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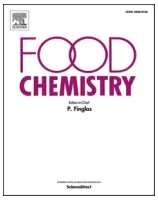

Short communication

Short-communication: Study of fatty acid metabolites in microbial conjugated fatty acids-enrichment of milk and discovery of additional undescribed conjugated linolenic acid isomers

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ABSTRACT

Microbially enriched food in conjugated linoleic (CLA) and conjugated linolenic (CLNA) acids is intensively studied nowadays. The conversion of linoleic (LA) and α-linolenic acids (α-LNA) into these compounds may involve different fatty acid (FA) intermediates. This research aimed to investigate potential FA byproducts in milk during microbial CLA/CLNA-enrichment using *Bifidobacterium breve* DSM 20091. Milk fermented with pure α-LNA showed a decrease in free myristic acid, while pure LA led to an increase in free stearic acid. No additional FA compounds were found alongside CLA/CLNA isomers. The strain produced several CLA isomers from LA, but only when administered alone. Nonetheless, when α-LNA was assayed, additional CLNA isomers, never reported before for bifidobacteria, were observed. In conclusion, except for stearic acid in the presence of LA, no side-FA metabolites were released during milk microbial CLA/CLNA-enrichment. Results suggest either CLA/CLNA production occurs in one single-step or intermediates biotransformation is very fast.

1. Introduction

In the last years, several food compounds with added-value properties have been identified and studied for their biological potential. Among these are the conjugated linoleic (CLA) and conjugated linolenic (CLNA) acids, which have been described to have potential anti-carcinogenic, anti-obesity and anti-inflammatory effects (Bauman et al., 2020; Yuan, 2022). Both compounds are 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), being produced from the biohydrogenation of dietary linoleic (LA) and α-linolenic (α-LNA) acids to stearic acid (C18:0) by ruminal bacteria, such as *Butyrivibrio fibrisolvens* (Salsinha et al., 2018). However, probiotic bacteria, such as *Bifidobacterium*, *Lactobacillus* and *Propionibacterium*, have shown the capacity to produce CLA and CLNA isomers when in the presence of LA and α-LNA, respectively (Gong et al., 2019). Indeed, a *Bifidobacterium breve* strain that could convert 31.2% of LA and 68.2% of α-LNA into respective conjugated isomers has already been reported (Fontes et al., 2018). Such transformation ability has been suggested as a detoxification mechanism from LA/α-LNA by bacteria (Salsinha et al., 2018).

As a strategy to increase daily consumption up to effective doses of CLA (3–6 g/day) and CLNA (2–3 g/day) (Kung & Lin, 2021), some research works have studied microbial CLA and CLNA-enrichment of food matrices, like sucuk (Özer et al., 2016), blackcurrant press residue (Vahvaselkä et al., 2021) or walnut milk (Mao et al., 2022). Moreover, it was previously reported the *in situ* production of up to 0.13 mg/mL of microbial CLA or 0.11 mg/mL of microbial CLNA, using pure LA or α-LNA, respectively, in a UHT semi-skimmed cows' milk (Fontes et al., 2018). More recently, pasteurized semi-skimmed cows' milk was enriched with up to 0.88 mg/g CLNA by growing *B. breve* DSM 20091

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with lipase-hydrolyzed flaxseed oil (FSO), a rich source of the precursor α-LNA (Fontes et al., 2023).

However, in the abovementioned works (Fontes et al., 2018, 2023), there was always a difference between substrate reduction percentage and the percentage that was converted into conjugated fatty acids which, in some cases, could reach ~30%. This suggests that LA and α-LNA biotransformation goes through other metabolites/intermediates, lacking information about their identity. In fact, it has been proposed that some lactobacilli strains produce CLA isomers through a multi-enzymatic system involving hydration/dehydration, dehydrogenation/hydrogenation and isomerization steps, with hydroxy and oxo fatty acid (FA) intermediates (Kishino et al., 2013; Yang et al., 2017), even though, linoleate isomerase (LAI) is described as the enzyme responsible for LA and α-LNA bioconversion since a long time ago (Kepler & Tove, 1967). Thus, we hypothesized that the conversion of LA/α-LNA by the *B. breve* strain previously employed for milk microbial enrichment with CLA/CLNA isomers involves the release of other FA metabolites.

In this regard, the aim of the present work was to study possible FA side-products in microbial CLA/CLNA-enrichment of milk. To do so, cows' milk, added with pure precursor substrates or from hydrolyzed FSO, was fermented with the previously characterized *B. breve* DSM 20091 (Fontes et al., 2018). The FSO was further tested at a higher concentration (2 mg/mL of free α-LNA) that was previously selected as optimal for the development of a CLNA-enriched fermented milk product (Fontes et al., 2023).

2. Materials and methods

2.1. Chemicals and resources

Hexane, methanol and dimethylformamide for free FA (FFA) analysis were purchased from VWR Chemicals (Radnor, PA, USA), while hexane, methanol, and dichloromethane for esterified FA (EFA) analysis were obtained from Fisher Scientific (Loughborough, UK). Sulphuric acid and potassium hydroxide were from Honeywell (Charlotte, NC, USA), while sodium methoxide and methyl acetate were from Acros Organics (Geel, Belgium). Sodium chloride was purchased from BDH Prolabo (VWR Chemicals). 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). Supelco 37 FAME mix, LA, α-LNA and Candida rugosa type VII lipase (CRL) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Flaxseed oil (FSO) and pasteurized semi-skimmed cows' milk were bought in local markets (Porto, Portugal). Milk's physico-chemical composition as stated by the manufacture is presented in Table S1.

2.2. Substrate emulsion preparation

The FSO was hydrolyzed with CRL and an oil emulsion prepared at approximately 50 mg/mL of free α-LNA (from hydrolyzed FSO) as described in Fontes et al. (2023).

Stock solutions of pure LA and α-LNA were prepared at 50 mg/mL according to Fontes et al. (2018).

2.3. Milk fermentation with pure substrates or hydrolyzed oil

Bifidobacterium breve DSM 20091 (DSMZ, Braunschweig, Germany), stored at −80 °C in glycerol 30% (w/w) (Fisher Scientific), 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), and incubated overnight at 37 °C. The activated culture was then transferred at 10% (v/v) to fresh cys-MRS medium and incubated at 37 °C for 16 h. Afterward, the strain was inoculated at 1% (v/v) in cows' milk (100 mL), supplemented with 0.05% (w/v) cysteine, and at the following precursor substrate conditions: *Control* – no substrate; *SLA* – 0.5 mg/mL LA; *SLNA* – 0.5 mg/mL α-LNA; *SLA + LNA* – 0.15 mg/mL LA + 0.5 mg/mL α-LNA; *S0.5FSO* – 0.5 mg/mL α-LNA from hydrolyzed FSO; *S2FSO* – 2 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 85% nitrogen, 5% hydrogen and 10% carbon dioxide). Milk samples were collected before and after fermentation for further viable cell numbers enumeration, pH measurement and FA analysis (sections 2.4 and 2.5).

2.4. Sample preparation for fatty acid analysis

For EFA analysis of milk (200 mg) samples (i.e., milk inoculated with B. breve DSM 20091, collected at 0 h and 24 h, with or without substrate addition), lipid isolation was performed through the Folch method as described by Matyash et al. (2008) and then fatty acid methyl esters (FAME) extracts were prepared according to Reis et al. (2013).

For FFA analysis, FSO emulsion (500 μL) and milk (500 mg) samples were prepared according to Pimentel et al. (2015) with some modifications, as reported in Fontes et al. (2023).

2.5. Gas chromatography conditions

2.5.1. Gas chromatography – flame ionization detector

For quantification of free α-LNA in hydrolyzed FSO emulsion, as previously reported by Fontes et al. (2018), FAME extracts were analyzed in a Hewlett-Packard HP6890A gas chromatograph (Avondale, PA, USA), equipped with a flame-ionization detector (GLC-FID) and a BPX70 capillary column (60 m × 0.32 mm × 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 was 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. 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).

2.5.2. Gas chromatography – mass spectrometry

For EFA and FFA profiling of milk samples, FAME extracts were analyzed in an Agilent Technologies 8860 gas chromatograph (Santa Clara, CA, USA) equipped with a DB-FFAP capillary column (30 m × 0.32 mm × 0.25 μm; J&W Scientific, Folsom, CA, USA) and coupled to an Agilent 5977B mass spectrometer (MS) detector (Agilent Technologies). The analysis conditions were as follows: injector temperature 220 °C, injection volume 2 μL (splitless); detector (MS) temperature 230 °C; helium as carrier gas at 1.4 mL/min; oven temperature program – started at 58 °C (held for 2 min), then raised at 25 °C/min to 160 °C, 2 °C/min to 210 °C and, finally, 20 °C/min to 225 °C (held for 15 min). Mass spectra were obtained at an electron impact ionization of 70 eV and a mass range of m/z 50–550 in 1-second cycles using the full scan acquisition mode. The FA compounds' identification was carried out using Agilent MassHunter Qualitative Analysis 10.0 (Agilent Technologies) based on mass spectra NIST database and previous works (Fontes et al., 2018, 2023). Results are expressed as relative abundance percentages.

2.6. Statistical analysis

Results are reported as mean values ± standard deviation of triplicate samples. Data were first analyzed for normality distribution by Shapiro-Wilk test. Differences between time 0 h and the end of fermentation (24 h) were analyzed by *t*-student paired samples test (normality guaranteed) or Wilcoxon (normality not guaranteed). The

level of significance was set, in general, at 0.05; for growth experiments, CFU differences had to be $\geq$1 $\log_{10}$ and of pH $\geq$0.5 units. Analyses were performed using IBM SPSS Statistics 28 (SPSS Inc., IBM Corporation, NY, USA).

3. Results and discussion

3.1. Bacterial growth and pH

To confirm that *B. breve* DSM 20091 growth was not affected by any tested condition, which might impair milk enrichment with CLA/CLNA isomers, viable cell numbers and pH value were determined. Concerning viable cell counts, there was a significant increase (>1 $\log_{10}$ difference) after 24 h, with no differences (<1 $\log_{10}$ difference) between substrates (from 7.42 to 7.54 to 9.48–9.58 $\log_{10}$ CFU/mL) (Fig. S1). Accordingly, milk pH value decreased significantly (>0.5-unit difference) after fermentation, and no differences (<0.5-unit difference) were detected between substrates (from pH 6.37–6.50 to pH 4.19–4.39) (Fig. S2). This is in accordance with previous studies, where the growth of this strain was not affected by pure precursor substrates at 0.5 mg/mL in semi-skimmed milk (Fontes et al., 2018) and could tolerate higher concentrations when these were delivered via vegetable oil (Fontes et al., 2023). Moreover, viable cell counts of *Lacticaseibacillus casei* 1.574 in milk fermented at increasing LA concentrations tended to be higher than control (no substrate), even though differences were not >1 $\log_{10}$ CFU/mL (Qing et al., 2023). Dairy matrices effectively exhibit a protective effect on probiotic bacteria viability (de Almeida et al., 2018; Meira et al., 2015).

3.2. Fatty acid byproducts

Regarding milk FA composition, some alterations were found in the FFA profile, beyond the expected reduction of CLA/CLNA precursor substrates (i.e., LA/α-LNA) (Table S2). The obtained results pinpoint a decrease in myristic acid (C14) proportion when using pure α-LNA, alone (SLNA; from 7.24% to 5.70%, $p < 0.05$) or in combination with LA (SLA + LNA; from 6.24% to 4.80%, $p < 0.05$), as in control (from 12.66 to 10.83%), even though not statistically different ($p = 0.074$) (Table S2). Moreover, although not statistically significant ($p > 0.05$), the stearic acid (C18) percentage increased with pure LA (SLA; from 4.52% to 7.30%) and α-LNA (SLNA; from 4.59% to 6.01%), similarly to control, where it increased significantly in ca. 30% (from 8.55% to 11.02%, $p < 0.05$), while changes were less noticeable when applying both substrates (SLA + SLNA, S0.5FSO or S2FSO) (Table S2). Stearic acid increase in control and SLNA (i.e., using pure α-LNA alone) could be related to myristic acid decrease, which might have been elongated into palmitic acid (C16) and this further elongated into stearic acid. In SLA (i.e., using pure LA alone), part of the LA could have been metabolized into stearic acid, as this FA is suggested as the final product of LA metabolization pathway (Kishino et al., 2013). These results show that *B. breve* DSM 20091 enzymatic behavior differs depending on the type of substrate that is exposed to. As for EFA composition, no considerable differences were observed after fermentation at the different substrate conditions (Table S3).

No further compounds were found in the milk FA profile that could have derived from LA or α-LNA metabolization, like oxo or hydroxy FA. This suggests that for *B. breve* DSM 20091 i) biotransformation of LA and α-LNA into CLA and CLNA might occur in one single-step or ii) intermediates biotransformation is very fast. Moreover, the lack of alterations in the EFA fraction shows that this strain has no capacity to transform the free substrates into the complex triglycerides or phospholipids, for instance. In fact, produced metabolites were only detected in the FFA fraction, being observed interesting results on yielded conjugated FA isomers (Table S2).

3.3. Conjugated fatty acid isomers

From pure LA (SLA), *B. breve* DSM 20091 was able to produce four different CLA isomers, namely C18:2 c9t11 and C18:2 t10c12. The other two isomers were identified as C18:2 CLA c,c and C18:2 CLA t,t (Figs. 1 and 2), based on previous results by GC-FID (Fontes et al., 2018). In fact, these FA shared typical fragmentation pattern of conjugated dienes, with fragments at *m/z* 67, 81, 95, 109 and 123 (Christie & Han, 2010). According to Christie and Han (2010), the only distinction from their precursor substrate (LA) spectrum is the higher intensity of molecular ion signal, which was indeed verified (Fig. 2).

Other research works have reported the production of C18:2 t9t11 by different *B. breve* strains (Raimondi et al., 2016), including the one studied herein (Hennessy et al., 2012), thus, the *trans/trans*-CLA isomer detected could be C18:2 t9t11. It was shown that different probiotic strains (e.g. *L. casei*, *Latilactobacillus sakei, Lactococcus lactis* or *Bifidobacterium animalis* subsp. *lactis*) can convert LA mainly into C18:2 c9t11, C18:2 t10c12 and/or C18:2 t9t11 CLA isomers (Terán et al., 2015). Despite not being very common for probiotic strains to produce *cis/cis*-CLA isomers, the ruminal bacteria *B. fibrisolvens*, which possesses a C12 LAI (catalyzes LA transformation into C18:2 c9,t11) similarly to probiotic species (Kepler & Tove, 1967; Luo et al., 2013), can further

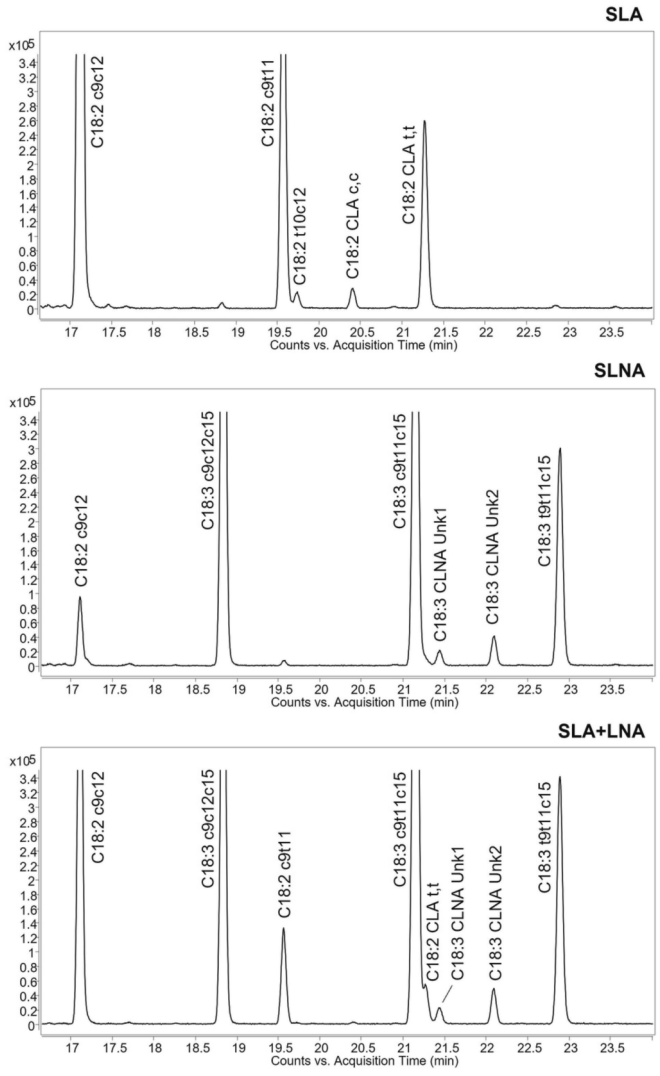

Fig. 1. Chromatograms of SLA (0.5 mg/mL linoleic acid – LA), SLNA (0.5 mg/mL α-linolenic acid – α-LNA) and SLA + LNA (0.15 mg/mL LA + 0.5 mg/mL α-LNA) pasteurized semi-skimmed milk after fermentation (24 h) with *Bifidobacterium breve* DSM 20091.

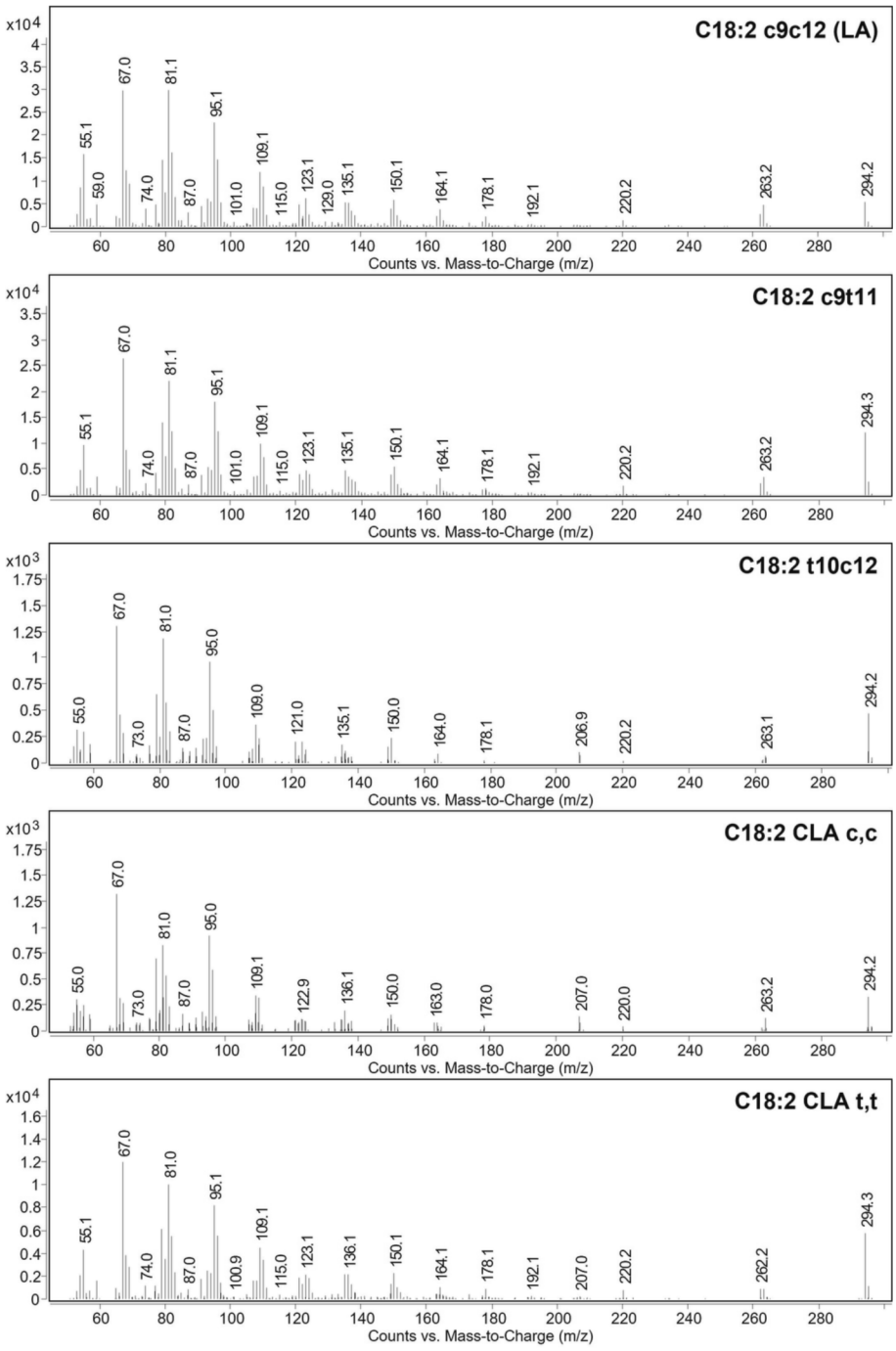

Fig. 2. Mass spectra of linoleic acid (LA) and conjugated linoleic acid (CLA) isomers detected in SLA (0.5 mg/mL LA) pasteurized semi-skimmed milk after fermentation (24 h) with *Bifidobacterium breve* DSM 20091.

produce the isomer C18:2 c9c11 (Wallace et al., 2007). Moreover, *Lactiplantibacillus plantarum* strains have shown the capacity to produce C18:2 c9c11 isomer, in addition to the ones mentioned above (Ribeiro et al., 2018). Therefore, the *cis/cis*-CLA isomer produced by *B. breve* DSM 20091 could be C18:2 c9c11. Recently, a C12-like LAI has been described and heterologously expressed from different *Bifidobacterium* species, including *B. breve* (Mei et al., 2023). Thus, it makes sense that the assayed strain produces the above-mentioned set of CLA isomers like other probiotic strains with similar LAI, as stated previously.

Fermentation with pure α-LNA (SLNA), revealed the production not only of the two isomers previously reported for this strain by GC-FID (Fontes et al., 2018), namely C18:3 c9t11c15 and C18:3 t9t11c15, but also two other compounds with a fragmentation pattern and molecular ion (*m/z* 292) similar to the above-mentioned ones, which most probably are other CLNA isomers (Figs. 1 and 3). In fact, these compounds shared typical fragmentation spectra of trienoic FA with conjugated dienes, with fragments at *m/z* 67, 81, 95, 107 and 121 (Christie & Han, 2010). Moreover, fragmentation spectra match with Rumelenic acid (C18:3 c9t11c15), for instance, was further confirmed by CFM-ID spectra prediction tool (Wang et al., 2021) for both presumed CLNA isomers, with scores of 0.39 (CLNA Unk1; Fig. S3) and 0.37 (CLNA Unk2; Fig. S4), where match score of detected Rumelenic acid spectrum with predicted one was 0.56 (Fig. S5). Yang et al. (2020) observed that *L. plantarum* ZS2058 could produce a third CLNA isomer, although at

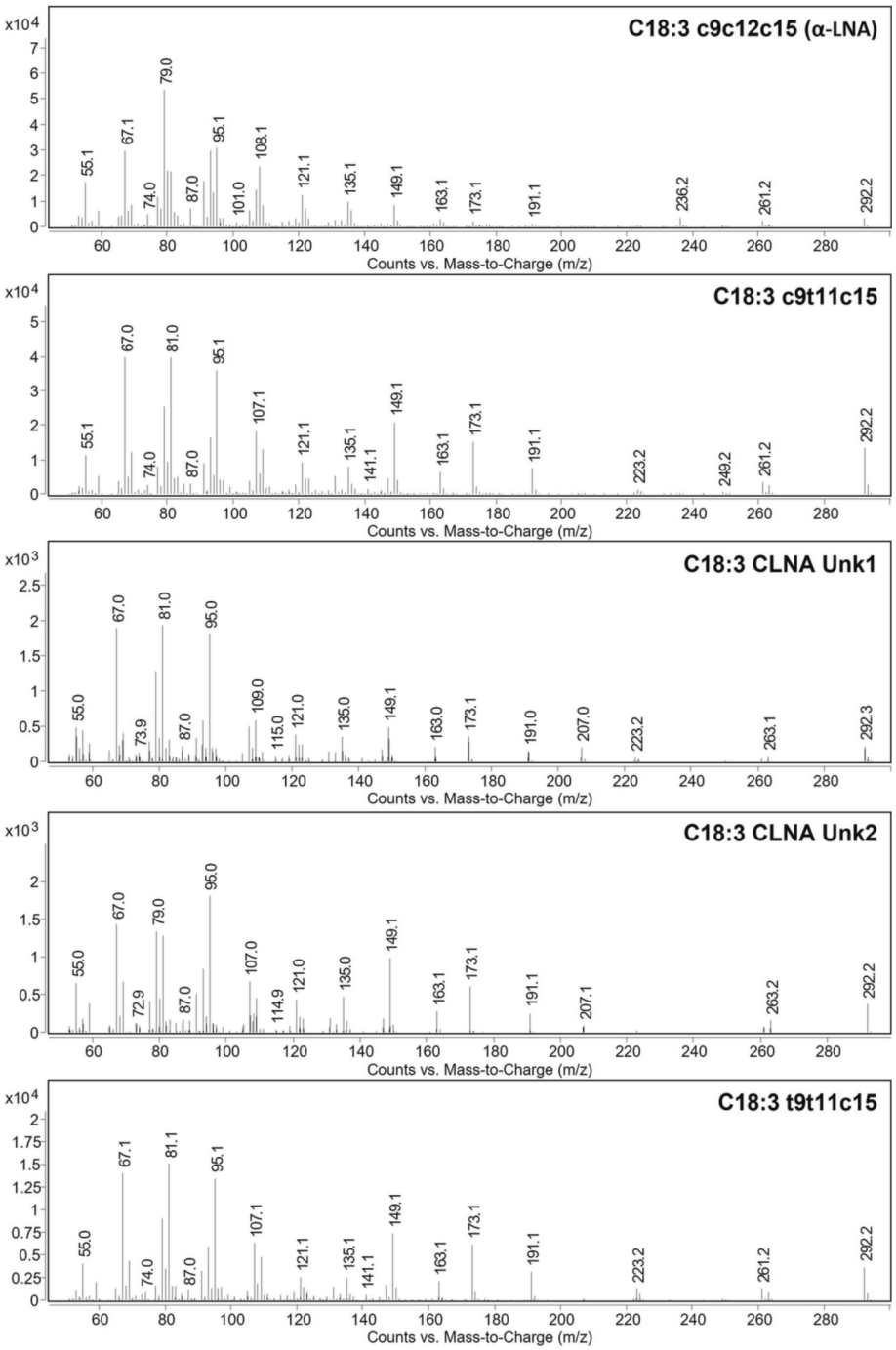

Fig. 3. Mass spectra of α-linolenic acid (α-LNA) and conjugated linolenic acid (CLNA) isomers detected in SLNA (0.5 mg/mL of α-LNA) pasteurized semi-skimmed milk after fermentation (24 h) with *Bifidobacterium breve* DSM 20091.

lower concentrations than C18:3 c9t11c15 and C18:3 t9t11c15. Nonetheless, the mass fragments formed were mostly different from these former CLNA isomers. In the current work, unknown CLNA isomers mass spectra revealed few different fragment ions at *m/z* 73, 79, 109, 207 and/or 263 *m/z*, compared to C18:3 c9t11c15 and C18:3 t9t11c15 (Fig. 3).

Considering the proposed CLA isomers configuration and elution order (C18:2 c9t11, C18:2 t10c12, C18:2 c9c11 and C18:3 t9t11), it is suggested that CLNA isomers might consist of C18:3 c9t11c15, C18:3 t10c12c15 (Unk1), C18:3 c9c11c15 (Unk2) and C18:3 t9t11c15. In fact, CLNA Unk2 shared a particular feature of all-*cis* CLA isomer, i.e., a lower intensity at ion 81 *m/z*, close to 79 *m/z* (Figs. 2 and 3). However, further analyses have to be performed in the future to confirm the structural configuration of these unknown CLNA isomers, like DMOX derivatization, since such information is not possible to retrieve from mass spectra alone (Christie & Han, 2010). Despite this, to the best of our knowledge, this is the first work to report the production of other CLNA isomers, besides C18:3 c9t11c15 and C18:3 t9t11c15, by a *Bifidobacterium* strain.

In the presence of both precursor substrates, either in pure form (SLA + LNA) or from hydrolyzed FSO (S0.5FSO), among the reported CLA isomers, only C18:2 c9t11 and C18:2 CLA t,t were detected, while all four above-mentioned CLNA isomers were observed (Fig. 1). This seems to support the previously discussed fact (Fontes et al., 2023), where *B. breve* DSM 20091 might have a preference to convert α-LNA than LA,

either because of a toxicity matter or LAI enzyme specificity (Fontes et al., 2018). Even though the initial proportion of LA was lower than α-LNA, these results also suggest that *B. breve* DSM 20091 enzymatic activity is affected by the type of substrate, expressing probably a higher affinity for α-LNA. At higher substrate concentration (S2FSO) C18:2 CLA t,t isomer could not be detected. This was probably because this isomer co-eluted with the C18:3 c9t11c15, as retention times were very close (21.15 vs. 21.27 min) (Fig. 1), and at this condition a higher amount of C18:3 c9t11c15 was produced when comparing with lower substrate concentrations (Fontes et al., 2023), enlarging peak area and retention time (21.19 min).

Ruminal bacteria have shown the ability to produce CLA isomers directly from α-LNA (Lee & Jenkins, 2011). In the current work, no CLA isomers were detected when using α-LNA alone, then, the assayed strain could only produce CLA from LA. In fact, to the best of our knowledge, no probiotic strains have demonstrated such above-mentioned capacity.

4. Conclusion

Apart from apparent LA metabolization into stearic acid, when applied alone, microbial enrichment of milk with CLA or CLNA isomers was not accompanied by further side-FA metabolites production, either because the biotransformation process does not involve other compounds or due to high enzymatic kinetics. Analysis of other lipid classes might elucidate what happens to the fraction of substrate that is not isomerized, thus, comprehensive lipidomic analysis must be further undertaken.

Despite this, the current work corroborated previous studies on the production of CLA and CLNA isomers by *B. breve* DSM 20091 and the influence of substrates in its enzymatic activity, namely, the reduced LA bioconversion due to possible higher affinity for α-LNA. Moreover, this study enabled the discovery of other two CLNA isomers that have never been reported for *Bifidobacterium* strains. These two new isomers could provide interesting health benefits, however further studies are necessary to assess their biological importance, namely their structural configuration to understand their chemical properties.

CRediT authorship contribution statement

Ana Luiza Fontes: Formal analysis, Investigation, Writing – original draft, Writing – review & editing, Visualization. **Bruna Neves:** Investigation, Writing – review & editing. **Tiago Conde:** Investigation, Writing – review & editing. **Daniela Couto:** Investigation, Writing – review & editing. **Lígia Leão Pimentel:** Conceptualization, Methodology, Supervision, Writing – original draft, Writing – review & editing. **Luis Miguel Rodríguez-Alcalá:** Conceptualization, Methodology, Supervision, Writing – original draft, Writing – review & editing, Funding acquisition. **M. Rosário Domingues:** Supervision, Writing – review & editing, Funding acquisition. **Ana Maria Gomes:** Conceptualization, Methodology, Supervision, Writing – review & editing, Funding acquisition.

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.2023.137276.

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