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## Article

# CLA-Producing Probiotics for the Development of a Yogurt-Type Beverage

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**Abstract:** This study examined the ability of four beneficial strains (*Lactobacillus rhamnosus* LbRE-LSAS, *Bifidobacterium animalis* subsp. *lactis* Bb12, and two yogurt starters TA040 and LB340) to ferment MRS or milk containing free linoleic acid (0, 0.5, or 1 mg/mL). The goal was to produce an enriched conjugated linoleic acid (CLA) isomers' yogurt-type beverage. Linoleic acid (LA) at 0.5 mg/mL did not interfere with the growth of the assayed bacteria on de Man Rogosa and Sharpe broth (MRS) or milk. On the other hand, increasing the content of LA in the MRS or yogurt-type beverage to 1 mg/mL slightly inhibited all strains and prevented accumulating high biomasses. A gas chromatography analysis of the fatty acid profiles confirmed the bioconversion of LA. The yogurt starters TA040 and LB340 had the highest bioconversion rates in the yogurt-type beverages, whereas the probiotic Bb12 strain was the most interesting at converting LA into its active CLA. CLA from the MRS supernatants of TA040, Bb12, and LbRE-LSAS had maximum antibacterial activities against *S. typhimurium*, *E. coli*, and *S. aureus*, respectively. Whey from the Bb12 beverage showed an inhibitory effect against all pathogens. These results suggest that all strains could be used as starter cultures in the proposition of a yogurt-type beverage with a high CLA content and antibacterial potential.

**Keywords:** beneficial bacteria; linoleic acid; CLA; yogurt-type beverage; antibacterial activity



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## 1. Introduction

The global revenue of functional probiotic products and beverages has shown a constant increase [1]. Probiotics, marketed as our partners for health, are gradually becoming a vital component of “healthy” food. The World Health Organization and the Food and Agriculture Organization of the United Nations [2] describe probiotics as “live microorganisms which, when administered in adequate amounts, confer a health benefit on the host”. These organisms are eligible to become probiotics if they meet a number of very specific functional requirements. Modification of the microbiota's composition and activity, improvement of the epithelial barrier function, immune system modulation, systemic metabolic response modification, and central nervous system signaling are some of the various positive effects of probiotics [3]. Moreover, probiotic bacteria like *Bifidobacterium* spp. and *Lactobacillus* spp. have been found to secrete acids like lactic acid, propionic acid, and acetic acid, which lower the pH and inhibit harmful bacteria.

Probiotics can be incorporated into a variety of merchandises, including foods, beverages, pharmaceuticals, and nutritional supplements. Probiotic fermented milk beverages

are produced by specific strains of probiotic bacteria with or without starter cultures, resulting in the synthesis of beneficial metabolites. Fermented milk promotes the growth and targeted transfer of probiotic bacteria to the host's gastrointestinal tract. A previous study suggested that consuming fermented milk and probiotics could restore and maintain gut microbial equilibrium [4,5].

Among other bioactive compounds in fermented milk, conjugated linoleic acid (CLA) isomers represent a particular group of bioactive fatty acids with multiple positive effects on human health, including anti-cancer, anti-atherogenic, anti-adipogenic, anti-diabetic, anti-inflammatory, cholesterol regulation, and immune function stimulation activities [6]. CLA isomers are found in animal-derived foods that humans routinely consume, notably those from ruminants such as dairy products. Several customers are already interested in these lipids, which have strong nutraceutical potential. Indeed, CLA has been utilized as a supplement since 1997, after it was shown in mice that its administration leads to a decrease in body fat content and an increase in lean mass [7]. Aside from the effects of CLA on body composition (fat reduction), these bioactive compounds have anti-cancer, anti-atherogenic, anti-adipogenic, anti-diabetic, and anti-inflammatory effects, as well as a potential impact on cholesterol levels and immune function stimulation [8,9]. The available data on daily CLA intake vary greatly among different populations around the world, from 15 to 440 mg/day [10].

In recent years, different bacteria have been confirmed to produce CLA, including lactobacilli, bifidobacteria, propionic bacteria, pediococci, enterococci, streptococci, and lactococci. One of the possibilities for increasing systemic CLA concentrations in a host is the bio-hydrogenation of linoleic acid by intestinal bacteria. Nevertheless, such bioconversion has also been described in lactic acid bacteria [11]. Thus, we intend to explore the ability of four strains to convert linoleic acid into CLA in a milk beverage in order to propose a healthy yogurt-type beverage rich in CLA. The interest in such a study aims to fulfill the numerous criteria that a given strain must meet before acquiring the very rigorous probiotic status. We also explore in this bioconversion the production of other fatty acids that have been suggested to exert an impact on lipid metabolism, such as stearic and oleic acids [12]. Given that milk originally contains a small amount of CLA, the present study was conducted on two media, MRS and a milk beverage, and free bacterial CLA were also tested for any antibacterial effects towards some virulent pathogens.

## 2. Materials and Methods

### 2.1. Bacteria

*Lactobacillus rhamnosus* LbRE-LSAS is a probiotic strain from LMBAFS's laboratory collection (LMBAFS, Abdelhamid Ibn Badis University, Algeria). It was isolated from the feces of a healthy, breastfed newborn. Bb-12, or *Bifidobacterium animalis* subsp. *lactis*, is the well-known probiotic strain from Chr. Hansen (Horsholm, Denmark). Danisco (NEUILLY-SUR-SEINE, France) supplied the yogurt starters, *Lactobacillus delbrueckii* subsp. *bulgaricus* LB 340 and *Streptococcus thermophilus* TA 040. Stock cultures were kept at  $-70\text{ }^{\circ}\text{C}$ .

Bacteria were serially cultured three times in the appropriate broth (Biomérieux, Craponne, France) prior to experimentation: MRS with Cysteine-HCl (Sigma, St. Louis, MO, USA) for LbRE-LSAS and Bb12, MRS for LB340, and M17 for TA040. Cultures were centrifuged (Beijing Medical Centrifuge Company, Beijing, China) at  $4000\times g$  for 5 min and rinsed with phosphate-buffered saline (Sigma, St. Louis, MO, USA). The cell suspensions were then centrifuged at  $4000\times g$  for 5 min. The bacterial cell counts (four monocultures + one associated culture of TA040 + LB340; 1 v:1 v) were adjusted to  $\sim 7.0\text{ log CFU/mL}$ .

## 2.2. Linoleic Acid (LA)

Linoleic acid (L1012, linoleic acid for culture, Sigma, Lezennes, France) was diluted in tween 80 solution (0.05%) and used at 0, 0.5, and 1 mg/mL based on previous published works [13,14] and our preliminary results.

## 2.3. Preparation of Yogurt-Type Beverage

The cell pellets were resuspended individually in ultra-high-temperature “UHT” skim milk (18% DW) for use in the preparation of yogurt-type beverages or in MRS broth (M17 broth was used for TA040 monoculture). At least three replications for each sample were performed. After cooling, each individual culture or the associated culture of starters was then added individually, and the samples containing LA or not were incubated at 37 °C overnight. After fermentation, the MRS broths and yogurt-type beverages were kept at 4 °C for subsequent analyses.

## 2.4. Biomass and pH

The number of viable cells from each MRS broth (CFU/mL) or yogurt-type beverage (CFU/g), determined three times, was calculated from the colonies obtained on appropriate media (log CFU/mL) [15]. The acidifying activities of the strains were estimated by measuring the pH using a digital pH meter (WTW, pH meter 330, Weilheim, Germany).

Different control cultures were conducted at the same conditions with or without LA, with or without probiotic strain addition, and fermented or not (0 h). The results were subtracted from their corresponding samples.

## 2.5. CLA Contents Released from the Bacterial Conversion of LA

The produced CLAs were quantified in the bacterial supernatants (pellets discarded), as it was discovered that only the released CLAs were systematically available to exert a probable positive activity [13]. After fermentation, the cultures were centrifuged at 23,500× g for 10 min at 5 °C. Lipids were recovered from the bacterial supernatants and methylated using the Alonso et al. [13] technique with some modifications.

### 2.5.1. Extraction

An amount of 6 mL of the supernatant was combined with 12 mL of isopropanol (99.7% purity; Sigma-Aldrich, St. Louis, MO, USA) and 60 µL of an internal standard (64.4 mg of heptadecanoic acid C17:0, 99% purity, in 10 mL of hexane). After 30 s of vigorous shaking with a vortex, 9 mL of pure hexane (99%, Sigma-Aldrich, St. Louis, MO, USA) was added, mixed for 3 min, then centrifuged at 1900× g for 5 min at 5 °C. After being aspirated, the supernatant was filtered through pure anhydrous sodium sulfate (99%, Sigma-Aldrich, St. Louis, MO, USA). An extra 7 mL of hexane was added to the filtrate and used to rinse the filter. A 25 mL glass conical tube was used to collect the lipid fraction, which was then dried at 37 °C in a dry bath using nitrogen gas. After adding 100 µL of sodium methoxide 1 N (Sigma, St. Louis, MO, USA), the mixture was vortexed for one minute and heated for 15 min at 40 °C. The same extraction procedures were used for the non-inoculated MRS supernatants or unfermented milk (the whey was obtained by acidification to pH 4.5 by adding 1 N lactic acid, Sigma, St. Louis, MO, USA).

### 2.5.2. Methylation

At an ambient temperature, 50 µL of toluene (Sigma-Aldrich, St. Louis, MO, USA) and twice 500 µL of 14% boron trifluoride (BF<sub>3</sub>) (Sigma-Aldrich, St. Louis, MO, USA) in methanol (Sigma-Aldrich, St. Louis, MO, USA) were added to free fatty acids to methylate them for 30 min. A saturated sodium bicarbonate solution (5 mL, Sigma-Aldrich, St. Louis, MO, USA) and hexane (2 mL, Sigma-Aldrich, St. Louis, MO, USA) were then added in that

order. The preparations underwent a vigorous vortexing process before being centrifuged for 10 min at 4 °C at 1000× g. After separation, the hexane phase was kept in airtight glass microtubes at −20 °C until gas chromatography (GC) analysis.

### 2.5.3. Gas Chromatography (GC) Analysis

Fatty acid methyl esters were analyzed in an Agilent 6890 (Agilent, CA, USA) chromatograph coupled with a flame ionization detector (FID, Agilent, CA, USA) according to the program previously described by Loor et al. [16]. One microliter of each sample was automatically injected in a 1:10 split ratio. Hydrogen was used as the carrier gas through a specific CP-Sil88 capillary column (100 m × 0.25 mm × 0.2 μm; i.d., Varian Inc., Lake Forest, CA, USA). The chromatograph oven was initially set at a temperature of 70 °C after the sample injection, then linearly increased at 5 °C per minute for 1 min until reaching 100 °C and stabilized at this value for 2 min. After this, the temperature was gradually increased at a rate of 10 °C per min until 175 °C and maintained at this level for 42 min and then at 5 °C per min until a final temperature of 225 °C was reached and maintained for 25 min. The measurements, repeated three times and recorded, were processed using Agilent ChemStation software (**OpenLab CDS ChemStation** edition c.01.10, Agilent, CA, USA).

### 2.5.4. Linoleic Acid's Bacterial Conversion Ability

The strains were compared by determining the conversion percentages of the added free linoleic acid (0.5 or 1%) into CLAs using the formula below [16]:

*LA Conversion Rate (%)*

$$= \left[ \frac{(\sum \text{Total Produced CLAs (9c11t CLA + 8t10c CLA + 11c13t CLA + 10t12c CLA + C21 : 0 + 9c11c CLA + 10c12c CLA + 11c13c CLA + 11t13t CLA + tt CLA)})}{(\text{Detected LA} + \sum \text{Total Produced CLAs})} \right] \times 100 \quad (1)$$

where *Detected LA* is the concentration of LA that was actually extracted and quantified in relation to the internal standard.

### 2.6. Antibacterial Activity

Cultures from the MRS or yogurt beverage were centrifuged at 8000× g for 15 min at 4 °C (Thermo Scientific, Waltham, MA, USA), and cell-free supernatants were yielded. The pH was adjusted to 6.5 with 6 mol/L NaOH (Sigma-Aldrich, St. Louis, MO, USA) and then prepared as described in Section 2.5.1. Sterile disks containing 100 μL of the free CLA-supernatant solution were placed on an MH agar (Fisher Scientific SAS, Strasbourg, France) surface, previously spread with one pathogen indicator (100 μL, 1 × 10<sup>7</sup> CFU/mL): *Staphylococcus aureus* ATCC 29523, *Escherichia coli* ATCC 25922, or *Salmonella typhimurium* ATCC 14028. Following incubation at 37 °C for 24 h, the diameters of the clear inhibitory zone were measured and compared to positive controls consisting of azithromycin for *S. aureus* and *E. coli* and cefoperazone for *S. typhimurium*.

### 2.7. Statistical Analysis

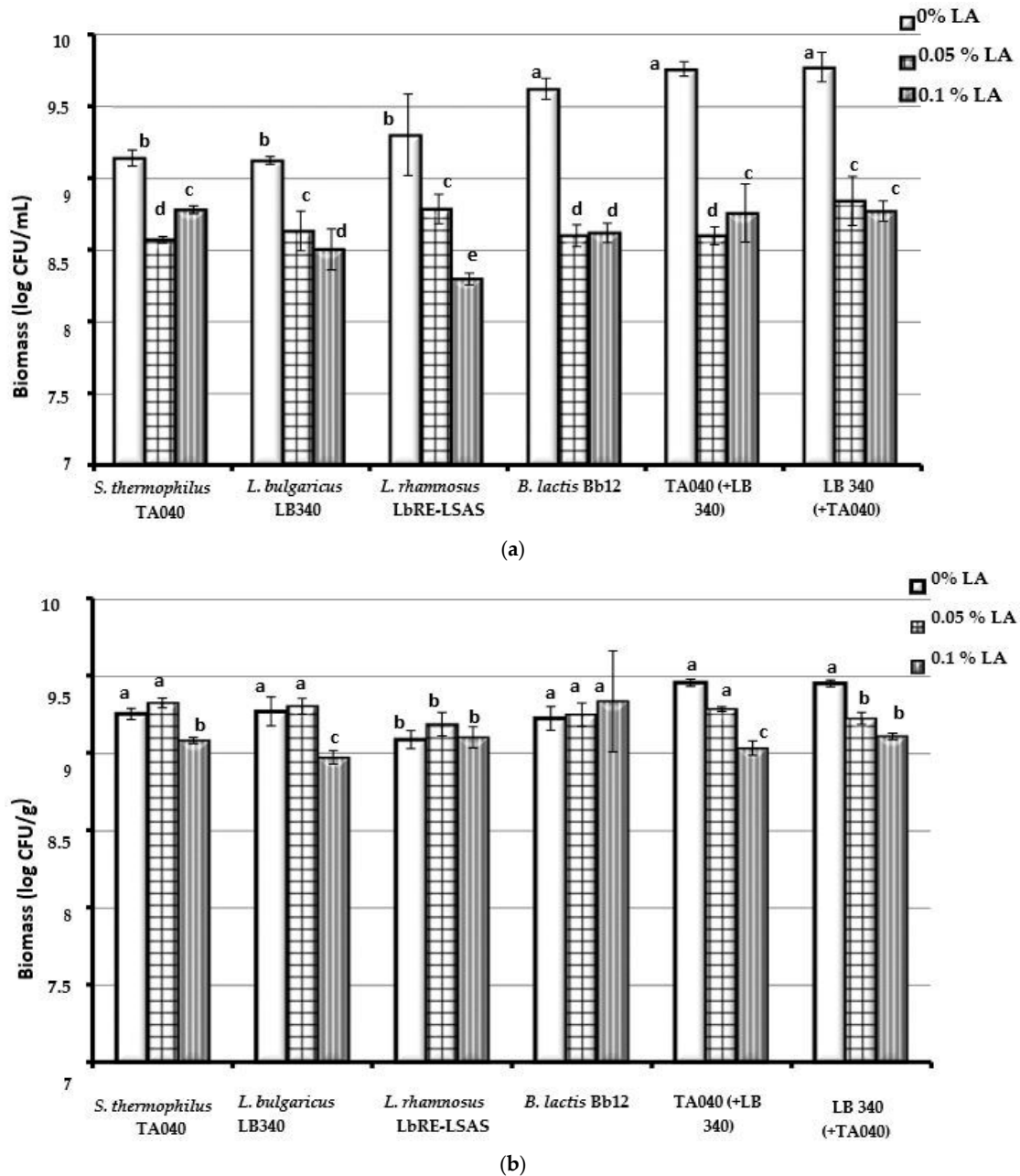
The results of the strains' fermentative capacities in the presence of linoleic acid were statistically treated using an analysis of variance (ANOVA). The means were compared using the Tukey test. The results are the averages of three independent studies (*n* = 9). The percentages of linoleic acid conversion to CLA were also compared using an ANOVA (SPSS statistical software, version 15.0, Chicago, IL, USA) with the Duncan post hoc test. In all situations, a significance level of 0.05 was fixed.

### 3. Results and Discussion

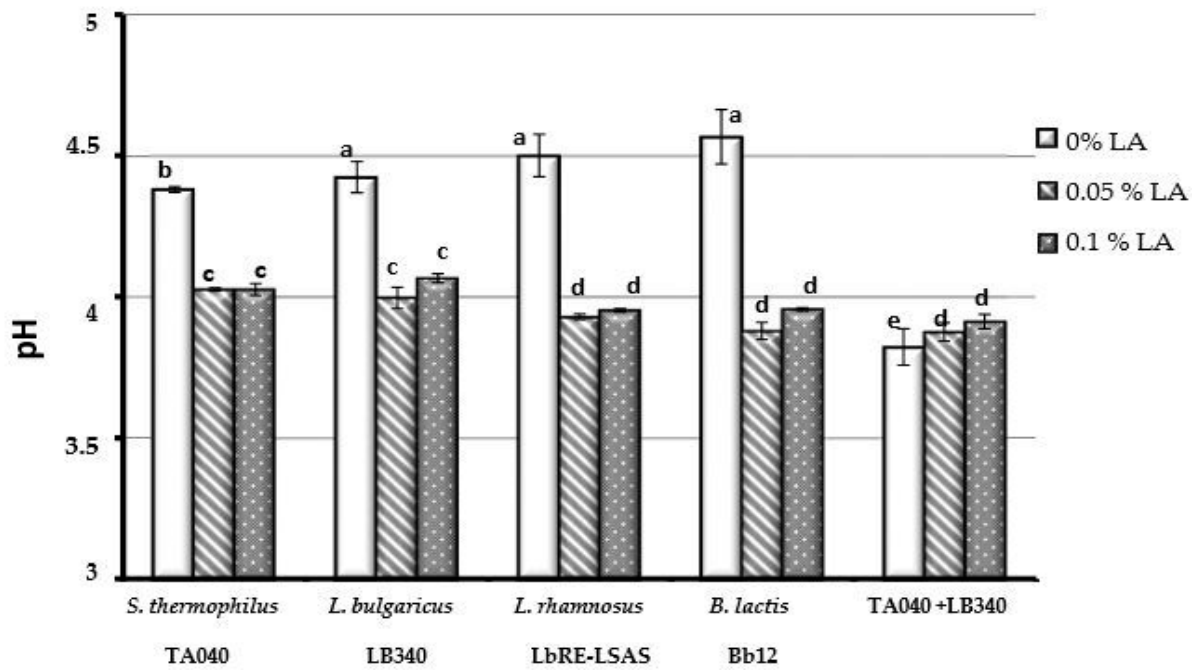
#### 3.1. All Probiotic Bacteria Were Able to Convert Linoleic Acid into CLA

##### 3.1.1. Bacterial Growth Was High for All Strains and Affected by Linoleic Acid in MRS Medium Only

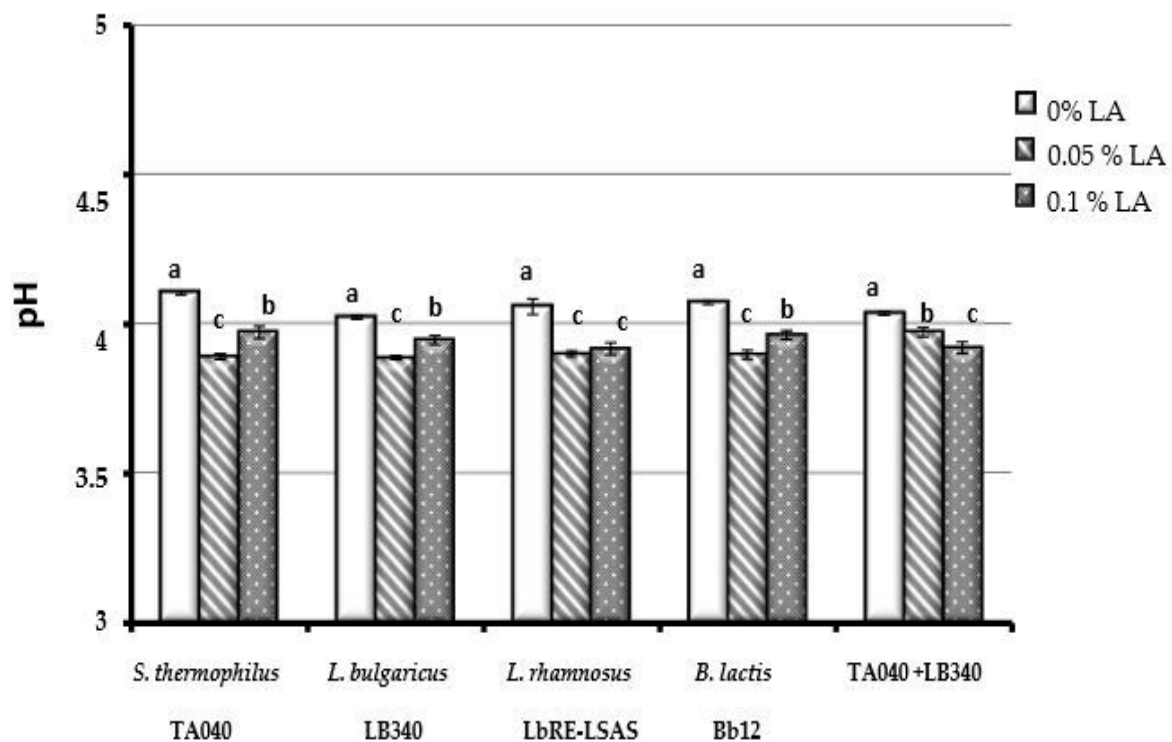
Figures 1 and 2 show the capacities of TA040, LB340, LbRE-LSAS, and Bb12 to ferment MRS or milk supplemented with linoleic acid used at final concentrations of 0.5 or 1 mg/mL. The data are represented by the biomass (Figure 1) (log CFU/mL or log CFU/g) and the acidifying activity (Figure 2) of the culture after 12 h at 37 °C and in anaerobiosis.



**Figure 1.** Biomass (log CFU/mL or log CFU/g) after 12 h of monoculture at 37 °C in anaerobiosis of *S. thermophilus* TA040, *L. delbrueckii* subsp. *bulgaricus* LB340, *L. rhamnosus* LbRE-LSAS, or *B. animalis* subsp. *lactis* Bb 12 and of the co-culture of the two yogurt starters (1:1) in the MRS (M17 for TA040) medium (a) or skim milk (18% DW) yogurt beverage (b) containing or not (0, 0.5, or 1 mg/mL) linoleic acid (LA). The values represent the mean of three independent experiments  $\pm$ SD ( $n = 9$ ). Different lowercase letters (a–e) stand for significantly various mean values of bacterial growth per one strain according to the performed one-way ANOVA with the post hoc test ( $p > 0.05$ ).



(a)



(b)

**Figure 2.** pH after 12 h of monoculture at 37 °C in anaerobiosis of *S. thermophilus* TA040, *L. delbrueckii* subsp. *bulgaricus* LB340, *L. rhamnosus* LbRE-LSAS, or *B. animalis* subsp. *lactis* Bb 12 and of the co-culture of the two yogurt starters (1:1) in the MRS (M17 for TA040) medium (a) or skim milk (18% DW) yogurt beverage (b) containing or not (0, 0.5, 1 mg/mL) linoleic acid (LA). The values represent the mean of three independent experiments  $\pm$ SD ( $n = 9$ ). Different lowercase letters (a–e) stand for significantly various mean values of pH per one strain according to the performed one-way ANOVA with the post hoc test ( $p > 0.05$ ).

After 12 h, all strains exhibited significant growth capacities. The amounts of biomass detected in the LA-supplemented medium varied between 8.57 and 8.84 log CFU/mL (0.5 mg/mL), 8.3 to 8.78 log CFU/mL (1 mg/mL) in the MRS (Figure 1a), and between 9.22 and 9.32 log CFU/g (0.5 mg/L) and 8.97 and 9.10 log CFU/g (1 mg/mL) in the yogurt-type beverages (Figure 1b). In the MRS-LA medium, all the strains had a biomass higher than 8 log CFU/mL. The symbiosis established between the two starter cultures displayed high biomasses, reflecting their tolerance to linoleic acid.

In general, linoleic acid partially inhibited the development of all the assayed bacteria in the MRS medium. After 12 h in MRS medium (M17 for *S. thermophilus*) and compared to the related control culture without linoleic acid, the decline in biomass ranged from  $-0.28$  ( $p > 0.05$ ) to  $-0.9$  ( $p < 0.05$ ) log units.

The behavior of the assayed strains was highly variable on the yogurt-type beverages enriched with LA (fermented milks-LA) (Figure 1b). LA had no negative impact on milk fermentation by *L. rhamnosus* or *B. lactis*, while the development of starter strains in pure cultures was slightly ( $p > 0.05$ ) inhibited ( $-0.2$  log unit on average) in the presence of 1 mg/mL LA. In co-culture, LA significantly disrupted the symbiosis between the two starters when added at 1 mg/mL (average decrease of  $-0.38$  log unit,  $p < 0.05$ ) compared to 0.5 mg/mL (average drop of  $-0.2$  log unit,  $p > 0.05$ ).

Rodríguez-Alcalá et al. [17] found that adding 1 mg/mL LA to milk did not significantly affect the growth of *B. animalis* (Bb12-1), *L. acidophilus*, or *L. lactis*. The related results to the probiotic strain *B. lactis* Bb12 presented in this study are consistent with their findings.

LA is an antimicrobial agent, and strains that can grow in its presence have the ability to convert it into other fatty acids, thus neutralizing its toxicity [18].

In summary, our findings indicate that LA provided at a concentration of 0.5 mg/mL in milk, unlike the MRS medium, did not inhibit the bacterial growth. This distinctive feature of milk could be attributed primarily to its buffering quality but also to its lactose content, as stated by Lin [19].

In the work of Gao et al. [20], the authors reported that LA inhibits the growth of *Bifidobacterium breve*, but its conjugated isomers do not. Furthermore, Kankaanpaa et al. [21] found that adding polyunsaturated fatty acids (PUFAs) like linoleic,  $\alpha$ -linolenic, arachidonic, and docosahexaenoic acids to the growth medium changes the lipid cell composition of *Lactobacillus rhamnosus*, *L. casei*, and *L. delbrueckii*. In fact, the stereochemistry of double bonds in fatty acids can have a significant impact on microbial development. However, Kankaanpaa [21] found that all free fatty acids rich in *trans*-type configuration, regardless of carbon chain length or double bond location, can enhance *Lactobacilli* development. Gibson et al. [22] claimed that combining long-chain PUFAs with probiotics may be advantageous for newborns because it promotes probiotic colonization in the microbiota by increasing their adherence to intestinal cells.

It is important to underline that the majority of research in this context has been focused on bacterial CLA synthesis rather than accumulating biomasses or acidifying activities in the presence of LA.

### 3.1.2. The Acidifying Activity of Bacterial Strains Was Not Affected by Linoleic Acid

Figure 2 shows the pH values after 12 h in MRS or milk enriched or not with LA. In control MRS cultures (without LA), the assayed strains significantly acidified ( $p < 0.05$ ) the broth, lowering the pH from 6.62 to 4.38–4.56. The mix between the yogurt starters resulted in a more acidic environment (pH 3.82) compared to their relative monocultures ( $p < 0.05$ ).

The acidity was higher in the presence of LA in MRS medium (Figure 2a) than in its absence. The addition of 0.5 mg/mL of LA resulted in more acidic pH values (4.02 to 3.88)

( $p < 0.05$ ) compared to 1 mg/mL (4.06 to 3.95). An exception was monitored in *S. thermophilus* cultures, where the acidity was nearly identical (pH of 4.02 each,  $p > 0.05$ ) regardless of the LA concentration.

These findings are consistent with those of the biomass in the presence of both LA concentrations. The culture combining the two yogurt starters behaved similarly to TA040 or LB340 monocultures where the acidity was higher at 0.5 than at 1 mg/mL of LA. Nonetheless, the acidity remained lower than that of the control-associated culture (Figure 2a).

In the yogurt beverages (Figure 2b), the acidifying activities of all strains were high: 4.02 to 4.10 in the absence of LA (controls) and 3.88 to 3.90 and 3.91 to 3.97 in the presence of LA at 0.5 and 1 mg/mL, respectively.

The pH of the yogurt beverage supplemented with 0.5 mg/mL of LA was more acidic than that obtained with 1 mg/mL, with the exception of *L. rhamnosus* LbRE-LSAS cultures ( $p > 0.05$ ) and those containing the associated yogurt starters ( $p > 0.05$ ).

Furthermore, the flavorful scent of the yogurt beverage was appealing in all cultures, particularly in the yogurt starter monocultures and in those of *B. lactis* Bb12 supplemented with LA, where the exhibited pH values were the lowest ( $p < 0.05$ ), 3.88 on average.

The results of the acidifying activities suggested that the fermentative capacities of the examined bacteria might be greatly boosted by the addition of 0.5 mg/mL of LA in the medium.

In the literature, pH variations were investigated and connected to CLA synthesis. The little existing data demonstrated that *Lactobacilli* have the ability to generate CLA at a wide pH range, from lower (pH = 5.0) to higher (pH = 7.0) values, and that this ability was also culture-condition- and strain-dependent [23].

### 3.2. Bioproduction of CLA Under Yogurt-Type Beverage Conditions Was Good

After 12 h of culture at 37 °C and in the MRS medium, the strains were able to convert 35.2 to 50.3% of the initial free LA at 0.5 mg/mL. This bioconversion mostly produced C18:3 *n*-3 fatty acid and CLA isomers. Furthermore, modest amounts of elaidic acid 9-*trans* C18:1 and C18:3 *n*-6 were generated (Table 1a).

The addition of LA to the culture medium did not increase the formation of stearic acid (SA, C18:0), as the fatty acid profiles of the control and LA-supplemented cultures were not significantly ( $p > 0.05$ ) different from each other.

In the MRS supernatants, rumenic acid (RA) or the 9-*cis*, 11-*trans* CLA isomer was the most common CLA identified ( $p < 0.05$ ), whereas the 10-*trans*, 12-*cis* CLA isomer was found in low concentrations (Table 1a). Moreover, extremely low levels ( $p < 0.05$ ) of *trans*, *trans*-type CLA isomers were solely found in the *L. bulgaricus* supernatants in pure culture or associated with *S. thermophilus*.

The non-fermented and non-added skim milk had an average CLA level of 0.0063 mg/mL. After 12 h of fermentation, the rate rose ( $p < 0.05$ ) to an average of 0.0132 to 0.0175 mg/mL, depending on the bacterial culture added to the milk enriched with 0.5 mg/mL free LA. In the yogurt beverages, only the culture containing the two yogurt starters did not generate CLA isomers from 0.5 mg/mL free LA (Table 1b).

The *Bifidobacterium* strain produced the greatest percentage of total CLAs ( $p < 0.05$ ), with the RA isomer accounting for almost 55%. *L. bulgaricus* accumulated around half of the total.

Similarly, Coakley et al. [18] examined the CLA production by different strains of LAB and *Bifidobacteria* in a CLA-free LA medium and found that *B. breve* and *B. dentium* were the most competent CLA producers among all the used strains, where 65% of the LA was converted to CLA-type rumenic acid. Probiotics mainly generate *cis*-9, *trans*-11 CLA isomers, which account for 90% of total CLA isomers [24]. The production of CLA by

microorganisms is a promising approach for obtaining natural sources of CLA, as it is a sustainable and cost-effective method. Foods high in CLA had a positive impact on the whole lipid profile when compared to CLA supplementation from the market; however, that effect was only statistically significant for LDL cholesterol [25].

**Table 1.** (a) Bacterial bioconversion of 0.5 mg/mL LA into CLA isomers (mg/mL) in the supernatants of 12 h MRS cultures. (b) Bacterial bioconversion of 0.5 mg/mL LA into CLA isomers (mg/mL) in the whey of 12 h yogurt beverages.

(a)									
Strain	% Bioconversion	9t C18:1	C18:3 n-6	C18:3 n-3	Total CLAs	9c, 11t CLA	10t, 12c CLA	tt CLA Mixture	
<i>S. thermophilus</i> <sup>1</sup> TA 040	42.49 ± 2.02	ND	ND	0.1652 <sup>b</sup>	0.1502 <sup>a</sup>	0.0825 <sup>a</sup>	0.0677 <sup>a</sup>	ND	
<i>L. bulgaricus</i> LB 340	45.34 ± 4.72	0.0013 <sup>b,*</sup>	0.0029 <sup>c</sup>	0.2512 <sup>a</sup>	0.1225 <sup>a</sup>	0.0512 <sup>b</sup>	0.0423 <sup>b</sup>	0.0289 <sup>a</sup>	
<i>B. lactis</i> Bb12	40.62 ± 3.11	0.0018 <sup>b,*</sup>	0.0064 <sup>b</sup>	0.0874 <sup>c</sup>	0.1106 <sup>b</sup>	0.0523 <sup>b</sup>	0.0607 <sup>a</sup>	ND	
<i>L. rhamnosus</i> LbRE-LSAS	50.32 ± 5.44	0.0031 <sup>a</sup>	0.0143 <sup>a</sup>	0.0788 <sup>c</sup>	0.0728 <sup>c</sup>	0.0364 <sup>c</sup>	0.0364 <sup>b</sup>	ND	
TA 040 + LB 340	35.23 ± 3.33	0.0018 <sup>b,*</sup>	0.0126 <sup>a</sup>	0.0522 <sup>d</sup>	0.0822 <sup>bc</sup>	0.0424 <sup>b</sup>	0.0321 <sup>b</sup>	0.0064 <sup>b</sup>	
(b)									
Strain	% Bioconversion	9t C18:1	11t C18:1	C18:3 n-6	C18:3 n-3	Total CLAs	9c, 11t CLA	10t, 12c CLA	ttCLA Mixture
<i>S. thermophilus</i> <sup>1</sup> TA 040	64.74 ± 2.66	0.0190 <sup>b</sup>	ND	0.0240 <sup>c</sup>	0.2150 <sup>c</sup>	0.0300 <sup>c</sup>	0.0182 <sup>d</sup>	0.0118 <sup>b</sup>	ND
<i>L. bulgaricus</i> LB 340	87.92 ± 4.55	0.0400 <sup>a</sup>	0.0380 <sup>a</sup>	0.0460 <sup>b</sup>	0.3190 <sup>b</sup>	0.0881 <sup>b</sup>	0.0545 <sup>b</sup>	ND	0.0336
<i>B. lactis</i> Bb12	46.65 ± 4.87	0.0320 <sup>a</sup>	0.0320 <sup>a</sup>	0.0450 <sup>b</sup>	0.4160 <sup>a</sup>	0.1893 <sup>a</sup>	0.1035 <sup>a</sup>	0.0454 <sup>a</sup>	0.0404
<i>L. rhamnosus</i> LbRE-LSAS	60.58 ± 5.22	0.0080 <sup>c</sup>	0.0230 <sup>b</sup>	0.0270 <sup>c</sup>	0.2000 <sup>c</sup>	0.0496 <sup>c</sup>	0.0354 <sup>c</sup>	0.0142 <sup>b</sup>	ND
TA 040 + LB 340	72.76 ± 3.33	0.0140 <sup>b</sup>	0.0260 <sup>b</sup>	0.1350 <sup>a</sup>	0.2400 <sup>c</sup>	ND	ND	ND	ND

<sup>1</sup> M17 for *S. thermophilus* TA040. \* Not different from those of control cultures ( $p > 0.05$ ). ND: not detected (detection limit was 0.0001 mg/mL). <sup>a-c</sup>: Significant differences in the same column.

In the present study and unlike MRS cultures, vaccenic acid 11-*trans* C18:1 was found in the whey of all milk cultures supplemented with 0.5 mg/mL of LA (with the exception of *S. thermophilus* monoculture). The bioconversion rate of LA was around 87.92% for *L. bulgaricus*, 64.74% for *S. thermophilus*, 60.58% for *L. rhamnosus*, and 46.65% for *B. lactis* (Table 1b).

The amount of CLA in food can be enhanced using either industrial or natural techniques. Microbial CLA synthesis is recognized as a natural, ecologically safe technique [26]. The naturally produced forms of CLA have more positive health effects than its chemically manufactured form, because the majority of the natural isomers are of the *cis*9, *trans* 11 configuration, while the chemical synthesis of CLA leads to various types of isomers, predominantly *trans*, *trans* isomers [27]. Furthermore, high doses of CLA supplements from the market may lead to side effects such as digestive issues, hepatic steatosis, the induction of colon carcinogenesis in humans, and potential liver problems [28,29].

In the presence of a two-fold dose of LA (1 mg/mL), our results showed that the 11-*cis*, 13-*trans* CLA isomer was solely generated in the *S. thermophilus* MRS supernatant (Table 2a). Likewise, the concentration of the RA isomer changed according to the bacterial culture.

**Table 2.** (a) Bacterial bioconversion of 1 mg/mL LA into CLA isomers (mg/mL) in the supernatants of 12 h MRS cultures. (b) Bacterial bioconversion of 1 mg/mL LA into CLA isomers (mg/mL) in the whey of 12 h yogurt beverages.

(a)									
Strain	% Bioconversion	9 <i>t</i> C18:1	C18:3 <i>n</i> -6	C18:3 <i>n</i> -3	Total CLAs	9 <i>c</i> , 11 <i>t</i> CLA	10 <i>t</i> , 12 <i>c</i> CLA	<i>tt</i> CLA Mixture	
<i>S. thermophilus</i> <sup>1</sup> TA 040	37.7 ± 3.33	0.2917 <sup>a</sup>	0.0534	ND	0.0623 <sup>‡b</sup>	ND	ND	ND	ND
<i>L. bulgaricus</i> LB 340	61.74 ± 1.66	0.0140 <sup>c</sup>	ND	ND	0.0628 <sup>b</sup>	0.0304 <sup>bc</sup>	0.0302 <sup>a</sup>	ND	ND
<i>B. lactis</i> Bb12	39.74 ± 2.99	0.0336 <sup>b</sup>	ND	ND	0.0619 <sup>b</sup>	0.0619 <sup>a</sup>	ND	ND	ND
<i>L. rhamnosus</i> LbRE-LSAS	24.92 ± 1.66	0.0068 <sup>d</sup>	ND	ND	0.0855 <sup>a</sup>	0.0461 <sup>ab</sup>	0.0393 <sup>a</sup>	ND	ND
TA 040 + LB 340	46.74 ± 3.33	0.0065 <sup>d</sup>	ND	ND	0.0432 <sup>c</sup>	0.0233 <sup>c</sup>	0.0199 <sup>b</sup>	ND	ND
(b)									
Strain	% Bioconversion	9 <i>t</i> C18:1	11 <i>t</i> C18:1	C18:3 <i>n</i> -6	C18:3 <i>n</i> -3	Total CLAs	9 <i>c</i> , 11 <i>t</i> CLA	10 <i>t</i> , 12 <i>c</i> CLA	<i>tt</i> CLA Mixture
<i>S. thermophilus</i> <sup>1</sup> TA 040	49.69 ± 2.66	0.0220 <sup>a</sup>	0.0340 <sup>a</sup>	0.0390 <sup>b</sup>	0.2590 <sup>b</sup>	0.0723 <sup>a</sup>	0.0493 <sup>a</sup>	0.0230 <sup>b</sup>	ND
<i>L. bulgaricus</i> LB 340	57.27 ± 0.33	0.0180 <sup>a</sup>	0.0240 <sup>b</sup>	0.0470 <sup>b</sup>	0.2170 <sup>c</sup>	0.0539 <sup>b</sup>	0.0330 <sup>b</sup>	0.0209 <sup>b</sup>	ND
<i>B. lactis</i> Bb12	48.22 ± 0.66	0.0031 <sup>b</sup>	0.0380 <sup>a</sup>	0.0420 <sup>b</sup>	0.1050 <sup>d</sup>	0.0837 <sup>a</sup>	0.0536 <sup>a</sup>	0.0301 <sup>a</sup>	ND
<i>L. rhamnosus</i> LbRE-LSAS	61.33 ± 1.75	0.0030 <sup>b</sup>	0.0390 <sup>a</sup>	0.0380 <sup>b</sup>	0.3910 <sup>a</sup>	0.0572 <sup>b</sup>	0.0429 <sup>a</sup>	0.0143 <sup>c</sup>	ND
TA 040 + LB 340	63.90 ± 0.66	0.0020 <sup>b</sup>	0.0320 <sup>a</sup>	0.0620 <sup>a</sup>	0.2080 <sup>bc</sup>	0.0751 <sup>a</sup>	0.0361 <sup>b</sup>	0.0390 <sup>a</sup>	ND

<sup>1</sup> M17 for *S. thermophilus* TA040. ND: not detected (detection limit was 0.0001 mg/mL). <sup>a-c</sup>: Significant differences in the same column. <sup>‡</sup> 11*c*, 13*t* CLA was exclusively produced.

The *Bifidobacterium* strain produced that form of CLA exclusively, while the two *lactobacilli* produced lower quantities of this isomer but accumulated more total CLAs ( $p < 0.05$ ). It should be emphasized that *S. thermophilus* was the only strain that did not produce the RA-type CLA.

A noteworthy observation is the absence of the *trans*, *trans*-CLA geometric configuration in all of the cultures used in this experiment.

The studied strains also had varying bioconversion activities of LA: 37.7% for *S. thermophilus*, 24.9% and 39.7% for *L. rhamnosus* and *B. lactis*, respectively; *L. bulgaricus* and its culture associated with *S. thermophilus* had the highest conversion percentages, 61.7% and 46.7%, respectively (Table 2a).

In the yogurt beverages, all bacterial cultures successfully converted free LA at 1 mg/mL ( $p < 0.05$ ). The bioconversion rates varied between 48.2 and 63.9% (Table 2b). The high rates could be due to the protective effect of milk proteins. In the milk matrix, CLA could be shielded from oxidation by alkyl radicals [30].

The fatty acid profiles found by the GC analysis were similar among the four mono-cultures. 9-*cis*, 11-*trans* C18:2 was the most abundantly generated CLA isomer (61–75% of the total CLAs,  $p < 0.05$ ). In comparison, the isomer 10-*trans*, 12-*cis* C18:2 accounted for just 25–38% of total CLAs. In addition, these two isomers were formed in nearly equal amounts ( $p > 0.05$ ) in the co-culture of the two yogurt starters.

Interestingly, the *trans-trans* configuration was entirely absent in all whey cultures of beverages containing 1 mg/mL of LA (Table 2b). At this concentration, LA was transformed into vaccenic acid (VA or 11-*trans* C18:1) and to a lesser amount to 9-*trans* C18:1.

It is well acknowledged that CLA isomers are predominantly produced via anaerobic conditions [13]. Meanwhile, numerous investigations on *Lactobacilli* strains have been

conducted. The results of Alonso et al. [13] using two *Lactobacillus acidophilus* strains and two *L. casei* strains showed a maximum level of CLA reaching 0.130–0.180 mg/mL after 24 h in medium containing LA.

Gorissen et al. [31] found that a high bioconversion of LA into CLA is not always associated with a high fermentative capacity in *Bifidobacteria*. The authors observed that *B. breve* produced a high amount of CLA despite its low biomass in the presence of LA. Our findings were consistent with these observations.

Different *Lactobacilli* strains have been shown to convert LA into CLA. *L. acidophilus* was reported to be the most efficient in accumulating good amounts of CLA [11,13,32]. Khan et al. [33] found that *Lactiplantibacillus plantarum* efficiently converted LA to CLA in cheddar cheese.

Yahla et al. [34] found that the human-origin probiotic strain *L. rhamnosus* LbRE-LSAS and the well-known probiotic strain of *B. lactis* Bb12 could both produce CLA in vitro and in vivo. Both probiotic strains demonstrated anti-obesity activity in fat-induced Wistar rats receiving a high-calorie diet for 8 weeks. The results showed that other CLA isomers, in addition to the *10-trans,12-cis* CLA isomer, might be involved in this anti-obesity effect. Indeed, in the case of *L. rhamnosus* LbRE-LSAS, the yield of the *10-trans,12-cis* CLA isomer was five times higher than that recorded in vitro.

Likewise, the four probiotic strains used in the present study were previously reported to resist digestive tract conditions and to assimilate cholesterol [15].

### 3.3. Good Antibacterial Effect Against Virulent Bacteria

The mechanism of PUFAs' antibacterial action is not well understood. Furthermore, it has been demonstrated that these fatty acids could exhibit bactericidal activity against certain important pathogenic microorganisms, including methicillin-resistant *Staphylococcus aureus* [35], *Helicobacter pylori*, and mycobacteria [36,37].

Table 3a,b displays the results from the antibacterial activities of supernatants obtained from the MRS or fermented milk supplemented with 0.5 or 1 mg/mL LA. CLAs were released in the medium (the free form) from the probiotic conversion of LA and were tested against some virulent bacteria.

From the MRS medium with 0.5 or 1 mg/mL LA, bioactive substances in the *S. thermophilus* TA040 supernatant showed good antibacterial action, but only ( $p < 0.05$ ) against *S. typhimurium* with a diameter of 25 and 35 mm, respectively. The *L. rhamnosus* LbRE-LSAS strain's supernatant from the 1 mg/mL LA culture had the greatest ( $p < 0.05$ ) inhibitory zone against *E. coli*, measuring 45 mm in diameter. The conversion of LA in *B. lactis* Bb12 produced powerful substances against *S. aureus*. An inhibition halo with a diameter of 30 and 40 mm ( $p < 0.05$ ) was calculated when adding the supernatants from the 0.5 and 1 mg/mL LA cultures, respectively (Table 3a,b).

*L. bulgaricus* LB340 has no inhibitory action ( $p < 0.05$ ) against the three assessed pathogenic strains. However, its associated culture has less antibacterial activities compared to *S. thermophilus* TA040 monocultures.

Using whey from yogurt-type beverages containing 0.5 or 1 mg/mL LA (Table 3a,b), the *B. lactis* Bb12 culture revealed antagonistic activity against all the tested pathogenic strains, with inhibition diameters of ( $p > 0.05$ ) 8 and 10 mm against *S. typhimurium* and ( $p < 0.05$ ) 18 and 20 and 18 and 25 mm against *E. coli* and *S. aureus*, respectively. The *L. bulgaricus* LB40 culture produced antibacterial compounds against *S. typhimurium* and *S. aureus*. The diameters of the inhibition zones were 20–25 mm ( $p < 0.05$ ) and 10–12 mm ( $p > 0.05$ ), respectively, and were higher in the whey from the 1 mg/mL LA beverages than that from 0.5 mg/mL.

The whey from the *L. rhamnosus* LbRE-LSAS beverages demonstrated significant inhibitory zones ( $p < 0.05$ ) against *E. coli* (30 and 40 mm, from 0.5 and 1 mg/mL LA cultures, respectively) and moderately significant ( $p > 0.05$ ) against *S. aureus* (8 and 10 mm, from 0.5 and 1 mg/mL LA cultures, respectively).

**Table 3.** (a) Antibacterial activities of supernatants from 0.5 mg/mL LA-enriched MRS cultures. (b) Antibacterial activities of supernatants from 1 mg/mL LA-enriched MRS cultures.

(a)						
Zone of Inhibition (mm)						
Supernatants from LA Probiotic Cultures	<i>S. typhimurium</i>	<i>E. coli</i>	<i>S. aureus</i>	<i>S. typhimurium</i>	<i>E. coli</i>	<i>S. aureus</i>
	MRS			WHEY		
TA 040	25 ± 0.66	-	-	-	-	-
LB 340	-	-	-	20 ± 0.11	-	10 ± 0.66
TA040 + LB340	20 ± 0.05	-	-	18 ± 0.05	-	18 ± 0.02
LbRE-LSAS	-	40 ± 0.33	-	-	30 ± 0.66	8 ± 0.11
Bb12	-	-	30 ± 0.67	8 ± 0.08	18 ± 0.11	18 ± 0.02
(b)						
Zone of Inhibition (mm)						
Supernatants from LA Probiotic Cultures	<i>S. typhimurium</i>	<i>E. coli</i>	<i>S. aureus</i>	<i>S. typhimurium</i>	<i>E. coli</i>	<i>S. aureus</i>
	MRS			WHEY		
TA 040	35 ± 0.12	-	-	-	-	-
LB 340	-	-	-	25 ± 0.05	-	12 ± 0.15
TA040 + LB340	25 ± 0.33	-	-	20 ± 0.01	-	18 ± 0.87
LbRE-LSAS	-	45 ± 0.66	-	-	40 ± 0.11	10 ± 0.22
Bb12	-	-	40 ± 0.33	10 ± 0.66	20 ± 0.33	25 ± 0.67

*Streptococcus thermophilus* TA040, *Lactobacillus delbrueckii* ssp. *bulgaricus* LB340, *L. rhamnosus* LbRE-LSAS, and *B. animalis* subsp. *lactis* Bb 12. -: no antibacterial effect. The values are represented as the means ± SD in triplicates obtained from two independent experiments.

The *S. thermophilus* TA040 whey tested negative for all pathogenic strains, but whey from its associated culture had similar trends to *L. bulgaricus* LB40 (Table 3a,b).

Xia et al. [38] claimed that free fatty acids generally disturb the permeability of the cytoplasmic membrane in Gram-positive bacteria and that the conversion of these free fatty acids into CLA could serve as a detoxification mechanism for CLA producers.

The ability of beneficial bacteria to suppress the growth and proliferation of harmful germs has been identified as one of the probiotic properties. Antimicrobial activity can be multifactorial, involving the synthesis of bacteriocins, defensins, short-chain fatty acids, nitric oxide, and hydrogen peroxide [39].

In the present study, the pH effect was neutralized using NaOH. The CLA released might be involved in the observed antibacterial effect, since improved results were observed with cultures containing the double LA concentration versus those with 0.5 mg/mL. Nonetheless, the data in Table 3a,b could also be related to other potent compounds in addition to CLA.

To the best of our knowledge, there are fewer studies on CLA's antimicrobial effect. Most of the research has been focused on reporting the amount of CLA produced and the suitable conditions for better yields [33,40].

#### 4. Conclusions

The increased interest in fermented dairy products emanates from their health benefits. Using a probiotic strain with beneficial health attributes as a starter culture results in a

more nutritious and biologically beneficial product. The four strains used in this study, all from different species and origins, were able to grow in the presence of linoleic acid, and the biomass was above the level of 6 log CFU recommended for human use. Their LA bioconversion rates to CLA were noticeable in the milk beverage. Therefore, the present study could be regarded as an overview of some potent CLA-producing strains with superior antibacterial activities in order to be used for the development of functional yogurt-type beverages. Hence, milk fermentation seems to be a feasible alternative to increase the CLA content in dairy foods. This research also identified four potential probiotic candidates for use in the milk-product industries. Large-scale studies will be required to confirm these experimental findings.

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## Abbreviations

The following abbreviations are used in this manuscript:

CLA	Conjugated linoleic acid isomer
LA	Linoleic acid
LAB	Lactic acid bacteria
RA	Rumenic acid
PUFAs	Polyunsaturated fatty acids

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