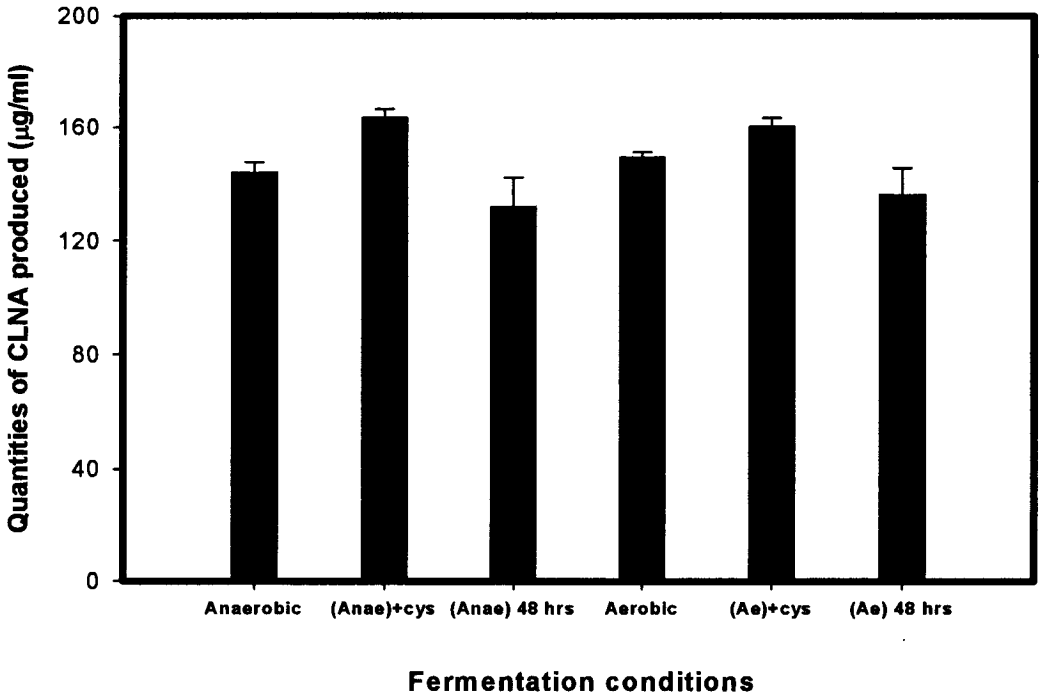

Figure 3.3 CLNA production by *Bifidobacterium breve* JKL03 in skim milk.

* CLNA (μgmL^{-1}) converted from 200 μgmL^{-1} free linolenic acid

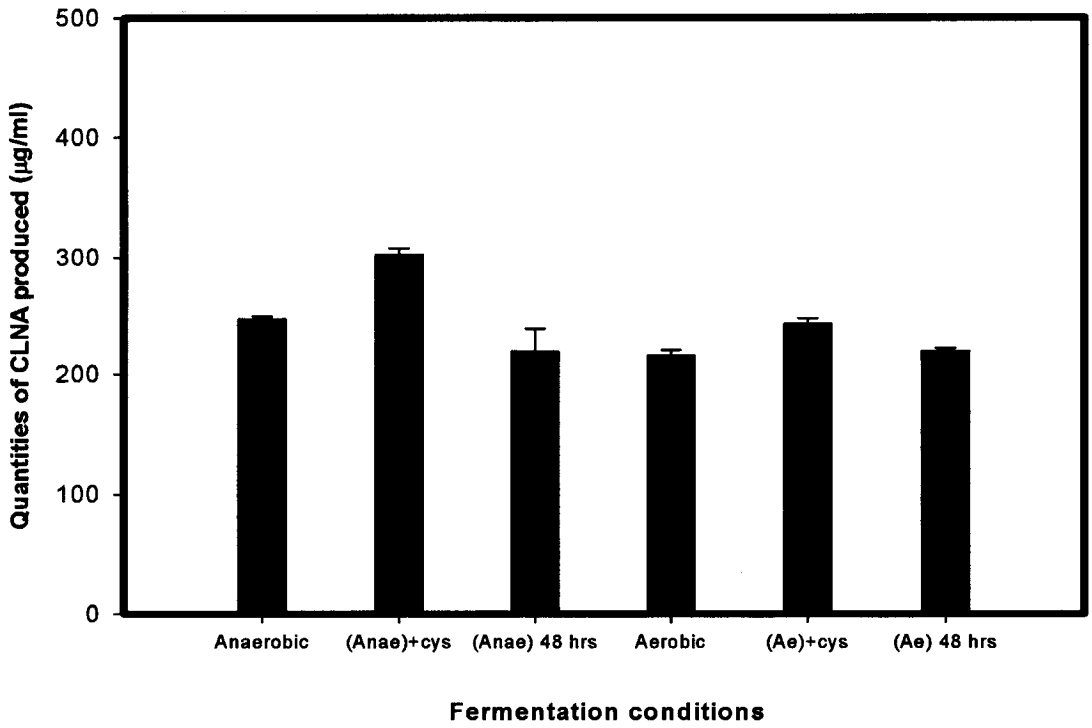

Figure 3.4 CLNA production by *Bifidobacterium breve* JKL03 in skim milk.

* CLNA (μgmL^{-1}) converted from 500 μgmL^{-1} free linolenic acid

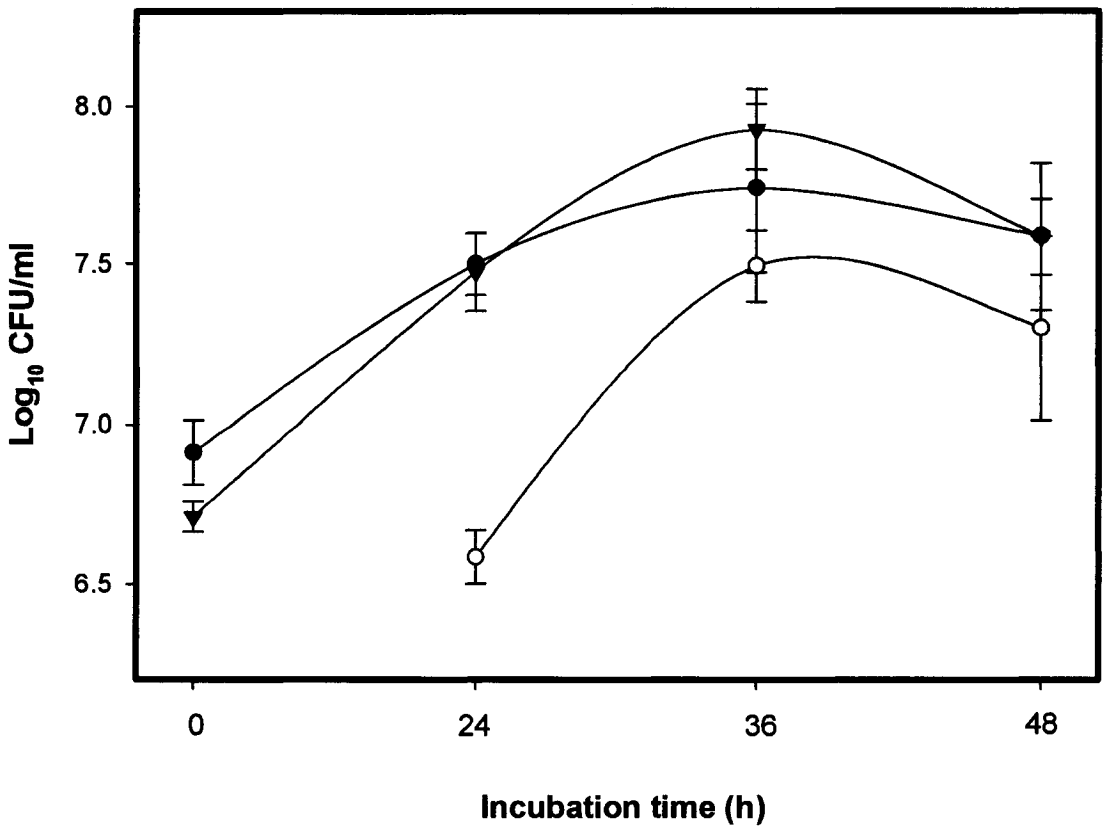

Figure 3.5 Growth of *Bifidobacterium breve* JKL03 (●), *Lactobacillus acidophilus* (○), and total (▼) in skim milk supplemented with LA.

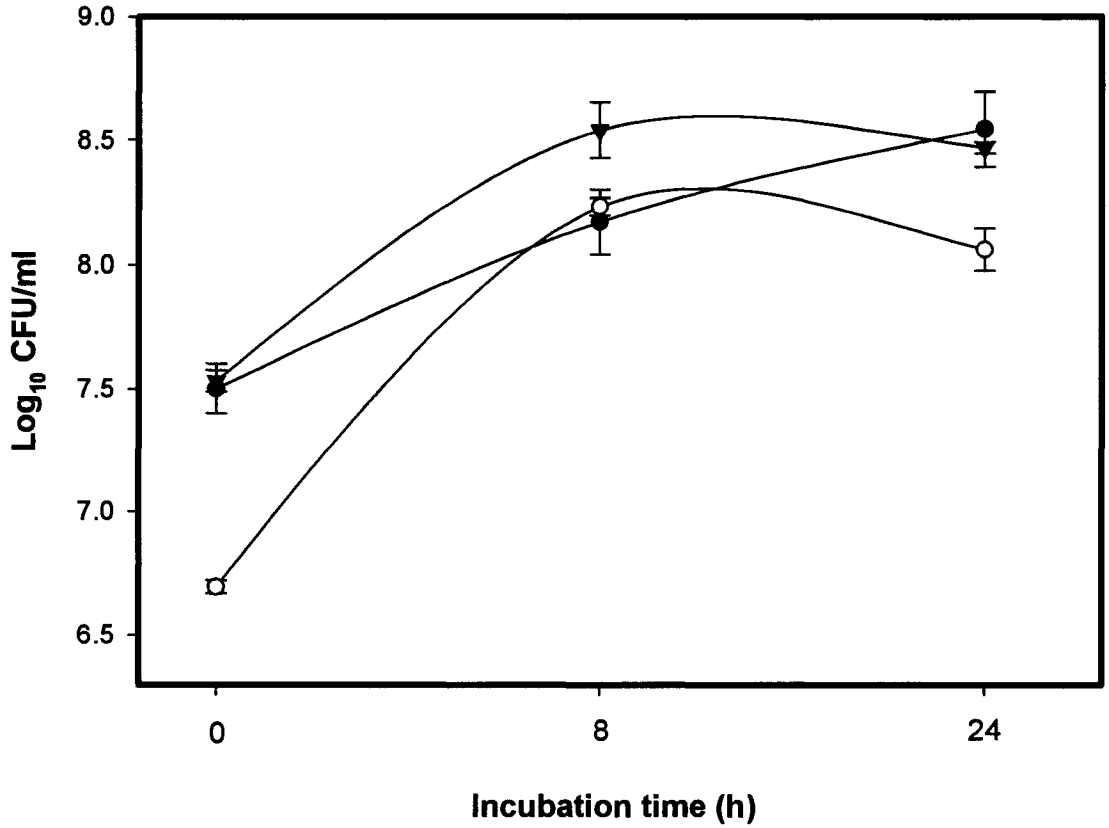

Figure 3.6 Growth of *Bifidobacterium breve* JKL03 (●), *Lactobacillus acidophilus* (○), and total (▼) in skim milk supplemented with LNA.

Table 3.1 CLA productions & pH development by *B. breve* JKL03 and *L. acidophilus* grown in skim milk (two-step fermentation).

Time (h)	pH	Total CLA	Remaining LA	Recovery
0	6.52	0.000	183.732 ± 7.159	91.866 ± 3.580
24	4.89	27.670 ± 3.666	156.050 ±19.807	91.860 ± 9.190
36	4.46	31.167 ± 2.432	149.270 ± 3.320	90.218 ± 1.660
48	4.33	30.486 ± 1.635	153.949 ± 9.357	92.217 ± 4.679

* NFDM supplemented with LA (0.2 mg/ml) under aerobic condition.

Table 3.2 CLNA productions & pH development by *B. breve* JKL03 and *L. acidophilus* grown in skim milk.

Time (h)	pH	Total CLNA	Remaining LNA	Recovery
0	6.50	0.000	179.326 ± 8.432	89.663 ± 5.015
8	4.69	150.190 ± 4.964	25.943 ± 0.457	88.067 ± 2.254
24	4.01	152.184 ± 4.497	23.142 ± 0.636	87.663 ± 2.567

* NFDM supplemented with LNA (0.2 mg/ml) under aerobic condition.

GENERAL CONCLUSION

Bifidobacterium breve JKL03 was the best CLA producer among the strains we have tested. Optimization of the concentration of substrates, fermentation time, and the presence of oxygen could increase the productivity of CLA and CLNA.

For the CLA production, fermentation in cys-MRS containing 1.0 mg/ml of LA for 12 h under anaerobic condition was the most efficient. For the CLNA production, 0.5 mg/ml of LNA and 24 h fermentation under anaerobic condition gave the best results.

The optimal growth conditions in skim milk were 0.2 mg/ml of substrates and 24 h fermentation time for LA, and 8 h for LNA. Under these conditions, the conversion rate of linoleic acid to CLA was up to 23.87 % (up to 27.97 % under aerobic condition), and that of linolenic acid to CLNA was up to 72.00 % (up to 74.74 % under aerobic condition).

The presence of oxygen partially inhibited the conversions of LA and LNA. Two-step fermentation was necessary to produce CLA during yogurt production, because *Lactobacillus acidophilus* NCFM inhibited on the growth of *Bifidobacterium breve* JKL03.

This study demonstrated that fermentations of linoleic acid and linolenic acid with *Bifidobacterium breve* JKL03 could be useful to increase levels of CLA and CLNA in dairy foods such as milk, yogurt, and cheese, however further studies are required to validate these results.

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