Study of the association between genotypic potential and linoleic acid tolerance with microbial production of conjugated linoleic acid

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ABSTRACT

Probiotic bacteria have shown to produce the bioactive conjugated linoleic acid (CLA) from linoleic acid (LA), this being associated with a detoxifying mechanism. Primers employed in genotypic selection are generally species-specific. Therefore, this work aimed to identify CLA-producers by previously screening genes encoding enzymes involved in LA isomerization with primers designed based on conserved motifs among different species. Moreover, it was intended to evaluate LA-tolerance relation to CLA production potential. Genotypically-positive strains were further cultured with LA and CLA quantified. At least one of the screened genes was detected in 39 out of 85 strains, where designed primers allowed to identify more potential producers than reported ones. The LA-tolerance (<1-5 mg/mL) showed to be independent of genotypical-positivity/negativity. Only four strains exhibited CLA production (4.94-312.39 µg/mL). Thus, primers targeting conserved motifs are more efficient in the selection of potential CLA-producers, however, this identification cannot rely solely on genotypic screening and LA-tolerance.

Keywords: probiotics; conjugated linoleic acid; linoleate isomerase; linoleic acid-tolerance.

1. INTRODUCTION

Conjugated linoleic acid (CLA) isomers have been attracting much attention due to their bioactive properties, which have been related to anti-carcinogenic, anti-obesity, anti-diabetic, anti-inflammatory or anti-atherogenic effects.[1] As a result, this group of conjugated fatty acid isomers is considered a promising functional ingredient. The only natural sources of CLA isomers for humans are meat (1.2-17.0 mg CLA/g fat) and ruminants' milk (2.0-33.0 mg CLA/g fat).[2] However, to obtain any beneficial effect from these bioactive fatty acids (FA), it has been recommended an effective dose of 3-6 g/day for a 70 Kg person,[3] which is not feasible to get through CLA natural sources. To overcome that problem, studies focused on increasing CLA content in food products through *in situ* bacterial production have been carried out.[4,5]

The CLA isomers are intermediates of rumen biohydrogenation pathway of dietary linoleic acid (LA; C18:2 c9,c12) to stearic acid (C18). For a long time, *Butyrivibrio fibrisolvens* was the only bacteria described as responsible for this process, but several other ruminal bacteria are also involved.[6] The CLA is also synthesized in the mammary gland of lactating cows through the conversion of trans-vaccenic acid (C18:1 t11; TVA) by Δ9-desaturase enzyme.[7] However, besides ruminal bacteria, other species isolated from dairy products and the human gastrointestinal tract have also demonstrated the capacity to produce CLA isomers in the presence of a LA source, with bifidobacteria and lactobacilli strains being the most well-characterized on this matter.[8] It has been further suggested that this capacity might consist of a detoxification mechanism from LA.[9]

During LA biohydrogenation, linoleate isomerase (LAI) is the enzyme responsible for its conversion into conjugated forms and was first described by Kepler and Tove from ruminal *B. fibrisolvens*.[10] There are two categories of LAIs: i) C12 isomerase that catalyzes the conversion of LA to C18:2 c9,t11 and ii) C9 isomerase that catalyzes the conversion of LA to C18:2 t10,c12.[11] C12 isomerase has been found in different

76 species of bacteria, including *B. fibrisolvens*, *Clostridium sporogenes* and *Lactobacillus*
77 *acidophilus*.[10–12] Moreover, C12 isomerase activity has been detected in several
78 bifidobacteria and lactobacilli species.[4,13,14] Ineffective attempts to solubilize this
79 isomerase suggest that it is a membrane-associated enzyme.[10,12] On the other hand,
80 C9 isomerase is a soluble cytoplasmic protein and has been detected in
81 *Propionibacterium acnes*, being also denominated as PAI.[15] However, it has been
82 suggested that in lactobacilli the bioconversion of LA is performed by a multi-
83 component enzyme system, composed of enzymes belonging to
84 dehydrogenase/oxidoreductase, acetoacetate decarboxylase and hydratase families,
85 including myosin-cross-reactive antigene (MCRA), whose combined action allows the
86 formation of CLA isomers, with hydroxy and oxo FAs as intermediates.[16] A
87 bifidobacterial MCRA has shown to catalyze the reversible conversion between LA and
88 10-hydroxy-cis-12 octadecenoic acid (10-HOE). Moreover, bifidobacteria strains have
89 been able to produce CLA from 10-HOE.[17]
90 The classical identification method of CLA-producing strains, i.e., strains culturing with
91 substrate followed by CLA detection and quantification through chromatographic or
92 spectrophotometric techniques, is laborious and time-consuming. To overcome that,
93 some authors have performed a prior selection of potential CLA-producers through
94 molecular detection of genes encoding the enzymes involved in LA isomerization.[18,19]
95 However, primers have been generally designed based on enzyme sequences of
96 certain species, becoming too species-specific. Thus, we hypothesized that primers
97 designed based on conserved motifs among different species would be more efficient
98 in the identification of potential CLA-producers. Also, strains containing such genes
99 should therefore tolerate the presence of LA better if this transformation is indeed a
100 detoxification mechanism.
101 Thus, the aims of the present work were: (i) to identify CLA-producing strains through
102 previous selection by molecular detection of genes encoding enzymes involved in LA

isomerization using previously reported and designed-by-us primers; and (ii) to study the relationship between LA-tolerance and CLA production potential.

2. MATERIALS AND METHODS

2.1. Analytical reagents

Hexane, methanol, dimethylformamide and acetonitrile were HPLC grade (VWR Chemicals, West Chester, PA). GLC-Nestlé36 FAME mix and glyceryl tritridecanoate (99.9%) were obtained from Nu-Chek Prep, inc. (Elysian, Minnesota, USA), undecanoic acid (99.9%) and N, O-Bis(trimethylsilyl)trifluoroacetamide (BSTFA) from Alfa Aesar (Haverhill, MA, USA) and butterfat CRM-164 (EU Commission; Brussels, Belgium) from Fedelco Inc. (Madrid, Spain). Sulphuric acid was from Fisher Scientific (Hampton, NH, USA), while sodium methoxide was from Acros Organics (Geel, Belgium). Supelco 37 FAME mix, bacterial FAME (BAME mix) and LA were purchased from Sigma-Aldrich (St. Louis, MO, USA).

2.2. Culture conditions

A total of 85 strains (belonging to *Lactobacillus*, *Bifidobacterium*, *Propionibacterium*, *Lactococcus* and *Enterococcus* genera) (Table S1), stored at –80 ºC in glycerol 30% (w/w) (Fisher Scientific), were inoculated at 2% (v/v) in de Man-Rogosa-Sharpe (MRS) broth (Biokar Diagnostics, Beauvais, France) and incubated overnight at 37 ºC. About 10% (v/v) of the activated culture was then subcultured in fresh MRS medium and incubated at 37 ºC for 16 h. Afterwards, 2% (v/v) was spiked into new medium (10 mL) for the following experiments. For bifidobacteria and propionibacteria, the culture medium was supplemented with 0.05% (w/v) L-cysteine-HCl (Sigma-Aldrich, St. Louis, MO, USA) and strains were grown in an anaerobic workstation (Whitley DG 250; Don Withley Scientific, Yorkshire, UK.) under a mixture of 80% nitrogen, 10% hydrogen and

10% carbon dioxide. Lactobacilli, lactococci and enterococci strains were cultured under aerobic conditions.

2.3. Genotypic screening

To detect the presence of the putative genes encoding enzymes involved in the LA isomerization pathway (i.e., LAI, MCRA and fatty acid hydratase – FA-HY), each strain was inoculated in fresh MRS broth (10 mL) at 2% (v/v) and incubated for 24 h at 37 ºC. Afterwards, pellets were recovered through centrifugation (1250 × *g*, 18 ºC, 5 min) and washed twice in 0.85% (w/v) NaCl. Then, DNA was extracted from the bacterial strains as described by Alimolaei and Golchin in their P3 protocol,[20] and subjected to PCR analysis to assess the presence of LAI, MCRA and/or FA-HY genes, using different sets of primers, depending on the strain (Table 1). More specifically, primers LISO3 and LISO4,[18] were used to screen the lactobacilli strains for the presence of a putative LAI gene. These primers were designed based on the partial nucleotide sequence obtained for *Latilactobacillus sakei* 23K and a nucleotide sequence of the LAI gene of *Lactiplantibacillus plantarum* AS1.555 (GenBank accession code DQ227322). In addition, the Entrez Gene tool (http://www.ncbi.nlm.nih.gov/gene) from the National Center for Biotechnology Information (NCBI) database was used to select the LAI sequences of lactobacilli (amino acid sequence alignment results in Supporting Information – Lactobacilli LAI alignment) and based on these sequences two primers were designed for their amplification: INIA-Lb1 (gaygayccrcayatsttccarac) and INIA-Lb2 (gtraagctgrcccaytcrct). In the case of the MCRA gene, primers Ev1a and Ev2a,[21] were used to screen its presence in the bifidobacteria, propionibacteria, lactococci and enterococci strains included in this study. These degenerated primers were originally designed after a comparison of the *Limosilactobacillus reuteri* PYR8 LAI protein with other sequences available in databases. In addition, the Entrez Gene tool from the NCBI database was used to select the MCRA sequences of bifidobacteria (amino acid sequence alignment results in Supporting Information – Bifidobacteria MCRA

158 alignment) and based on these sequences two primers were designed for their
159 amplification: INIA-Bf1 (gcgyacbatgttcgcbttyg) and INIA-Bf2 (cytccatrcckgtrcgcatsga).
160 Finally, two sets of primers based on the genome of *L. acidophilus* strains were used
161 for the screening of two FA-HY genes (*fa-hy1* and *fa-hy2*), described as encoding
162 enzymes with FA hydratase activity, among the *L. acidophilus* strains included in this
163 study.[22]
164 For PCR amplification a Techne TC-512 Thermocycler (Keison Products, England) was
165 used. All amplifications were carried out in a final volume of 25 µl. Each PCR mixture
166 (*Taq* DNA polymerase 5U/µL PCR kit, Thermo Fischer Scientific) contained 2.5 µL 10×
167 *Taq* buffer, 2 mM dNTP mix, 20 pmol of each primer, 1.5 mM $MgCl_2$, 1.25 U Taq DNA
168 polymerase, 1.25 µL DMSO and 5 µL of the diluted DNA solution. The PCR program
169 consisted of an initial denaturation at 95 ºC for 3 minutes, followed by 40 cycles of
170 denaturation at 95 ºC for 30 s, annealing at a specific temperature for the primers used
171 (Table 1) for 30 s, extension at 72 ºC for 60-110s. The last step consisted of a final
172 extension at 72 ºC for 10 min.
173 To assess the presence of PCR products, 5 µL of each PCR reaction was subjected to
174 electrophoresis using a 1% (w/v) agarose gel in 1X Tris-acetate-EDTA (TAE) buffer
175 (GRiSP, Porto, Portugal), stained with GreenSafe Premium (NZYTech, Lisbon,
176 Portugal). After electrophoresis, gels were illuminated under UV light.
177
178 **2.4. Determination of maximum tolerance to LA**
179 A stock solution of LA was prepared at 50 mg/mL with 2% (w/v) Tween 80 (Sigma-
180 Aldrich, St. Louis, MO, USA) and homogenized using an Ultra-Turrax (IKA Works, Inc.,
181 Wilmington, NC, USA) at 15,000 rpm during 150 s (five 30 s-intervals separated by 30
182 s-pauses, to not heat-up the emulsions), before filter-sterilization through a 0.20 µm-
183 pore size membrane (Millipore, Burlington, MA, USA).
184 To find out the LA maximum tolerance of each strain, tests were conducted according
185 to Romero-Pérez et al.[23] with slight modifications. Briefly, after activation, cultures were

plated in MRS agar (Biokar Diagnostics) containing 1, 2 or 5 mg/mL of LA and incubated for 48 h at 37 ºC in duplicate. Plates devoid of LA were used as controls. For bifidobacteria and propionibacteria, MRS was supplemented with L-cysteine-HCl (0.05%, w/v) (cys-MRS). From LA-containing plates, colonies were assayed by spiking well plates containing 250 µL of MRS (lactobacilli, lactococci and enterococci) or 200 µL of cys-MRS (bifidobacteria and propionibacteria) and LA at the same concentration present in the agar plates from which they had been isolated, in triplicate. For each strain, inoculated wells with LA-devoid medium were used as control. Paraffin (50 µL) was added to the top of the wells where bifidobacteria and propionibacteria were assayed. Growth was monitored in a plate reader (model FLUOSTAR optima; BMG labtech, Ortenberg, Germany) at 600 nm for 48 h at 37 ºC.

2.5. Phenotypic screening

To determine which of the positive strains for LAI, MCRA and/or FA-HY genes were able to produce CLA isomers, each strain was inoculated at 2% (v/v) in fresh MRS broth (10 mL) without substrate (control) or containing 0.5 mg/mL of LA. Samples were incubated at 37 ºC for 48 h. Bacterial growth was analyzed through plating on MRS agar plates of sequential decimal dilutions followed by viable cell numbers determination. The pH was measured through a digital pH meter (Basic 20, Crison, Barcelona, Spain). The supernatant was collected after centrifugation at 1250 × *g* and 18 ºC for 5 min for further FA analysis. Later, producing strains with high LA-tolerance were further tested for CLA production at their maximum tolerance (medium supplemented with 5 mg/mL of LA).

The LA reduction percentage was calculated as follows:

$$LA\ reduction\ (\%) = \frac{([LA_i] - [LA_f]) \times 100}{[LA_i]}$$

Being LA_i the initial amount (0 h) of LA and LA_f the final amount of LA after 24 h or 48 h of incubation. The LA conversion percentage was calculated as follows:

$$LA\ conversion\ (\%) = \frac{([CLA_f] - [CLA_i]) \times 100}{[LA_i]}$$

Being CLA_f the final amount (after 24 h or 48 h) of CLA, CLA_i the initial amount (0 h) of CLA and LA_i the initial amount (0 h) of LA.

2.6. Fatty acid and hydroxy fatty acid analyses

For the FA analysis, stock solutions and supernatants (500 µL) were prepared according to Pimentel et al.[24] Briefly, for quantification purposes, samples were added with 100 µL of tritridecanoin (1.5 mg/mL) and undecanoic acid (1.5 mg/mL) before derivatization. Then 2.26 mL of methanol was added, followed by 1 mL of hexane and 240 µL of sodium methoxide (5M). Samples were homogenized and incubated at 80 ºC for 10 min. After cooling in ice, 1.25 mL of dimethylformamide was added before 1.25 mL of sulphuric acid 3M (prepared daily in methanol). Samples were homogenized and incubated at 60 ºC for 30 min. Finally, after cooling, 1 mL of hexane was added, and samples were homogenized and centrifuged (1250 ×*g*; 18 ºC; 5 min). The upper layer containing methyl esters (FAME) was collected for further analysis.

For the analysis of hydroxyl FAs as trimethylsilyl (TMS) derivatives in supernatant samples, the solvent was evaporated in a nitrogen stream after obtaining the FAME fraction as previously described elsewhere.[25] Then, 150 µL of BSTFA and 500 µL of acetonitrile were added and the mix was incubated at 70 ºC for 30 min.[26] Finally, acetonitrile was evaporated in a nitrogen stream and the extract was resuspended in 500 µL of hexane.

2.7. Gas Chromatography conditions

As previously reported by Fontes et al.,[25] FAME and TMS were both 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 x 0.32 mm x 0.25 μm; SGE Europe Ltd, Courtaboeuf, France). Analysis conditions were as follows: injector temperature 250 ºC, split 25:1, injection volume 1 μL; detector (FID) temperature 275 ºC; hydrogen was carrier gas at 20.5 psi; oven temperature program – started at 60 ºC (held 5 min), then raised at 15 ºC/min to 165 ºC (held 1 min) and, finally, at 2 ºC/min up to 225 ºC (held 2 min). Supelco 37, FAME from CRM-164 and BAME mix 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).

2.8. Statistical analysis

Results are reported as mean values ± standard deviation of duplicate samples. The LA reduction and conversion degrees and CLA amount data were first analyzed for normality distribution. Levene's test was applied to verify the homogeneity of the variances. Afterwards, t-Student test was applied when comparing between incubation times (24 and 48 h). The LA-tolerance and genotypic screening frequencies were compared by Qui-squared test. The level of significance was set in general at 0.05; for growth experiments CFU differences had to be ≥ 1 $\log_{10}$ unit and of pH ≥ 0.5 units. Analyses were performed using IBM SPSS Statistics 28 (SPSS Inc., IBM Corporation, NY, USA).

3. RESULTS AND DISCUSSION

3.1. Potential CLA-producing strains

Some studies have detected the presence of LAI gene in lactobacilli strains with associated CLA production capacity.[18,19] Among the 70 strains of lactobacilli assayed, the putative LAI gene was detected with the designed primers in 32 of them. In contrast, with the primers described by Gorissen et al.,[18] the LAI gene was only detected in 14 of them (Table S1). Different LAI gene sequences corresponding to different species are available in GenBank,[27] but the designed primers, which were constructed targeting conserved motifs of the lactobacilli LAI protein, showed to be more efficient in identifying potential CLA-producing strains than the primers described by Gorissen et al.,[18] which were constructed based on the LAI gene sequence of only one strain of *L. plantarum* and one strain of *L. sakei*.

As previously mentioned, LAI activity may consist of a multi-enzymatic system, and it has been suggested that the hydration/dehydration steps are catalyzed by the MCRA protein.[16] Despite the significant homology existing between LAI and MCRA,[28] hydratase activity has been associated only with MCRA, with hydroxy FAs production from LA.[21] The MCRA role in LA isomerization is not clear, but its high homology with LAI suggests that detection of the MCRA gene may be associated with CLA production. Within the 15 strains belonging to *Bifidobacterium*, *Enterococcus*, *Lactococcus* and *Propionibacterium* genera, an MCRA-like gene was detected only in 6 bifidobacteria strains (Table S1). The designed primers were less efficient in detecting this gene (3 out of the 6 bifidobacteria), even though both were constructed based on conserved motifs. In fact, the MCRA sequence is highly conserved among different bacterial species.[29]

A LA hydratase (CLA-HY) that catalyzes the hydration and dehydration steps of the CLA production pathway has been further described.[30] Consequently, the detection of the corresponding structural gene could allow the identification of potential CLA-

296 producers. Hirata et al.[22] identified two proteins in the genome of *L. acidophilus*
297 ATCC4796 that shared 32% amino acid identity with CLA-HY and were able to clone
298 the respective structural genes (*fa-hy1* and *fa-hy2*). But since both sets of primers were
299 designed based on a single *L. acidophilus* genome, only the *L. acidophilus* strains were
300 screened with them. However, only one strain (out of 12) was positive and contained
301 both hydratase genes (Table S1).
302 Accordingly, a total of 39 strains were identified as potential CLA-producers.
303
304 **3.2. LA-tolerance vs. CLA production potential**
305 Approximately 33% (28/85) of the strains tested in this study were not able to grow at
306 the lowest LA concentration assayed (i.e., 1 mg/mL). Among the remaining strains,
307 about 18% (15/85) tolerated 5 mg/mL of LA, 27% (23/85) tolerated 2 mg/mL, and 22%
308 (19/85) tolerated 1 mg/mL (Table S1). As for the genotypically-positive strains, i.e., the
309 potential CLA-producers, a large fraction (~38%; 15/39) revealed LA tolerance < 1
310 mg/mL. The number of strains that tolerated 1 and 5 mg/mL was similar (10/39 and
311 9/39, respectively), while ~13% (5/39) tolerated 2 mg/mL (Table S1). According to Qui-
312 square test, LA-tolerance level is independent of genotypical-positivity/negativity ($\chi 2$ (3,
313 N = 85) = 7.62; $P > 0.05$) (Table S2), even when frequencies of levels < 1 and 1 mg/mL
314 were grouped as “low tolerance” and of 2 and 5 mg/mL as “high tolerance” ($\chi 2$ (1, N =
315 85) = 2.26; $P > 0.05$) (Table S3).
316 The LA conversion to CLA isomers may consist of a detoxification mechanism since
317 the presence of LA impairs microbial growth.[31] Xu et al.[32] observed that, among
318 different probiotic strains, the best CLA producer could tolerate up to 3 mg LA/mL after
319 42 h of incubation. Therefore, it was expected that the genotypically-positive strains,
320 i.e. the potential CLA-producers, would reveal a high LA-tolerance (≥2 mg/mL), and the
321 negative ones a low LA-tolerance (<2 mg/mL). However, results showed that strain
322 tolerance level to LA has no relation to their CLA production potential. This might
323 happen because i) the LA bioconversion into CLA is not in fact a detoxifying

mechanism, and only some strains genetically possess that potential while others do not, or ii) it is a detoxifying mechanism but the degree of tolerance to LA is particular to each potential CLA producing-strain, which would merely reflect on different production capacities, i.e., the high-tolerant strains would yield more CLA than the low-tolerant ones.

3.3. CLA production among genotypically-positive strains

From the group of microorganisms containing CLA-related genes, only 4 strains showed the capacity to transform LA into CLA: *Bifidobacterium breve* DSM 20091, *Lactobacillus gasseri* MP551, *L. plantarum* DSM 20205 and *L. sakei* DSM 20017 (Table 2). Results of molecular detection of putative LAI or MCRA genes for these strains are presented in Figure 1, and those of maximum LA-tolerance determination can be found in Figures S1-4 or Fontes et al.[25]

About CLA production, *B. breve* DSM 20091 converted 51% and 47% of LA after 24 and 48 h of incubation, yielding 312 and 287 µg CLA/mL, respectively. The CLA isomers produced were essentially C18:2 c9t11 and an all *trans* CLA (Figure 2). The above conversion percentages are in accordance with those reported for other B*. breve* strains (> 40%) at equal temperature and LA concentration conditions.[33,34] However, it has been widely verified that CLA production capacity is not only species-specific, but also strain-dependent,[4,35] existing *B. breve* strains that can achieve up to 90% of LA conversion at similar culture conditions.[4,31]

As for *L. gasseri* MP551, the strain was able to convert 1.08% of LA, only after 48 h, resulting in 5 µg CLA/mL (Table 2). A higher conversion percentage (~11%) has been reported for *L. gasseri* CCFM5115 after 48 h under similar assay conditions.[13] Furthermore, *L. gasseri* strains isolated from human infant feces showed higher CLA amounts as well, approximately 11-29 µg/mL after 48 h.[35]

Lactiplantibacillus plantarum DSM 20205 and *L. sakei* DSM 20017 converted just over 1% LA after 24 h of incubation, which marginally decreased after 48 h, releasing about

352 5 to 7 µg CLA/mL (Table 2). Slightly higher conversion percentages (about 3% to 5%)
353 have been obtained for *L. sakei* strains when incubated at a lower temperature (30
354 ºC).[14] On the other hand, *L. plantarum* DSM 20205 values are within the range reported
355 by this latter study for other *L. plantarum* strains grown at 37 ºC (c.a. 0.4-3.5%), but
356 other research works have detected higher yields of CLA (approximately 10 to 55
357 µg/mL), whether at equal or lower incubation temperature (30 ºC) than that employed
358 in our work.[35,36] The above-mentioned lactobacilli strains produced the same CLA
359 isomers as *B. breve* DSM 20091, plus C18:2 t10c12, with exception to *L. plantarum*
360 sakei DSM 20017 (data not shown).
361 Since *L. gasseri* MP551 and *L. sakei* DSM 20017 showed a high tolerance to LA (5
362 mg/mL), and considering the detoxification mechanism previously stated, it was
363 hypothesized that these strains were not stressed enough at 0.5 mg/mL LA to produce
364 higher CLA amounts. However, when these strains were further tested at 5 mg/mL LA,
365 no CLA production was detected at all (data not shown). Therefore, the data presented
366 here do not corroborate the idea of CLA production as a detoxifying strategy.
367 Two further factors can exert an influence on CLA production: (a) the antimicrobial
368 activity of LA; and (b) the pH. Kishino et al.[37] observed that when bacterial growth of *L.*
369 *plantarum* AKU 1009a was inhibited by LA, CLA production decreased. In the current
370 study, viable cell counts were significantly lower (≥ 1 $\log_{10}$ unit difference) with LA for *L.*
371 *gasseri* MP551 (48 h) and *B. breve* DSM 20091 (24 h), compared to control (Figure 3).
372 Thus, LA affected the bacterial growth of *L. gasseri* MP551 only when this strain
373 showed CLA production, and interfered with *B. breve* DSM 20091, which was, in fact,
374 the best producer. Therefore, growth inhibition does not seem an influencing factor in
375 CLA production. Concerning pH, it decreased (> 0.5 units difference) from around pH
376 6.0 to pH 3.8-4.6 (24/48 h) independently of LA presence/absence (Figure 4). Suteebut
377 et al.[38] observed that CLA production by *L. plantarum* GSI 303 was higher when the
378 initial pH of the culture medium was set at pH 6.5. Moreover, when *B. breve* LMC 520
379 was grown elsewhere under buffered pH, a maximal level of CLA production was

achieved between pH 5.5 and 6.0.[39] Thus, this may suggest that pH could have critically influenced CLA production in our study, but further assays would be needed to confirm that assumption.

The LA conversion into other compounds may be an additional reason explaining low CLA production levels by the identified producers, because LA reduction percentages reached considerable values (up to 86%; Table 2). As mentioned earlier, CLA production may include multiple reactions with hydroxy and oxo FAs intermediates of LA conversion into CLA isomers,[9] therefore it was performed a hydroxy FA analysis, but no other compounds were detected at levels that could justify LA reduction amounts. However, Aziz et al.[40] have identified different metabolites produced from LA by *L. plantarum* YW11, such as *trans*/*trans*-9,12-octadecadienoic acid propyl ester, *trans*-9-octadecenoic acid ethyl ester and linoelaidic acid, among others. Thus, comprehensive lipidomic analysis should be performed in the future.

Contrary to what was expected, few positive strains identified in the genotypic screening could produce CLA isomers. Another previous study also reported that some strains of lactobacilli did not convert LA despite the presence of the LAI gene.[41] This suggests that genotypic screening stills not a reliable technique for the identification of CLA-producing strains. On the other hand, among the non-CLA-producers, LA reduction percentages were considerably high (up to ~70%; data not shown), suggesting that other compounds might have been produced instead, as stated above.

There are, at least, four hypotheses that could explain the lack of LA conversion into CLA isomers by the genotypically-positive strains: i) low or no expression of the genes encoding required enzymes; ii) inhibition of expressed enzymes; iii) production of other compounds from LA; and iv) non-optimal growth conditions for CLA production.

In conclusion, the development of LAI primers based on conserved motifs revealed more efficiency in the identification of potential CLA-producing strains. However, very few strains possessing putative genes encoding enzymes involved in LA isomerization

could yield CLA isomers, even though LA reduction percentages were considerably high. Moreover, no association was found between CLA production potential and LA tolerance that could corroborate the hypothesized detoxifying mechanism behind LA conversion.

Thus, the main outcome of the present research work is that selection of potential CLA-producing bacteria cannot be performed relying solely on genotypic screening and/or substrate tolerance. Further works should focus on the key factors that play a critical role in the LA transformation, including LAI and other involved enzymes expression, as well as deepen into the metabolite intermediates with a strain-dependent approach.

ABBREVIATIONS

CLA – Conjugated linoleic acid, FA – Fatty acid, FA-HY - Fatty acid hydratase, LA – Linoleic acid, LAI – Linoleate isomerase, MCRA – Myosin-cross-reactive antigen.

SUPPORTING INFORMATION

List of all strains assessed in this study, their sources, and respective outcome from LA-tolerance and genotypic screening assays (Excel).

Results of Qui-square test, regarding strains LA-tolerance association with CLA production potential, and LA-tolerance assay results of the identified CLA-producing strains (Word).

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CONFLICT OF INTEREST

The authors declare no competing financial interest.

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607

608 **FIGURES CAPTIONS**

609

610 **Figure 1.** Detection of the putative genes encoding LAI or MCRA in the identified producing
611 strains. Each lane corresponds to the following samples: 1 and 6 – SmartLadder small fragments
612 (Eurogent); 2 – *Bifidobacterium breve* DSM 20091 MCRA gene amplification using Ev1a and Ev2a
613 primers; 3 – *Bifidobacterium breve* DSM 20091 MCRA gene amplification using INIA-Bf1 and INIA-
614 Bf2 primers; 4 – Primers Ev1a and Ev2a negative control; 5 – Primers INIA-Bf1 and INIA-Bf2
615 negative control; 7 – *Lactobacillus gasseri* MP551 LAI gene amplification using primers LISO3 and
616 LISO4; 8 – *Lactobacillus gasseri* MP551 LAI gene amplification using primers INIA-Lb1 and INIA-
617 Lb2; 9 – *Lactiplantibacillus plantarum* DSM 20205 LAI gene amplification using primers LISO3 and
618 LISO4; 10 – *Lactiplantibacillus plantarum* DSM 20205 LAI gene amplification using primers INIA-
619 Lb1 and INIA-Lb2; 11 – *Latilactobacillus sakei* DSM 20017 LAI gene amplification using primers
620 LISO3 and LISO4; 12 – *Latilactobacillus sakei* DSM 20017 LAI gene amplification using INIA-Lb1
621 and INIA-Lb2; 13 – Primers LISO3 and LISO4 negative control; 14 – Primers INIA-Lb1 and INIA-
622 Lb2 negative control.

623

624 **Figure 2.** Chromatogram profile by GC-FID of *Bifidobacterium breve* DSM 20091 cultured with 0.5
625 mg/mL of linoleic acid (LA) at 0 h (A), 24 h (B) and 48 h (C) of incubation. DMF –
626 dimethylformamide, IS – Internal standard, CLA – Conjugated linoleic acid.

627

628 **Figure 3.** Viable cell counts of *Bifidobacterium breve* DSM 20091 (A), *Lactobacillus gasseri* MP551
629 (B), *Lactiplantibacillus plantarum* DSM 20205 (C) and *Latilactobacillus sakei* DSM 20017 (D) grown
630 with or without 0.5 mg/mL of linoleic acid (LA) (n=2). Different superscript letters for significant
631 differences (≥ 1 $\log_{10}$ unit difference) between incubation times. *Significant differences (≥ 1 $\log_{10}$
632 unit difference) between LA presence/absence within incubation time. nd = bacterial growth below
633 the countable range.

634

635 **Figure 4.** pH of *Bifidobacterium breve* DSM 20091 (A), *Lactobacillus gasseri* MP551 (B),
636 *Lactiplantibacillus plantarum* DSM 20205 (C) and *Latilactobacillus sakei* DSM 20017 (D) cultures

637 with or without 0.5 mg/mL of linoleic acid (LA) (n=2). Different superscript letters for significant
638 differences (≥ 0.5 units difference) between incubation times.

639 **TABLES**

640

641 **Table 1.** Primers used in this study for the detection of the putative genes encoding LAI, MCRA and FA-HY.

Primer set designation	Target strains (Genera/Species)	Target gene annotation	Annealing temperature (ºC)	Amplification product length (kb)	Reference
LISO3 and LISO4	Lactobacilli	Linoleate isomerase gene (*lai*)	56	≈1.0	Gorissen et al.[18]
INIA-Lb1 and INIA-Lb2			52	≈0.6	Designed based on conserved domains
Ev1a and Ev2a	Bifidobacteria, Propionibacteria, Enterococci and Lactococci	Myosin cross-reactive-antigen gene (*mcra*)	45	≈1.0	Rosberg-Cody et al.[21]
INIA-Bf1 and INIA-Bf2			57	≈1.0	Designed based on conserved domains
Fa-hy1-F and Fa-hy1-R	*Lactobacillus acidophilus*	Fatty acid hydratase 1 (*fa-hy1*)	51	≈1.8	Hirata et al.[22]
Fa-hy2-F and Fa-hy2-R		Fatty acid hydratase 2 (*fa-hy2*)	51	≈1.8	

642 **Table 2.** Linoleic acid reduction and conversion percentages and conjugated linoleic acid yield by the identified producing strains.

Strain	LA reduction (%)[a]		CLA production (µg/mL)[a]		LA conversion (%)[a]	
	24 h[b]	48 h[b]	24 h[b]	48 h[b]	24 h[b]	48 h[b]
Bifidobacterium breve DSM 20091	85.23 ± 0.48	86.49 ± 0.16	312.39 ± 3.42 a	287.30 ± 5.81 b	50.81 ± 0.55 a	46.73 ± 0.94 b
Lactobacillus gasseri MP551	39.08 ± 3.91	34.19 ± 0.52	ND	5.49 ± 0.76	ND	1.08 ± 0.15
Lactiplantibacillus plantarum DSM 20205	61.19 ± 0.45 b	67.24 ± 1.61 a	6.12 ± 0.27	4.94 ± 1.48	1.23 ± 0.05	0.99 ± 0.30
Latilactobacillus sakei DSM 20017	41.65 ± 1.00 b	58.69 ± 1.83 a	6.85 ± 1.52	5.10 ± 0.77	1.22 ± 0.27	0.91 ± 0.14

643 [a]LA – Linoleic acid; CLA – Conjugated linoleic acid; ND - no CLA production was verified at detectable levels.

644 [b]Average value ± standard deviation (n=2). Different superscript letters for significant differences between 24 and 48 h ($P < 0.05$).

FIGURES

Figure 1

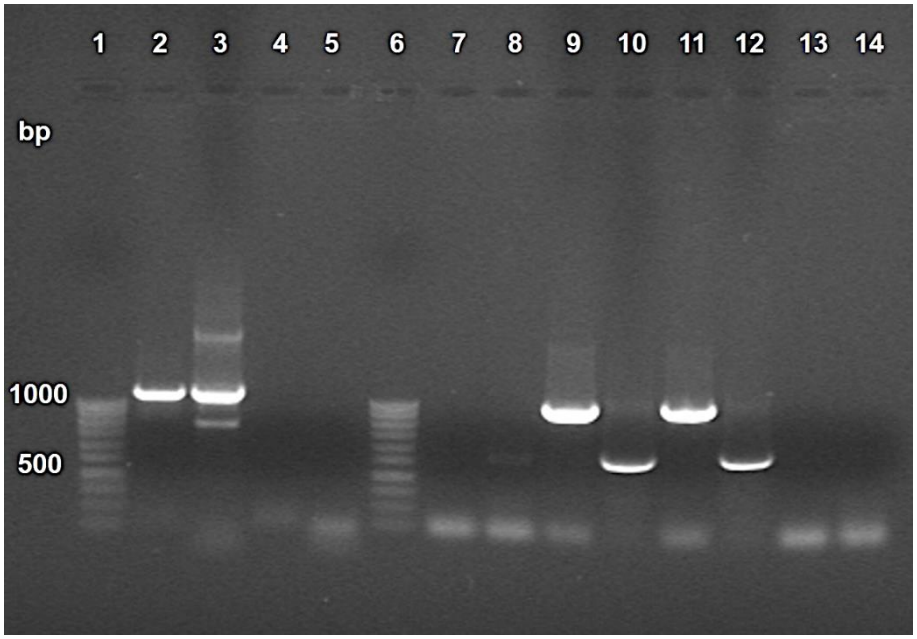


Figure 2

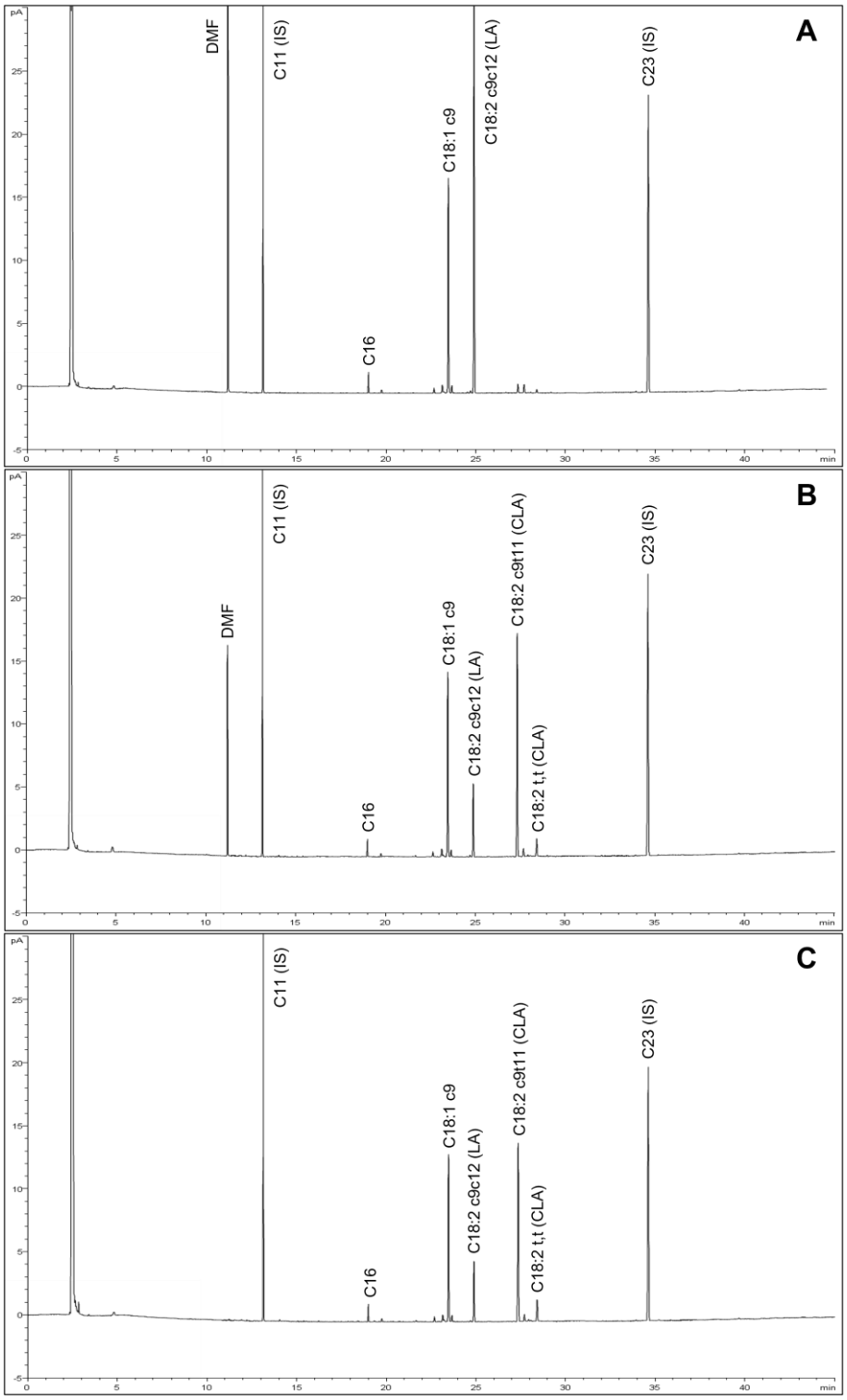

A
DMF
C11 (IS)
C16
C18:1 c9
C18:2 c9c12 (LA)
C23 (IS)
B
DMF
C11 (IS)
C16
C18:1 c9
C18:2 c9c12 (LA)
C18:2 c9t11 (CLA)
C18:2 t,t (CLA)
C23 (IS)
C
C11 (IS)
C16
C18:1 c9
C18:2 c9c12 (LA)
C18:2 c9t11 (CLA)
C18:2 t,t (CLA)
C23 (IS)
pA
min

Figure 3

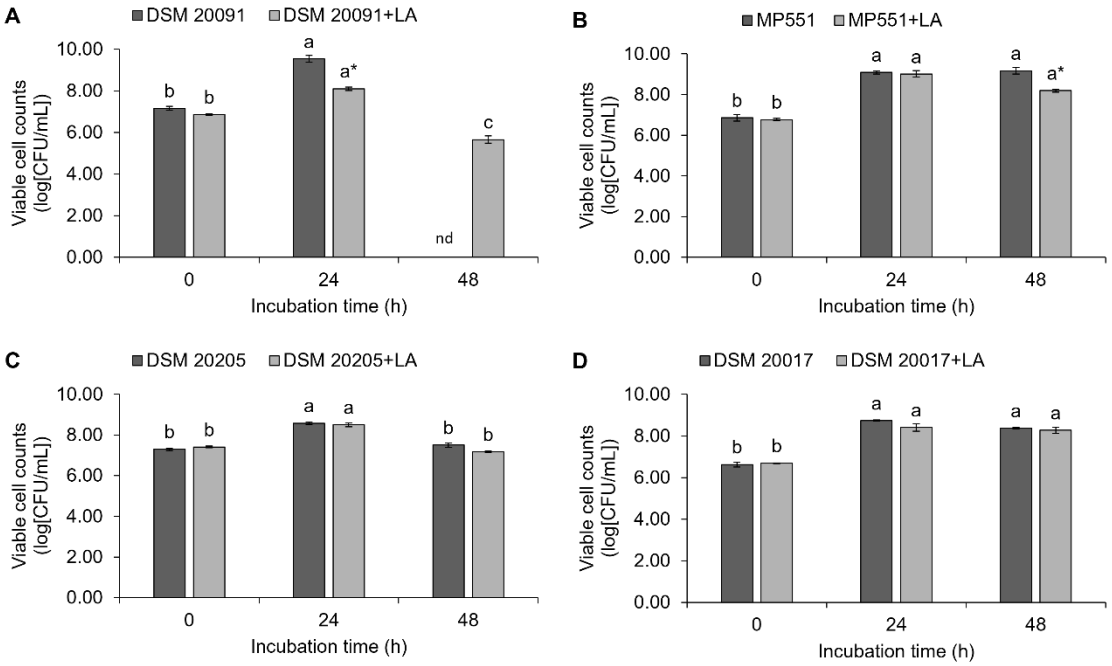
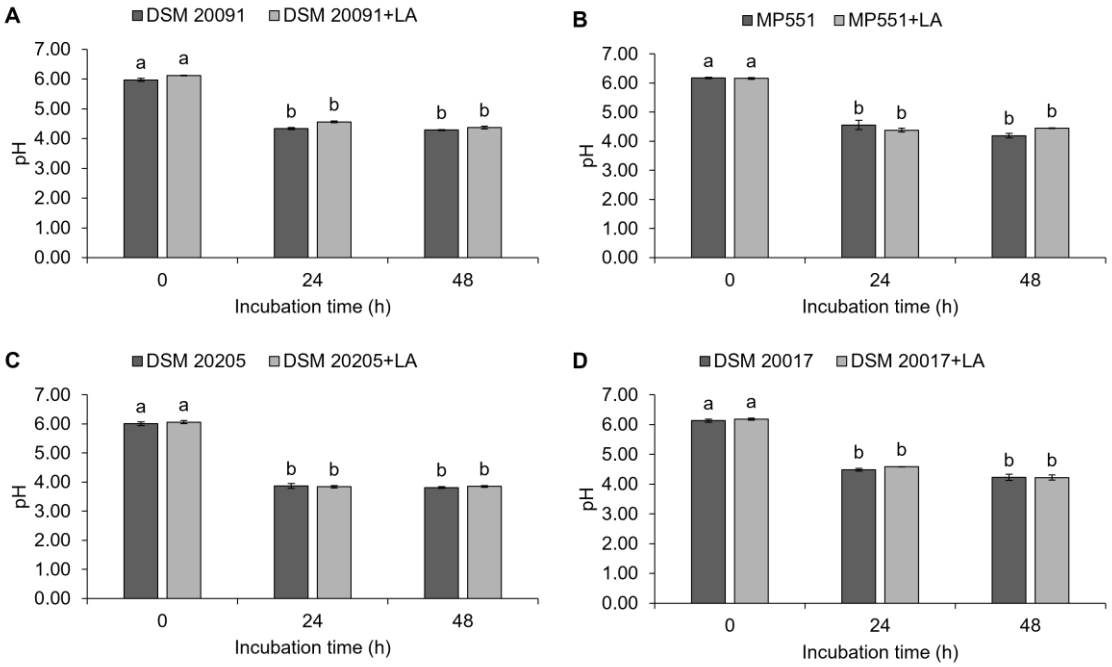
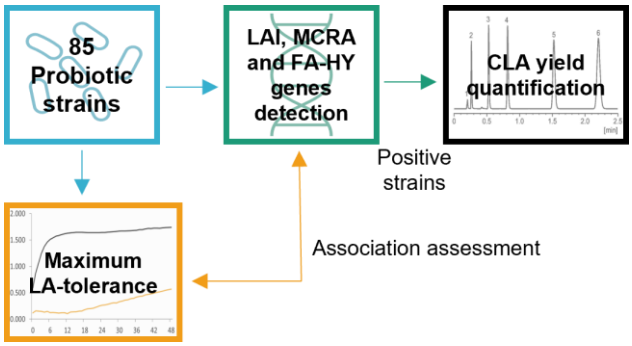


Figure 4



663 **For Table of Contents only**

664



665