CONJUGATED LINOLEIC ACID PRODUCTION OF *Lactobacillus plantarum* BIOTECH 1066 IN MILK

Angelo M. Tapia[1], Jose Arceo N. Bautista[1], Bernadette C. Mendoza[2], Laura J. Pham[3], Ione G. Sarmago[4] and Maria Cynthia R. Oliveros[1]

ABSTRACT

This study was conducted to quantify conjugated linoleic acid production (CLA) of *Lactobacillus plantarum* BIOTECH 1066 as well as its viable count and percent lactic acid in sterile cow, buffalo and goat milk. The sterile medium was inoculated with 1% *L. plantarum* BIOTECH 1066 and then incubated at 37ºC for 18 hours. Lipids were extracted from the reaction mixture with choloroform/methanol (1:2 v/v) and were analyzed using a GC-14 gas chromatograph. Triplicate data were statistically analyzed using *t*-test and analysis of variance (ANOVA) in completely randomized design at 5% level of significance. Comparison of treatment means was done using Least Significant Difference. Viable cell count (cfu/ml) and developed acidity of *L. plantarum* 1066 had no significant differences after 18 hours of incubation on different media while CLA produced has increased significantly. Using different milk media, *L. plantarum* 1066 was able to produce significant amounts of CLA (0.32 to 0.91 mg/ml) with significant concentration of 0.62 mg/ml synthesized in buffalo's milk. This could be attributed to the relatively high fat content of buffalo milk (7.2%).

Key words: conjugated linoleic acid, *Lactobacillus plantarum*, linoleic acid

INTRODUCTION

Conjugated linoleic acids (CLA) are isomers of the polyunsaturated fatty acid, linoleic acid. It is a mixture of positional and geometric isomers of linoleic acid (c-9, c-12 C-18:2-LA) with two conjugated unsaturated double bonds at various carbon positions (c-9, c-11 and c-10, c-12). It is formed as an intermediate during the biohydrogenation of linoleic acid to stearic acid by *Butyrivibrio fibrisolvens* (Kepler *et al*., 1966) and other rumen bacteria (Kritchevsky, 2000) or from the endogenous conversion of transvaccenic acid (t-11 C, 18:1 TVA) by Δ9-desaturase in the mammary gland (Corl *et al*., 2001).

The health benefits of CLA for man have been reported (Lawson *et al*., 2002). Animal studies and clinical trials indicated the possibility that CLA could be useful in improving human health in a number of areas like controlling body fat gain and enhancing

[1]Institute of Animal Science, College of Agriculture and Food Science (CAFS), University of the Philippines Los Baños (UPLB), [2]Institute of Biological Sciences, College of Arts and Sciences (CAS), UPLB; [3]National Institute of Molecular Biology and Biotechnology, UPLB; [4]Dairy Training and Research Institute, CAFS, UPLB (email: amtapia@up.edu.ph).

immunity while also reducing inflammation and other adverse effects typically associated with immune enhancement (Pariza, 2004). CLA may also influence the onset and severity of several chronic diseases, including various cancers, atherosclerosis, obesity, bone density loss, and diabetes (McGuire and McGuire, 2000). Supplementation with CLA had an adverse effect on insulin and glucose metabolism. CLA also had positive effects on high density lipoprotein (HDL) metabolism (Moloney *et al*., 2004). Important isomer-specific metabolic actions of CLA have seen significant effects on abdominally obese humans. A CLA-induced insulin resistance has previously been described only in lip dystrophic mice (Riserus *et al*., 2002). Numerous studies confirmed the anti-carcinogenic activity of CLA in both *in vivo* and *in vitro* models (Cook and Pariza, 1998). Mechanisms of inhibition of carcinogenesis may include reduction of cell proliferation, alterations in the components of the cell cycle and induction of apoptosis. In addition, CLA modulates markers of immunity and eicosanoid formation in numerous species as well as lipid metabolism and gene expression. It is likely that CLA exerts inhibitory properties in carcinogenesis via one or more of these pathways with some tissue specificity (Belury, 2002). Though human requirement has not been established, the most commonly reported intake recommendation of CLA for humans is 0.8 g/day (from 0.6 to 3.0 g/day) (Siruana and Calsamiglia, 2016). These findings are of special interest to the agriculture sector because dietary sources of CLA are almost exclusively beef and dairy products (McGuire and McGuire, 2000). Microbial CLA production, in addition to rumen microflora, has also been reported (Jiang *et al*., 1998). The advantageous nutritional properties and health benefits associated with CLA have important implications also for food industries whose challenge is the production of functional foods with health-promoting properties (Alcala and Fontecha, 2007).

L. plantarum 1066 was isolated from a fermented mungbean and was obtained from the Philippine National Collection of Microorganisms (PNCM) at BIOTECH, UPLB. *L. plantarum* 1066 is a rod-shaped, gram-positive, lactic acid bacterium. It was among the 10 strains of lactic acid that were screened and was positive for CLA production. *L. plantarum* 1066 has produced 327.67 µg/ml of CLA in DeMan-Rogosa-Sharpe (MRS) broth after 18 hours of incubation. However, concentration decreased from 327.67 µg/ml to 259.33 µg/ml after 24 hours of incubation (Tapia, 2017).

The objectives of the study were to: 1) quantify and compare the oleic acid, linoleic acid and CLA produced by *L. plantarum* 1066 using cow, buffalo and goat milk substrates; and 2) evaluate the growth behavior of *L. plantarum* 1066 using viable cell count and lactic acid production parameters using cow, buffalo and goat milk.

MATERIALS AND METHODS

Sterile cow, buffalo and goat milk (10 ml) were used. Sterilization of the media was done by autoclaving at 10 psi for 10 minutes. The sterile milk medium was inoculated with 1% *L. plantarum* BIOTECH 1066 (grown for 18 hours in 12% reconstituted skim milk medium) and then incubated at 37ºC for 18 hours. Viable counts were obtained by plating serial dilutions of the suspension in MRS agar after incubation and the plates were incubated at 37ºC for 48 hours.

Five (5) ml of milk sample was obtained and was added with phenolphthalein indicator and was titrated with 0.1N NaOH (endpoint of titration light pink).

Percent lactic acid was computed as follow:

$$\%\ lactic\ acid = \frac{Vol\ NaOH_{(used\ for\ titration)}\ x\ 0.1N\ NaoH\ x\ \frac{90}{1000}}{Vol\ of\ sample} x100$$

One (1) ml of milk sample was obtained in a separate tube and was analyzed for oleic acid, linoleic acid and CLA (9-cis, 11-trans and 10-trans, 12-cis) content before and after incubation. Lipids were extracted from the reaction mixture with choloroform/ methanol (1:2 v/v) according to the procedure of Bligh and Dyer (1959) with modifications. Briefly, ten ml of reaction mixture was added with 37.5 ml of choloroform/methanol (1:2, v/v) and was mixed using a vortex mixer for 10 minutes. Then 12.5 ml of chloroform was added with mixing for 1 minute, after which, 12.5 ml 1M NaCl was added and mixed for another minute and then centrifuged at 3000 rpm for five minutes. The upper layer was discarded and the lipid extract (lower phase) was trans methylated with 10% methanolic HCl at 50°C for 20 min. The resultant fatty acid methyl esters were extracted with n-hexane and were analyzed by gas chromatography for oleic acid, linoleic acid and conjugated linoleic acid content. A Shimadzu GC-14 (Shimadzu Corp., Kyoto Japan) gas chromatograph was used. As described by Pham and Gregorio (2008), it was equipped with SUPELCOWAX 10 (30m) column, a flame ionization detector, and interphased to a GC-workstation. Detection and injection ports were maintained at 260ºC and 250ºC, respectively, while the column temperature was at 140-240ºC and programmed at 4ºC/min. The carrier flow was 5mL/min and peaks were identified by comparing them with CLA standard (Sigma Aldrich-O5507). CLA was quantified using an external standard (myristic acid); the formula used to compute for CLA content was:

$$Fatty\ Acid\ \left(\frac{mg}{ml}\right) = \frac{A_i\ x\ (is)}{A_{is}\ x\ V_{ml}}$$

where: A_i = Area of fatty acid
is = concentration of myristic acid (mg/ml)
A_{is} = area of the standard
V_{ml} = volume of sample suspension (ml)

Triplicate data were gathered and the results were expressed as means and standard deviations (SD). Data were statistically analyzed using *t*-test and analysis of variance (ANOVA) in completely randomized design (CRD) at 5% level of significance. Comparison of treatment means was done using Least Significant Difference.

RESULTS AND DISCUSSION

Viable cell count (cfu/ml) and developed acidity (% lactic acid) of *L. plantarum* 1066 grown on different milk media (sterile cow, buffalo and goat) had no significant differences after 18 hours of incubation as shown in Table 1. Results showed that given any of the 3 media, *L. plantarum* 1066 exhibited the same behavior in terms of growth and lactic acid production. Viable cell count has ranged from $2.1x10^7$ to $1.9x10^8$ cfu/ml, while developed acidity has ranged from 0.25% to 0.28% lactic acid among milk media. Milk fat has proven to be one of the richest source of CLA. The total CLA content of raw milk has been reported to range from 2 to 37 mg/g in fat (Csapo and Varga, 2015).

Large variation in CLA can be attributed to several factors especially the feeding system (Wahle *et al*., 2004). The inclusion of microbial cultures in fermentation in dairy processing may result in additional health benefits of the product (Csapo and Varga, 2015). In addition, starter cultures commonly used in dairy products could show significant improvement in CLA concentration (Lin, 2003). Table 2 shows the oleic acid, linoleic acid and CLA production of *L. plantarum* 1066 on different milk media at 0 and 18 hours of incubation without linoleic acid supplementation. Among the fatty acids quantified, there is significant increase after incubation except for the oleic acid production in goat's milk. However, it can still be observed that with the different milk media, *L. plantarum* 1066 was able to produce significant amounts of CLA. After 18 hour of incubation, the CLA produced from the three milk media ranged from 0.32 to 0.91 mg/ml. Yadav *et al*. (2007) mentioned that L. acidophilus and L. casei increased CLA content of Dahi (Indian yoghurt) during fermentation by lipolysis of natural milk fat and the increased CLA was produced using internal linoleic acid. Serafeimidou *et al*. (2013) also reported that cow milk processed as yoghurt using S. salivarius spp. thermophilus and L. delbrueckii ssp. bulgaricus produced a total of 510 µg/ml of CLA after 24-hour incubation. In another study, Serafeimidou *et al*. (2012) also described the CLA content of Greek yoghurt. CLA ranged from 2.79 to 4.62 mg/ml and concluded that there was no significant difference in the CLA content of Greek yoghurt made from cow, goat and low-fat sheep milk. Possible differences in the CLA content of the fermented dairy products were the use of different starter cultures (Csapo and Varga, 2015). Sosa-Castañeda *et al*. (2015) also reported CLA production of 4 strains of lactic acid bacteria from 13.44 to 50.9 µg/ml using skim milk medium. However, Lin *et al*. (1995) found no significant difference in the CLA content between milk and yoghurt. Commercially used dairy starter bacteria have only a minor contribution in the CLA level of fermented dairy products while using appropriate strains may significantly increase the CLA content (Csapo and Varga, 2015). Table 3 shows the amount of fatty acid synthesized by *L. plantarum* 1066 on different milk media after 18 hours of incubation. Generally, buffalo milk exhibited significant concentrations of oleic acid, linoleic acid and CLA after incubation, which is 4.16 mg/ml, 1.00 mg/ml and 0.62 mg/ml, respectively. This can be attributed to the high fat content of buffalo milk, which is 7.20%. Kim and Liu (2002)

Table 1. Growth and physico-chemical properties of milk inoculated with *L. plantarum* 1066 in cow, buffalo and goat's milk.

Parameters	Treatment			*P*-value
	Cow	Buffalo	Goat	
Fat (%)	3.40 ± 0.25^{a}	7.20 ± 0.32^{b}	3.20 ± 0.19^{a}	<0.0001
Acidity (% lactic acid)				
0 hour^{ns}	0.15 ± 0.02	0.17 ± 0.01	0.15 ± 0.01	0.2399
18 hour^{1ns}	0.25 ± 0.02	0.28 ± 0.03	0.27 ± 0.03	0.2274
Viable Count (cfu/ml)^{1ns}	1.9×10^{8}	2.1×10^{7}	1.7×10^{8}	0.1742

[1]Incubation for 18 hours at 37°C.

[abc]means with different superscripts are significantly different at $P<0.05$

[ns]not significant at $P>0.05$

Table 2. Oleic, linoleic and conjugated linoleic acid (CLA) production by *L. plantarum* 1066 in cow, buffalo and goat's milk.

Medium	Fatty Acid (mg/ml)			*P*-value
	Fatty Acid	0 h	18 h[1]	
Cow's Milk	Oleic Acid*	7.96 ± 0.55	10.12 ± 0.67	<0.0001
	Linoleic Acid*	0.17 ± 0.01	0.28 ± 0.05	<0.0001
	CLA*	0.13 ± 0.01	0.51 ± 0.04	<0.0001
Buffalo's Milk	Oleic Acid*	6.08 ± 0.75	10.24 ± 0.14	<0.0001
	Linoleic Acid*	0.74 ± 0.35	1.74 ± 0.50	<0.0001
	CLA*	0.29 ± 0.07	0.91 ± 0.37	<0.0001
Goat's Milk	Oleic Acid[ns]	3.86 ± 0.20	3.44 ± 0.20	0.2567
	Linoleic Acid*	0.35 ± 0.03	0.70 ± 0.41	<0.0001
	CLA*	0.13 ± 0.01	0.32 ± 0.02	<0.0001

[1]Incubation for 18 hours at 37°C
*significant at $P<0.05$
[ns]not significant at $P>0.05$

Table 3. Fatty acid (oleic, linoleic and conjugated linoleic acid) synthesized by *L. plantarum* 1066 in cow, buffalo and goat's milk after incubation[1].

Fatty Acid (mg/ml)	Treatment			*P*-value
	Cow	Buffalo	Goat	
Oleic Acid	2.16[b]	4.16[c]	-0.42[a]	<0.0001
Linoliec Acid	0.11[a]	1.00[c]	0.35[b]	<0.0001
CLA	0.38[b]	0.62[c]	0.19[a]	<0.0001

[1]Incubation for 18 hours at 37°C
[abc]means with different superscripts are significantly different at $P<0.05$

investigated the CLA content of milk by fermentation using *L. lactis* and concluded that CLA production was influenced by several factors such as substrate composition, incubation time, culture condition as well as pH. Identifying the specific role of each bacterial species in pure culture was necessary in order to understand the biochemical mechanism to increase CLA content of fermented dairy products (Florence *et al*., 2012).

It can be concluded that milk from cow, goat, and buffalo can be used as a substrate for the synthesis of CLA by *L. plantarum* 1066. Where highest amount of CLA synthesized (0.62 mg/ml) was exhibited using buffalo's milk. Further studies can be conducted, specifically on understanding the metabolic pathway for CLA production by identifying specific enzymes and fatty acid precursor that favors CLA production. Studies can also be done to evaluate the CLA production of *L. plantarum* 1066 as adjunct culture for the production of different types of fermented dairy products.

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