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Production of conjugated dienoic and trienoic fatty acids by lactic acid bacteria and bifidobacteria

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ABSTRACT

The production of conjugated linoleic (CLA) and linolenic (CLNA) acids by lactic acid bacteria (LAB) and bifidobacteria strains was assessed. The initial selection was based on optical density measurements by the UV method and GC analysis. The LAB strains showed a lower ability to synthesise CLA and CLNA. Yet, two *Lactobacillus sakei* strains were the best producers. Moreover, the production of CLA and CLNA by *Lactobacillus mucosae* is reported here for the first time. In contrast, bifidobacteria strains were more efficient to synthesise CLA and CLNA. Among these, the *t9, t11*-CLA was the main isomer, which is a potent anticarcinogenic compound. All tested strains produced the *c9,t11,c15*-CLNA isomer. High substrate concentrations of LA and LNA negatively affected the conversion rate of CLA and CLNA by *Bifidobacterium animalis* subsp. *lactis* INL2, even though the bacterial growth was not affected.

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1. Introduction

The conjugated linoleic (CLA) and linolenic (CLNA) acids are both defined as bioactive compounds that include a mixture of positional and geometric isomers of linoleic (LA, C18:2) and α-linolenic (LNA, C18:3) acids, respectively. The CLA is formed as an intermediate product of the biohydrogenation of dietary fat in the rumen and by the Δ-9 desaturase activity in the mammary gland of ruminants. Among the isomers, *c9,t11* and *t10,c12* are the most important due to their biological action. In addition, *t9,t11* is also defined as an anticarcinogenic compound against tumour cells (Coakley et al., 2006).

Isomers of CLNA are mainly present in the oils obtained from certain seeds. For instance, the pomegranate seed oil contains punicic acid (*c9,t11,c13*-CLNA; >70 g/100 g of oil); the tung seed oil is rich in α-eleostearic acid (*c9,t11,t13*-CLNA; Özgül-Yücel, 2005; Shinohara et al., 2012; Suzuki et al., 2001);

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and the jacarandá (*Jacaranda mimosifolia*) seed oil contains high amounts of jacaric acid (*c8,t10,c12*-CLNA) (36 g/100 g of oil; Gasmi & Thomas Sanderson, 2013). Likewise, lower amounts of CLNA are also present in the milk and meat of ruminants.

Different biological functions are attributed to the conjugated fatty acids, most importantly, their anticarcinogenic activity. Despite this well-established activity for both CLA and CLNA (Bhattacharya, Banu, Rahman, Causey, & Fernandes, 2006; Chin, Liu, Storkson, Ha, & Pariza, 1992; Gasmi & Thomas Sanderson, 2013; Tsuzuki, Tokuyama, Igarashi, & Miyazawa, 2004), the underlying mechanism remains uncertain. In addition, CLNA exhibits a stronger cytotoxic effect on tumour cells than the CLA isomers (Igarashi & Miyazawa, 2000).

The daily intake of CLA recommended to obtain biological effects on human health is approximately 1–3 g/day (Ip et al., 1994; MacDonald, 2000), while the recommended dose of CLNA is approximately 2–3 g/day (Shinohara et al., 2012). However, the usual human daily intake is lower than those values (Lin, Hung, & Cheng, 2005).

The most important sources of both conjugates for humans are milk and meat of ruminants. However, the levels of both conjugated fatty acids in these food products are significantly lower than the level required to obtain health benefits. The CLA content in milk is approximately 3 to 15 mg/g of fat (Van Nieuwenhove, Oliszewski, & González, 2009), whereas the CLNA content is even lower, reaching values from 0.3 to 0.5 mg/g of fat (Van Nieuwenhove, Terán, & González, 2012). Hence, several researchers are trying to increase the CLA and CLNA levels in the food through diet supplementation to animals or by using selected cultures to produce conjugated fatty acids during fermentation processes. The ability to synthesise CLA was first discovered in the anaerobic ruminant bacteria *Butyrivibrio fibrisolvens* (Kepler, Hirons, McNeill, & Tove, 1966). However, certain strains of lactic acid bacteria (LAB), bifidobacteria and propionibacteria were thought to be CLA-producing microorganism (Coakley et al., 2003; Gorissen et al., 2010; Lee & Lee, 2009; Van Nieuwenhove, Cano, Chaia, & González, 2007). Nevertheless, only few studies reported the CLNA production by these microorganisms (Coakley et al., 2003, 2006; Gorissen et al., 2010; Park et al., 2012).

Recent studies have shown higher CLA levels in fermented dairy products after the addition of cultures. Rodrigues et al. (2012) determined higher CLA levels in probiotic and synbiotic cheeses manufactured with bacteria able to produce CLA *in vitro* (Rodríguez-Alcalá et al., 2011). Moreover, Taboada et al. (2015) determined an increased CLA content in goat cheese manufactured with autochthonous strains.

The administration of bacteria able to produce CLA at the intestinal level is important for human health (Lee et al., 2007; Wall et al., 2009). Therefore, the selection of bacteria able to synthesise CLA and/or CLNA *in vitro* is required as a first step to assess the potential impact on their production during food fermentation or at the intestinal level. The percentage of conversion and the isomer patterns depends on several factors such as the strain and culture conditions (e.g., temperature and substrate concentration).

The aims of this work were to evaluate the production of CLA and CLNA by different food-grade lactic acid bacteria (LAB) and bifidobacteria strains, and to determine the influence of the substrate concentration on the fatty acid production.

2. Materials and methods

2.1. Bacterial strains and growth conditions

A total of 64 food-grade LAB and bifidobacteria strains were used in this study. All of the employed bacteria could potentially be considered as probiotic strains or as starter cultures for meat or milk fermentation. Certain CRL strains were obtained from the Culture Collection of the Centro de Referencia para Lactobacilos (CERELA, Tucumán, Argentina), while other LABs belong to the Ecophysiology Technological Laboratory from the same institute (EFL). The bifidobacteria strains were provided by the INLAIN (UNL-CONICET, Santa Fe, Argentina).

The initial selection of strains (36 LABs and 4 bifidobacteria) was based on the CLA and/or CLNA production measured by the UV method, as shown in Table 1. The LAB strains were subcultured twice in MRS broth (Biokar Diagnostics, Allonne, Beauvais, France) before each experiment. Afterwards, 2% (v/v) of inoculum was cultured into MRS broth in the presence of each substrate; then, the cultures were incubated during 24 h at 37 °C, except for *Lactobacillus sakei* and *Lactobacillus curvatus*, which were cultured at 30 °C. The bifidobacteria strains were cultured in an MRS medium supplemented with 0.05% (v/v) of L-cysteine (Sigma-Aldrich, St. Louis, MO, USA; cys-MRS) and anaerobically incubated using Anaerocult A (Merck KGaA, Darmstadt, Germany) at 37 °C during 48 h. The counting of bifidobacteria was carried out in MRS-agar plates (Biokar Diagnostics). All of the strains were kept at –70 °C in an MRS medium supplemented with 25% of glycerol as cryoprotectant.

2.2. Fatty acid substrates

The substrates were prepared as stock solutions with a concentration of 25 mg/mL of linoleic or α-linolenic acid (99% pure; Sigma, St. Louis, MO, USA) dissolved in distilled water with 2% (v/v) Tween 80 (Merck). The fatty acid stock solutions were filtered-sterilised through a 0.45-μm Ministar filter (Sartorius AG, Goetting, Germany) and kept in the dark at –20 °C until use. Each substrate was initially added to the medium together with the inoculums. Cultures without the addition of substrates were used as controls and processed as independent samples. Furthermore, the MRS broth was LA/LNA-free.

2.3. Determination of CLA and CLNA with UV spectrophotometry

The initial selection of strains was based on its CLA and/or CLNA production measured with the UV method. The bacteria were inoculated at 2% (v/v) in the corresponding culture media with 100 μg/mL of LA or LNA as a substrate. Subsequently, the bacteria were tested for the production of CLA/CLNA with the UV method at $\lambda = 233$ nm, according to Xu et al. (2008). Then, 5 mL of growing culture were centrifuged (5000 × *g*, 10 min), the supernatant was mixed with 10 mL of isopropanol and the organic phase was dried using anhydrous Na_2SO_4. After the addition of 5 mL of hexane, the mixture was centrifuged at 3000 × *g* for 20 min. The amount of CLA or CLNA in the hexane phase was determined by measuring the absorbance at 233 nm with a spectrophotometer (VersaMax™ Tunable

Table 1 – Conjugated fatty acid production by UV method (233 nm).

Strain	Origin	Reaction Condition	CLA production	CLNA production
Lactobacilli				
L. acidophilus CRL44	Regional cheese	24 h, 37 °C	+	+
L. acidophilus CRL258	Commercial cheese	24 h, 37 °C	+	–
L. acidophilus CRL800	Unknown	24 h, 37 °C	+	–
L. acidophilus CRL1063	Cheese	24 h, 37 °C	+	–
L. casei CRL 66	Commercial yoghurt	24 h, 37 °C	+	–
L. casei CRL117	Regional cheese	24 h, 37 °C	+	–
L. casei CRL238	Roquefort cheese	24 h, 37 °C	–	+
L. casei CRL239	Cheese	24 h, 37 °C	+	–
L. curvatus CRL81	Regional cheese	24 h, 30 °C	+	+
L. curvatus CRL1284	Meat	24 h, 30 °C	+	–
L. curvatus CRL1465	Fermented sausage	24 h, 30 °C	+	–
L. curvatus CRL1629	Salami	24 h, 30 °C	+	–
L. fermentum CRL574	Child faeces	24 h, 37 °C	+	–
L. fermentum CRL1446	Goat cheese	24 h, 37 °C	+	–
L. fermentum EFL2	Cheese	24 h, 37 °C	+	–
L. fermentum EFL3	Cheese	24 h, 37 °C	+	–
L. mucosae CRL573	Child faeces	24 h, 37 °C	+	+
L. murinus EFL4	Unknown	24 h, 37 °C	+	–
L. murinus EFL5	Unknown	24 h, 37 °C	+	–
L. paracasei subsp. *paracasei* CRL577	Child faeces	24 h, 37 °C	+	–
L. paracasei subsp. *paracasei* CRL731	Child faeces	24 h, 37 °C	+	–
L. pentosus CRL1630	Salami	24 h, 37 °C	+	–
L. plantarum CRL41	Regional cheese	24 h, 37 °C	–	+
L. plantarum CRL100	Regional cheese	24 h, 37 °C	+	+
L. plantarum CRL355	Soil and mountain flora	24 h, 37 °C	–	+
L. plantarum CRL1920	Chicha	24 h, 37 °C	+	–
L. plantarum CRL1935	Cheese	24 h, 37 °C		
L. plantarum EFL6	Cheese	24 h, 37 °C	+	–
L. plantarum EFL7	Unknown	24 h, 37 °C	+	–
L. reuteri EFL8	Cheese	24 h, 37 °C	+	–
L. sakei CRL1424	Vacuum packaged meat	24 h, 30 °C	+	–
L. sakei CRL1468	Fermented sausage	24 h, 30 °C	+	+
L. sakei CRL1470	Fermented sausage	24 h, 30 °C	+	+
L. salivarius EFL9	Unknown	24 h, 37 °C	+	–
Lactococci				
Lactococcus lactis biovar-*diacetylactis* CRL967	Cheese	24 h, 37 °C	+	+
Lactococcus lactis biovar-*diacetylactis* CRL1061	Cheese	24 h, 37 °C	+	+
Bifidobacteria				
B. animalis subsp. *lactis* INL4	Human breast milk	48 h, 37 °C	+	+
B. animalis subsp. *lactis* INL2	Human breast milk	48 h, 37 °C	+	+
B. dentium LM8a	Human breast milk	48 h, 37 °C	+	+
B. longum LM7a	Human breast milk	48 h, 37 °C	+	+

(–): no production; (+): production of conjugated fatty acid; $n = 3$; EFL: Ecophysiology Technological Laboratory.

Microplate Reader, Molecular Devices, Radnor, PA, USA). A standard curve of pure c9,t11-CLA was obtained. The pomegranate oil was used as the standard for the CLNA curve because it has 77% of α-eleostearic acid (data not shown). To avoid interferences in the optical density (OD) measurements of this sensitive method, a low substrate concentration (100 μg/mL) was used. All of the organic solvents were HPLC-grade (Sintorgan, Buenos Aires, Argentina).

2.4. *Assessment of the CLA and/or CLNA production by the microbial community with gas chromatography*

According to the conjugated fatty acid production, certain strains were selected for the CLA/CLNA-isomer production and assessed by gas chromatography. After two subcultures, the strains were transferred with a concentration of 2% (v/v) to a broth (10 mL) with each substrate at a final concentration of

500 μg/mL and incubated during 24–48 h at 30–37 °C. Then, samples were taken at the beginning (time 0) and at the end of the incubation period (24 h for LABs and 48 h for bifidobacteria, respectively), which was the time where they reached their exponential growth phase. The lipid extraction was carried out from the supernatant and pellet, which was obtained after the centrifugation of cultures at 5000 × *g* for 15 min.

2.5. *Fatty acid analysis*

The total lipids from the pellet and supernatant were extracted following the methods of Folch, Lees, and Sloane Stanley (1957) using a chloroform/methanol (2:1, v/v). The lipids were hydrolysed with the addition of 3 mL of 0.9% (w/v) NaOH in methanol at 50 °C for 35 min. The fatty acids were methylated using 4% (v/v) HCl in methanol at 60 °C for 20 min, then extracted with hexane and evaporated under a nitrogen stream. The fatty acid methyl esters were dissolved in hexane and injected into an Agilent Technologies (Palo Alto, CA, USA) gas chromatograph (model 6890N) equipped with a flame ionization detector, an automatic injector (model 7683) and an HP-Sil88 (100 m × 0.25 mm × 0.2 μm) GC capillary column. Then 1 μL of fatty acid methyl esters dissolved in hexane was injected in splitless mode. The gas chromatograph conditions were as follows: injector temperature, 255 °C; initial oven temperature, 75 °C, which was increased to 165 °C at 8 °C/min and held for 25 min; then, the temperature was increased to 210 °C at a rate of 10 °C/min and held for 15 min; finally, it was increased to 240 °C at a rate of 15 °C/min and held for 3 min. The total time was 64.75 min and the temperature detector was set at 255 °C. Nitrogen was used as the carrier gas at a flow rate of 18 mL/min and at 38 psi. As an internal standard, pentadecanoic acid (C15:0) was added to the medium at a final concentration of 500 μg/mL. The fatty acids were identified by comparison of retention times with the methylated standards (Sigma, 99% pure), and the results were measured in μg/mL. To confirm that the production of *t9,t11* was not the result of an artefact of the methylation technique of the *c9,t11* isomer (Raes, De Smet, & Demeyer, 2001), an aliquot of the *c9,t11* pure standard was processed alone under the same conditions and, then, evaluated by GC. No other isomer was determined (data not shown).

2.6. *CLA and CLNA production*

The CLA and CLNA production was estimated by quantifying the content of fatty acids present in the cultures based on the following equations:

$$\text{CLA production} = \Sigma\,\text{CLA}/(\text{LA} + \Sigma\,\text{CLA}) \times 100$$

$$\text{CLNA production} = \Sigma\,\text{CLNA}/(\text{LNA} + \Sigma\,\text{CLNA}) \times 100$$

2.7. *Influence of substrate concentration on the CLA/CLNA production by* B. animalis *subsp.* lactis *INL2*

To evaluate the influences of LA and LNA on the metabolism of the best CLA/CLNA-producer bacteria, *B. animalis* subsp. *lactis* INL2 was cultured at 2% (v/v) in a cys-MRS broth (10 mL) with increasing concentrations of each substrate (0, 200, 400, 500 and 1000 μg/mL). The fermentations were performed under anaerobic conditions at 37 °C for 48 h. The pH was measured and the viable cell count was assessed by the pour-plating method in cys-MRS agar plates. Cultures without substrates were used as controls. The fatty acid analysis was performed by GC.

The selection of the 500 μg/mL concentration was based on the highest percentage of conversion obtained of CLA and CLNA. Thus, to evaluate the time when the conjugated fatty acid production began, *B. animalis* subsp. *lactis* INL2 was cultured at 37 °C during 48 h under anaerobic conditions, and samples were taken at time 0, or after 4, 8, 12, 24 and 48 h to determinate the pH, cell count and fatty acid profile.

2.8. *Statistical analysis*

The samples were analysed by triplicate, and the results were expressed as the mean ± standard deviation (SD) values. The results were statistically evaluated with the analysis of variance test (MINITAB Release 14.1 Statistical Software, Minitab Inc., State College, PA, USA), using the Duncan post-hoc test. Differences were considered significant at $P < 0.05$.

3. Results

3.1. *Screening of the CLA and/or CLNA production by the UV method*

A total of 64 bacteria were cultured in MRS broth or cys-MRS broth enriched with 100 μg/mL of LA or LNA and cultured during 24–48 h. The bacteria were classified as positive or negative according to their conjugated fatty acid production by the UV method. Forty strains (Table 1) were classified as CLA and/or CLNA producers.

3.2. *Conjugated fatty acids production by GC analysis*

After the selection using UV absorbance, bacteria were grown in an MRS medium in the presence of high concentrations of LA or LNA (500 μg/mL) to promote the conjugated fatty acids production for the lipid analysis by the GC method. The results of the CLA and CLNA production and the isomer profile are shown in Table 2. The CLA and CLNA concentrations correspond to the sum of the content obtained from the pellet and the supernatant. Interestingly, more than 90% of the CLA and CLNA concentrations were detected in the supernatant for all of the evaluated strains (data not shown). Of all of the 40 selected bacteria, only *Lactobacillus acidophilus* CRL800 was not confirmed as a CLA-producer strain probably due to the high substrate concentration used in this assay.

The range of LA and LNA conversion rate varied among strains, and the LABs exhibited a lower conjugated fatty acids production than the bifidobacteria. Among the 35 LABs, both *L. sakei* CRL1468 and CRL1470 reached the highest CLA (4.12 and 4.88%, respectively) and CLNA (2.45 and 2.78%, respectively) production. Moreover, *L. acidophilus* strains were able to produce CLA with a conversion rate that ranged from 0.44 to 3.36%. Only

Table 2 – Conjugated fatty acid production by GC method.

Strain	MRS medium					
	CLA (μg/mL)			Total production (%)	CLNA (μg/mL)	Total production (%)
	c9,t11	*t10,c12*	*t9,t11*		*c9,t11,c15*	
Lactobacilli						
L. acidophilus CRL44	ND	ND	5.61	1.12 ± 0.02	3.09	0.62 ± 0.11
L. acidophilus CRL258	7	5.14	4.65	3.36 ± 0.09	ND	ND
L. acidophilus CRL1063	ND	2.20	ND	0.44 ± 0.01	ND	ND
L. casei CRL66	5.87	4.72	2.25	2.56 ± 0.04	ND	ND
L. casei CRL117	7.47	6.48	4.79	3.75 ± 0.03	ND	ND
L. casei CRL238	ND	ND	ND	ND	4.33	0.87
L. casei CRL239	5.51	6.93	1.94	2.88 ± 0.11	ND	ND
L. curvatus CRL81	5.30	4.50	4.18	2.80 ± 0.06	3.17	0.63 ± 0.03
L. curvatus CRL1284	6.20	4.54	5.13	3.18 ± 0.12	ND	ND
L. curvatus CRL1465	5.85	5.14	4.83	3.17 ± 0.06	ND	ND
L. curvatus CRL1629	6.96	6.04	5.21	3.64 ± 0.02	ND	ND
L. fermentum CRL574	6.77	5.20	4.64	3.32 ± 0.03	ND	ND
L. fermentum CRL1446	5.03	6.32	2.19	2.71 ± 0.05	ND	ND
L. fermentum EFL2	0.32	ND	0.18	0.10 ± 0.01	ND	ND
L. fermentum EFL3	0.82	ND	1.30	0.42 ± 0.01	ND	ND
L. mucosae CRL573	4.41	3.86	6.33	2.92 ± 0.11	11.02	2.26 ± 0.09
L. murinus EFL4	ND	0.25	0.77	0.20 ± 0.02	ND	ND
L. murinus EFL5	0.98	0.11	0.95	0.41 ± 0.01	ND	ND
L. paracasei subsp. *paracasei* CRL577	6.23	5.74	3.86	3.17 ± 0.07	ND	ND
L. paracasei subsp. *paracasei* CRL731	6.33	5.90	6.30	3.71 ± 0.02	ND	ND
L. pentosus CRL1630	4.52	4.61	5.66	2.95 ± 0.06	ND	ND
L. plantarum CRL41	ND	ND	ND	ND	3.08	0.62 ± 0.02
L. plantarum CRL100	ND	ND	4.19	0.84 ± 0.03	10.38	2.08 ± 0.03
L. plantarum CRL355	ND	ND	ND	ND	2.68	0.54 ± 0.02
L. plantarum CRL1920	6.95	5.11	5.28	3.47 ± 0.08	ND	ND
L. plantarum CRL1935	7.26	5.22	5.04	3.50 ± 0.15	ND	ND
L. plantarum EFL6	0.33	0.39	1.09	0.37 ± 0.01	ND	ND
L. plantarum EFL7	0.93	0.75	0.69	0.48 ± 0.02	ND	ND
L. reuteri EFL8	1.04	0.89	ND	0.39 ± 0.02	ND	ND
L. sakei CRL1424	5.98	4.68	4.67	3.07 ± 0.17	ND	ND
L. sakei CRL1468	8.79	4.57	7.26	4.12 ± 0.22	12.26	2.45 ± 0.19
L. sakei CRL1470	11.72	5.22	7.50	4.88 ± 0.34	13.89	2.78 ± 0.06
L. salivarius EFL9	ND	ND	0.35	0.07 ± 0.01	ND	ND
Lactococci						
L. lactis biovar-*diacetylactis* CRL967	ND	ND	2.52	0.50 ± 0.03	4.91	0.98 ± 0.04
L. lactis biovar-*diacetylactis* CRL1061	ND	ND	4.88	0.98 ± 0.03	3.06	0.61 ± 0.02
Bifidobacteria		ND				
B. animalis subsp. *lactis* INL2	20.30	ND	44.08	12.88 ± 0.25	327.18	65.44 ± 0.45
B. animalis subsp. *lactis* INL4	ND	ND	0.94	0.19 ± 0.03	17.26	0.62 ± 0.00
B. longum LM7a	25.62	ND	45.05	14.14 ± 0.30	81.52	16.30 ± 0.18
B. dentium LM8a	ND	ND	2.40	0.48 ± 0.02	3.57	0.71 ± 0.01

Isomer values are expressed as the mean, $n = 3$. Total production (%) is expressed as the mean ± SD. ND: not detected; CLA: conjugated linoleic acid; CLNA: conjugated linolenic acid; EFL: Ecophysiology Technological Laboratory.

L. acidophilus CRL44 produced 0.62% of CLNA. Three *Lactobacillus casei* strains produced between 2.56 and 3.75% of CLA, and only the *L. casei* CRL238 produced 0.87% of CLNA. All of the *L. curvatus* strains were able to produce small amounts of CLA, and only the CRL81 strain produced low levels of CLNA (0.63%). Four *Lactobacillus fermentum* strains showed a CLA production that ranged from 0.1 to 3.32%, but none of them produced CLNA. *Lactobacillus mucosae* CRL573 was able to produce 2.92% of CLA and 2.26% of CLNA. Both *Lactobacillus murinus* strains produced small amounts of CLA but did not produce CLNA. The *Lactobacillus pentosus* CRL1630 strain and two *Lactobacillus paracasei* subsp. *paracasei* strains showed a CLA production that ranged from 2.95 to 3.71%, but they did not produced CLNA. Of the seven *Lactobacillus plantarum* tested strains, five of them were able to conjugate LA until a maximum of 3.5%, and three of them produced between 0.68 and 2.08% of LNA. Of the five *L. plantarum* strains evaluated, only the CRL100 strain synthetised CLA (0.35–3.5%) and CLNA (0.54–2.08%). Small amounts of CLA were detected for *Lactobacillus reuteri*, *Lactobacillus salivarius* and two lactococci strains, but no CLNA production was observed in these bacteria.

The bifidobacteria strains were the highest CLA-producers because they showed a wide range of conversion from 0.2 to 14.14%. Regarding the LNA conversion rate, *Bifidobacterium*

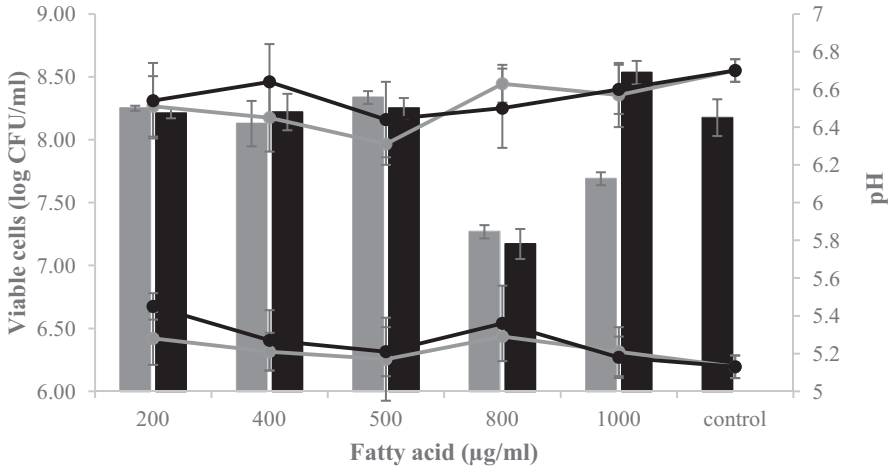

Fig. 1 – Growth of *B. animalis* ssp. *lactis* INL2 in MRS-cys broth at different concentrations of LA (■) and LNA (■) after 48 h of incubation at 37 °C under anaerobic conditions. The culture without substrate addition was used as control. Measurement of initial pH (–■–) and final pH (–●–) after 48 h of incubation. Data represent mean ± SD, *n* = 3.

animalis subsp. *lactis* INL4 and *Bifidobacterium dentium* LM8a produced low levels of CLNA (0.62–0.71%, respe ctively), whereas in *Bifidobacterium longum* LM7a, the conversion rate was 16.30%. *B. animalis* subsp. *lactis* INL2 showed the highest CLNA concentration (65.44%), and it was selected for further assays.

The isomer profile was different for each strain. In general, the main CLA isomers produced by the LABs were *c9,t11* followed by *t10,c12*. Instead, the main isomer detected was *t9,t11* for the bifidobacteria. The CLNA production was evidenced with the *c9,t11,c15* isomer in all of the evaluated strains.

3.3. *Influence of the substrate on the CLA and CLNA production by* B. animalis *subsp. lactis INL2*

B. animalis subsp. *lactis* INL2 was grown in a cys-MRS medium (50 mL) with different concentrations of substrate (0, 200, 400, 500, 800 and 1000 µg/mL). The bacterial growth and pH values after 48 h of incubation were evaluated (Fig. 1). The viable cell counts after the culture in the cys-MRS broth with 200, 400 and 500 µg/mL of LA or LNA increased approximately 2 log units (6 log CFU/mL to 8.5 log CFU/mL and 8.0 log CFU/mL, respectively) after 48 h of cultivation. Higher concentrations of LA or LNA (800 and 1000 µg/mL) significantly inhibited the bacterial growth except with 1000 µg/mL of LNA. No significant differences in pH with these different concentrations of substrates were observed, and the final pH reached values between 5 and 5.4.

The percentage of LA and LNA conversion with the different concentrations of substrates is shown in Table 3. The highest percentages of conversion for both LA and LNA were determined with a substrate concentration of 500 µg/mL of (13% and 65%, respectively), and these percentages decreased with higher concentrations. These results show that 500 µg/mL is the optimal substrate concentration to produce CLA and CLNA for this strain, which will be subsequently used to evaluate the production of CLA and CLNA over time.

3.4. *Production of conjugated fatty acids over time*

To evaluate the effect of the substrates on the kinetics of CLA and CLNA production, *B. animalis* subsp. *lactis* INL2 was cultivated for 48 h in a cys-MRS broth with 500 µg/mL of LA or LNA. Both the viable cell number (Fig. 2) and the CLA/CLNA production (Table 4) gradually increased during the incubation period in presence of LA or LNA. The viable cell count was similar in all cultures with (8.2–8.4 log CFU/mL) or without (8 log CFU/mL) the addition of substrates. The pH values decreased over time and reached a value of 5 at the end of the incubation period (Fig. 2).

The production of CLA and CLNA began after 8 h of incubation and maintained the isomer proportion (31% of *c9,t11* and 69% of *t9,t11*) until the end of the experiment (data not shown).

4. Discussion

For several years, the production of dienoic and trienoic conjugated fatty acid by food-grade bacteria was reported with the

Table 3 – Percentages of conversion of *Bifidobacterium animalis* subsp.*lactis* INL2 at different substrate concentrations.

Substrate addition (µg/mL)	Total production (%)	
	CLA	CLNA
200	2.76 ± 0.15^{a}	11.04 ± 1.16^{a}
400	5.46 ± 1.33^{b}	21.12 ± 1.92^{b}
500	12.88 ± 0.96^{c}	65.44 ± 1.56^{c}
800	10.01 ± 2.01^{c}	48.90 ± 1.90^{d}
1000	9.20 ± 2.12^{c}	53.20 ± 3.09^{d}

Values are expressed as mean ± SD; *n* = 3. Different letters in the same column correspond to statistically significant differences ($P < 0.05$).

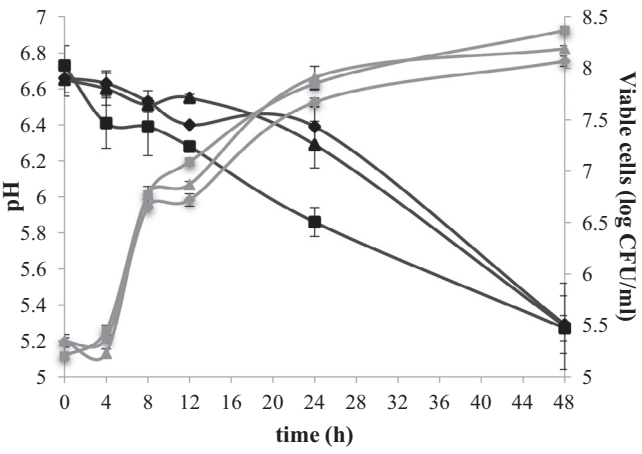

Fig. 2 – Viable cell counts (—) and pH (—) of *B. animalis* ssp. *lactis* INL2 cultured in cys-MRS broth plus 500 μg/mL of LA (■■) or LNA (▲▲) under anaerobic conditions at 37 °C for 48 h. The culture without substrate addition was used as control (◆◆). Data represent mean ± SD, *n* = 3.

aim of its possible application to produce fermented products with increased CLA or CLNA content or to be used as a probiotic (Van Nieuwenhove et al., 2012).

In sausage production, both *L. sakei* and *L. curvatus* are involved in the fermentation process (Cocolin, Dolci, & Rantsiou, 2011). Few studies demonstrated the CLA production by these strains. We found four *L. curvatus* strains that were able to produce CLA at higher levels than previously reported and one strain with the ability to produce CLNA but with lower values (Gorissen et al., 2011). In the present study, three *L. sakei* strains produced similar CLA amounts to the values reported by Gorissen et al. (2011). Only two of them were able to produce lower levels of CLNA than the ones reported by the mentioned studies.

To the best of our knowledge, this is the first study to report the production of CLA and CLNA by *L. mucosae*, a strain whose probiotic potential has been demonstrated (Valeriano, Parungao-Balolong, & Kang, 2014).

The bifidobacteria are GRAS microorganisms and are regarded as probiotic because its consumption improves different biological parameters of human health, especially in newborns and infants (Di Gioia, Aloisio, Mazzola, & Biavati, 2014). Several authors reported a great variability among bifidobacteria with regard to the production of conjugated fatty acids (Coakley et al., 2003; Gorissen et al., 2010; Villar-Tajadura et al., 2014). In this study, we demonstrated the ability of four human-breast isolated bifidobacteria to synthesise CLA and CLNA at different levels: *B. animalis* subsp. *lactis* INL2 and INL4 and *B. longum* LM7a and *B. dentium* LM8. The CLA production evidenced by *B. animalis* subsp. *lactis* INL2 is in agreement with earlier studies (Coakley et al., 2003; Gorissen et al., 2010). However, higher values (74–77%) were informed by Villar-Tajadura et al. (2014).

Table 4 – Production of CLA and CLNA over time by *Bifidobacterium animalis* subsp. *lactis* INL2 in cys-MRS broth.

Time (h)	Total production (%)	
	CLA	CLNA
0	0.24 ± 0.13[a]	0.18 ± 0.72[a]
4	0.37 ± 0.11[a]	0.29 ± 0.18[a]
8	1.44 ± 0.39[b]	9.10 ± 1.03[b]
12	4.65 ± 1.07[b]	17.73 ± 3.01[c]
24	8.24 ± 2.31[c]	27.35 ± 3.92[d]
48	12.2 ± 1.09[d]	64.3 ± 1.56[e]

Values were expressed as mean ± SD; *n* = 3. Different letters in the same column correspond to statistically significant differences ($P < 0.05$).

A great variability in the CLA isomer profile produced by each strain was found in this study. LABs were able to synthesise *c9,t11*, *t10,c12* and *t9,t11* CLA isomers, which agrees with the data published in the literature (Yang et al., 2014). Bifidobacteria strains showed *t9,t11*-CLA as the main isomer (69% of total CLA), followed by *c9,t11* (31%) and no *t10,c12* was detected. An earlier study reported 19 to 44% of the total CLA produced as the *t9,t11*- isomer, which is in agreement with our results (Gorissen et al., 2010). The production of the *t9,t11*-CLA isomer by bifidobacteria was demonstrated by Coakley et al. (2003), and this isomer seems to be the final metabolite of the CLA production pathway in bifidobacteria (Coakley et al., 2006). However, Villar-Tajadura et al. (2014) informed that 81–85% of the total CLA was synthesised as the *c9,t11* isomer. The *t9,t11*-CLA isomer showed a more potent effect as antiproliferative in the viability of two cancer cell lines (SW480 and HT-29) than the *c9,t11* isomer (Coakley et al., 2006).

We only identified the *c9,t11,c15* as the unique CLNA isomer produced by LABs and bifidobacteria strains, which is in agreement with earlier studies (Gorissen et al., 2010; Villar-Tajadura et al., 2014).

The reason why bacteria exhibit different LA and LNA metabolisms is still unclear. A mechanism of detoxification was proposed by Jiang, Björck, and Fondén (1998) to explain the conjugation of LA. Ruminal bacteria are able to transform LA and LNA into stearic acid (C18:0). In our study, the C18:0 content did not change over time, and the levels of vaccenic acid (C18:1 *t*11) were determined (data not shown). Perhaps, the bifidobacteria are unable to complete the biohydrogenation pathway to obtain the final product as ruminal bacteria do. This result agrees with those of other authors (Coakley et al., 2003; Gorissen et al., 2010; Park et al., 2012).

The CLA and CLNA isomers were mainly found in the supernatant (almost 90% of the total production) compared with the pellets (less 10%; data not shown). In almost every bacteria, the CLA production is primarily located in the extracellular phase (Rainio, Vahvaselkä, Suomalainen, & Laakso, 2002), but it can also be found in the cellular membrane as structural lipids in a lesser amount (Oh et al., 2003).

The consumption of bifidobacteria is beneficial for human health. Therefore, the selection of appropriate strains to be included into fermented foods could be considered a critical step to design new functional foods with probiotic strains that produce CLA and CLNA. Thus, for the *B. animalis* subsp. *lactis* INL2, the optimal conditions for CLA and/or CLNA production were determined. The substrate concentrations showed a strong influence in the LA/LNA conversion rate, and high levels of these substrates in the medium negatively affected the LA/

LNA rate of conversion. This result is coincident with previous studies (Li et al., 2013; Van Nieuwenhove et al., 2007; Xu et al., 2008). The CLA and CLNA production began after 8 h of incubation and reached the highest levels at 48 h, which is similar to the data reported for *Bifidobacterium breve* M 7–70 (Villar-Tajadura et al., 2014).

Genetic studies on the regulatory mechanisms of the CLA and CLNA production in *B. animalis* subsp. *lactis* INL2 are currently being performed.

5. Conclusion

The concentration and the dienoic and trienoic fatty acid production were shown to be strain-dependent. Overall, the evaluated LABs produced lower levels of conjugated acids with respect to the bifidobacteria. The *B. animalis* subsp. *lactis* INL2 strain exhibited a high CLA and CLNA production depending on the concentration of the substrate present in the culture medium. Hence, this strain could be used for the development of functional dairy products with high concentrations of conjugated fatty acids or as a probiotic to promote the synthesis of these bioactive lipids in the intestine.

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REFERENCES

Bhattacharya, A., Banu, J., Rahman, M., Causey, J., & Fernandes, G. (2006). Biological effects of conjugated linoleic acids in health and disease. *The Journal of Nutritional Biochemistry*, *17*(12), 789–810. doi:10.1016/j.jnutbio.2006.02.009.

Chin, S. F., Liu, W., Storkson, J. M., Ha, Y. L., & Pariza, M. W. (1992). Dietary sources of conjugated dienoic isomers of linoleic acid, a newly recognized class of anticarcinogens. *Journal of Food Composition and Analysis*, 5(3), 185–197. doi:10.1016/0889-1575(92)90037-K.

Coakley, M., Johnson, M. C., McGrath, E., Rahman, S., Ross, R. P., Fitzgerald, G. F., & Stanton, C. (2006). Intestinal bifidobacteria that produce trans-9, trans-11 conjugated linoleic acid: A fatty acid with antiproliferative activity against human colon SW480 and HT-29 cancer cells. *Nutrition and Cancer*, *56*(1), 95–102. doi:10.1207/s15327914nc5601_13.

Coakley, M., Ross, R. P., Nordgren, M., Fitzgerald, G., Devery, R., & Stanton, C. (2003). Conjugated linoleic acid biosynthesis by human-derived *Bifidobacterium* species. *Journal of Applied Microbiology*, *94*(1), 138–145.

Cocolin, L., Dolci, P., & Rantsiou, K. (2011). Biodiversity and dynamics of meat fermentations: The contribution of molecular methods for a better comprehension of a complex ecosystem. *Meat Science*, *89*(3), 296–302.

Di Gioia, D., Aloisio, I., Mazzola, G., & Biavati, B. (2014). Bifidobacteria: Their impact on gut microbiota composition and their applications as probiotics in infants. *Applied Microbiology and Biotechnology*, *98*(2), 563–577. doi:10.1007/s00253-013-5405-9.

Folch, J., Lees, M., & Sloane Stanley, G. H. (1957). A simple method for the isolation and purification of total lipides from animal tissues. *The Journal of Biological Chemistry*, *226*(1), 497–509.

Gasmi, J., & Thomas Sanderson, J. (2013). Jacaric acid and its octadecatrienoic acid geoisomers induce apoptosis selectively in cancerous human prostate cells: A mechanistic and 3-D structure–activity study. *Phytomedicine: International Journal of Phytotherapy and Phytopharmacology*, *20*(8–9), 734–742. doi:10.1016/j.phymed.2013.01.012.

Gorissen, L., Raes, K., Weckx, S., Dannenberger, D., Leroy, F., De Vuyst, L., & De Smet, S. (2010). Production of conjugated linoleic acid and conjugated linolenic acid isomers by *Bifidobacterium* species. *Applied Microbiology and Biotechnology*, *87*(6), 2257–2266. doi:10.1007/s00253-010-2713-1.

Gorissen, L., Weckx, S., Vlaeminck, B., Raes, K., De Vuyst, L., De Smet, S., & Leroy, F. (2011). Linoleate isomerase activity occurs in lactic acid bacteria strains and is affected by pH and temperature. *Journal of Applied Microbiology*, *111*(3), 593–606. doi:10.1111/j.1365-2672.2011.05087.x.

Igarashi, M., & Miyazawa, T. (2000). Newly recognized cytotoxic effect of conjugated trienoic fatty acids on cultured human tumor cells. *Cancer Letters*, *148*(2), 173–179. doi:10.1016/S0304-3835(99)00332-8.

Ip, C., Scimeca, J. A., & Thompson, H. J. (1994). Conjugated linoleic acid. A powerful anticarcinogen from animal fat sources. *Cancer*, *74*(3 Suppl.), 1050–1054.

Jiang, J., Björck, L., & Fondén, R. (1998). Production of conjugated linoleic acid by dairy starter cultures. *Journal of Applied Microbiology*, *85*(1), 95–102.

Kepler, C. R., Hirons, K. P., McNeill, J. J., & Tove, S. B. (1966). Intermediates and products of the biohydrogenation of linoleic acid by *Butyrinvibrio fibrisolvens*. *The Journal of Biological Chemistry*, *241*(6), 1350–1354.

Lee, K., Paek, K., Lee, H. Y., Park, J. H., & Lee, Y. (2007). Antiobesity effect of *trans*-10, *cis*-12-conjugated linoleic acid-producing *Lactobacillus plantarum* PL62 on diet-induced obese mice. *Journal of Applied Microbiology*, *103*(4), 1140–1146. <http://dx.doi.org/10.1111/j.1365-2672.2007.03336.x>.

Lee, K., & Lee, Y. (2009). Production of *c9,t11*- and *t10,c12*-conjugated linoleic acids in humans by *Lactobacillus rhamnosus* PL60. *Journal of Microbiology and Biotechnology*, *19*(12), 1617–1619.

Li, J., Zhang, L., Han, X., Yi, H., Guo, C., Zhang, Y., Du, M., Luo, X., Zhang, Y., & Shan, Y. (2013). Effect of incubation conditions and possible intestinal nutrients on *cis*-9, *trans*-11 conjugated linoleic acid production by *Lactobacillus acidophilus* F0221. International Dairy Journal, *29*, 93–98.

Lin, T. Y., Hung, T. H., & Cheng, T. S. J. (2005). Conjugated linoleic acid production by immobilized cell of *Lactobacillus delbrueckii* ssp. *bulgaricus* and *Lactobacillus acidophilus*. *Food Chemistry*, *92*, 23–28.

MacDonald, H. B. (2000). Conjugated linoleic acid and disease prevention: a review of current knowledge. *Journal of American College of Nutrition*, *19*, 1–13. <http://dx.doi.org/10.5772/50321>.

Oh, D. K., Hong, G. H., Lee, Y., Min, S., Sin, H. S., & Cho, S. K. (2003). Production of conjugated linoleic acid by isolated *Bifidobacterium* strains. *World Journal of Microbiology and Biotechnology*, *19*, 907–912.

Özgül-Yücel, S. (2005). Determination of conjugated linolenic acid content of selected oil seeds grown in Turkey. *Journal of the American Oil Chemists' Society*, *82*(12), 893–897. doi:10.1007/s11746-005-1161-7.

Park, H. G., Cho, H. T., Song, M.-C., Kim, S. B., Kwon, E. G., Choi, N. J., & Kim, Y. J. (2012). Production of a conjugated fatty acid by *Bifidobacterium breve* LMC520 from α-linolenic acid: Conjugated linolenic acid (CLnA). *Journal of Agricultural and Food Chemistry*, *60*(12), 3204–3210. doi:10.1021/jf2041559.

Raes, K., De Smet, S., & Demeyer, D. (2001). Effect of double-muscling in Belgian Blue young bulls on the intramuscular fatty acid composition with emphasis on conjugated linoleic acid and polyunsaturated fatty acids. *Animal Science*, (Pt. 2), 253–260.

Rainio, A., Vahvaselkä, M., Suomalainen, T., & Laakso, S. (2002). Production of conjugated linoleic acid by *Propionibacterium freudenreichii* ssp. *shermanii*. *Le Lait*, *82*(1), 91–101.

Rodrigues, D., Rocha-Santos, T. A. P., Gomes, A. M., Goodfellow, B. J., & Freitas, A. C. (2012). Lipolysis in probiotic and synbiotic cheese: The influence of probiotic bacteria, prebiotic compounds and ripening time on free fatty acid profiles. *Food Chemistry*, *131*, 1414–1421.

Rodríguez-Alcalá, L. M., Braga, T., Xavier Malcata, F., Gomes, A., & Fontecha, J. (2011). Quantitative and qualitative determination of CLA produced by Bifidobacterium and lactic acid bacteria by combining spectrophotometric and Ag +-HPLC techniques. *Food Chemistry*, *125*(4), 1373–1378.

Shinohara, N., Ito, J., Tsuduki, T., Honma, T., Kijima, R., Sugawara, S., Arai, T., Yamasaki, M., Ikezaki, A., Yokoyama, M., Nishiyama, K., Nakagawa, K., Miyazawa, T., & Ikeda, I. (2012). Jacaric acid, a linolenic acid isomer with a conjugated triene system, reduces stearoyl-CoA desaturase expression in liver of mice. *Journal of Oleo Science*, *61*(8), 433–441.

Suzuki, R., Noguchi, R., Ota, T., Abe, M., Miyashita, K., & Kawada, T. (2001). Cytotoxic effect of conjugated trienoic fatty acids on mouse tumor and human monocytic leukemia cells. *Lipids*, *36*(5), 477–482.

Taboada, N., Van Nieuwenhove, C., López Alzogaray, S., & Medina, R. (2015). Influence of autochthonous cultures on fatty acid composition, esterase activity and sensory profile of Argentinean goat cheeses. *Journal of Food Composition and Analysis*, *40*, 86–94.

Tsuzuki, T., Tokuyama, Y., Igarashi, M., & Miyazawa, T. (2004). Tumor growth suppression by alpha-eleostearic acid, a linolenic acid isomer with a conjugated triene system, via lipid peroxidation. *Carcinogenesis*, *25*(8), 1417–1425. doi:10.1093/carcin/bgh109.

Valeriano, V. D., Parungao-Balolong, M. M., & Kang, D. K. (2014). *In vitro* evaluation of the mucin-adhesion ability and probiotic potential of *Lactobacillus mucosae* LM1. *Journal of Applied Microbiology*, *117*(2), 485–497.

Van Nieuwenhove, C., Cano, P. G., Chaia, A. P., & González, S. (2007). Chemical composition and fatty acid content of buffalo cheese from Northwest Argentina: Effect on lipid composition of mice tissues. *Journal of Food Lipids*, *14*(3), 232–243.

Van Nieuwenhove, C. P., Oliszewski, R., & González, S. N. (2009). Fatty acid composition and conjugated linoleic acid content of cow and goat cheese from the northwest of Argentina. *Journal of Food Quality*, *32*(3), 303–314. doi:10.1111/j.1745-4557.2009.00258.x.

Van Nieuwenhove, C. P., Terán, V., & González, S. (2012). Conjugated linoleic and linolenic acid production by bacteria: Development of functional foods. In E. Rigobelo (Ed.), *Probiotics*. InTech. <http://www.intechopen.com/books/probiotics/conjugated-linoleic-and-linolenic-acid-production-by-bacteria-development-of-functional-foods>.

Villar-Tajadura, M. A., Rodríguez-Alcalá, L. M., Martín, V., Gómez de Segura, A., Rodríguez, J. M., Requena, T., & Fontecha, J. (2014). Production of conjugated linoleic and conjugated α-linolenic acid in a reconstituted skim milk-based medium by bifidobacterial strains isolated from human breast milk. *BioMed Research International*, *2014*, 725406. doi:10.1155/2014/725406.

Wall, R., Ross, R. P., Shanahan, F., O'Mahony, L., O'Mahony, C., Coakley, M., Hart, O., Lawlor, P., Quigley, E. M., Kiely, B., Fitzgerald, G. F., & Stanton, C. (2009). Metabolic activity of the enteric microbiota influences the fatty acid composition of murine and porcine liver and adipose tissues. *American Journal of Clinical Nutrition*, *89*(5), 1393–1401.

Xu, H., Lee, H. Y., Hwang, B., Nam, J. H., Kang, H. Y., & Ahn, J. (2008). Kinetics of microbial hydrogenation of free linoleic acid to conjugated linoleic acids. *Journal of Applied Microbiology*, *105*(6), 2239–2247. doi:10.1111/j.1365-2672.2008.03937.x.

Yang, B., Chen, H., Gu, Z., Tian, F., Ross, R. P., Stanton, C., & Zhang, H. (2014). Synthesis of conjugated linoleic acid by the linoleateisomerase complex in food-derived lactobacilli. *Journal of Applied Microbiology*, *117*(2), 430–439. doi:10.1111/jam.12524.