

## Influence of free linoleic acid on the fatty acids profile of fermentation by selected probiotic bacteria

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### Summary

The ability of strain *Lactobacillus acidophilus* La5 and mixed starter culture ABT-2 to convert linoleic acid to its conjugated form was tested in fermentative MRS medium and low-fat (0.5%) milk. The addition of free linoleic acid to the fermentative media changed the fatty acids profile of biomass; it also changed fat composition of tested media. The content of conjugated linoleic acid increased to 2.5–2.8 mg per 1 g of dry mass and 21.1–68.9 mg per 1 l of MRS medium. The content of conjugated linoleic acid in fermented milk increased to 2%. By fermentation of milk with addition of free linoleic acid, the content of oleic acid increased by 3% while the content of stearic acid decreased by 11%.

### Keywords

conjugated linoleic acid; probiotic; vaccenic acid; fatty acid; *Lactobacillus*

Conjugated linoleic acid (CLA) is the name given to a group of positional and geometric isomers of a long chain, polyunsaturated fatty acid - linoleic acid (LA). The isomers have conjugated double bonds which may be of *cis* or *trans* configuration and may be located at positions 6, 8 up to 12, 14, most commonly found at carbons 9 and 11 or 10 and 12 [1]. It is believed that *cis*-9, *trans*-11 (CLA1) and *trans*-10, *cis*-12 (CLA2) isomers of CLA are the most active forms. An increasing interest in CLA has been attributed to its potential health benefits such as anticarcinogenic, antiatherogenic, antidiabetic and antiadipogenic effects [2-4]. Its role in vitamin A metabolism [5], bone modelling [6] and immune response [7] has also been reported.

The dietary sources of CLA are milk fat, natural and processed cheeses, meat products and plant oils. Animal sources are richer in CLA than are plant sources [8].

Basically CLA can be produced by chemical synthesis or biosynthesis. Synthetic CLA is synthesized from linoleic acid by alkaline isomerization [9]. Also, a large quantity of *trans*-*cis*/*cis*-*trans* CLA isomers has been prepared from synthetic CLA by low-temperature precipitation with [10] or without [11] urea treatment. CLA, which is sold in

health food stores as a muscle builder, is made by hydrogenation of oils, the process being somewhat difficult, as some of the CLA isomers are very unstable [12].

In case of biosynthesis of CLA, the first reaction in linoleic acid biohydrogenation is the isomerization, where the double bond at carbon 12 position is transferred to carbon 11 position forming *cis*-9, *trans*-11 CLA. It is followed by rapid hydrogenation of the *cis*-9 bond, giving rise to the *trans*-vaccenic acid (TVA). The enzyme responsible for the conjugation of *cis*-9, *cis*-12 double bonds was identified as linoleic acid isomerase (EC 5.3.1.5). It is a particulate enzyme bound to the bacterial cell membrane [13] and demonstrates an absolute substrate requirement for a *cis*-9, *cis*-12 diene system and a free carboxyl group [14]. Biohydrogenation of linolenic acid (LNA) is proceeding in a similar way. A common intermediate during the biohydrogenation of both LA and LNA was found to be the TVA [15].

CLA can be produced also microbially. *Butyrivibrio fibrisolvens* is the best known producer of CLA from LA. Numerous studies on *Butyrivibrio fibrisolvens* have been published [9, 16, 17]. The *Butyrivibrio fibrisolvens* is not the only producer of CLA, strains belonging to the genera *Lacto*-

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*bacillus*, *Lactococcus*, *Pediococcus*, *Enterococcus* and *Bifidobacterium* too were tested for their ability to generate CLA from free LA [18]. *Bifidobacterium breve* and *Bifidobacterium dentium* were the most efficient CLA producers in the study of COAKLEY et al. [19]. Washed cells of *Lactobacillus acidophilus* AKU 1137 produced CLA from LA under microaerobic conditions, with a preceding accumulation of hydroxy fatty acids (HY) as 10-hydroxy-*cis*-12-octadecaenoic acid and 10-hydroxy-*trans*-12-octadecaenoic acid. The 10-hydroxy-*cis*-12-octadecaenoic acid was transformed into CLA during incubation with washed cells, suggesting that this hydroxy fatty acid is one of the intermediates of CLA production from LA [20]. At Oklahoma State University four different cultures were tested, two strains of *Lactobacillus acidophilus* (016 and L1) and *Lactobacillus casei* (E5 and E10), for their ability to produce CLA from different concentrations of LA. The experiments were successful [21]. HAM et al. isolated 34 lactic acid bacteria cultures from 19 faeces samples of healthy babies. From these isolates, culture identified as *Lactobacillus fermentum* showed the highest ability to form CLA [22]. Researchers from Kyoto University used 14 genera of lactic bacteria from different culture collections for screening. All of the tested strains were able to produce CLA. *Lactobacillus plantarum* AKU 1009a had the highest yield of CLA and was therefore selected as the potential strain for CLA production from LA [18, 23]. The strain *L. plantarum* AKU 1009a was tested for CLA formation starting from different form of the ricinoleic acid (12-hydroxy-*cis*-9-octadecaenoic acid) and castor oil (triacylglycerol of fatty acids - 88.2% ricinoleic acid, 4.8% linoleic acid and 7.0% others) as substrates. Ricinoleic acid was found to be a effective substrate for the production of CLA [24]. The effect of various compounds on CLA isomer production by *L. plantarum* AKU 1009a was investigated (sugars, amino acids, metal ions, salts, enzyme cofactors and enzyme inhibitors) added to the reaction mixture. These compounds reduced CLA<sub>2</sub> production, thus increasing the apparent selectivity for CLA<sub>1</sub>. Additions of L-serine or AgNO<sub>3</sub> were effective for selective production of CLA<sub>1</sub>. Interestingly, D-serine did not have such an effect [25]. Under optimal conditions immobilization of *Lactobacillus reuteri* ATCC 55739 on silica gel increased the accumulation of CLA, [26]. Among the 13 screened lactic acid bacteria, 6 strains showed CLA producing ability in MRS medium and 10 strains increased CLA formation in whole milk, using sunflower oil (containing 70% of LA) as a substrate. Cells usually produced more CLA in whole milk than in MRS medium. *Lacto-*

*coccus lactis* I-01 showed the highest CLA producing ability. The optimal concentration of sunflower oil for CLA production was 0.1 g.l<sup>-1</sup> in whole milk, which accounted for 0.25% of the total milk fat. Results of this work demonstrated that CLA formation in fermented milk could be affected by numerous factors such as bacterial strain, number of cells, optimal substrate concentration, and the period of incubation at optimal pH [27]. Three lactic cultures, *Lactobacillus acidophilus* (CCRC 14079), yoghurt bacteria (*L. delbrueckii* ssp. *bulgaricus* and *Streptococcus thermophilus*) and mixed cultures of *L. acidophilus* and yoghurt bacteria, were tested for the effect of addition of 5% of fructo-oligosaccharides and 0.1% LA on CLA production in non-fat yoghurt. Sensory attributes were also evaluated. Inoculation of mixed cultures comprising *L. acidophilus* and yoghurt bacteria with 0.1% LA addition showed significant improvement in the content of *cis*-9, *trans*-11-CLA, without decreasing product acceptability, and makes therefore a suitable method for CLA-rich non-fat yoghurt production [28].

## MATERIAL AND METHODS

The strain *L. acidophilus* La5 and mixed starter culture ABT-2 (Chr. Hansen, Bratislava, Slovakia) were used for verification of the ability to convert linoleic acid to its conjugated form. Bacteria were cultivated in MRS medium (*Lactobacillus*-selective medium, BIOMARK Laboratories, Pune, India) for 24 h at 37 °C.

### Conversion

Conversion was studied in three different conditions:

1. Conversion in the buffer solution: 50 mg of bacterial cells were added to 5 cm<sup>3</sup> of the conversion solution, consisting of the phosphate buffer (pH 6.5) with addition of 0.001% bovine serum albumin (BSA) (Imuna Pharm Holding, Šarišské Michalany, Slovakia) and 0.2% free linoleic acid (LA) (99%, Sigma, St. Louise, Missouri, USA). Conversion run stationary, at the temperature of 37 °C, under the nitrogen atmosphere, for 48 h.
2. Conversion in MRS medium: 50 mg of bacterial cells were added to 5 cm<sup>3</sup> of MRS medium with addition of 0.01% L-cystein, 0.4% Tween 80 and 0.2% free linoleic acid. Conversion was done stationary, at the temperature of 37 °C, under the nitrogen atmosphere, for 48 h.
3. The determination of conjugated linoleic acid content in the milk: low-fat (0.5%) UHT

milk (Tatranské mlieko nízkotučné, Tatranská mliekareň, Kežmarok, Slovakia) was enriched with 0.2% free linoleic acid (99% Sigma, St. Louise, Missouri, USA) and 0.4% Tween 80 and inoculated with 50 mg of bacterial cells. Conversion was done stationary, at the temperature of 37 °C, under the nitrogen atmosphere, for 48 h.

#### Extraction of lipids from cells and esterification

After the conversion, cells were centrifuged (1950 g, 5 min). The supernatant was discarded 1 cm<sup>3</sup> of dichloromethane with internal standard (0.001% heptadecenoic acid) and 2 cm<sup>3</sup> of methanolic HCl were added to the centrifuged cells and thoroughly mixed. After 20 min of incubation under the argon atmosphere at 50 °C in capped tubes, 1 cm<sup>3</sup> of distilled water was added and methylesters were extracted to 4 cm<sup>3</sup> of *n*-hexane. The extract was dried with anhydrous sodium sul-

phate and evaporated. The residue was dissolved in small amount of *n*-hexane and determined by capillary gas chromatography.

#### Extraction and esterification of milk fat

5 cm<sup>3</sup> of the extraction mixture of chloroform: methanol (2:1) were added to 1 cm<sup>3</sup> of milk. The mixture was incubated 5 min at 30 °C and after stirring, the layers were separated by centrifugation (1430 g, 5 min). The lower chloroform layer was then taken away and dried with anhydrous sodium sulfate and the extraction was repeated. Then both extract were gathered and evaporated in rotary evaporator at 30 °C until dry. Lipids were methylated by method of [29].

#### Capillary gas chromatography

Fatty acid methylesters were determined by capillary gas chromatography. Instrumentation used for the analysis was as follows: GC Hewlett-

**Tab. 1A.** The comparison of the fatty acid profile of cell biomass of mixed starter culture ABT-2 in different conversion solutions.

Fatty acid [μg.g <sup>-1</sup> of cell dry mass]	Conditions of conversion			
	background	conversion solution with BSA	conversion solution with 0.4% Tween	MRS medium with 0.4% Tween
C18:0	294.7	212.2	470.6	1 573.9
C18:1(9t)	–	–	–	458.2
C18:1(9c)	1 932.7	1 089.0	6 429.9	48 801.7
C18:1(11t)	–	–	235.3	909.5
C18:1(11c)	138.4	–	122.2	1 056.9
C18:2 (9c,12c)	264.6	117 013.0	4 475.1	43 919.5
CLA	–	–	461.5	2 828.7
HY1	–	1 704.0	8 800.0	2 618.8

**Tab. 1B.** The comparison of the fatty acid profile of cell biomass of strain *L. acidophilus* La5 in different conversion solutions.

Fatty acid [μg.g <sup>-1</sup> of cell dry mass]	Conditions of conversion			
	background	conversion solution with BSA	conversion solution with 0.4% Tween	MRS medium with 0.4% Tween
C18:0	1 407.2	1 398.3	702.8	4 442.3
C18:1(9t)	–	–	–	634.2
C18:1(9c)	605.3	530.9	18 139.2	41 270.7
C18:1(11t)	–	–	147.3	609.8
C18:1(11c)	1 320.8	1 253.0	241.0	3752.1
C18:2(9c,12c)	586.6	31 902.7	7 603.7	58 492.9
CLA	–	–	1 840.9	2 536.6
HY1	–	7 520.0	3 200.0	2 380.0

Conditions of conversion: 50 mg of 24 h biomass, linoleic acid 0.2%, temperature 37 °C.

C18:0 - stearic acid, C18:1(9t) - elaidic acid, C18:1(9c) - oleic acid, C18:1(11c) - *cis*-vaccenic acid, C18:1(11t) - *trans*-vaccenic acid, C18:2 (9c,12c) - *cis,cis*-linoleic acid, CLA - conjugated linoleic acid, HY1 - 10-hydroxy-*trans*-12-octadecenoic acid.

Packard 5890 Series II Plus (Hewlett Packard, Little Falls, Delaware, USA). Column DB-23 (60 m × 0.25 mm × 0.25 μm) (J&W Scientific, Folsom, California, USA), injector 250 °C, split 1 μl, flame ionization detector 250 °C, carrier gas: helium, flow rate 20 cm.min<sup>-1</sup>.

Oven temperature program: 130 °C held for 1 min, then increased to 170 °C at a rate of 20 °C.min<sup>-1</sup>, then increased to 230 °C at a rate of 7 °C.min<sup>-1</sup>.

Standards of fatty acid methylesters: a mixture of fatty acids C4-C24 FAME Mix and linoleic acid methylester mix *cis/trans* were purchased from Supelco (distributor Lamda, Bratislava, Slovakia), conjugated linoleic acid methylester, *cis*-9-octadecenoic methylester, *trans*-9-octadecenoic methylester, *cis*-11-octadecenoic methylester, *trans*-11-octadecenoic methylester and ricinoleic acid methylester, all of them 99% pure, were purchased from Sigma (St. Louise, Missouri, USA).

## RESULTS AND DISCUSSION

The experiment was aimed on monitoring the ability of commercial probiotic culture *Lactobacillus acidophilus* La5 and mixed starter culture ABT-2 to produce conjugated acids. ABT-2 is mixture of strains: *Streptococcus thermophilus* ST-20Z, *Lactobacillus acidophilus* La5 a *Bifidobacterium* BB-12. According to bibliography [2-7, 30] it is assumed that CLA has an influence on human health. The first step of the experiment was to determine the ability of 24 h biomass to transform externally added free linoleic acid under defined conditions of the conversion and the following step was the determination of this ability in fermentation of low-fat milk.

Determination of ability to biosynthesize free linoleic acid was carried out in different conditions. Conversion run in the phosphate buffer (pH 6.5) or in MRS medium. Although after 56 h of conversion in the phosphate buffer with addition of bovine serum albumin of the reaction mixture contained 10-hydroxy-*trans*-12-octadecenoic acid (HY1), reactions leading to the formation of CLA were not running. After the addition of non-ionic detergent Tween 80, the use of which in food is permitted by the Food Codex of the Slovak Republic, CLA was found in the studied medium. CLA was not determined in biomass of cells before conversion (background), even though linoleic acid occurred in the extract of cell lipids. These results support the notion that the conversion reaction runs only on the free linoleic acid. After the addition of free linoleic acid and non-ion detergent

Tween 80 to the conversion medium, CLA was determined in the dry matter of cells in concentrations of 2.5–2.8 mg.g<sup>-1</sup>. Results obtained are shown in Table 1A for mixed starter culture ABT-2 and in Table 1B for *L. acidophilus* La5.

These results proved the ability of tested cultures to biosynthesize CLA from externally added linoleic acid. Considering these findings, next experiments were focused on increasing the CLA content in the fermented product.

Low-fat milk (content of fat 0.5%) was selected as a matrix for study of fermentation. The content of CLA that naturally occurs in milk, was determined to reach the values of 4.8–5 mg.g<sup>-1</sup> of milk fat. Milk was then inoculated with 50 mg of 24 h biomass of tested cultures and 0.2% free linoleic acid and 0.4% Tween 80 were added as well. Stationary milk fermentation was run at 37 °C for 72 h. Changes in the CLA content were checked at 24 h intervals. It was discovered that the total content of CLA in milk increased during fermentation from 5 mg.g<sup>-1</sup> to 18.7 mg.g<sup>-1</sup> of fat. The results obtained are shown in Table 2.

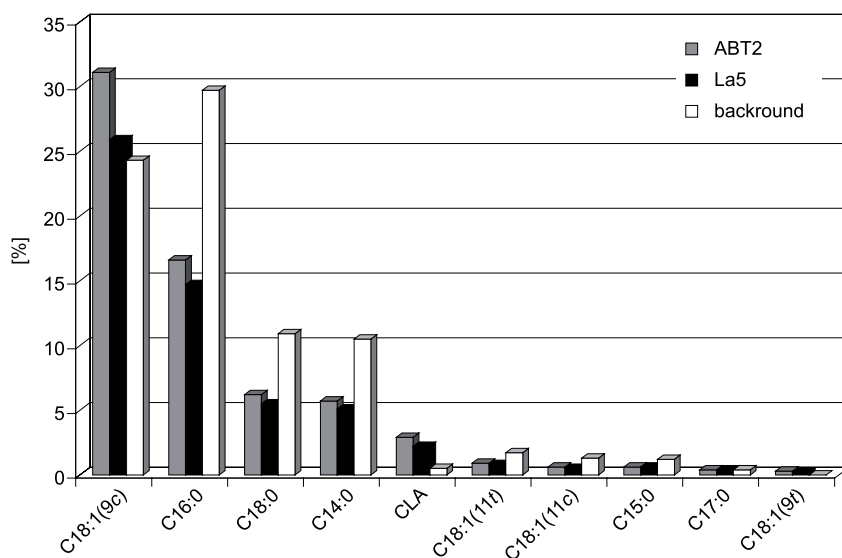
Only the isomer CLA1 was determined in non-fermented milk. Amount of CLA1 increased by 3.1 mg.g<sup>-1</sup> of fat during fermentation. Other isomers were also formed. These were CLA2 (6.2 mg.g<sup>-1</sup> of fat) and CLA3 (4.4 mg.g<sup>-1</sup> of fat). Isomers CLA1, CLA2 and CLA3 accounted for approximately 43, 33 and 24% of total amount of determined CLA, respectively. The presence of ricinoleic acid, in the amount of 0.9–2.0 mg.g<sup>-1</sup> of fat, was also found in lipid extract. This acid did not occur in the non-fermented sample. This supports the suggestion that ricinoleic acid could be the precursor of CLA [31]. The presence of this acid was not determined in every experiment and therefore this finding demands further study and explanation of kinetics of CLA production.

Considering the results shown in Table 2, the effect of 72 h fermentation on milk fat composi-

**Tab. 2.** The kinetics of the production of CLA during the fermentation of low-fat milk enriched with 0.2% free linoleic acid by *L. acidophilus* La5.

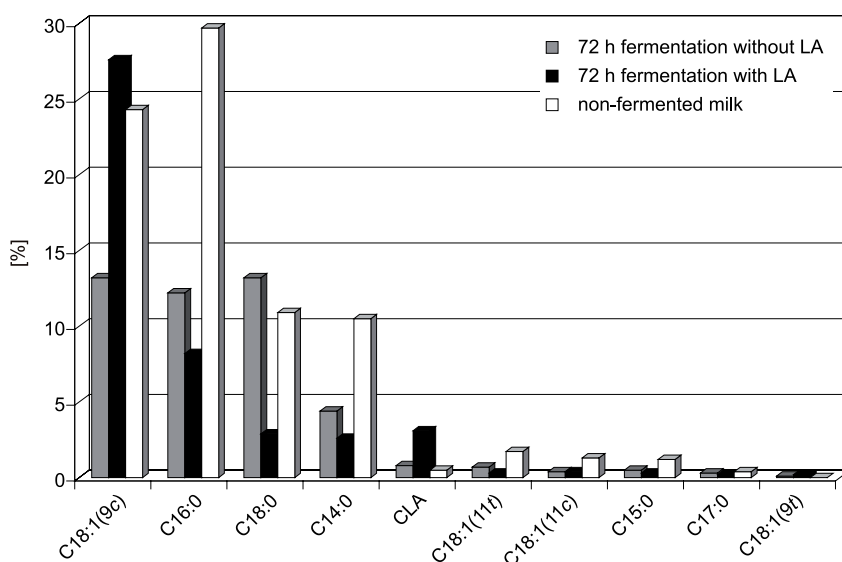
Time [h]	Fatty acid [μg.g <sup>-1</sup> of fat]			
	CLA1	CLA2	CLA3	ΣCLA
0	5.0	–	–	5.0
24	4.3	2.0	1.1	7.4
48	6.4	3.9	1.9	12.2
72	8.1	6.2	4.4	18.7

CLA - conjugated linoleic acid. CLA1 - *cis* 9, *trans* 11 CLA, CLA2 - *cis* 10, *trans* 12 CLA, CLA3 - *trans* 11, *trans* 13 CLA.



**Fig. 1.** Influence of the fermentation by strain *Lactobacillus acidophilus* La5 and mixed starter culture ABT-2 on the fat composition of milk with addition 0.2% free linoleic acid.

C18:1(9c) - oleic acid, C16:0 - palmitic acid, C18:0 - stearic acid, C14:0 - myristic acid, CLA - conjugated linoleic acid, C18:1(11t) - *trans*-vaccenic acid, C18:1(11c) - *cis*-vaccenic acid, C15:0 - pentadecanoic acid, C17:0 - heptadecanoic acid, C18:1(9t) - elaidic acid.



**Fig. 2.** Influence of an addition of 0.2% free linoleic acid on the fatty acid profile of fermented milk by strain *L. acidophilus* La5.

C18:1(9c) - oleic acid, C16:0 - palmitic acid, C18:0 - stearic acid, C14:0 - myristic acid, CLA - conjugated linoleic acid, C18:1(11t) - *trans*-vaccenic acid, C18:1(11c) - *cis*-vaccenic acid, C15:0 - pentadecanoic acid, C17:0 - heptadecanoic acid, C18:1(9t) - elaidic acid.

tion was further studied. Results obtained from fermentation of milk are shown in Fig. 1.

The increase in CLA content was effected by fermenting the milk by both cultures; at the same time the decrease in content of unsaturated acids (palmitic and stearic acid) was observed. Content of oleic acid increased from 23.4% to 25.9% (La5) and from 23.4% to 31.1% (ABT-2). The content of CLA determined in the milk fermented by La5 and by ABT-2 was 2.2% and 2.9%, respectively. It is possible that the higher amount of CLA determined in ABT-2 fermented milk is caused by the fact, that this starter culture is a mixed culture and contains strains *Lactobacillus acidophilus* La5 a *Bifidobacterium lactis* Bb12. Both these strains are known to be producers of CLA [19].

*fidobacterium lactis* Bb12. Both these strains are known to be producers of CLA [19].

A comparison of milk with and without addition of free linoleic acid fermented by strain La5 is given in Fig. 2. The content of oleic acid decreased after fermentation from 24.3% to 13.2% in milk with the addition of free linoleic acid and increased from 24.3% to 27.2% in milk without addition of free linoleic acid. The presence of elaidic acid was determined in every sample of fermented milk. This acid did not occur in milk before fermentation. According to authors [32], in acidic conditions (pH under 5.0) the stearic acid is formed from the intermediate, which is the elaidic



acid. This reaction mechanism could explain the presence of elaidic acid in fermented milk, because pH dropped below 4.2 during the fermentation.

## CONCLUSIONS

Studied commercial probiotic cultures La5 and ABT-2 are able to produce conjugated linoleic acid. The presence of free linoleic acid is a necessary prerequisite for the conversion reaction producing CLA after 48 h of fermentation. During fermentation of low-fat milk with addition of free linoleic acid the amount of unsaturated fatty acids decreases and the amount of unsaturated acids increases. Thus content of conjugated linoleic acid increased from 0.5% to 2.2% by strain *L. acidophilus* La5 and from 0.5% to 2.9% by mixed starter culture ABT-2. It was discovered that only isomer CLA1 occurs in the primary non-fermented milk, whereas isomers CLA2 and CLA3 were formed during fermentation of milk that was enriched with 0.2% of free linoleic acid.

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