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Selection of Lactic Acid Bacteria for the Optimized Production of Sheep's Milk Yogurt with a High Conjugated Linoleic Acid Content

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Abstract

Thirty-five different lactic acid bacteria (LAB) strains were screened for their conjugated linoleic acid (CLA) isomers (C18:2 *cis*-9, *trans*-11 and C18:2 *trans*-10, *cis*-12) producing ability from linoleic acid (LA) in sheep's milk. Preliminary experiments revealed that *Lactobacillus delbrueckii* ssp. *bulgaricus* 2230 and *Streptococcus thermophilus* St 360 among the screened strains had the highest CLA-producing ability. This two strains were assayed in an 11-run fractional factorial design to investigate the effect of four variables included glucose, powdered sheep milk, LA and inoculum ratio on CLA production in a sheep's milk yogurt. The optimum conditions for producing the highest levels of CLA (42.86%) were obtained by adding 10.00 mg/mL of glucose, 30.00 mg/mL of powdered sheep milk, 0.90 mg/mL of LA and a 1:2 (*St:Lb*) ratio of bacterial strains in the inoculum. This CLA-rich sheep's milk yogurt could be an important supplementary food source for increasing the CLA in the human diet.

Keywords: dairy product, functional food, *Lactobacillus delbrueckii* ssp. b*ulgaricus, Streptococcus thermophilus*

1. Introduction

Bovine milk is the most commonly consumed type of milk. However, in many countries, milk from other animal species is a significant share of the milk consumed. Ewe milk, for example, has been proposed as an alternative milk source to feed infants who are allergic to milk from other species (Balthazar et al., 2017). Compared to bovine and caprine milk, ovine milk is richer in fat, proteins, ash, calcium, iron, manganese, phosphorus, zinc, all essential amino acids, most vitamins, medium-chain fatty acids and monounsaturated fatty acids (MUFA) (Balthazar et al., 2015; Park, 2007).

Ovine milk is favored in terms of fatty acids (FA); they have a high proportion of saturated fatty acids (SFA) with both short and medium chains (Corrêa, Rohenkohl, & Osório, 2014; Mayer & Fiechter, 2012). Ovine milk has also the highest conjugated linoleic acid (CLA) content (*i.e.* 1.08% of ovine against 1.01% and 0.65% of bovine and caprine milks respectively) and 3.21% of linoleic acid (LA) (Jahreis et al., 1999).

The acronym CLA is used to describe a family of isomers of octadecadienoic acid (18:2), which have a pair of conjugated double bonds along the alkyl chain (Garcia et al., 2017). Recent studies have shown that isomers of CLA present in dairy products improve human health at a dosage of 1.00 to 3.00 g/day (MacDonald, 2000), through their anti-cancer (Thuillier et al., 2013), anti-diabetic (Malinska, Hüttl, Oliyarnyk, Bratova, & Kazdova, 2015), anti-atherosclerotic (Stachowska et al., 2012) and anti-osteoporosis (DeGuire et al., 2012) properties, as well as their ability to prevent increased body fat (Chen et al., 2012) and stimulate of the immune system (Bassaganya-Riera et al., 2012). We recently discussed these properties in a review (Kuhl and De Dea Lindner, 2016).

CLA may be formed in the rumen by anaerobic bacteria as an intermediate in the biohydrogenation of LA and/or in the mammary glands through desaturation of vaccenic acid (*trans*-11 octadecenoic acid) from Δ9-desaturase (Serafeimidou, Zlatanos, Laskaridis, & Sagredos, 2012). Biohydrogenation pathway of LA was firstly demonstrated by Kepler, Hirons, and Tove (1966) using *Butyrivibrio fibrisolvens*. LA was firstly isomerized to a conjugated 18:2 and then hydrogenated, leaving a *trans*-18:1 as the final product. Two categories of LA isomerase enzymes involved in this process were characterized to date. The C12 isomerase, which was predominantly found in *Lactobacillus* species, catalyzes the conversion of LA to C18:2 *cis*-9, *trans*-11 isomer. While the C9 isomerase, found mainly in *Propionibacterium*, catalyzes LA to C18:2 *trans*-10, *cis*-12 isomer (Luo et al., 2013).

The production of CLA by rumen bacteria led to the speculation that other microorganisms may also be able to synthesize this metabolite (Lucatto, Brandão, & Drunkler, 2014). Several studies reviewed by Gorissen, Leroy, De Vuyst, De Smet, and Raes (2013) reported the production of CLA during lactic fermentation by lactic acid bacteria (LAB). According to these studies, some strains of bacteria were able to change the FA profile of milk, in addition to producing functional FAs during fermentation as a result of its metabolism.

There are several important factors that can lead to the increased production of CLA by LAB. Among them, studies have cited variables such as fermentation time and temperature, as well as protein concentration and LA source (Gholami & Khosravi-Darani, 2014; Khosravi, Safari, Khodaiyan, & Gharibzahedi, 2015; Terán et al., 2015). According to Kim and Liu (2002) a glucose source is more efficient for CLA production than sucrose or lactose supplementation. Besides, the presence of LA isomerase and its activity in the culture may improve CLA production by adding LA to the medium (Dahiya & Puniya, 2017). Lin, Lin, and Lee (1999) reported the addition of 1.00 mg/mL (w/v) LA to the medium led to an effective CLA conversion during fermentation by *Lactobacillus delbrüeckii* ssp. *bulgaricus* and *Streptococcus thermophilus* in a single culture. The addition of dairy-based additives such as sodium caseinate, whey powder, or nonfat milk powder were described to CLA concentration enhancing in dairy products (Shantha & Decker, 1993). Overall, the CLA conversion by lactic acid fermentation may be influenced by numerous factors like bacterial strain, cell density, incubation time and cell age (Gholami & Khosravi-Darani, 2014).

The purpose of the present study was to evaluate strains of *L. bulgaricus* and *S. thermophilus* for their ability to produce CLA from LA. It is well documented that these strains are able to synthesize CLA using LA under single culture conditions. However, there is little information on CLA production during co-culture. Therefore, the effect of processing variables (glucose, powdered sheep's milk, LA and inoculum ratio) was investigated for the first time with regards to production of a sheep's milk yogurt with a high CLA content.

2. Materials and Methods

2.1 Bacterial Strains and Growth Conditions

A total of 35 LAB strains obtained from the Food Microbiology Laboratory collection culture of the Food and Drug Science Department (University of Parma, Italy) were used in this study (Table 1). All of the employed strains are potential starter cultures for milk fermentation. The initial screening of strains aimed to determine their ability to produce CLA from free LA in supplemented sheep's milk. *L. bulgaricus* strains were cultured in Man Rogosa Sharpe (MRS) medium (Merck, Darmstadt, Germany) and *S. thermophilus* strains were cultured in M17 medium (HiMedia, Mumbai, India) with incubation at 37 ℃ and 42 ℃, respectively, for 48 h under anaerobic conditions. Stock solutions for maintenance of the strains were prepared with 1 mL of bacterial suspension in 0.5 mL of glycerol solution (1:1 v/v) and kept at -80 ℃.

Table 1. Strains used in screening for their ability to produce CLA from LA in sheep's milk.

Strain	Origin
Lactobacillus delbrueckii* ssp. *bulgaricus	
2214; 2259; 2260; 2230	Grana Padano cheese
1865; Lb 260; Lb 261; Lb 263; Lb 264; Lb 265; Lb 308; Lb 309; Lb 313	Unknown
Streptococcus thermophilus	
1688; 1689	Parmigiano Reggiano cheese whey starter
St 360; St 362; St 356	Valtellina Casera cheese milk starter
St 393	Gorgonzola cheese milk starter
St 410	Provolone cheese whey starter
St 383	Provolone cheese
St 233; St 234	Grana Padano whey starter
St 451	Pecorino Toscano cheese whey starter
St 257; St 258; St 508; St 509	Pecorino Toscano cheese
St 357; St 361; St 363; St 366; St 387; St 388; St 390	Unknown

2.2 Screening of Strains for CLA Production

Raw sheep's milk was provided by the Pinheiro Seco Farm (Bom Retiro, SC, Brazil). The milk originated from Lacaune × Texel sheep crosses was pasteurized at 82 ℃ for 15 min in batches, hot filled in bottles previously sanitized and immediately cooled to 4 ℃ in an ice bath before being frozen until further use.

The stock solution of LA (30.00 mg/mL) was composed by 1.20 g of pure LA (Neon, São Paulo, Brazil), 40.00 mL of sheep's milk with 1.00% (v/v) Tween 80 (polyoxyethylene sorbitan monooleate) (Synth, Diadema, Brazil) to improve its solubility (Ando, Ogawa, Kishino, & Shimizu, 2004; Van Nieuwenhove, Oliszewski, González, & Pérez Chaia, 2007). The strains were reactivated overnight under the same conditions described above. Bacterial cells were harvested by centrifugation (4000 × g, 10 min) and suspended in 0.10% (w/v) peptone water until reaching a 0.10 OD at 600 nm as assessed by a SP-2000 UV spectrophotometer (Spectrum, Shanghai, China).

For the screening, excepted in control trial (none strain added), each strain was inoculated in milk supplemented with 1.66 mL of previously prepared LA stock solution, reaching a concentration of 0.50 mg/mL LA, 1.00% (w/v) glucose and 5.00% (w/v) ovine skim milk powder. These mixtures were incubated at 37 ℃ (*L. bulgaricus*) or 42 ℃ (*S. thermophilus*), until reaching pH 4.65 and maintained at -20 ℃ until FA analysis.

2.3 Fatty Acid Analysis

Fermented milk samples were collected to determine the FA profile by gas chromatography (GC). Samples were carried out in duplicate, expressing results as mean value. Lipids were extracted following the Hara and Radin (1978) method using a hexane/isopropanol solution (3:2, v/v) and derivatized to methyl ester using methyl acetate and sodium methoxide (1 M) in a methanol solution at room temperature, according to (Christie, 1982).

Fatty acid methyl esters (FAME) were analyzed by a GC (model GC 2010-Plus, Shimadzu, Kyoto, Japan) equipped with a flame ionization detector, an automatic injector (model AOC-i-20) and a CP-SIL 88 (100 m × 0.25 mm × 0.2 μm) GC capillary column (Agilent Technologies, Palo Alto, USA). The injection volume was 1.00 μL in a split mode 1:50 ratio. GC operating conditions were set according to Cruz-Hernandez et al. (2007): injector and detector temperature set to 250 ℃, initial oven temperature (45 ℃) increased to 175 ℃ at 13 ℃/min and held for 27 min, temperature increased to 215 ℃ at a rate of 4 ℃/min and held for 35 min. Hydrogen was used as a carrier gas at a flow rate of 1.5 mL/min. Fatty acids were identified by comparison with the retention times of methylated standards of Supelco 37 Component FAME Mix (Sigma, St Louis, MO, USA). A mixture of *cis*-9, *trans*-11 and *trans*-10, *cis*-12 octadecadienoic acid methyl esters was used as CLA standard (Sigma).

Data were calculated as normalized area percentages of fatty acid and converted to mg/mL of fermented milk by considering the total fatty acids in 1 mL of fermented milk as reference. Because milk supplemented with LA was used as a fermentation medium, total FAs were quantified and CLA production was calculated by subtracting the natural CLA content (Van Nieuwenhove et al., 2007). Percentages increase in CLA was calculated by using equation 1. Only one strain of each *L. bulgaricus* and *S. termophilus* which showed the greatest abilities to produce CLA, was selected for the next steps of this work.

$$[(CLAs \times 100/CLAm) - 100] \quad (1)$$

Note. CLAs= mg of CLA in the sample, CLAm= naturally present mg of CLA in the sheep's milk.

2.4 Influence of LA Concentration on Bacterial Growth

To evaluate the inhibitory potential and ability of bacteria to grow in the presence of LA, overnight activated strains were inoculated in MRS or M17 broth, according to specie. These broths were chosen to avoid turbidity interference in the OD measurement from milk matrix. The media were supplemented with 0.25, 0.50, 1.00, 1.50 or 2.00 mg/mL LA and incubated for 12 h at 37 ℃ or 42 ℃ under anaerobic conditions. The growth of selected strains was determined by measuring OD values at 600 nm using a spectrophotometer. Cultures without LA supplementation served as controls.

2.5 Experimental Design

After the initial screening, the selected strains that demonstrated major ability to produce CLA from LA were chosen for fermentation by protocooperation in co-culture to produce yogurt with a high CLA content. *S. thermophilus* St 360 and *L. bulgaricus* 2230 were assayed in a fractional design to evaluate the effect of four independent variables, including LA (Neon, São Paulo, Brazil); powdered sheep's milk, provided by Universidade Regional Integrada do Alto Uruguai e das Missões (Erechim, RG, Brazil); glucose (Sigma-Aldrich, Saint Louis, USA); and inoculum ratio, on CLA production (Table 2).

Table 2. Level of coded and real values for fractional factorial design.

Factors	Level		
	-1	0	1
Glucose (mg/mL) (X_1)	10.00	20.00	30.00
Powdered sheep's milk (mg/mL) (X_2)	10.00	20.00	30.00
LA (mg/mL) (X_3)	0.10	0.50	0.90
Inoculum ratio (*St*:*Lb*) (X_4)	1:2	1:1	2:1

The experiments were performed according to Table 3 to optimize the parameters. Experimental data were fitted to a quadratic polynomial model, and the model proposed for predicting the response variables was explained by the equation 2.

$$Y = A_0 + \sum_{i=1}^{n} A_i X_i + \sum_{i=1}^{n} \sum_{j=i+1}^{n} A_{ij} X_j X_i \quad (2)$$

Note. Where **Y**= predicted response, $\mathbf{A_0}$= regression coefficients for the intercept, **Ai=** linearity, $\mathbf{A_iX_i}$= square, $\mathbf{A_{ij}}$= interaction, $\mathbf{X_i}$ and $\mathbf{X_j}$= coded independent factors.

The data were analyzed using Statistic software version 7.0 (StatSoft Inc., Tulsa, OK, USA), and the coefficients were interpreted using the *F*-test. Analysis of variance (ANOVA), regression analysis and plotting of the function that correlated CLA production with the evaluated factors (glucose content, powdered sheep's milk concentration, LA concentration and inoculum ratio) were performed to establish the optimum conditions for CLA production.

2.6 Yogurts Production

The eleven runs (Table 3) were performed by adding specific proportions of each variable to the milk according to the experimental design (Table 2). Before the treatment at 90 ºC for 5 min, glucose and powdered sheep's milk were added to the milk in each batch. Afterwards, the mixtures were cooled to 42 ℃ in an ice bath for LA addition (from previously prepared stock solution) and culture inoculation in the ratios described within the experimental design. The mixtures were incubated at 42 ℃ for 15 h. After the fermentation period, the yogurts were stirred and stored at 4 ℃ until further analysis.

Table 3. Experimental design used to test the influence of X_1 (glucose), X_2 (powder sheep's milk), X_3 (LA) and X_4 (Inoculum ratio).

Run	Coded Variables			
	X_1	X_2	X_3	X_4
1	-1	-1	-1	-1
2	1	-1	-1	1
3	-1	1	-1	1
4	1	1	-1	-1
5	-1	-1	1	1
6	1	-1	1	-1
7	-1	1	1	-1
8	1	1	1	1
9	0	0	0	0
10	0	0	0	0
11	0	0	0	0

2.7 Identification and Quantification of FAs

Lipid extraction, derivatization of FA to FAME and the GC conditions were the same as described above. As an internal standard, tricosanoic acid (C23:0 Me) was added to the medium to a final concentration of 1.00 mg/mL. Identification of the FAME peaks was performed by comparing the retention times of the standard Supelco 37 Component FAME Mix and mixture of *cis*-9, *trans*-11 and *trans*-10, *cis*-12 octadecadienoic acid methyl esters. C18:2 *cis*-9, *trans*-11 isomer concentrations in the yogurt were calculated, as described by Visentainer (2012), and expressed as mg/g of total lipids.

2.8 Physico-chemical Analysis

Moisture content within the different yogurts was determined by weight loss using an oven at 102 ℃ and weighed until constant weight (Brazil, 2006). Ashes were determined by the incineration method in a muffle at 550 ℃ according to the AOAC 945.46 method (AOAC, 2007). Crude protein was determined via the micro Kjeldahl method with nitrogen (%) × 6.38 following the Brazilian normative instruction No. 68 (Brazil, 2006). The pH was measured using a pH meter (model DM-22, Digimed, São Paulo, Brazil). Acidity was assayed by a simple titration method (0.1 M NaOH) according to the AOAC 947.05 method (AOAC, 2007). The analyses were performed in triplicate and the values reported are the mean ± standard deviation. The analysis of variance (ANOVA) and Tukey's studentized range test (5% significance) were carried out to identify significant differences between the results. The data were analyzed using Statistic software.

3. Results and Discussion

3.1 Screening of Strains for CLA Production

Strains tested in our study either increased or decreased CLA levels (Table 4). Jiang, Björck, and Fondén (1998) found similar results using an *in vitro* screening for dairy starter cultures, by varying the concentration of LA free in medium. The depletion of the milk's natural CLA content observed for some strains could be potentially associated with lipolytic metabolism or explained by fermentation temperature, as observed by Gorissen et al. (2011). To optimize microorganism ability to grow at different temperature, a thermal control system allows the regulation of the phospholipid membrane fluidity by incorporating unsaturated fatty acids (Hernandez-Mendoza, Lopez-Hernandez, Hill, & Garcia, 2009; Kishino, Ogawa, Omura, Matsumura, & Shimizu, 2002).

Table 4. Increase or decrease in CLA concentrations in sheep's milk fermented by LAB strains.

Specie	Strain	CLA (mg/mL)	Increase/Decrease (%)
Lactobacillus delbrueckii ssp. *bulgaricus*	2214	0.82	25
	2259	0.61	-7
	2260	0.88	34
	2230	1.15	74
	1865	0.84	27
	Lb 260	0.88	34
	Lb 261	0.79	20
	Lb 263	1.03	56
	Lb 264	0.94	42
	Lb 265	0.71	7
	Lb 308	1.01	53
	Lb 309	0.90	36
	Lb 313	0.80	21
Streptococcus thermophilus	1688	0.50	-24
	1689	0.57	-14
	St 360	1.02	54
	St 362	0.95	44
	St 356	0.85	30
	St 393	0.84	28
	St 410	0.64	-3
	St 383	0.53	-20
	St 233	0.77	17
	St 234	0.92	39
	St 451	0.88	34
	St 257	0.38	-42
	St 258	0.57	-13
	St 508	0.60	-9
	St 509	0.53	-20
	St 357	0.89	35
	St 361	0.75	14
	St 363	0.64	0
	St 366	0.77	17
	St 387	0.71	8
	St 388	0.74	12
	St 390	0.82	24

Wang, Delettre, Guillot, Corrieu, and Béal (2005) used a low fermentation temperature for *Lactobacillus acidophilus* and *S. thermophilus* and the result showed an increased incorporation of unsaturated fatty acids in the cell membrane. Furthermore, Jenkins and Courtney (2003) reported a preference for the incorporation of CLA to LA into the cell membrane, which may account in part for the depletion of CLA naturally present in milk by "homeoviscous adaptation" (Ernst, Ejsing, & Antonny, 2016).

According to Hernandez-Mendoza et al. (2009), a part of the isomerized product from LA could be incorporated into cell membranes while another part could be released into the growth medium. Besides, because the enzyme responsible for bioconversion of LA to CLA (linoleate isomerase - EC 5.2.1.5) is anchored to the cell membrane, authors suggests that the range of biohydrogenation may decrease if some membrane damage occurs or the exposure to a low pH, for example.

Among the microorganisms screened in our work, *L. bulgaricus* 2230 and *S. thermophilus* St 360 demonstrated the highest potential for producing CLA in sheep's milk; the total CLA quantified was 1.15 mg/mL (74.00% increase from the original CLA content in the milk) and 1.02 mg/mL (54.00% increase), respectively (Table 4).

Several authors reported the ability of different LAB strains to produce CLA (Jiang et al., 1998; Kim & Liu, 2002; MacOuzet, Lee, & Robert, 2009; Rodríguez-Alcalá, Braga, Xavier Malcata, Gomes, & Fontecha, 2011;

Terán et al., 2015). Lin et al. (1999) demonstrated the production of CLA by *S. thermophilus* and *L. bulgaricus* as single strains and observed an increase in levels by adding 1.00 mg/mL LA in a culture medium containing 12.00% (w/v) skim milk powder, during a 24 h fermentation at 37 ℃. *L. bulgaricus* and *S. thermophilus* strains produced 0.09 and 0.07 mg/mL of CLA from LA under these conditions, respectively. In addition, Van Nieuwenhove et al. (2007) demonstrated a conversion of 30.30% by *S. thermophilus* in fermented buffalo milk supplemented with 0.80 mg/mL of LA, while *L. bulgaricus* did not exhibit any ability to produce CLA from LA.

3.2 Influence of LA Concentration on Bacterial Growth

To evaluate the inhibitory potential and ability of bacteria to grow in the presence of LA, the selected *L. bulgaricus* and *S. thermophilus* grew in MRS and M17 media in the presence of different concentrations of LA for 12 h (Figure 1). Both strains tested (data not shown for *S. thermophilus* St 360) suffered inhibition of growth in the presence of more than 1.00 mg/mL LA. As stated by Van Nieuwenhove et al. (2007), different strains tolerate different concentrations of LA. Additionally, the inhibitory dose depends on the availability of the FA (Gorissen, Raes, De Smet, De Vuyst, & Leroy, 2012; Trigueros & Sendra, 2015). According to Kim and Liu (2002), the growth of *Lactococcus lactis* was completely inhibited at LA concentrations higher than 0.50 mg/mL. In a similar study, *Bifidobacterium animalis* ssp. *lactis* was tested for growth in a MRS-cysteine medium supplemented with 0.20 - 1.00 mg/mL LA. Higher concentrations of LA (0.80 and 1.00 mg/mL) significantly inhibited bacterial growth (Terán et al., 2015). According to Lin et al. (1999) the inhibitory effect of high LA concentrations could be due to an antimicrobial effect of free LA.

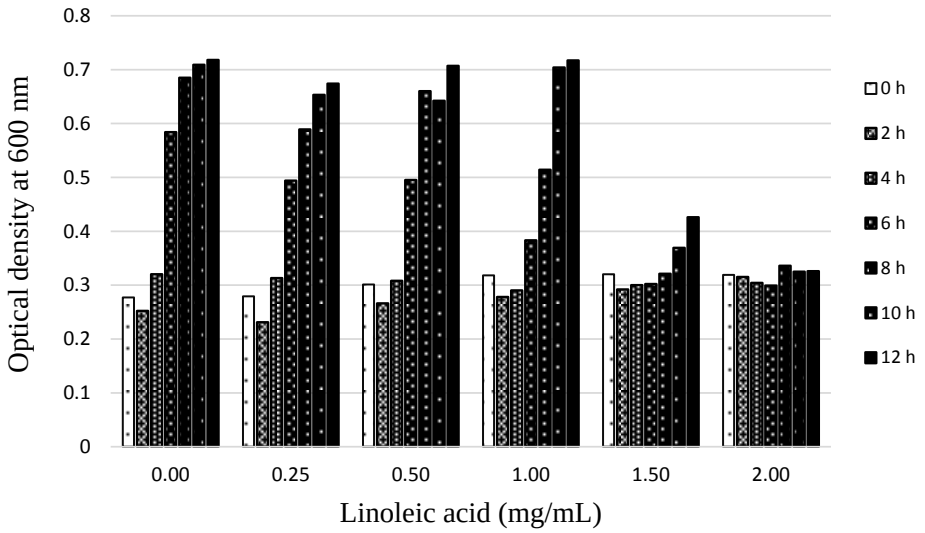

Figure 1. Inhibition of *L. bulgaricus* 2230 growth in MRS broth by the presence of different concentrations of LA (mg/mL). OD at 600 nm was determined during 12 h.

3.3 Optimization of CLA Production in Yogurt

The optimal mixture for producing CLA by selected bacterial strains consisted of adding 10.00 mg/mL (w/v) glucose, 30.00 mg/mL (w/v) powdered sheep's milk, 0.90 mg/mL (w/v) LA and a 1:2 (*St:Lb*) (v/v) ratio of bacterial strains (Run 7) (Table 5). The highest CLA level found (70.41 mg/g fat) was increased 42.86% over those in raw sheep's milk. Table 5 shows the actual response values obtained from experimental data and the predicted response values based on the quadratic polynomial model.

Table 5. Microbial CLA production (%) in yogurt under different conditions based on a fractional factorial design.

Run	CLA experimental production (%)	CLA predicted production (%)
1	36.82	36.16
2	26.92	26.26
3	01.74	01.07
4	11.34	10.67
5	04.32	03.66
6	30.23	29.57
7	42.86	42.19
8	38.66	37.99
9	19.60	23.45

Analysis of variance (ANOVA) (Table 6) showed that the quadratic polynomial model was significant ($p < 0.015$), revealing a viability of the constructed model and also was sufficient to represent the actual relationship between the response and significant parameters. A high value of R^2 (0.97) is an indication that the fitted model can be used for prediction with reasonable precision. The *F*-value (20.16) implies that the model is significant at a 95.00% confidence level. The value of the lack of fit test (0.99) was higher than 0.42, which is not significant relative to the pure error, and indicates that the fitting model is adequate to describe the experimental data. The variation for CLA synthesis according different treatments might be linked to the independent variables.

Table 6. Analysis of variance (ANOVA) for the fitted factorial polynomial model for optimization of CLA production and model equation.

Model	$Y (\% CLA) = 23.44+2.68X_1 + 4.91X_3 - 6.20X_4 + 12.20X_1X_4$				
Source	Sum of Squares (SS)	Degree of Freedom (DF)	Mean of Squares (MS)	*F*-value	*p*-Value
Model	1838.14	7	142.09	20.16	<0.015
Lack of fit	12.89	1	12.89	0.99	0.42
Pure error	25.87	2	12.94		

Note. $R^2 = 0.97$.

CLA production was shown to be significant ($p < 0.05$) depending on glucose concentration (X_1), inoculum ratio (X_4) and LA content (X_3). Powdered sheep's milk as a protein source does not appear to be a significant variable in CLA production. Second order interactions between independent variables were not found to be significant, except for glucose and the inoculum ratio represented in the response surface methodology (RSM) (Figure 2). The interaction of glucose and inoculum ratio in minimum levels enhances the CLA percentage production in a confidence level of 95.00%. The RSM showed that CLA production increase with inoculum ratio inversion (2:1 to 1:2) during the decreasing of glucose content.

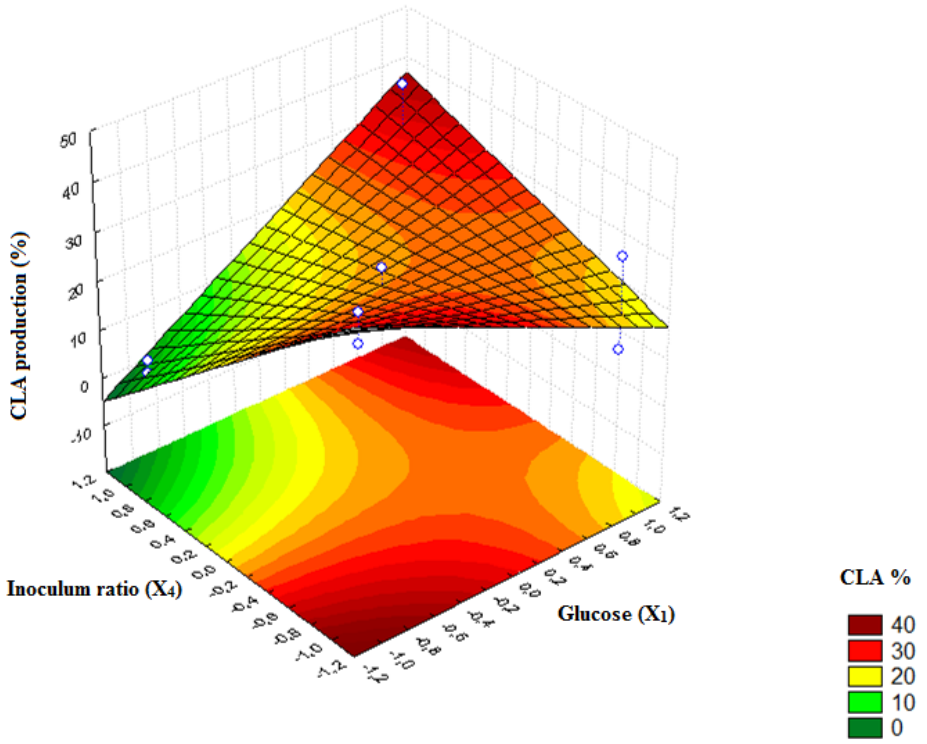

Figure 2. Response surface plots showing the significant ($p < 0.05$) interaction effects (glucose and inoculum ratio) on the CLA produced (%) by yogurt co-culture.

Kim and Liu (2002), in a previous research, had used different model systems to optimize conversion conditions from LA to CLA by diverse bacterial species. Recent studies also used independent variables in the production of fermented milk to determine the best conditions for increasing CLA levels in products. Khosravi-Darani, Reihani, and Feili (2014) analyzed the effect of processing variables with regards to CLA production in probiotic yogurt containing strains of *L. acidophilus*, *Bifidobacterium bifidum* and *Propionibacterium freudenerchii*. Variables included the addition of supplements, fermentation conditions and both inoculum size and viability. The highest concentration of CLA (11.03 mg/g fat) was obtained by the addition of 4.00% (w/v) whey powder, 4.00% (v/v) grape seed oil, inoculation of 0.80% (v/v), 36 h of incubation and 27 h of fermentation at 35 ℃. The research showed that under the optimized conditions, the amount of CLA in probiotic yogurt increased by 40.00% from an average of 8.01 mg/g fat.

Khosravi et al. (2015) evaluated the ability of different *Lactobacillus* strains to produce CLA from LA. Experiments revealed that *Lactobacillus plantarum* had the highest CLA-producing potential (0.09 mg/mL). The results showed that the use of yeast extract and glucose could significantly increase cell growth and CLA production. RSM was also applied to investigate the effects of three independent variables (LA, yeast extract concentrations and inoculum size) on CLA formation. The optimum conditions to achieve the highest CLA production (0.24 mg/mL) were obtained using 3.00 mg/mL LA, 4.00 mg/mL yeast extract and a 4.00% (v/v) inoculum size.

The highest CLA content was produced by adding 0.90 mg/mL LA. According to Gorissen et al. (2011), the conversion of CLA depends on the amount of free LA in the medium. Gorissen et al. (2012) used *Bifidobacteria* and *Lactobacillus sakei* strains, which are able to produce CLA *in vitro*, as starter cultures for milk fermentation, and no significant increase in CLA content was observed, even with sufficient amounts of LA. This result is in accordance with the prior study (Gorissen et al., 2011), suggesting that the availability of free FA was likely too low. Xu, Boylston, and Glatz (2004) tested *Propionibacterium freudenreichii* ssp. *shermanii* inoculum in a model system containing milk-hydrolyzed soybean oil to obtain CLA. They obtained 1.45 mg of CLA (C18:2 *cis*-9, *trans*-11 isomer) per gram of fat after 24 h of fermentation. Lin et al. (1999) evaluated six lactic cultures for their ability to generate CLA from LA. The results showed no significant differences in CLA levels without added LA, suggesting that addition of a LA source is effective at enhancing CLA conversion during fermentation by LAB.

In the present study, we showed for the first time that *L. bulgaricus* and *S. thermophilus* could be co-cultured to produce CLA in yogurt. Surprisingly, a substantial increase in the amount of CLA was obtained by using an inoculum ratio of 1:2 (*St:Lb*). A 1:1 (*St:Lb*) ratio might not be effective for CLA synthesis as observed for these two strains. In yogurt production technology, if the 1:1 ratio is not respected, the ratio is generally unbalanced in coccus favor that drives the early stages of fermentation. Ye et al. (2013) found that a 1:1 ratio is optimal for the co-culture of *L. acidophilus* and *S. thermophilus* using hydrolyzed safflower oil as a substrate in a skim milk-based medium. However, a co-culture of *L. acidophilus* and *L. plantarum* led to an even greater production of CLA, which suggests that different factors influence CLA production.

Glucose was also an important variable in the production of CLA in yogurt samples. According to Kim and Liu (2002) supplementation of glucose improves CLA production even than compared to sucrose or lactose sources, which authors have associated with biohydrogenation pathway activation or bacterial cell growth by an easier energy source.

The addition of the lowest tested concentration (10.00 mg/mL) of glucose had a positive effect on CLA production. As shown in Figure 2, the variation in the CLA yield could be explained as a polynomial function of the glucose concentration and inoculum ratio. Two hypotheses can be used to explain why the low glucose concentration in the model positively affects the production of CLA. The first one is that the high availability of glucose in the mixture can cause metabolic stress by the generation of osmotic pressure. The second hypothesis comes from the presence of LA in high amounts, up to a specific limit that might potentially activate a metabolic response (detoxification) to convert LA into a less toxic molecule for cell growth. A better understanding of these two hypotheses will be investigated in future studies using comparative proteomics. Lin (2000) tested *L. bulgaricus* and *S. thermophilus* cultures for the effects of different carbohydrates (sucrose, lactose and fructose) at 60.00 mg/mL in skim milk medium on CLA (C18:2 *cis*-9,*trans*-11 isomer) production. All carbohydrates at this concentration inhibited CLA produced by *L. bulgaricus*.

3.4 Fatty Acid Composition of Yogurts

The C18:2 *cis*-9, *trans*-11 was the most abundant isomer in all different runs (Table 7). According to Jenkins, Wallace, Moate, and Mosley (2008), C18:2 *cis*-9, *trans*-11 isomer is usually considered the main health-promoting CLA for human consumption. The FAs involved in the biohydrogenation indicate an interesting pattern of oscillation between C18:2 *cis*-9, *trans*-11 and C18:2 *trans*-10, *cis*-12 isomers, and the amount of LA added to the yogurts appears to influence this ratio. Treatments containing 0.10 mg/mL LA (runs 1 to 4), disfavored the synthesis of the C18:2 *trans*-10, *cis*-12 isomer, which was 0.00-10.00% of the total CLA quantified. Whereas, treatments containing 0.90 mg/mL LA (runs 5 to 8) favored the C18:2 *trans*-10, *cis*-12 isomer, which was 22.00-25.00% of the total CLA quantified. However, in both cases, C18:2 *cis*-9, *trans*-11 was the major isomer produced. Furthermore, it was observed that the common factor between the two treatments able to form C18:2 *trans*-10, *cis*-12 isomer at low LA concentrations, was a 2:1 inoculum ratio of *St:Lb.* Thus, the production of these two isomers depend not only the enzyme sort, but also LA concentrations and appears to be strain dependent.

Table 7. Profile of the FAs involved in the biohydrogenation from different treatments used to produce sheep's milk yogurt (mg/g fat). Runs 1 to 9 are correspondent to the experimental design.

Fatty Acid	Run								
	1	2	3	4	5	6	7	8	9
Vaccenic acid (C18:1 t11)	157.45	125.30	121.10	130.49	149.01	146.34	131.34	174.85	115.95
LA (C18:2n6c)	26.93	105.25	78.74	94.45	88.88	113.32	133.05	119.53	86.11
CLA (c9, t11)	67.43	62.55	50.15	54.87	51.41	64.18	70.41	68.34	58.94
CLA (t10, c12)	0.00	4.90	5.0	0.00	14.59	21.42	24.18	19.64	9.25

Among the FAs involved in LA biohydrogenation, a purely empirical observation showed an inverse proportion to variations corresponding to LA and vaccenic acid levels, which can be explained by the reversible characteristic of the metabolic pathways (isomerization and hydrogenation processes). However, this issue has not been fully explored and needs further clarification.

To determine whether the storage time affects the fatty acid composition, especially in light of the CLA concentrations, in yogurt produced under different treatment conditions, the percent of FA in different samples was analyzed on the first day of fermentation and after 14 days of storage at 5 ℃ (Table 8). The FA composition was classified into saturated (SFA), monounsaturated (MUFA) and polyunsaturated (PUFA) FA according to the

saturation degree. In addition, the levels of C18:2 *cis*-9, *trans*-11 were represented in relation to the storage period as well.

Table 8. CLA value (mg/g fat) and FA percentage (% total FAME) from yogurts at day one of fermentation and after 14 days of storage at 5 ℃.

Run	SFA (%)		MUFA (%)		PUFA (%)		CLA (mg/g) (*cis*-9, *trans*-11 isomer)	
	1 day	14 day	1 day	14 day	1 day	14 day	1 day	14 day
1	71.70	71.30	25.11	24.55	03.17	04.15	67.43	55.22
2	72.77	71.37	23.80	24.40	03.43	04.24	62.55	48.42
3	72.20	71.48	23.82	24.40	04.00	04.15	50.14	50.42
4	72.54	71.07	23.53	24.67	03.93	04.25	54.87	48.20
5	73.47	71.80	22.78	23.94	03.75	04.28	51.41	48.26
6	72.02	71.21	23.76	24.60	04.21	04.20	64.18	55.17
7	72.15	71.27	23.70	24.47	04.13	04.26	70.41	47.95
8	72.56	71.20	23.51	24.54	03.92	04.26	68.34	47.02
9	72.16	71.15	23.81	24.60	04.03	04.25	58.94	49.31

Note. SFA= saturated fatty acids, MUFA= monounsaturated fatty acids, PUFA= polyunsaturated fatty acids.

The PUFA levels ranged from 03.17% to 04.26%. Serafeimidou et al. (2012) observed lower PUFA levels (02.80%) in sheep's milk yogurt, while in a similar study, Serafeimidou, Zlatanos, Kritikos, and Tourianis (2013) found equivalent PUFA levels (04.50%). In our work, the percentage of CLA generally decreased during cold storage in the samples analyzed. Kim and Liu (2002) suggested that this behavior might be related to the activation of reduction steps in the biohydrogenation pathway. Florence et al. (2012) suggested a possible strategy to increase the amount of CLA during cold storage by co-inoculating probiotic bacteria with yogurt starter cultures. The authors found a significant increase in relative CLA levels after 7 days of storage at 4 ℃ in fermented milk with a co-culture of *B. animalis* ssp. *lactis*, *S. thermophilus* and *L. bulgaricus*. In accordance with these results, Xu, Boylston, and Glatz (2005) screened four probiotic bacteria in single or co-culture with traditional yogurt cultures to evaluate CLA production in fermented milk. The combination of most bacteria with the yogurt cultures produced higher levels of CLA isomers than yogurt culture alone after 14 days of storage, which suggests that incorporation of probiotic bacteria could be key to keeping the CLA levels stable during storage periods of 14 days.

In a similar study, Serafeimidou et al. (2013) described changes in the CLA concentrations, FA profile and chemical composition of yogurt from cow's and sheep's milk, produced using traditional methods, during 14 days of storage at 5 °C. Refrigerated storage resulted in a significant decrease in CLA in cow's milk yogurt, while in sheep's milk yogurt there was the opposite trend, indicating a contradiction compared to results obtained in the present study.

The influence of storage time on CLA levels in dairy products is still controversial (Serafeimidou et al., 2013). Some authors have suggested that the accumulation of lactic acid has a detrimental effect on CLA levels (Khosravi-Darani et al., 2014; Kim & Liu, 2002). In our work, a co-culture of *L. bulgaricus* 2230 and *S. thermophilus* St 360 provided a more suitable condition for acid production (post-acidification), which may explain the two-week storage results. Dairy products can be impacted by several physical and chemical characteristics resulting from a modified acid and FA profile because the risk of oxidation and off-flavors increases with a high concentration of unsaturated FAs (Collomb, Schmid, Sieber, Wechsler, & Ryhänen, 2006).

3.5 Physico-chemical Analysis of Yogurts

The analytical results of the physical-chemical characteristics of sheep's milk yogurts are shown in Table 9. Moisture values ranged between 75.38 and 81.79%. Although there were no significant differences ($p > 0.05$) in the moisture content, this variation appears to be associated with glucose content into the samples. The incorporation of glucose as an ingredient in the prepared yogurt, leads to an increase in the osmolarity which would attract water to the yogurt-forming casein micelles. Serafeimidou et al. (2013) measured similar values in sheep's milk yogurt (79.37%), and no significant change was observed during storage. Prandini, Sigolo, Tansini, Brogna, and Piva (2007) found no correlation between CLA levels and the moisture of yogurt in their studies.

Table 9. Physico-chemical characteristics of sheep's milk yogurts.

Run	Moisture (%)	Ash (%)	Protein (%)	pH	Titratable acidity (% lactic acid)
1	81.79 ± 0.58^{a}	1.02 ± 0.00^{a,b}	5.96 ± 0.17^{a,b}	4.34 ± 0.07^{a,b}	1.64 ± 0.07^{a,b,c}
2	75.38 ± 6.16^{a}	1.00 ± 0.02^{a,b,c}	5.72 ± 0.26^{a,b}	4.33 ± 0.15^{a,b}	1.59 ± 0.06^{a,b}
3	76.28 ± 1.38^{a}	1.08 ± 0.03^{a}	6.25 ± 0.27^{a,b}	4.28 ± 0.31^{a,b}	1.58 ± 0.00^{a}
4	77.01 ± 3.98^{a}	1.03 ± 0.03^{a,b}	6.11 ± 0.22^{a,b}	4.58 ± 0.13^{a,b}	1.35 ± 0.01^{d}
5	81.27 ± 0.27^{a}	0.92 ± 0.06^{b,c}	5.70 ± 0.14^{a}	4.64 ± 0.21^{b}	1.75 ± 0.01^{a,b,c}
6	77.93 ± 1.04^{a}	0.89 ± 0.07^{c}	5.06 ± 0.99^{a}	4.16 ± 0.20^{a}	1.76 ± 0.08^{b,c}
7	79.96 ± 0.57^{a}	0.99 ± 0.06^{a,b,c}	6.84 ± 0.62^{b}	4.26 ± 0.17^{a,b}	1.64 ± 0.01^{a,b,c}
8	76.95 ± 0.15^{a}	1.06 ± 0.02^{a}	6.15 ± 0.12^{a,b}	4.47 ± 0.05^{a,b}	1.60 ± 0.00^{a,b,c}
9	79.73 ± 0.50^{a}	1.02 ± 0.02^{a}	5.90 ± 0.10^{a,b}	4.31 ± 0.14^{a,b}	1.77 ± 0.07^{c}

Note. ^{a,b,c} Means of three replicates ± SD with different letters in the same column are significantly different ($p < 0.05$).

There were significant ($p < 0.05$) differences in ash between yogurt samples. The highest ash levels were noted in run 3 (1.08%), whereas the lowest were found in run 6 (00.89%). Bano, Abdullah, Nadeem, Babar, and Khan (2011) used different concentrations of goat's and sheep's milk to develop a functional yogurt. The ash content increased significantly for all levels of sheep's milk added to the yogurt.

Generally, there were no significant ($p > 0.05$) differences for protein content in treatments of the yogurt samples, except between run 7 (06.84%) and runs 5 (05.70%) and 6 (05.06%). Katsiari, Voutsinas, and Kondyli (2002) and Voutsinas, Katsiari, Pappas, and Mallatou (1996) found similar protein levels in sheep's milk yogurt. Based on the results of Khosravi-Darani et al. (2014), the addition of 4.00% whey powder to whole milk increased CLA concentrations. The authors suggest that this occurs due to proteins acting as hydrogen donors during the first step of biohydrogenation, improving isomerization of LA and facilitating CLA formation. Kim and Liu (2002) also observed enhanced CLA production with the addition of nonfat dry milk powder. LAB partially hydrolyzes proteins, which increases the amount of free amino acids in fermented dairy products, making yogurt proteins more easily digestible than the proteins found in liquid milk, although the amount of proteins in each are similar (Hossain, 2015).

The analytical results showed a significant ($p < 0.05$) difference between run 5 (04.64%) and run 6 (04.16%) in terms of pH values. This finding is in agreement with Balthazar et al. (2015) who produced a sheep's milk yogurt with a pH of 04.41. These authors also found an acidity (lactic acid % w/v) of 00.94, similar to our results.

Significant ($p < 0.05$) differences in titratable acidity were also found in some yogurt samples. The highest lactic acid was observed in run 9 (01.77%), whereas the lowest was found in run 4 (01.35%). According to Jay (2005), *S. thermophilus* can produce approximately 00.50% lactic acid, while *L. bulgaricus* can produce 00.60 to 00.80% (pH 04.20-04.50). However, if the incubation time is longer, the pH may fall, increasing lactic acid to 02.00%.

In conclusion, among screened *L. bulgaricus* strains, 12 exhibited an ability to produce CLA (C18:2 *cis*-9, *trans*-11 isomer) in sheep's milk. The percentage of CLA produced varied from 07.00 to 74.00%. For the *S. thermophilus* strains, 13 of them showed an increase in CLA (C18:2 *cis*-9, *trans*-11 isomer) levels (from 8.00 to 54.00%). Therefore, when placed together in co-culture, *L. bulgaricus* 2230 and *S. thermophilus* St 360 should elevate the CLA content in sheep's milk yogurt. The optimum conditions for producing the highest CLA levels in sheep's milk yogurt consisted of adding 10.00 mg/mL (w/v) glucose, 30.00 mg/mL (w/v) powdered sheep's milk, 0.90 mg/mL (w/v) LA and a 1:2 (*St:Lb*) (v/v) ratio of bacterial strains. Nevertheless, the CLA levels in sheep's milk yogurt decreased after storage at 5 ℃ for 14 days.

To confer health benefits to humans, one must consume 1.00 to 3.00 g of CLA per 70 kg of human body weight per day. To reach this recommended value, approximately four servings per day of 250 mL each of the high-CLA yogurt produced in this work would be sufficient. To estimate the number of recommended dose, calculation was based on total fat (4.28%) and total CLA (70.41 mg/g fat) of treatment 7. However, further investigation into the ingestion of CLA is necessary considering the fact that there are other sources of CLAs in the human diet. Further studies are also needed to characterize the screened CLA producer strains to explore their potential functional properties, which could be used to the benefit of consumers. Furthermore, the model obtained in this work may allow for optimization in the development of CLA-rich yogurts by the dairy industry, although an expansion to a proper technological and sensory evaluation is required.

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