Composition of conjugated linoleic acid isomers formed by *Lactobacillus* and *Bifidobacterium* spp. in conversion media

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Summary
The ability to produce conjugated linoleic acid (CLA) by bacteria *Lactobacillus plantarum*, *Lactobacillus acidophilus* and *Bifidobacterium lactis* was studied. In conversion media with neutral pH containing the detergent Tween 80, CLA was produced at 0.3 $mg \cdot g^{-1}$ – 0,5 $mg \cdot g^{-1}$ (dry mass). Presence of Tween 80 was essential for conversion of linoleic acid to CLA. Also, pH of the conversion medium affected CLA contents and the proportion of individual isomers (*cis*-9, *trans*-11 CLA, *trans*-10, *cis*-12 CLA, *trans*-9, *trans*-11 CLA). At pH lower than 6.5, contents of CLA isomers ranged between 0.04 $mg \cdot g^{-1}$ and 0.44 $mg \cdot g^{-1}$ (dry mass). At pH lower than 6.5, more *trans*-10, *cis*-12 isomer and also *trans*-9, *trans*-11 isomer was formed. Isomer *trans*-9, *trans*-11 was formed probably at isolation of fatty acids from the biomass and/or at their esterification.

Keywords
conjugated linoleic acid; *Lactobacillus*; *Bifidobacterium*; pH; isomer; Tween 80

Conjugated linoleic acid (CLA) is the name of 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, they are 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 biologically active forms. An increasing interest in CLA has been attributed to its potential health benefits such as anticarcinogenic, antiatherogenic, antidiabetic and antiadipogenic effects [2–14]. Its role in vitamin A metabolism [15], bone forming [16] and immune response [5, 17] has also been reported. In addition to the increased interest in the possible physiological effects on humans following CLA consumption, there has been a concomitant increase in interest in the isolation of novel human-derived or dairy starter cultures with the ability to produce the bioactive fatty acid [18–24].

The dietary sources of CLA are milk fat, natural and processed cheeses, meat products and vegetable oils. Animal sources are richer in CLA than are plant sources [25]. Dairy products are the principal dietary source of CLA (0.55–9.12 $mg \cdot g^{-1}$ fat), but the current human intake is less than the daily intake recommended for optimal beneficial effects [26]. The level of CLA in dairy products may vary due to different processing parameters, such as the heat treatment during pasteurization and starter cultures used [27, 28].

It has been reported that certain starter cultures may increase the CLA contents in cheese and yoghurt. Ability to produce CLA from free linoleic acid occurs in different bacterial strains. Studies showed that this ability occured in *Propionibacterium freudenreichii* (CCRC 11076), *Lactobacillus acidophilus* (CCRC 14079, 1.1854), *L. casei* (Bs5, Bs7), *L. lactis* (CCRC 12586), and *L. lactis* subsp. *lactis* (CCRC 10791) [27–33]. This work studies the possibility of formation of CLA in cultivation media fortified with linoleic acid using *Lactobacillus* and *Bifidobacterium* strains.

MATERIALS AND METHODS

Bacterial strains

L. acidophilus 405*, L. acidophilus* 984*, L. plantarum* 198 and *L. plantarum* 195 were provided

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by the collection Laktoflóra (Praha, Czech Republic). Commercial strain *Bifidobacterium lactis BB12* was purchased from Christian Hansen (Hørsholm, Denmark).

Cultivation

Strains of *L. plantarum* were cultivated in de Man-Rogosa-Sharpe (MRS) medium (Biomark Laboratories, Pune, India) for 24 h at 30 °C. Strains of *L. acidophilus* were cultivated in MRS medium for 24 h at 37 °C. Strains of *Bifidobacterium* and starter culture strains were cultivated stationary for 24 h at 37 °C in 100 cm^3 of Viande-Foie medium with the following composition: 5 g yeast autolysate, 0.5% Viande-Foie broth (Imuna Pharm Holding, Šarišské Michaľany, Slovakia), 0.25 g of liver extract, 2% glucose and 0.02% L-cysteinhydrochloride.

Conversion studies

Conversion was studied at different conditions:

1. Bacterial cells were washed with physiological solution and centrifuged (1950 *g*, 5 min) and then were added to the conversion medium.
2. Conversion in the buffer solution: 100 mg of washed cells were added to 5 cm^3 of the conversion solution, consisting of the potassium phosphate buffer (pH 6.5) with 0.2% free linoleic acid (99%; Sigma, St. Louis, Missouri, USA), 0.001% bovine serum albumin (BSA) (Imuna Pharm Holding).
3. Conversion in the buffer solution with addition of Tween 80: same as 2. with 0.4% Tween 80 (Imuna Pharm Holding).
4. Conversion in the buffer solution with complex linoleic acid-albumine: 100 mg of washed cells were added to 5 cm^3 of the conversion solution, consisting of the potassium phosphate buffer (pH 6.5) and 0.4% linoleic acid-albumin from bovine serum albumin (Sigma).
5. Conversion in MRS medium: 100 mg of washed cells were added to 5 cm^3 of MRS medium with addition of 0.01% L-cystein (Imuna Pharm Holding), 0.4% Tween 80 and 0.2% free linoleic acid.

Conversions were carried out at 37 °C under the nitrogen gas for 56 h.

Extraction of lipids from cells and esterification (direct analysis)

After the conversion, cells were centrifuged (1950 *g*, 5 min). The supernatant was discarded, 1 cm^3 of dichloromethane with internal standard (0.001% heptadecenoic acid) and 2 cm^3 of methanolic HCl were added to the centrifuged cells, and thoroughly mixed. After 20 min of incubation under argon at 50 °C in capped tubes, 1 cm^3 of distilled water was added and methylesters were extracted to 4 cm^3 of *n*-hexane. The extract was dried with anhydrous sodium sulphate and evaporated. The residue was dissolved in a small amount of *n*-hexane and analysed by gas chromatography (GC).

Modified esterification method (Christopherson & Glass [34], complete extraction)

A sample of lipids was dissolved in 1 cm^3 of *n*-hexane. A volume of 0.1 cm^3 of the transesterification agent (sodium methanolate in benzene) was added, mixed and left at the laboratory temperature for 20 min. Then, 0.5 cm^3 of methanolic HCl was added and thoroughly mixed until the mixture was colourless. When needed, the amount of methanolic HCl added was increased so that the mixture became colourless. Then the mixture was left at the laboratory temperature for 45 min. The upper hexane layer containing methylesters of fatty acids was transferred to a vial and analysed by GC.

GC for the analysis of fatty acid methylesters

Gas chromatograph: Hewlett Packard 5890 Series II Plus (Hewlett Packard, Wilmington, Delaware, USA).

Column: DB-23 (Agilent Technologies, Santa Clara, California, USA) – 60 m × 0.25 mm × × 0.25 μm.

Analysis conditions: pressure 230 kPa at 230 °C, velocity 24 $cm \cdot s^{-1}$, inlet temperature > 250 °C, split ratio 20 : 1.

Gas: helium.

Injector: Hewlett Packard 7673 (Hewlett Packard), 1 μl.

Oven: 130 °C (1 min), 20 $°C \cdot min^{-1}$ to 170 °C (0 min), 2 $°C \cdot min^{-1}$ to 235 °C (4.4 min).

Detector: flame ionization detector, operating at 250 °C.

GC-MS for the analysis of 10-hydroxy-*trans*-12-octadecenoic acid (HY)

Gas chromatograph: Agilent GC 6890 (Agilent, Waldbronn, Germany).

Column: DB-1 (Agilent Technologies) – 30 m × × 0.32 mm × 1 μm.

Analysis conditions: pressure 60 kPa, velocity 31 $cm \cdot s^{-1}$, initial flow 1.8 $ml \cdot min^{-1}$, inlet temperature 280 °C, split ratio 5 : 1, split flow 9.2 $ml \cdot min^{-1}$, total flow 13.7 $ml \cdot min^{-1}$, saver flow 20.0 $ml \cdot min^{-1}$.

Gas: helium.

Injector: Hewlett Packard 7673, 1 μl.

Oven: 40 °C (0 min), 15 °C·min^{-1} to 240 °C (0 min).
Detector: mass selective detector, scan mode, mass 35.0–800.0, threshold: 100.

GC-MS for the analysis of CLA isomers

Gas chromatograph: Agilent GC 6890.
Column: HP-88 (Agilent Technologies) – 100 m × 0.25 mm × 0.2 μm.
Analysis conditions: presure 240 kPa, velocity 20 cm·s^{-1}, initial flow 1.2 ml·min^{-1}, inlet temperature 280 °C, split ratio 10:1, split flow 12.2 ml·min^{-1}, total flow 15.8 ml·min^{-1}, saver flow 20.0 ml·min^{-1}.
Gas: helium.
Injector: Hewlett Packard 7673, 1 μl.
Oven: 70 °C (1 min), 100 °C·min^{-1} to 160 °C (78 min), 25 °C·min^{-1} to 200 °C (5 min).
Detector: mass selective detector, SIM mode, ions mass 55, 67, 294, 296, 298, MS quad 150 °C, MS source 230 °C.

Independent peak identification of *cis*-9, *trans*-11 CLA and *trans*-10, *cis*-12 CLA

In order to confirm the identity of the peak of CLA isomers, the independent method of Blaško et. al. [35] was used.

RESULTS AND DISCUSSION

The aim of this study was to obtain data on the ability of selected lactic acid bacteria to form conjugated linoleic acid from externally added linoleic acid. Three different modes of application of linoleic acid to conversion medium were studied. From comparison of the profiles of the biomass that was subjected to conversion and the biomass which was not, it follows that the biomass was not capable of transforming linoleic acid to a conjugated system, even if the strain *Bifidobacterium lactis* BB12, which is known to produce conjugated linoleic acid [36] was used (Tab. 1).

The profile of fatty acids determined by GC-MS (Tab. 2) involved the product, which matched with hydroxy acid HY (10-hydroxy-*trans*-12-octadecenoic acid). This acid is, according to the Japanese authors [37], an intermediate of the bioconversion of linoleic acid to its conjugated form. The presence of the intermediate suggests the ability of the studied strains to form conjugated linoleic acid. HY was formed in course of the conversion in the buffer with pH 6.5. However, under these conditions, neither CLA was formed, nor *trans*-vaccenic acid considered to be another intermediate of the CLA conversion, was detected [37]. This finding leads to a suggestion, that HY is just an intermediate of the conversion and the finalization of the conversion is not possible unless the activator of the enzyme reaction is present. However, it was essential to find the conditions, at which the complete enzymatic isomerization reaction may run.

From the results in Tab. 2 it follows that even if free linoleic acid was added, the conversion to the conjugated form did not take place. The condition of the successful conversion was the presence of a detergent. When Tween 20, a mixture of a polysorbate of lauric acid (40%) and minor ingredients (myristemic acid, palmitic acid and stearic acid), was applied, the conversion did not run. In the case of the addition of Tween 80, a mixture of polysorbate of oleic acid (70%) and minor ingredients (linoleic acid, palmitic acid and stearic acid), traces of CLA were determined in the biomass.

The further interest was to study the role of the presence of an emulgator in the formation

Tab. 1. C18 fatty acid profile in the 24 h biomass.

Fatty acids [mg·g^{-1}]	Strain			
	BB 12	LA 405	LP 189	LA 984
C18:0	0.29 ± 0.08	0.27 ± 0.07	0.37 ± 0.10	0.29 ± 0.08
C18:1 (*t*-9)	–	–	–	–
C18:1 (*c*-9)	1.23 ± 0.49	0.44 ± 0.17	1.46 ± 0.58	1.23 ± 0.49
C18:1 (*t*-11)	–	–	–	–
C18:2 (*c*-9,*c*-12)	4.94 ± 1.95	3.39 ± 1.34	2.03 ± 0.80	4.94 ± 1.95
CLA	–	–	–	–

Cultivation: stationary, in 100 cm^3 of medium, under optimal conditions for individual strains.
Fatty acids are expressed in mg of fatty acids per g of cell dry mass. Values are means ± standard deviation (n = 9).
C18:0 – stearic acid, C18:1 (*t*-9) – elaidic acid, C18:1 (*c*-9) – oleic acid, C18:1 (*t*-11) – *trans*-vaccenic acid, C18:2 (*c*-9,*c*-12) – *cis*-9, *cis*-12-linoleic acid, CLA – conjugated linoleic acid, BB – *Bifidobacterium lactis*, LA – *Lactobacillus acidophilus*, LP – *Lactobacillus plantarum.*

Tab. 2. Influence of the addition of Tween 80 on C18 fatty acid profile in the biomass after the conversion of linoleic acid to conjugated linoleic acid.

Fatty acids [mg·g^{-1}]	Strain							
	BB 12		LA 405		LP 189		LA 984	
	–T	+T	–T	+T	–T	+T	–T	+T
C18:0	0.21 ± 0.07	0.47 ± 0.15	1.43 ± 0.46	1.57 ± 0.51	0.29 ± 0.09	0.47 ± 0.15	2.05 ± 0.67	1.40 ± 0.46
C18:1 (*t*-9)	–	–	–	–	–	–	–	–
C18:1 (*c*-9)	1.09 ± 0.47	6.43 ± 2.75	1.32 ± 0.56	7.38 ± 3.16	1.44 ± 0.61	154.90 ± 66.30	2.84 ± 1.21	9.30 ± 3.98
C18:1 (*t*-11)	–	0.24 ± 0.09	–	0.10 ± 0.04	–	0.10 ± 0.04	–	0.32 ± 0.21
c-9,*t*-11CLA	–	0.15 ± 0.02	–	0.27 ± 0.17	–	0.47 ± 0.3	–	0.32 ± 0.02
t-10,*c*-12 CLA	–	0.13 ± 0.01	–	0.25 ± 0.15	–	0.49 ± 0.29	–	0.31 ± 0.03
t-9,*t*-11 CLA	–	0.19 ± 0.03	–	0.34 ± 0.21	–	0.35 ± 0.21	–	0.54 ± 0.03
HY	5.76 ± 3.60	4.43 ± 0.66	3.58 ± 2.24	1.55 ± 1.00	98.72 ± 61.67	143.35 ± 89.60	5.94 ± 3.71	0.32 ± 0.21

Conditions: 56 h conversion in the buffer (pH 6.5), 0.2% linoleic acid, 0.001% BSA, 0.4% Tween 80.
Fatty acids are expressed in mg of fatty acids per g of cell dry mass. Values are means ± standard deviation (n = 9).
BB – *Bifidobacterium lactis*, LA – *Lactobacillus acidophilus*, LP – *Lactobacillus plantarum*. –T – without addition of Tween 80, +T – with addition of Tween 80.
C18:0 – stearic acid, C18:1 (*t*-9) – elaidic acid, C18:1 (*c*-9) – oleic acid, C18:1 (*t*-11) – *trans*-vaccenic acid, *c*-9,*t*-11 CLA- *cis*-9, *trans*-11-conjugated linoleic acid, *t*-10,*c*-12 CLA – *trans*-10, *cis*-12-conjugated linoleic acid, *t*-9,*t*-11 CLA – *trans*-9, *trans*-11-linoleic acid, HY – 10-hydroxy-*trans*-12-octadecenoic acid.

of conjugated fatty acids. It was observed that the presence of Tween 80 in the conversion medium was important for the conversion, while in the presence of Tween 20, the conversion did not run. Interestingly, the conversion ran also when linoleic acid was added to the conversion medium in the form of a complex with BSA (Fig. 1).

Taking into consideration these observations, it can be assumed that, in media that do not contain substances facilitating the access of a substrate to the active center of the enzyme linoleate isomerase, the conversion is not possible. The results obtained suggest that the role of Tween is not to make the substrate accesible to linoleate isomerase, but Tween 80 and BSA are the activators of the enzyme reaction.

The conversion was studied also in MRS medium (Tab. 3). It was found that, at a lower pH, a lower amount of CLA was formed. This fact is in correlation with the previously published optimal pH of the conversion [37].

The presence of the isomer with an unknown position of the conjugated bonds was determined in the fatty acid profile by GC-MS as *trans*-9,*trans*-11 isomer. We were interested whether the isomer was generated due to the presence of the enzymes responsible for the isomerization or as a consequence of a non-enzymatic reaction. It was observed that the individual isomers were formed in different ratios (Fig. 2). At a neutral pH, *trans*-10, *cis*-12 isomers were more abundant than *cis*-9, *trans*-11 isomers, and the percentage of *trans*-9, *trans*-11 CLA was lower. This lead us to an assumption that the transformation was not carried out by the sole enzyme linoleate isomerase, but it was carried out by several enzymes with various optimal pH conditions.

However, the results might have been influenced by the esterification method used. At acidic methanolic conditions of the esterification at 50 °C, the higher amount of *trans*-9, *trans*-11 isomer was detected, when compared to esterification at less harsh conditions with sodium benzoate at laboratory temperature [34]. We therefore assume that the non-enzymatic re-arrangement to the energetically favourable position took place in the extreme conditions. However, the same esterification method was used also with fatty acids. This fact was omitted when expressing the results, because the method used does not enable to distinguish whether the isomer *trans*-9, *trans*-11 was formed as a result of the esterification method used or as a result of the presence of the enzyme

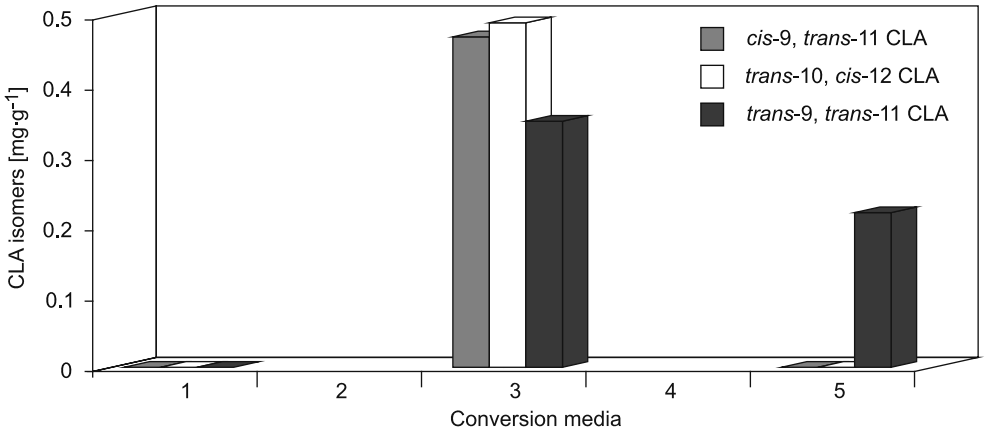

Fig. 1. The influence of substrate addition on the C18 fatty acid profile in the biomass of *L. plantarum* LP 189 after the conversion of linoleic acid.
Conditions: 56 h conversion in the buffer (pH 6.5). Fatty acids are expressed in mg of fatty acids per g of cell dry mass.
Substrate addition: 1 – 0.4% Tween 80, 2 – 0.4% Tween 20, 3 – 0.4% Tween 80 + linoleic acid, 4 – 0.4% Tween 20 + linoleic acid, 5 – 0.4% BSA + linoleic acid.

responsible for the isomerization at the position 9, 11. The instability of the studied isomers under the acidic conditions was observed when the known amount of CLA was added to the biomass and different methods of isolation and esterification were used. By repeated analysis, a higher content of *trans*-9, *trans*-11 isomer was detected using the direct analysis method (19.9 mg·g^{-1} of fat) than by the complete extraction method (45.6 mg·g^{-1} of fat).

Regarding the amount of *trans*-9, *trans*-11 isomers created in the drastic conditions, it may be assumed that these are formed from *trans*-10, *cis*-12 isomer and so the actual amount of *trans*-10, *cis*-12 in MRS under conversion conditions is lower than at pH 6.5. This supports the theory that various CLA isomerases with different pH optima are involved in the studied process.

During the conversion in MRS medium, elaidic acid (C18:1, *trans*-9) was found to be formed in the biomass. The presence of this acid was not detected in the biomass after the conversion in the buffer with an optimal pH. This finding corresponds with the results published by MORTIMER [38] stating

Tab. 3. C18 fatty acid profile in the cells after the conversion of linoleic acid to conjugated linoleic acid in MRS.

Fatty acids [mg·g^{-1}]	Strain			
	BB 12	LA 405	LP 189	LA 984
	pH 5.1	pH 3.7	pH 3.6	pH 3.6
C18:0	0.24 ± 0.07	0.39 ± 0.11	0.21 ± 0.06	0.37 ± 0.10
C18:1 (*t*-9)	0.10 ± 0.04	0.20 ± 0.08	0.13 ± 0.06	0.13 ± 0.06
C18:1 (*c*-9)	5.20 ± 1.89	6.49 ± 2.36	7.00 ± 2.53	6.23 ± 2.27
C18:1 (*t*-11)	0.08 ± 0.04	0.14 ± 0.07	0.04 ± 0.02	0.13 ± 0.06
c-9,*t*-11 CLA	0.08 ± 0.04	0.15 ± 0.08	0.04 ± 0.03	0.15 ± 0.07
t-10,*c*-12 CLA	0.17 ± 0.10	0.13 ± 0.10	0.06 ± 0.03	0.13 ± 0.08
t-9,*t*-11 CLA	0.16 ± 0.07	0.13 ± 0.11	0.44 ± 0.10	0.13 ± 0.10

Conditions: 56 h conversion in MRS medium with 0.4% Tween 80 and 0.2% linoleic acid with the initial pH 6.5. 37 °C. 100 mg of washed cells. Rotary shaker.
Fatty acids are expressed in mg of fatty acids per g of cell dry mass. Values are means ± standard deviation (n = 9).
BB – *Bifidobacterium lactis*, LA – *Lactobacillus acidophilus*, LP – *Lactobacillus plantarum.*
C18:0 – stearic acid, C18:1 (*t*-9) – elaidic acid, C18:1 (*c*-9) – oleic acid, C18:1 (*t*-11) – *trans*-vaccenic acid, *c*-9,*t*-11 CLA- *cis*-9, *trans*-11-conjugated linoleic acid, *t*-10,*c*-12 CLA – *trans*-10, *cis*-12-conjugated linoleic acid, *t*-9,*t*-11 CLA – *trans*-9, *trans*-11-linoleic acid.

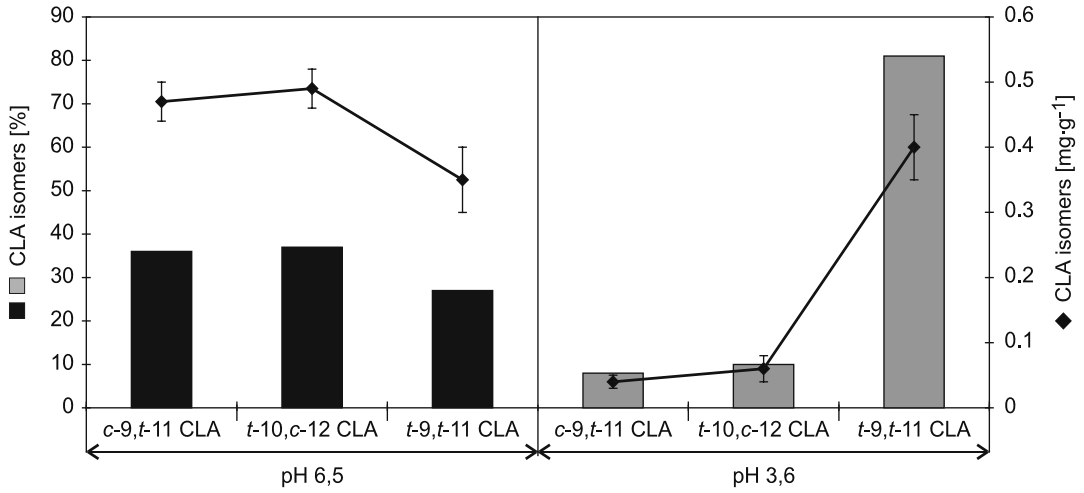

Fig. 2. Comparison of the contents of CLA isomers in the cell biomass after the conversion at pH 6.5 and pH 3.9 by strain *L. plantarum* 189.
Fatty acids are expressed in mg of fatty acids per g of cell dry mass. Values are means ± standard deviation (n = 9).
c-9,t-11 CLA - *cis*-9, *trans*-11-conjugated linoleic acid, t-10,c-12 CLA - *trans*-10, *cis*-12-conjugated linoleic acid, t-9,t-11 CLA - *trans*-9, *trans*-11-conjugated linoleic acid.

that, at pH below 7.0 during the cultivation of the bacterial strain *Pseudomonas* sNRRL 3266, *trans* acids were preferentially formed in the course of isomerization of oleic acid.

trans-Vaccenic acid (C18:1, *trans*-11) acid, which is assumed to be an intermediate of the conversion of conjugated acids to stearic acid, was detected in the biomass after the conversion at pH 6.5 [39]. As can be seen in Tab. 2 and Tab. 3, the amount of stearic acid was lower in acidic conditions than at the optimal pH, but the amount of oleic acid was higher in the acidic conditions than at a neutral pH. The equilibrium of the enzymatic reaction is so shifted in favour of formation of oleic acid in the acidic conditions. From the comparison of the amount of CLA produced at pH 6.5 and pH 3.6, it is apparent that the optimal pH for CLA production by strain *L. plantarum* 189 lied in the neutral region. It was found that, in the acidic conditions at pH 3.6, a higher proportional amount of *trans*-9, *trans*-11 isomer was produced compared to the neutral conditions of conversion, pH 6.5 (Fig. 2). This finding affirms the assumption that *trans*-9, *trans*-11 isomer is produced as a result of the instability of other CLA isomers through the effect of the acidic environment of the conversion, as this encourages the isomerization. The highest ability of the conversion of free linoleic acid was showed by strain *L. plantarum* 189, which seems to be a suitable strain for optimization of conditions for the conversion of linoleic acid to the conjugated form.

CONCLUSIONS

The experiments confirmed the ability of strains from the genera *Lactobacillus* and *Bifidobacterium* to produce conjugated linoleic acid under certain conditions. The presence of free linoleic acid together with the detergent Tween 80 was a prerequisite of the conversion. The addition of Tween 20 had no positive influence on CLA production. When free linoleic acid was not added to the conversion medium, the conversion reaction did not run.

The pH of the medium had an influence on the amount and the proportions of CLA isomers. The production of CLA was higher in neutral conditions in a buffer solution. In the acidic conditions, more *trans*-10, *cis*-12 isomer was formed. The unknown isomer identified by GC-MS as *trans*-9, *trans*-11 isomer, was most likely an artefact formed as a result of acidic methanolic conditions during the isolation and esterification of fatty acids. Optimization of the conditions of the production of fermented foods with increased contents of CLA should involve externally added effectors, but the aspect of organoleptic properties of the final product should be taken into account.

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