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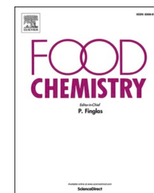

Study of the viability of using lipase-hydrolyzed commercial vegetable oils to produce microbially conjugated linolenic acid-enriched milk

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ABSTRACT

This work studied the viability of using vegetable oils as precursor substrates to develop a dairy product enriched in microbial conjugated linoleic (CLA) and conjugated linolenic (CLNA) acids.

Hydrolysis of hempseed, flaxseed (FSO) and soybean (SBO) oils was tested with *Candida rugosa* (CRL), *Pseudomonas fluorescens,* or Pancreatic porcine lipases. FSO and SBO, previously hydrolyzed with CRL, were further selected for cow's milk CLA/CLNA-enrichment with *Bifidobacterium breve* DSM 20091. Thereafter, higher substrate concentrations with hydrolyzed FSO were tested.

For all tested oils, CRL revealed the best degrees of hydrolysis (>90 %). Highest microbial CLA/CLNA yield in milk was achieved with hydrolyzed FSO, which led to the appearance of mainly CLNA isomers (0.34 mg/g). At higher substrate concentrations, maximum yield was 0.88 mg/g CLNA.

Therefore, it was possible to enrich milk with microbial CLNA using vegetable oil, but not with CLA, nor develop a functional product that can deliver a reliable effective dose.

1. Introduction

The study on the development of innovative and value-added food products has been an increasing tendency in the last years, and different compounds with potential bioactive properties have been identified. Conjugated linoleic acid (CLA) and conjugated linolenic acid (CLNA) isomers are described with potential anti-carcinogenic, anti-obesity and anti-inflammatory effects (Hennessy et al., 2016; Yang et al., 2015). Both compounds are naturally produced during dietary linoleic (LA) and α-linolenic (α-LNA) acids biohydrogenation to stearic acid (C18:0) by ruminal bacteria. CLA is also synthesized through the conversion of *trans*-vaccenic acid (C18:1 t11; TVA) by Δ9-desaturase, mainly in the mammary gland (Salsinha et al., 2018). These fatty acids (FA) are then naturally present in meat (0.3–17 mg/g fat) and milk (0.3–33 mg/g fat) of ruminants (Fontes et al., 2017; Shokryzadan et al., 2017), however not at levels high enough to cause any beneficial effect, since effective doses of 3–6 g/day for CLA and 2–3 g/day for CLNA have been recommended (Kung & Lin, 2021).

Strains isolated from dairy products and human gastrointestinal tract have shown the capacity to produce CLA and CLNA isomers when in the presence of LA and α-LNA, respectively. Indeed, bifidobacteria, lactobacilli and propionibacteria strains have revealed considerably high substrate conversion rates (50.5–98.0 %) in culture medium (Yang, Chen, et al., 2017, Yang, Gao, et al., 2017). Therefore, some research works have studied microbial CLA/CLNA production in different food products, like sucuk (Özer et al., 2016), walnut milk (Mao et al., 2022)

Abbreviations: LA, Linoleic acid; α-LNA, alfa-Linolenic acid; CLA, Conjugated linoleic acid; CLNA, Conjugated linolenic acid; FA, Fatty acid; CRL, *Candida rugosa* lipase; PFL, *Pseudomonas fluorescens* lipase; PPL, Porcine pancreatic lipase; HSO, Hemp seed oil; FSO, Flaxseed oil; SBO, Soybean oil.

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or blackcurrant press residue (Vahvaselkä et al., 2021), as a strategy to develop enriched sources of these conjugated fatty acids (CFA). In the case of dairy, Hennessy et al. (2009) have inoculated a 20 % (w/v) reconstituted skimmed milk, added with 0.35 mg/mL LA, with several bifidobacteria strains, obtaining conversion rates of 7.3–49.0 %. Moreover, our team has previously cultured a semi-skimmed UHT milk, added with 0.5 mg/mL α-LNA, with *B. breve* NCIMB 702258 and found that 21.6 % of the substrate was converted into CLNA isomers (Fontes et al., 2018). However, these former research works have been performed with pure LA or α-LNA, which are not safe for human consumption. An alternative solution could be the use of edible vegetable oils rich in LA or α-LNA, and among the few commercially available are flaxseed oil (16.7 % LA/59.3 % α-LNA of total FA (Teh & Birch, 2013)), soybean oil (54.2 % LA/5.2 % α-LNA of total FA (Dorni et al., 2018)) and hemp seed oil (56.1 % LA/16.0 % α-LNA of total FA (Vonapartis et al., 2015)), which were inclusively selected for this study. However, previous treatment with lipases would be necessary, since it has been reported much higher microbial CLA contents when lipases were applied (Ando et al., 2004). Indeed, without previous hydrolysis, bifidobacteria strains showed no yield of CLA or CLNA from different vegetable oils (Gorissen et al., 2012). This comes from the fact that such bacteria convert LA and α-LNA into conjugated isomers at the cell membrane level from their free forms (Salsinha et al., 2018). For this study three lipases were selected: *Candida rugosa* lipase, a non-specific lipase extensively studied, with high hydrolytic efficiency (Aziz et al., 2015; Nguyen et al., 2018), *Pseudomonas fluorescens* lipase and porcine pancreatic lipase, both 1, 3-specific lipases (Rodriguez et al., 2008; Rupani et al., 2012), that have shown good release rates of free LA/α-LNA (Freitas et al., 2007; Rupani et al., 2012), although, in vegetable oils, these FAs are also found in the *sn*-2 position of the triacylglyceride and at higher distribution levels (González-Fernández et al., 2017).

Taken all together, we hypothesized that a functional and edible dairy product enriched in microbial CLA and CLNA can be obtained by inoculating the previously identified *B. breve* NCIMB 702258 (or DSM 20091) strain with lipase-hydrolyzed vegetable oils rich in LA and α-LNA. Thus, the aim of this research was to i) establish the most favorable edible vegetable oil plus lipase combination, among those selected for this study, that would enable the release of the highest levels of free LA/α-LNA, ii) test the viability of using hydrolyzed commercial vegetable oils as precursor substrate source for milk microbial CLA/CLNA-enrichment, and iii) determine the maximum CLA/CLNA content that is possible to achieve in a dairy product through this strategy.

2. Material and methods

2.1. Chemicals and samples

Hexane and dimethylformamide (DMF) were HPLC grade (VWR Chemicals, West Chester, PA, USA), as well as methanol (Carlo Erba Reagents, Barcelona, Spain). Sulphuric acid was from Honeywell Fluka (Charlotte, NC, USA), while sodium methoxide and methyl acetate were from Acros Organics (Geel, Belgium). GLC-Nestlé36 FAME mix was obtained from Nu-Chek Prep, Inc. (Elysian, MN, USA) and butterfat CRM-164 (EU Commission; Brussels, Belgium) from Fedelco Inc. (Madrid, Spain). Undecanoic acid (98.0 %) was from Alfa Aesar (Haverhill, MA, USA), while glyceryl tritridecanoate (>99.0 %) was from Larodan (Solna, Sweeden). Supelco 37 FAME mix and methyl tricosanoate (≥99.0 %) were purchased from Sigma-Aldrich (St. Louis, MO, USA), as well as *Candida rugosa* (CRL) type VII, Pancreatic porcine (PPL) type II and *Pseudomonas fluorescens* (PFL) lipases. Tonalin CLA was kindly given by BASF Portuguesa S.A. (Porto, Portugal) and pomegranate seed oil (PSO) by Earthoil (Norfolk, United Kingdom). Pasteurized semi-skimmed cow's milk and soybean (SBO), flaxseed (FSO) and hemp seed (HSO) oils were bought in local markets (Porto, Portugal).

2.2. Vegetable oils hydrolysis

For each edible vegetable oil a 10 g reaction mixture was prepared in Erlenmeyer flasks of 100 mL according to Lu et al. (2018): water/oil ratio of 3:5 (w/w) and lipase 1 % (w/w) of the vegetable oil weight. The flasks were incubated in an orbital shaker-incubator (MaxQ SHKE6000; Thermo Fisher Scientific, Waltham, MA, USA) at 35 °C (PPL), 45 °C (CRL) or 55 °C (PFL) and 200 rpm for 24 h. Then, the reaction mixture was centrifuged at 5000 rpm and room temperature for 10 min. The vegetable oil was thereafter recovered (upper layer) and centrifuged again, at the same previous conditions, to remove any traces of water. Experiments were carried out in triplicate. Vegetable oil samples were taken before and after hydrolysis for further FA analysis (sections 2.5 and 2.6). The hydrolysis degree was calculated based on the initial and final amount of FA in the esterified fraction as follows:

$$Hydrolysis\ degree(\%) = \frac{([EFA_i] - [EFA_f]) \times 100}{[EFA_i]}$$

Being EFA_i the initial concentration of esterified FA (before hydrolysis) and EFA_f the final concentration of esterified FA (after hydrolysis).

2.3. Microbially CLA/CLNA-enriched milk

Stock oil emulsions were prepared at 50 mg/mL of free LA (with hydrolyzed SBO) or free α-LNA (with hydrolyzed FSO) with 2 % (w/v) Tween 80 (Sigma-Aldrich) and homogenized with an Ultra-Turrax (T 25 digital; IKA Works, Inc., Wilmington, NC, USA) at 13600 rpm for 150 s (five 30 s-intervals separated by 30 s-pauses, to not heat-up the emulsions). Then, oil emulsions were UV-sterilized in a Laminar Flow Hood (BIO-II-A; Azbil Telstar, Tokyo, Japan) for 20 min to guarantee asepsis when further added to milk.

A strain previously assayed as CLA/CLNA producer by these authors (Fontes et al., 2018), *Bifidobacterium breve* DSM 20091 (DSMZ, Braunschweig, Germany), stored at −80 °C in glycerol 30 % (w/w) (Fisher Scientific, Hampton, NH, USA), was activated at 2 % (v/v) in MRS broth (Biokar Diagnostics, Beauvais, France), supplemented with 0.01 % (w/v) bacteriological meat extract (Biokar) and 0.05 % (w/v) L-cysteine-HCl (Sigma-Aldrich), since microorganisms like bifidobacteria require cysteine as a sulfur source for their growth (Wada et al., 2021). After overnight incubation at 37 °C, the activated culture was then transferred to fresh cys-MRS medium at 10 % (v/v) and incubated at 37 °C for 16 h. Afterward, the strain was inoculated at 1 % (v/v) in milk (100 mL) supplemented with 0.05 % (w/v) cysteine and under the following substrate conditions: *Control* – No substrate; *S0.5LA* – 0.5 mg/mL LA from hydrolyzed SBO; *S0.5LNA* – 0.5 mg/mL α-LNA from hydrolyzed FSO; *S0.25LA/LNA* – 0.25 mg/mL LA plus 0.25 mg/mL α-LNA from combined hydrolyzed SBO and FSO; *S0.25LA/LNA'* – 0.25 mg/mL LA from hydrolyzed SBO plus 0.25 mg/mL α-LNA from hydrolyzed FSO; *S0.5LA/LNA* – 0.5 mg/mL LA plus 0.5 mg/mL α-LNA from combined hydrolyzed SBO and FSO; *S0.5LA/LNA'* – 0.5 mg/mL LA from hydrolyzed SBO plus 0.5 mg/mL α-LNA from hydrolyzed FSO. Inoculated milk was then incubated at 37 °C for 24 h. Cultures were always grown under anaerobic conditions (Whitley DG 250; Don Withley Scientific, Yorkshire, UK; gas mixture of 80 % nitrogen, 10 % hydrogen and 10 % carbon dioxide). Experiments were carried out in triplicate. Milk samples were collected before and after the incubation period for further viable cell numbers determination, pH measurement (Basic 20; Crison, Barcelona, Spain) and FA analysis (sections 2.5 and 2.6). The substrate reduction percentage was calculated as follows:

$$Substrate\ reduction(\%) = \frac{([Subs_i] - [Subs_f]) \times 100}{[Subs_i]}$$

Being $Subs_i$ the initial amount (0 h) of free substrate and $Subs_f$ the final amount (24 h) of free substrate. Linoleic acid and α-LNA conversion

percentages were calculated based on the assumption that all CLA and CLNA produced derived from their former substrates only:

$$Substrate\ \ conversion(\%) = \frac{([CFA_f] - [CFA_i]) \times 100}{[Subs_i]}$$

Being CFA_f the final amount (24 h) of free conjugated fatty acid, CFA_i the initial amount (0 h) of free conjugated fatty acid and $Subs_i$ the initial amount (0 h) of the corresponding free substrate (ie. LA for CLA and α-LNA for CLNA).

2.4. Microbially CLNA-enriched milk

Bifidobacterium breve DSM 20091 was activated and inoculated as described in section 2.3 using hydrolyzed FSO at the following substrate conditions: *Control* – No substrate; *S1LNA* – 1 mg/mL α-LNA; *S1.5LNA* – 1.5 mg/mL α-LNA; *S2LNA* – 2 mg/mL α-LNA; *S5LNA* – 5 mg/mL α-LNA; *S10LNA* – 10 mg/mL α-LNA. Experiments were carried out in triplicate. Milk samples were collected before and after the incubation period for further viable cell numbers enumeration, pH measurement and FA analysis (sections 2.5 and 2.6).

2.5. FA analysis

For the FA analysis, edible vegetable oil (15 mg), edible vegetable oil emulsion (500 μL), milk (500 mg) samples, or Tonalin CLA and PSO (5 mg) were prepared according to Pimentel et al. (2015). First, for quantification of esterified FA (EFA), samples were added with 200 μL of glyceryl tritridecanoate (1.5 mg/mL) and 100 μL of undecanoic acid (1.5 mg/mL) before derivatization. Then, 100 μL of methyl acetate was added, followed by 3.60 mL of hexane, 2.26 mL of methanol and 240 μL of sodium methoxide (5.4 M). Samples were vortexed and incubated at 40 °C for 10 min. After cooling on ice, 500 μL of deionized water was added (to enable phase separation), and samples were vortexed and centrifuged (1250 ×g, 18 °C, 5 min). The upper layer, containing methyl esters (FAME), was then collected in a 15 mL centrifuge tube, containing already 200 μL of methyl tricosanoate (1.5 mg/mL), for further analysis. To clean up, 2 mL of hexane was added to the remaining methanol, and samples were vortexed and centrifuged (1250 ×g, 18 °C, 5 min). The upper layer was collected into the centrifuge tube. Thereafter, for quantification of free FA (FFA), 100 μL of methyl tricosanoate (1.5 mg/mL) was added, followed by 1.25 mL of DMF, before 1.25 mL of sulphuric acid (3 M). Samples were vortexed and incubated at 60 °C for 30 min. Finally, after cooling, 700 μL of hexane was added, and samples were vortexed and centrifuged (1250 ×g, 18 °C, 5 min). The upper layer, containing FAME, was collected for further analysis.

2.6. Gas chromatography conditions

As previously reported (Fontes et al., 2018), FAME were analyzed in a gas chromatograph HP6890A (Hewlett-Packard, Avondale, PA, USA), equipped with a flame-ionization detector (GLC-FID) and a BPX70 capillary column (60 m × 250 μm × 0.25 μm; SGE Europe ltd, Courtaboeuf, France). Analysis conditions were as follows: injector temperature 250 °C, split 25:1, injection volume 1 μL; detector (FID) temperature 275 °C; hydrogen as the carrier gas at 20.5 psi; oven temperature program – started at 60 °C (held 5 min), then raised at 15 °C/min to 165 °C (held 1 min) and finally at 2 °C/min to 225 °C (held 2 min). Supelco 37 and FAME from CRM-164 were used for the identification of FAs. Tonalin CLA was applied to identify the chromatographic region of CLA isomers. CLA and CLNA isomers identification was based on previous works carried out with the same strain assayed herein (Coakley et al., 2009; Fontes et al., 2018). GLC-Nestlé36 was assayed for calculation of response factors and detection and quantification limits (LOD: 0.79 ng FA/mL; LOQ: 2.64 ng FA/mL).

2.7. UV spectroscopy

FAME extracts of milk FFA fraction before and after fermentation with hydrolyzed FSO at 2 mg/mL α-LNA (S2LNA) and of EFA fraction from Tonalin CLA and PSO were analyzed in a UV spectrophotometer UV-1900 (Shimadzu, Kyoto, Japan). The absorption spectra were recorded between 200 and 400 nm, at 1 nm intervals, using diluted solutions of each sample in hexane until absorbance was below 1.50. Hexane was further used for background measure.

2.8. Statistical analysis

Results are reported as mean values ± standard deviation (n = 3). Data were first analyzed for normality distribution. Levene's test was applied to verify the homogeneity of the variances. When the normality assumption was not violated, one-way ANOVA was performed to verify statistical differences between groups with equal variances, otherwise, it was applied the Welch test. When normality was not guaranteed, the Kruskal-Wallis test was carried out instead. For groups with statistical differences, pairwise comparisons were made through post-hoc Bonferroni (homogeneity of variances guaranteed) or Games-Howell (homogeneity of variances not guaranteed) for normal data, otherwise, the Mann-Whitney test was applied. Regarding the analysis of FA profiles, at each substrate condition, differences from 0 h to 24 h were compared through T-test paired samples when normality was guaranteed, otherwise, the Wilcoxon test was applied. The level of significance was set, in general, at 5 %; for growth experiments, CFU differences were considered when differing ≥ 1 log cycle and for pH when differing ≥ 0.5 units. Analyses were performed using IBM SPSS Statistics 28 (SPSS Inc., IBM Corporation, NY, USA).

3. Results and discussion

3.1. Vegetable oils hydrolysis

A preliminary analysis of the edible vegetable oils' composition revealed that HSO and SBO oils had a high amount of LA (49.57 and 43.71 g/100 g, respectively), while FSO was rich in α-LNA (41.32 g/100 g) (Table 1). A smaller proportion of α-LNA was also detected in HSO and SBO, and of LA in FSO (6.07–15.06 g/100 g) (Table 1). However, only a small amount of these FA was present as free form (<1.5 g/100 g), thus remaining FAs should be esterified in other lipids found in oils' composition, like triacylglycerides (data not shown).

After hydrolysis, it was observed that CRL lipase produced the best outcome, with hydrolysis percentages of at least 91.7 % for LA and α-LNA in all the edible vegetable oils tested (Table 2). Based on FFA release, hydrolysis degrees ranging from 79.6 % to 97.2 % have also been reported for other vegetable oils treated with this lipase, including flaxseed (Rupani et al., 2012), coconut (Nguyen et al., 2018) and palm

Table 1
HSO, FSO and SBO total FA composition (g/100 g oil).

Fatty acid	HSO	FSO	SBO
C14	0.05 ± <0.01	0.07 ± <0.01	0.10 ± <0.01
C16	6.70 ± 0.41	5.99 ± 0.22	10.66 ± 0.35
C16:1 c9	0.12 ± 0.01	0.09 ± <0.01	0.11 ± 0.01
C18	5.20 ± 0.74	6.96 ± 0.56	6.46 ± 0.19
C18:1 c9	14.62 ± 1.07	21.35 ± 0.96	23.59 ± 0.74
C18:2 c9c12	49.57 ± 4.60	15.06 + 0.76	43.71 ± 1.52
C18:3 c6c9c12	0.47 ± 0.04	ND	ND
C18:3 c9c12c15	12.83 ± 1.22	41.32 ± 2.04	6.07 ± 0.23
C20	0.80 ± 0.05	0.23 ± 0.02	0.40 ± 0.03
C20:1 c11	0.35 ± 0.03	0.15 ± 0.01	0.22 ± 0.01
C20:3 c11c14c17	0.31 ± 0.03	0.20 ± 0.01	0.41 ± 0.02

Average values ± standard deviation (n = 3).
HSO – Hemp seed oil; FSO – Flaxseed oil; SBO – Soybean oil; *c* – cis double bound; ND – Not detected.

Table 2
Hydrolysis degree (%) of esterified LA, α-LNA and total FA.

	HSO			FSO			SBO		
	CRL	PFL	PPL	CRL	PFL	PPL	CRL	PFL	PPL
LA	95.0 ± 0.1^a	68.7 ± 1.6^b	13.4 ± 0.1^c	$94.8 \pm <0.1^a$	67.8 ± 1.4^b	4.3 ± 1.5^c	92.6 ± 3.6^a	70.3 ± 1.8^b	$0.2 \pm <0.1^c$
α-LNA	94.3 ± 0.1^a	69.2 ± 1.5^b	17.5 ± 0.2^c	94.6 ± 0.1^a	68.5 ± 1.2^b	5.6 ± 1.5^c	91.7 ± 2.9^a	68.5 ± 2.3^b	0.5 ± 0.1^c
Σ FA	94.4 ± 0.1^a	67.2 ± 1.6^b	14.9 ± 0.1^c	94.5 ± 0.1^a	66.2 ± 1.4^b	5.4 ± 2.1^c	90.7 ± 5.7^a	68.2 ± 1.9^b	0.5 ± 0.1^c

Average values ± standard deviation (n = 3).
Different superscript letters for significant differences ($p < 0.05$) within rows of the same oil.
HSO – Hempseed oil; FSO – Flaxseed oil; SBO – Soybean oil; CRL – *Candida rugosa* lipase; PFL – *Pseudomonas fluorescens* lipase; PPL – Pancreatic porcine lipase; LA – Linoleic acid; α-LNA – α-Linolenic acid; Σ FA – Total fatty acids.

(Serri et al., 2008) oils. Pancreatic porcine lipase revealed the worst hydrolytic efficiency (0.2–17.5 %) (Table 2). In other studies, PPL exhibited a 23 % hydrolysis degree with SBO (Freitas et al., 2007), but, with safflower oil, it reached 91.6 % (Aziz et al., 2015). PPL hydrolytic efficiency seems oil-dependent, but differences in reaction conditions could have also influenced it. Concerning PFL, it was able to hydrolyze 67.8–70.3 % of LA or α-LNA in all the assayed oils (Table 2). A similar hydrolysis degree (~65 %) has been reported for blackcurrant oil by Vacek et al. (2000).

Given CRL's high hydrolytic efficiency, this lipase achieved the highest concentration of free LA in HSO (63.63 g/100 g), followed by SBO (53.85 g/100 g), and of α-LNA in FSO (48.94 g/100 g) (Table 3). Therefore, CRL was selected and tested in the following assays. For cost-effective reasons, HSO was not included in the following experiments, since it is more expensive than SBO, and this former oil can provide a good source of LA as well.

3.2. Microbially CLA/CLNA-enriched milk

Bifidobacterium breve DSM 20091 was able to grow well at the different substrate concentrations assayed, corroborated by the significant (>1 $\log_{10}$) increase in viable cell numbers after 24 h of incubation (from 7.10–7.22 to 9.44–9.61 $\log_{10}$; Fig. S1), followed by a significant (>0.5 units) decrease in pH level (from pH 6.23–6.50 to pH 4.23–4.42; Fig. S2), with no differences from control (ie. strain without precursor substrate). No differences were found between substrate conditions at 24 h as well (Figures S1 and S2). In a previous work (Fontes et al., 2018) it was shown that this strain could also grow with pure substrates as well as without them in semi-skimmed milk. Changing substrate source to vegetable oils revealed that these do not negatively affect *B. breve* DSM 20091. Besides, dairy matrices exhibit a protective effect on probiotic bacteria viability (de Almeida et al., 2018).

Regarding the CFA production, in control conditions, although milk has a certain amount of LA and α-LNA, the majority is in the esterified form (0.352 mg/g and 0.061 mg/g, respectively; Table S2), and therefore not bioavailable for the microorganism. Substrate reduction was minimal – 4.51 % for LA and 11.45 % for α-LNA (Table 4). Furthermore, the CLA initially present in the esterified fraction of control samples (0.049 mg/g) did not increase (Table S2). All this supports the fact that microbial production of CLA and CLNA can only occur from free substrates. Regarding CFA production in the presence of the assayed vegetable oils, the amount of the CLA released remained low at any of the tested substrate conditions (0.04–0.08 mg/g), being detected C18:2 c9t11 and C18:2 t,t (Table 4). With both vegetable oils added (ie. S0.25LA/LNA, S0.25LA/LNA', S0.5LA/LNA and S0.5LA/LNA'), CLNA production was higher than that of CLA (Table 4). The highest CFA production was achieved for S0.5LNA (0.37 mg/g), corresponding mainly to CLNA isomers (0.34 mg/g) – C18:3 c9t11c15 and C18:3 t9t11c15 (Table 4). Accordingly, α-LNA conversion degrees were higher than those of LA for all tested conditions, ranging from 43.31 to 61.69 mg/g for α-LNA and from 12.93 to 23.58 mg/g for LA (Table 4). Taken all together, it seems that *B. breve* DSM 20091 has a preference to convert α-LNA instead of LA. In a previous research work (Fontes et al., 2018), the authors reported similar behavior by this same strain when grown in culture medium or semi-skimmed milk supplemented with pure precursor substrates, either alone or in combination. In this latter case, two possible reasons were suggested for such findings: i) α-LNA is more toxic than LA, since CLA and CLNA production by bacteria may be part of a detoxification mechanism (Sosa-Castañeda et al., 2015); ii) linoleate isomerase (LAI), described as the enzyme responsible for LA and α-LNA isomerization (Salsinha et al., 2018), has a higher specificity for α-LNA. Comparing the conversion degrees, the percentages of the precursor substrate reduction were higher, especially for LA, with a nearly 2.4 to 3.3-fold difference (Table 4). Previous results (Fontes et al., 2018) suggested that both FA enter bacteria membrane, but α-LNA bioconversion to CLNA is faster.

The results of this second stage of the research work showed that it is not possible to simultaneously enrich milk with CLA and CLNA by *B. breve* DSM 20091 using vegetable oils as precursor substrate sources, and the best option would be to use just FSO and obtain a food product enriched in CLNA. Based on this rationale, the third stage was performed with only FSO.

Few studies have been carried out on dairy microbial CLA/CLNA-enrichment with vegetable oils so far. When different bifidobacteria strains were tested with rapeseed and sunflower oils as LA/α-LNA sources, no production was detected in milk (Gorissen et al., 2012). However, in this previous study, the vegetable oils were not hydrolyzed, thus, the substrate was not bioavailable for further conversion. On the other hand, a *Lactococcus lactis* strain has been able to produce up to 11 mg CLA/g fat from sunflower oil in milk (Kim & Liu, 2002). That could

Table 3
Free LA, α-LNA and total FA amounts (g/100 g) after hydrolysis.

	HSO			FSO			SBO		
	CRL	PFL	PPL	CRL	PFL	PPL	CRL	PFL	PPL
LA	63.63 ± 1.42^a	46.47 ± 0.86^b	13.81 ± 0.98^c	19.32 ± 0.77^a	14.26 ± 0.40^b	0.95 ± 0.03^c	53.85 ± 2.23^a	39.78 ± 0.92^b	1.00 ± 0.01^c
α-LNA	15.13 ± 0.36^a	11.27 ± 0.20^b	3.94 ± 0.29^c	48.94 ± 2.05^a	35.27 ± 1.00^b	3.01 ± 0.08^c	6.84 ± 0.22^a	5.03 ± 0.12^a	0.18 ± 0.01^b
Σ FA	114.15 ± 2.98^a	81.43 ± 1.43^b	28.24 ± 2.40^c	112.00 ± 4.65^a	79.95 ± 1.42^b	6.89 ± 0.14^c	108.70 ± 5.43^a	78.66 ± 2.23^b	2.38 ± 0.03^c

Average values ± standard deviation (n = 3).
Different superscript letters for significant differences ($p < 0.05$) within rows of the same oil.
HSO – Hempseed oil; FSO – Flaxseed oil; SBO – Soybean oil; CRL – *Candida rugosa* lipase; PFL – *Pseudomonas fluorescens* lipase; PPL – Pancreatic porcine lipase; LA – Linoleic acid; α-LNA – α-Linolenic acid; Σ FA – Total fatty acids.

Table 4
LA and α-LNA acids reduction and conversion percentages and amounts of CLA and CLNA isomers produced by *Bifidobacterium breve* DSM 20091 cultured in semi-skimmed milk supplemented with different substrate concentrations for 24 h at 37 °C (based on FA profile of the free fraction).

	Control	S0.5LA	S0.5LNA	S0.25LA/LNA	S0.25LA/LNA'	S0.5LA/LNA	S0.5LA/LNA'
LA reduction (%)	4.51 ± 0.45^{c}	41.56 ± 4.74^{ab}	32.31 ± 4.57^{b}	49.23 ± 4.56^{a}	50.25 ± 2.33^{a}	36.67 ± 3.86^{b}	38.86 ± 2.74^{b}
α-LNA reduction (%)	11.45 ± 2.52^{e}	59.85 ± 5.09^{d}	84.48 ± 0.47^{a}	79.60 ± 3.69^{ab}	79.08 ± 1.72^{ab}	72.75 ± 5.16^{bc}	69.95 ± 2.36^{c}
Δ CLA (mg/g)	ND	0.06 ± 0.01^{b}	$0.04 \pm <0.01^{c}$	0.06 ± 0.01^{b}	$0.06 \pm <0.01^{b}$	0.08 ± 0.01^{a}	$0.07 \pm <0.01^{ab}$
Δ CLNA (mg/g)	ND	$0.03 \pm <0.01^{d}$	0.34 ± 0.04^{a}	$0.11 \pm <0.01^{c}$	0.11 ± 0.01^{c}	0.20 ± 0.01^{b}	0.17 ± 0.01^{b}
Δ CFA (mg/g)	ND	0.09 ± 0.01^{d}	0.37 ± 0.04^{a}	0.17 ± 0.01^{c}	0.17 ± 0.01^{c}	0.28 ± 0.02^{b}	0.24 ± 0.01^{b}
LA conversion (%)	ND	14.69 ± 1.37^{bc}	19.21 ± 2.55^{ab}	23.58 ± 2.86^{a}	18.81 ± 0.73^{ab}	16.79 ± 1.83^{bc}	12.93 ± 0.49^{c}
α-LNA conversion (%)	ND	48.52 ± 4.57^{bc}	61.69 ± 5.90^{a}	56.46 ± 2.97^{ab}	49.36 ± 1.83^{b}	51.41 ± 3.47^{ab}	43.31 ± 2.06^{c}

Average values ± standard deviation (n = 3).
Different superscript letters for significant differences ($p < 0.05$) within rows.
Control – No substrate; S0.5LA – 0.5 mg/mL LA from hydrolyzed SBO; S0.5LNA – 0.5 mg/mL α-LNA from hydrolyzed FSO; S0.25LA/LNA – 0.25 mg/mL LA plus 0.25 mg/mL α-LNA from combined hydrolyzed SBO and FSO; S0.25LA/LNA' – 0.25 mg/mL LA from hydrolyzed SBO plus 0.25 mg/mL α-LNA from hydrolyzed FSO; S0.5LA/LNA – 0.5 mg/mL LA plus 0.5 mg/mL α-LNA from combined hydrolyzed SBO and FSO; S0.5LA/LNA' – 0.5 mg/mL LA from hydrolyzed SBO plus 0.5 mg/mL α-LNA from hydrolyzed FSO; LA – Linoleic acid; α-LNA – α-Linolenic acid; CLA – Conjugated linoleic acid (C18:2 c9t11 + C18:2 t,t); CLNA – Conjugated linolenic acid (C18:3 c9t11c15 + C18:3 t9t11c15); CFA – Conjugated fatty acids (CLA + CLNA); ND – not detected.

be associated with the fact that lactic acid bacteria (LAB) are described as possessing weak lipolytic activity (García-Cano et al., 2019). With hydrolyzed SBO, Xu et al. (2004) were able to enrich milk with 1.04 to 2.21 mg CLA/g lipid by different probiotic strains after 24 h, but none was a *B. breve* strain. Therefore, CLA production capacity from SBO could be also a strain-dependent feature. In a further study, with previously selected strains, Xu et al. (2005) could no longer detect CLA production in milk with hydrolyzed SBO, but that could be related to the high LA concentration tested (5 mg/mL). To the best of our knowledge, the study presented herein is the first research work to show microbial CLNA-enrichment of a dairy matrix using an edible substrate source.

3.3. Effect on the FA profile

When comparing the FFA composition at the beginning of the experiment (at 0 h) versus fermented samples (at 24 h), it was observed in most of the milk samples at the substrate conditions tested, a significant decrease ($p < 0.05$) in butyric (C4; from 0.018–0.021 mg/g to 0.013–0.015 mg/g), oleic (C18:1 c9; from 0.314–0.503 mg/g to 0.184–0.335 mg/g), total monounsaturated FA (MUFA; from 0.342–0.540 mg/g to 0.201–0.355 mg/g) and total FA levels (from 1.524–2.454 mg/g to 0.931–1.605 mg/g) (Table S1). Total polyunsaturated FA (PUFA) was also significantly changed ($p < 0.05$; from 0.450–0.952 mg/g to 0.340–0.704 mg/g), given the reduction in LA and α-LNA (Table S1). As for EFA, few significant changes were observed, although there was a general reduction in FAs for all tested conditions (Table S2), which may be due to their use during microbial growth (Fontes et al., 2018). Analyses of the FA composition of sucuk (Özer et al., 2016) and cow's milk (Vieira et al., 2017), fermented with LAB, have also revealed a decrease in oleic acid and MUFA when compared with control (i.e. without LAB). Vieira et al. (2017) reported that a decrease in MUFA, attributed to the consumption of oleic acid, occurs as a way to control bacterial membrane fluidity under stress conditions, such as fermentation. Furthermore, this former study observed an increase in stearic acid (C18), as did Özer and Kılıç (2021) in beef fermented with *Lactobacillus plantarum* strains and hydrolyzed safflower oil. It has been stated that it could be associated with the detoxification mechanism (Özer & Kılıç, 2021) since stearic acid is the final product of the LA biohydrogenation pathway (Fontes et al., 2017). Yet, a significant increment in stearic acid was never observed in the current work.

3.4. Microbially CLNA-enriched milk

At all tested α-LNA concentrations (with hydrolyzed FSO), *B. breve* DSM 20091 was able to grow well, even at 5 (S5LNA) or 10 (S10LNA) mg/mL, as seen by the significant (>1 $\log_{10}$) increase in viable cell numbers after 24 h (from 7.10–7.26 to 8.97–9.64 $\log_{10}$; Fig. S3), with no differences from the control (strain without precursor substrate), neither between tested α-LNA concentrations at 24 h (Fig. S3). At the beginning of incubation, the milk pH of S5LNA was more acidic (pH 6.02) than the control (pH 6.51), and S10LNA was more acidic (pH 5.82) than all conditions, except for S5LNA; this is related to the higher amount of FA provided. However, after 24 h of incubation, the pH decreased significantly for any condition tested to pH 4.02–4.43, and no differences were found from the control or between tested α-LNA concentrations at 24 h (Fig. S4), which is in accordance with the bacterial growth observed. This behavior is an upgrade to previous results where the assessed strain could only tolerate up to 1 mg/mL of pure LA (Fontes et al., 2018). According to free LA:α-LNA proportion in hydrolyzed FSO (1:2.53; Table 3), 10 mg/mL of α-LNA added from FSO would also provide nearly 4 mg/mL of LA. Within this framework of results, it seems that the use of edible vegetable oils, as substrate vehicles, in particular FSO, confers somehow a protective system to *B. breve* DSM 20091 against associated inhibitory effects at high substrate concentrations. Additionally, it could be related to the milk protective effect (de Almeida et al., 2018).

At the conditions assayed, it was possible to enrich milk with a maximum of 0.95 mg/g of CFA (S2LNA), being mainly CLNA isomers (0.88 mg/g) – C18:3 c9t11c15 and C18:3 t9t11c15 (Table 5; Fig. 1). The 2 mg/mL amount showed to be the limiting substrate concentration for *B. breve* DSM 20091 LA/LNA conversion ability since no significant differences ($p > 0.05$) were observed on CFA production at 5 (S5LNA) or 10 (S10LNA) mg/mL α-LNA (Table 5). Indeed, this strain maintained its substrate conversion rates at the same level of capacity until 2 mg/mL α-LNA (50.46–55.81 %) and then dropped these considerably to 15.50–17.03 % (Table 5). Moreover, from 2 mg/mL, inclusively, substrate reduction degree decreased in a concentration dependent-manner (Table 5). This strongly suggests that from that concentration LAI becomes saturated with substrate and therefore has no further capacity to produce more CFA. To the best of our knowledge, no other studies have reported such values in CLNA-enrichment of dairy products. Indeed, this is the first study to employ α-LNA concentrations above 0.5 mg/mL for dairy fermentation. Only Chung et al. (2008) had achieved comparable levels, but in CLA isomers, with a ~1.06 mg/mL production in skim milk fermented with a *B. breve* strain, as well, and 0.5 % (v/v) of monolinolein.

Regarding milk FFA composition, it was observed that PUFA level changed significantly ($p < 0.05$) from 0 h (1.065–2.045 mg/g) to 24 h (0.792–1.889 mg/g) of fermentation only until 2 mg/mL α-LNA (S2LNA; Table S3), which is explained by the low LA/α-LNA reduction and conversion rates verified at 5 (S5LNA) or 10 (S10LNA) mg/mL α-LNA (Table 5). As for EFA, although not always significant, there was a general decrease in FAs for all tested conditions (Table S4).

Analysis of milk composition revealed an amount of 0.05 mg/g of CLA only (Table S5). By culturing milk with *B. breve* DSM 20091 at 2

Table 5
LA and α-LNA reduction and conversion percentages and amounts of CLA and CLNA isomers produced by *Bifidobacterium breve* DSM 20091 cultured in semi-skimmed milk supplemented with different α-LNA concentrations from hydrolyzed FSO for 24 h at 37 °C (based on FA profile of the free fraction).

	Control	S1LNA	S1.5LNA	S2LNA	S5LNA	S10LNA
LA reduction (%)	6.77 ± 0.68^{c}	37.85 ± 8.62^{a}	26.30 ± 5.23^{a}	14.19 ± 1.42^{b}	6.85 ± 0.68^{c}	0.26 ± 0.03^{d}
α-LNA reduction (%)	12.78 ± 1.41^{e}	82.78 ± 8.67^{ab}	72.03 ± 8.21^{bc}	65.95 ± 6.59^{c}	20.89 ± 3.53^{d}	7.06 ± 0.71^{f}
Δ CLA (mg/g)	ND	0.05 ± 0.02^{ab}	0.05 ± 0.01^{ab}	0.07 ± 0.01^{a}	0.04 ± <0.01^{b}	0.04 ± <0.01^{b}
Δ CLNA (mg/g)	ND	0.44 ± 0.05 ^{cd}	0.61 ± 0.07^{bc}	0.88 ± 0.09^{a}	0.70 ± 0.05^{ab}	0.77 ± <0.01^{a}
Δ CFA (mg/g)	ND	0.49 ± 0.07 ^{cd}	0.67 ± 0.08^{bc}	0.95 ± 0.09^{a}	0.74 ± 0.06^{ab}	0.81 ± 0.01^{a}
LA conversion (%)	ND	19.67 ± 2.00^{a}	14.54 ± 2.47^{a}	14.77 ± 1.48^{a}	2.58 ± 0.16^{b}	2.70 ± 0.20^{b}
α-LNA conversion (%)	ND	54.57 ± 8.36^{ab}	50.46 ± 5.52^{b}	55.81 ± 5.58^{ab}	15.50 ± 1.22^{c}	17.03 ± 0.07^{c}

Average values ± standard deviation (n = 3).
Different superscript letters for significant differences ($p < 0.05$) within rows.
Control – No substrate; S1LNA – 1 mg/mL α-LNA; S1.5LNA – 1.5 mg/mL α-LNA; S2LNA – 2 mg/mL α-LNA; S5LNA – 5 mg/mL α-LNA; S10LNA – 10 mg/mL α-LNA; LA – Linoleic acid; α-LNA – α-Linolenic acid; CLA – Conjugated linoleic acid (C18:2 c9t11 + C18:2 t,t); CLNA – Conjugated linolenic acid (C18:3 c9t11c15 + C18:3 t9t11c15); CFA – Conjugated fatty acids (CLA + CLNA); ND – not detected.

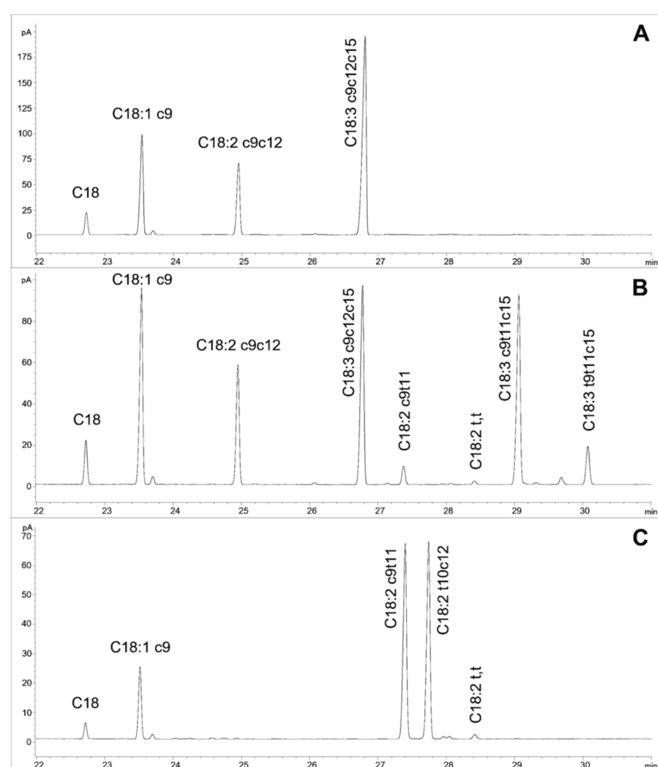

Fig. 1. Chromatogram profile by GC-FID of milk FFA at 0 h (A) and 24 h (B) of fermentation with *Bifidobacterium breve* DSM 20091 and hydrolyzed FSO at 2 mg/mL α-LNA (S2LNA), plus EFA profile of Tonalin CLA (C). Tonalin CLA isomers identification was based on results reported by Rodríguez-Alcalá and Fontecha (2007).

mg/mL α-LNA (with hydrolyzed FSO), it was possible to increase CFA contents (mostly CLNA isomers) to a total of 1.01 mg/g (0.060 mg/g free C18:2 c9t11 + 0.017 mg/g free C18: t,t + 0.722 mg/g free C18:3 c9t11c15 + 0.154 mg/g free C18:3 t9t11c15 + 0.054 mg/g esterified C18:2 c9t11; Tables S3 and S4). According to European Commission Regulation (EC) 983/2009, amended by Regulation (EC) 376/2010, α-LNA is recognized as a functional ingredient. Totalizing CFAs and α-LNA, the CLNA-enriched fermented milk obtained at 2 mg/mL α-LNA (with hydrolyzed FSO) could account for 1.69 mg/g of bioactive compounds (0.530 mg/g free C18:3 c9c12c15 + 0.060 mg/g free C18:2 c9t11 + 0.017 mg/g free C18: t,t + 0.722 mg/g free C18:3 c9t11c15 + 0.154 mg/g free C18:3 t9t11c15 + 0.155 mg/g esterified C18:3 c9c12c15 + 0.054 mg/g esterified C18:2 c9t11; Tables S3 and S4). Although at 10 mg/mL α-LNA (with hydrolyzed FSO) the CLNA-enriched fermented milk reached 9.47 mg/g in bioactive compounds (7.822 mg/g free C18:3 c9c12c15 + 0.041 mg/g free C18:2 c9t11 + 0.015 free C18:2 t,t + 0.0638 mg/g free C18:3 c9t11c15 + 0.130 mg/g free C18:3 t9t11c15 + 0.689 mg/g esterified C18:3 c9c12c15 + 0.092 mg/g esterified C18:2 c9t11 + 0.040 mg/g esterified C18:2 t,t; Tables S3 and S4), most part corresponded to the α-LNA that was not used by the strain (low conversion rate), therefore, the 2 mg/mL α-LNA condition revealed to be the most cost-effective regarding the development of a functional dairy product through this *in situ* microbial production strategy.

It has been suggested that to obtain a beneficial effect from CLNA isomers a human would need to consume 2–3 g/day (Kung & Lin, 2021). Considering the amount of CLNA present in the enriched fermented milk obtained in this work, undoubtedly, it is not an efficient way to achieve the required dose. As an alternative, it is being studied the possibility of concentrating the lipid content.

3.5. UV spectra

To confirm the structural nature of the conjugated isomers detected by GC-FID in the microbially CLNA-enriched milk, it was performed a UV scanning test. Before fermentation with hydrolyzed FSO at 2 mg/mL α-LNA (S2LNA), milk FFA revealed a maximum absorbance at 210 nm (Fig. S5). After fermentation, two maximums of absorbance at 211 and 233 nm (Fig. S5) were observed. As for Tonalin CLA, the highest absorption peak was detected at 233 nm, while for PSO three peaks of absorption −264, 274 and 285 nm (Fig. S5) - were observed.

Maximum absorbance at 232–235 nm is specific of conjugated dienes, independent of the total number of double bounds and chain length, as shown by Coakley et al. (2009) in their works regarding the microbial production of C18:3 c9t11c15 and C18:3 t9t11c15 isomers. Moreover, Czauderna et al. (2011) reported that *ct/tc* conjugated dienes are characterized by a maximum absorbance at 234 nm, and *ct* isomers were the major ones detected after fermentation (Table S3). Therefore, according to the obtained results, the CLNA isomers detected in the enriched fermented milk correspond to conjugated dienes.

4. Conclusion

CRL lipase turned out to have the best efficiency in releasing FFA from any of the edible vegetable oils assayed. Due to cost matters, SBO and FSO, which are rich in the precursor LA and α-LNA, respectively, were selected to study microbial CLA/CLNA-enrichment with *B. breve* DSM 20091, after previous hydrolysis with CRL. In a first stage, the best outcome was achieved with hydrolyzed FSO at 0.5 mg/mL α-LNA, being mainly produced CLNA isomers (0.34 mg/g). Higher α-LNA concentrations (with hydrolyzed FSO) were further tested, where maximum milk enrichment, of mostly CLNA (0.88 mg/g), was reached at 2 mg/mL α-LNA.

The obtained results suggest that it is possible to increase the

bioavailability of LA and α-LNA in milk for *B. breve* DSM 20091 to produce CFA, by using lipase-hydrolyzed commercial edible vegetable oils. However, this strain preferentially produces CLNA and in amounts that do not allow to reach the effective dose with a normal intake of the product.

CRediT authorship contribution statement

Ana Luiza Fontes: Conceptualization, Investigation, Formal analysis, Writing – original draft, Writing – review & editing. **Lígia Pimentel:** Conceptualization, Project administration, Supervision, Investigation, Writing – review & editing, Validation. **Ana Maria Silva Soares:** Investigation, Writing – review & editing. **M. Rosário Domingues:** Supervision, Writing – review & editing. **Luis Miguel Rodríguez-Alcalá:** Conceptualization, Formal analysis, Project administration, Resources, Supervision, Writing – review & editing, Funding acquisition. **Ana Maria Gomes:** Conceptualization, Supervision, Funding acquisition, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodchem.2023.135665.

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