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Effect of *Lactiplantibacillus plantarum* on the Conversion of Linoleic Acid of Vegetable Oil to Conjugated Linoleic Acid, Lipolysis, and Sensory Properties of Cheddar Cheese

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Abstract: Conjugated linoleic acid (CLA) is perceived to protect the body from metabolic diseases. This study was conducted to determine the effect of *Lactiplantibacillus plantarum* (*Lp. plantarum*) on CLA production and sensory characteristics of cheddar cheese. *Lp. plantarum* can convert linoleic acid (LA) to CLA. To increase CLA in cheddar cheese and monitor the conversion of LA to CLA by *Lp. plantarum*, the LA content of cheese milk (3.4% fat) was increased by partially replacing fat with safflower oil (85% LA of oil) at 0, 3, 6, and 9% concentrations (T₁, T₂, T₃, and T₄). Furthermore, *Lp. plantarum* 10⁸ colony-forming units (CFU)/mL (8 log CFU mL^{−1}) was added in all treatments along with traditional cheddar cheese culture (*Lactococcus lactis* ssp. *lactis* and *L. lactis* ssp. *cremoris*). After 30 days of ripening, *Lp. plantarum* in T₁, T₂, T₃, and T₄ was 6.75, 6.72, 6.65, and 6.55 log CFU g^{−1}. After 60 days of ripening, *Lp. plantarum* in T₁, T₂, T₃, and T₄ was 6.35, 6.27, 6.19, and 6.32 log CFU g^{−1}. After 60 days of ripening, *Lp. plantarum* in T₁, T₂, T₃, and T₄ was 6.41, 6.25, 6.69, and 6.65 log CFU g^{−1}. GC-MS analysis showed that concentrations of CLA in the 90 days' control, T₁, T₂, T₃, and T₄ were 1.18, 2.73, 4.44, 6.24, and 9.57 mg/100 g, respectively. HPLC analysis revealed that treatments containing *Lp. plantarum* and LA presented higher concentrations of organic acids than the control sample. The addition of safflower oil at all concentrations did not affect cheese composition, free fatty acids (FFA), and the peroxide value (POV) of cheddar cheese. Color flavor and texture scores of experimental cheeses were not different from the control cheese. It was concluded that *Lp. plantarum* and safflower oil can be used to increase CLA production in cheddar cheese.

Keywords: cheese; *Lp. plantarum*; conjugated linoleic acid; lipolysis

1. Introduction

The globalization of functional foods has brought remarkable changes in dietary patterns, especially in developing countries. Changes in dietary patterns have been shown to be an important underlying factor for the increasing prevalence of chronic disorders and metabolic diseases. Therefore, increasing knowledge about the nutritional and health issues associated with the consumption of traditional foods has led researchers and consumers to become increasingly interested in the development of functional foods [1]. Conjugated linoleic acid (CLA)-supplemented foods have attracted great interest from consumers because of their anticarcinogenic, anti-atherogenic, antidiabetic, anti-inflammatory, and anti-obesity properties [2]. CLA occurs naturally in ruminant food products (beef, lamb, and dairy) due to bacterial biohydrogenation of linoleic acid in the rumen [3]. It is recommended to intake 2 g CLA/day, but the CLA content of dairy products is approximately 0.5 g/g of fat [2]. To achieve the nutritional need of CLA, a significant portion of dairy products and meat must be included in the daily diet, which may lead to excessive fat intake leading to several other health-related issues. It is possible to increase CLA in milk and dairy products by altering feeding practices; CLA in milk can be increased by adding vegetable oils, grazing on lush green pastures, and oilseeds. However, this is not a practical solution to the problem, since every farm has a different feeding strategy and dairy industries collect milk from thousands of farmers which is blended during the collection; the amount of CLA in milk is also affected by the season, with winter milk having more CLA than summer milk [4,5].

CLA can be synthetically produced from vegetable oils. In a recent study, vegetable oils were used as substrates to produce CLA from linoleic acid (LA) by urea treatment. It has been found that the change of LA to CLA is dependent on the concentration of LA. Safflower oil had more than 80% LA and yielded 31% CLA [6].

Conversion of linoleic acid to CLA through microbiological processes has been reported in the literature [7]; the addition of 0.02% LA to fermentation media considerably increased the production of CLA [8]. Among 14 genera of lactic acid bacteria (LAB), *Lactiplantibacillus plantarum* (*Lp. plantarum*) was found to be the highest converter of LA to CLA [9]. Certain strains of *Lb. acidophilus* converted LA to CLA [10]. Other researchers reported that certain strains of food fermenting bacteria, such as *Propionibacterium freudenreichii* ssp. *Freudenreichii*, *Propionibacterium freudenreichii* spp. *Shermanii*, and *Lp. plantarum*, can convert LA to CLA [11]. Strains of *Lp. plantarum* are widely used to accelerate the ripening process of certain varieties of cheese as they possess potent groups of enzymes to initiate several metabolic processes [12]. Guidone et al. [13] reported that LA can be converted to CLA by chemical and microbiological methods. However, the magnitude of conversion of LA to CLA by *Lp. plantarum* in cheese matrix has not been studied in detail.

According to the above-mentioned facts, it is evident that CLA production in food matrixes depends largely on the concentration of LA in the substrates. The LA content of cheddar cheese is approximately 2–2.5%. With this lower level of LA, it is practically impossible to raise CLA content by *Lp. plantarum* to the extent that can fulfill the recommended dietary allowance of CLA. In view of the current situation of metabolic diseases, it is technically challenging for food scientists to develop sustainable CLA-enriched foods using the mixed milk of hundreds from farms using different feed and managerial practices.

Safflower is produced in several countries of the world; it has already been used for a large number of food and medicinal applications. In comparison to all dietary lipids, it possesses the highest concentration of LA 85% fatty acid [14]. Safflower oil has a pale yellow color with an almost bland flavor and its suitability as a substrate for the conversion of LA to CLA in cheddar cheese needs to be explored. The literature supports the impression

of using cheese as a potential vector for converting linoleic acid to CLA due to solid matrix, buffering capacity, and fat content. During the ripening of cheddar cheese, lactose metabolism, proteolysis, breakdown of lipids, production of free fatty acids (FFA), and flavoring compounds production [15]. The fatty acid composition of cheese before and after ripening was considerably different from each other [16]. CLA-enriched cheddar cheese may undergo lipid oxidation during the ripening phase; therefore, it is necessary to monitor lipid oxidation in CLA-enriched cheddar cheese. The use of safflower oil as a source of LA and *Lp. plantarum* as a microbiological converter of LA to CLA in cheese matrix has not been reported in the literature so far. The effect of four different concentrations of safflower oil and *Lp. plantarum* on the conversion of LA to CLA, fatty acids composition, organic acids, and lipolysis were determined with the latest analytical facilities.

2. Materials and Methods

2.1. Materials

Cheese starter culture, *L. lactis* ssp. *lactis* and *L. lactis* ssp. *cremoris* (DVS-RST-900), and *Lactiplantibacillus plantarum* “(*Lp. plantarum*)” (UALp-05) were purchased from Christian Hansen (Horsholm, Denmark). For analytical work, HPLC-grade chemicals were used (Sigma Aldrich, St. Louis, MO, USA). Safflower oil was obtained by mechanical expression of 20–25 °C; cold-pressed safflower oil was used for this study.

2.2. Cheddar Cheese Production and Experimental Plan

Milk (100 L for every treatment in replicate) from Holstein–Friesian cows having 3.4% fat and 3.2% protein content was pasteurized at 65 °C (30 min, batch pasteurization) and cooled to 31 °C. Cheese starter culture, *Lactococcus lactis* ssp. *lactis* and *Lactococcus lactis* ssp. *cremoris* (DVS culture @10 U/100 L milk), and *Lp. plantarum* and safflower oil were added as an experimental plan. Pre-acidification was conducted for 45 min (pH 6.4), and 17 mL calcium chloride (35%) and rennet 0.02% were mixed and left for 30 min for curd formation. Curd was then cut into 1.5–2 cm cubes, the temperature of the vat was increased to 39 °C in 45 min, and cooked at this temperature for 40 min. Whey was then drained, cheddaring was conducted till pH 5.2, followed by milling, salting (1.5%), pressing for 16 h (3.5 bar), and vacuum packaging. T1 comprised 100% milk fat and a (mixed/co-cultured) starter culture of *L. lactis* ssp. *lactis* and *L. lactis* ssp. *cremoris* and *Lp. plantarum* 10⁸ CfU/mL. T2 comprised 97% milk fat, 3% safflower oil, and a starter culture of *L. lactis* ssp. *lactis* and *L. lactis* ssp. *cremoris* and *Lp. plantarum* 10⁸ CfU/mL. T3 comprised 94% milk fat, 6% safflower oil, and a starter culture of *L. lactis* ssp. *lactis* and *L. lactis* ssp. *cremoris* and *Lp. plantarum* 10⁸ CfU/mL. T4 comprised 91% milk fat, 9% safflower oil, and a starter culture of *L. lactis* ssp. *lactis* and *L. lactis* ssp. *cremoris* and *Lp. plantarum* 10⁸ CfU/mL. The Control comprised 100% milk fat and a starter culture of *L. lactis* ssp. *lactis* and *L. lactis* ssp. *cremoris*. All kinds of cheeses were produced according to the standard protocol of cheddar cheese production and were ripened at 6–8 °C for the duration of 90 days and analyzed at pre-set intervals of 0, 45, and 90 days [14].

2.3. Analysis of Safflower Oil

Cold-extracted safflower oil was tested only for moisture, FFA, peroxide value (POV), iodine value, saponification value, and unsaponifiable matter by the standard protocols [17,18].

2.4. Milk and Cheese Composition

Fat, protein, solids-not-fat, total solids, pH, acidity, and mineral contents in cheese milk were determined by wet chemistry [19]. Moisture, fat, protein, and the pH of cheese were measured at 0, 45, and 90 days of ripening. Free fatty acids (FFA), color, moisture, peroxide value, refractive index, iodine value, and unsaponifiable matter were also tested [18].

2.4.1. CLA Isomers and Other Fatty Acids

The fatty acid composition was analyzed at 0 and 90 days of this study on a GC-MS (7890-B, Agilent Technologies, Santa Clara, CA, USA) using an SP-2560 column (100 m × 0.25 mm id) and FID. For ester preparation, a 50 mg sample was reacted with 2 mL methanolic HCl in C₂H₅OH (15%) at 100 °C/60 min in a heating block, then cooled to 20–25 °C followed by the addition of 2 mL each *n*-hexane (99.99%) and deionized H₂O. Test tubes were then mixed for 1 min; after 15 min, the upper layer was extracted and dried over Na₂SO₄ and put in GC-vials for injection (1 µL) by ALS at 1:50 split ratio. The temperature of the inlet and FID were 250 °C and He, O₂, and H₂ were flowing at 2, 4, and 40 mL/min. The total run time was 52 min. For comparison and quantification, FAME-37 (Supelco, St. Louis, MO, USA) standard was used, and for CLA isomers, SLB-IL111, and *cis*, the 11-*trans*-octadecadienoic acid solution was used [20].

2.4.2. Population of *Lactiplantibacillus plantarum* (*Lp. plantarum*)

Lp. plantarum was determined after inoculation, pre-acidification, cooking, cheddaring, and 30, 60, and 90 days of maturation. *Lp. plantarum* was determined by the serial dilution of the sample in 0.85% solution of sodium chloride in MRS agar with vancomycin (20 mg/L, pH 5.6). Samples were incubated at 30 °C for 48 h, and colonies were counted and calculated as CfU/mL. *Lp. plantarum* was determined after inoculation, pre-acidification, cooking, 156 cheddaring, and 30, 60, and 90 days of maturation [21].

2.4.3. Organic Acids

In cheese samples, acetic acid (AA), butyric acid (BA), citric acid (CA), and lactic acid (LCA) were quantified by HPLC with Varian 9012 delivery system, auto-sampler, UV detector, Aminex-HPX-87H column (300 mm × 7.8 mm). Cheese samples of 5 g were blended with sulfuric acid (H₂SO₄) and nitric acid (HNO₃, 25 mL, and 70 µL) and homogenized at 10,000 rpm. Furthermore, the fat layer was discarded and the central layer was extracted followed by heating at 50 °C/60 min, then centrifuged at 10,000 × g, filtered, and transferred to HPLC vials. The mobile phase comprised 0.009 N H₂SO₄ and organic acids were measured at 220 nm [22].

2.4.4. Lipolysis

Lipolysis in cheese samples was tested by analyzing the FFA, cholesterol, and peroxide value (POV) at 0, 45, and 90 days of refrigerated storage using standard methods [23].

2.5. Sensory Evaluation

Descriptive sensory analysis of CLA-enriched cheddar cheese samples was performed by 10 trained judges. For the pre-drafting of terminology (color, flavor, and texture), four sittings (each 2 h) were conducted. The color, flavor, and texture of cheddar cheese samples were analyzed in a well-ventilated and well-lit sensory evaluation laboratory at 20–25 °C using a nine-point scale and data were analyzed with FIZZ software (“FIZZ sensory software version 2.47B, Biosystèmes, Courtenon, France”). For palate cleansing, unsalted crisp bread was provided in individual sensory evaluation booths [24]. For sensorial testing, all applicable regulations were followed and informed consent was signed by all participants in the test.

2.6. Statistical Analysis

The experiment was designed in a completely randomized design and data were analyzed by two-way ANOVA to determine the impact of treatments. For the determination of significant differences among the means, Duncan’s multiple range (DMR) was applied using SAS 9.4 software at a 0.05 level of significance.

3. Results and Discussion

Free fatty acids (FFA) (0.14%), moisture content (0.19%), peroxide value (POV) (0.25 MeqO₂/kg), iodine value (141.5 cg/100 g), unsaponifiable matter (1.34%), saponification value (191.7 mg KOH/g), and refractive index (1.4745) were found in cold extracted safflower oil. Fat, protein, lactose, mineral, solids-not-fat, total solids contents, pH, and acidity in cow milk were 3.4%, 3.21%, 4.63%, 0.72%, 8.61, 11.96%, 6.67, and 0.14% (lactic acid). The results of the chemical characteristics of safflower oil are not different from the literature of Milesi et al. [25]. The addition of *Lactiplantibacillus plantarum* (*Lp. plantarum*) and safflower oil from 3 to 9% concentrations did not affect cheddar cheese composition (Table 1). Cheddar cheese was prepared using *Lp. plantarum* as an adjunct starter culture, with fat, protein, and moisture contents similar to standard cheddar cheese [12]. In another similar research work, starter cultures did not affect the chemical composition of all dry-salted cheeses over 4 months of ripening [26]. *Lp. plantarum* did not affect the chemical composition of cheddar cheeses [27].

Table 1. Composition of cheddar cheese.

Attribute	Control	T ₁	T ₂	T ₃	T ₄
Moisture%	40.52 ± 1.03 ^a	41.22 ± 1.05 ^a	40.16 ± 1.02 ^a	41.11 ± 1.06 ^a	40.85 ± 1.02 ^a
Fat%	30.47 ± 0.9 ^a	29.91 ± 0.9 ^a	30.17 ± 0.9 ^a	30.37 ± 0.9 ^a	29.78 ± 0.9 ^a
Protein%	26.19 ± 0.7 ^a	26.37 ± 0.7 ^a	26.14 ± 0.7 ^a	26.65 ± 0.7 ^a	26.88 ± 0.7 ^a
pH	5.24 ± 0.2 ^a	5.22 ± 0.2 ^a	5.21 ± 0.2 ^a	5.23 ± 0.2 ^a	5.22 ± 0.2 ^a

^a All means mentioned in the rows of moisture, fat, protein, and pH showed a non-significant ($p > 0.05$) variation.

Murtaza et al. reported that the addition of adjunct starter cultures did not affect the moisture, fat, protein, and pH of cheese [28]. The chemical composition of cheddar cheese prepared from *Lactobacillus acidophilus* and *Bifidobacterium bifidum* cultures was not different from standard cheese. In a relevant study, chia oil was added to cheddar cheese in 2.5–10% concentrations, and fat, protein, and moisture contents were similar to the standard cheddar cheese ($p > 0.05$) [29]. Ahmed et al. [30] prepared Gouda cheese with mango kernel oil, and researchers reported that up to a 10% level, the composition of Gouda cheese was not influenced. In a similar investigation, when low melting point fractions of milk were transformed to cheddar cheese, it was found that the addition of low melting point fractions did not alter the chemical composition of cheddar cheese [31].

3.1. Population of *Lactiplantibacillus plantarum* (*Lp. plantarum*)

The population of *Lp. plantarum* was determined at different stages of cheese production and ripening (Figure 1). *Lp. plantarum* was not affected by the three different concentrations of safflower oil, the processes of cheese production, and the ripening period. The results of the population of *Lp. plantarum* are highly significant from technological and industrial adaptation viewpoints, as no additional technique for safeguarding the bacterial strain is required. Moreover, cheese industries can easily adopt the technology of CLA-enriched cheddar cheese, as no alteration in the cheese production process and additional processing or packaging equipment is required. The population of *Lp. plantarum* in cheddar cheese has been reported; however, its population in cheese in the presence of safflower oil (as a source of linoleic acid) has not been previously studied.

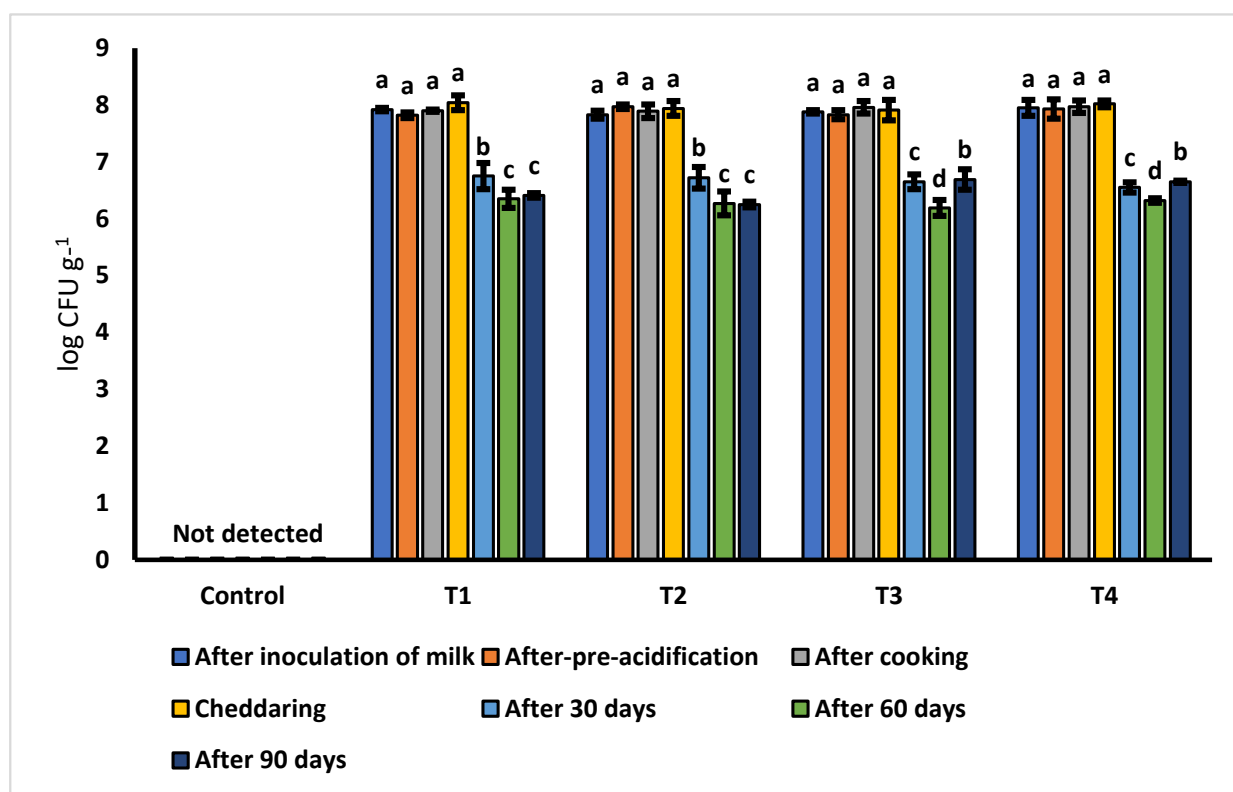


Figure 1. Survival of *Lp. plantarum* (log CFU g⁻¹) in ripening phase.

In a previous investigation, probiotic cheddar cheese was produced using *Lp. plantarum* in combination with traditional starter culture, and *Lp. plantarum* survived the cheese production and ripening phase with no decline in population till 12 weeks of storage [26]. In another study, the populations of *L. acidophilus* and *Lactobacillus* increased during the ripening period [32]. In a previous study, the populations of *Bifidobacterium lactis* B 94 and *L. casei* 01 were analyzed in cheese during the 60 days of ripening. In the first 15–30 days, the populations of both bacteria increased by two log cycles; however, their populations remained almost constant till 60 days of ripening [33]. Cheddar cheese was enriched with *Bifidobacterium* and *Lactobacillus* and the bacterial population was monitored till 10 weeks of ripening. It was found that the populations of both strains ranged from 10⁶ to 10⁷/g after the end of the ripening phase [34].

3.2. Fatty Acid Composition

In the current investigation effect of *Lactiplantibacillus plantarum* (*Lp. plantarum*), it was determined on the conversion of LA to CLA using safflower oil as substrate (Table 2). It was recorded that *Lp. plantarum* efficiently converted LA of milk (T₁) and safflower oil (T₂, T₃, and T₄) to CLA isomers. However, conversion was largely dependent upon the availability of LA in the reaction substrate. The LA content of safflower oil was 85.19%, which was the main reason for the rise of CLA content in T₂, T₃, and T₄. During the ripening of 90 days, concentrations of CLA in all the treatments and the control significantly increased; however, the rise in CLA was dependent upon the extent of LA in the substrate, and treatments having more linoleic acid underwent more CLA production.

Table 2. Fatty acids composition of cheddar cheese added with *Lp. plantarum* (mg/100 g).

Fatty Acid	Safflower Oil	Control		T ₁		T ₂		T ₃		T ₄	
		0-Day	90-Days	0-Day	90-Days	0-Day	90-Days	0-Day	90-Days	0-Day	90-Days
C4:0	Not Found	1.89 ± 0.01 a	1.90 ± 0.01 a	1.85 ± 0.02 a	1.80 ± 0.01 a	1.79 ± 0.03 a	1.75 ± 0.02 a	1.77 ± 0.01 a	1.70 ± 0.02 b	1.78 ± 0.02 a	1.72 ± 0.01 b
C6:0	Not Found	2.36 ± 0.03 a	2.32 ± 0.06 a	2.34 ± 0.04 a	2.28 ± 0.05 a	2.26 ± 0.01 a	2.23 ± 0.01 a	2.25 ± 0.04 a	2.13 ± 0.01 a	2.21 ± 0.01 a	2.10 ± 0.01 b
C8:0	Not Found	2.51 ± 0.06 a	2.49 ± 0.05 a	2.48 ± 0.03 a	2.42 ± 0.07 a	2.40 ± 0.04 a	2.25 ± 0.03 b	2.37 ± 0.02 a	2.23 ± 0.02 b	2.31 ± 0.03 a	2.18 ± 0.06 b
C10:0	Not Found	2.77 ± 0.10 a	2.74 ± 0.10 a	2.75 ± 0.07 a	2.70 ± 0.09 a	2.67 ± 0.02 a	2.62 ± 0.02 b	2.75 ± 0.04 a	2.61 ± 0.07 b	2.70 ± 0.04 a	2.57 ± 0.02 c
C12:0	Not Found	2.94 ± 0.12 a	2.93 ± 0.13 a	2.93 ± 0.11 a	2.87 ± 0.02 a	2.86 ± 0.22 a	2.74 ± 0.06 b	2.83 ± 0.06 a	2.70 ± 0.08 b	2.77 ± 0.03 a	2.53 ± 0.07 c
C14:0	0.11 ± 0.01 f	11.25 ± 0.23 a	11.13 ± 0.02 a	10.65 ± 0.16 b	10.49 ± 0.24 b	10.33 ± 0.17 b	9.98 ± 0.42 b	9.14 ± 0.16 c	8.55 ± 0.02 d	8.51 ± 0.20 d	7.66 ± 0.11 e
C16:0	1.22 ± 0.02 h	26.74 ± 0.29 a	26.25 ± 0.74 a	25.16 ± 0.33 b	24.75 ± 0.45 c	24.37 ± 0.31 c	23.38 ± 0.51 d	22.74 ± 0.31 e	21.27 ± 0.52 f	21.20 ± 0.08 f	18.41 ± 0.28 g
C18:0	4.74 ± 0.15 f	8.19 ± 0.15 a	8.13 ± 0.20 a	7.81 ± 0.17 b	7.44 ± 0.35 b	7.16 ± 0.10 c	6.62 ± 0.09 d	6.11 ± 0.09 d	5.43 ± 0.19 e	5.35 ± 0.13 e	4.11 ± 0.16 g
C18:1	6.89 ± 0.13 h	23.94 ± 0.34 a	21.17 ± 0.16 c	23.51 ± 0.54 a	22.98 ± 0.77 b	22.36 ± 0.29 b	21.87 ± 0.39 c	20.19 ± 0.37 d	19.13 ± 0.22 e	18.98 ± 0.09 f	16.14 ± 0.18 g
C18:2	85.19 ± 0.22 a	2.56 ± 0.21 e	1.87 ± 0.16 g	2.52 ± 0.09 e	2.18 ± 0.04 f	5.98 ± 0.05 d	1.34 ± 0.42 h	8.25 ± 0.24 c	0.98 ± 0.17 j	11.49 ± 0.12 b	1.11 ± 0.02 i
Δ9c,11t-18:2	Not Found	0.23 ± 0.09 f	0.37 ± 0.02 e	0.22 ± 0.03 f	0.58 ± 0.10 d	0.22 ± 0.02 f	1.12 ± 0.03 c	0.21 ± 0.01 f	1.34 ± 0.04 b	0.26 ± 0.04 f	1.91 ± 0.03 a
Δ10t,12c-18:2	Not Found	0.10 ± 0.06 f	0.16 ± 0.01 e	0.11 ± 0.01 f	0.42 ± 0.02 d	0.08 ± 0.01 f	0.79 ± 0.15 c	0.07 ± 0.01 f	1.12 ± 0.02 b	0.12 ± 0.02 f	1.54 ± 0.06 a
Δ9c,11c-18:2	Not Found	0.09 ± 0.02 f	0.14 ± 0.03 e	0.08 ± 0.01 f	0.47 ± 0.05 d	0.07 ± 0.01 f	0.91 ± 0.01 c	0.09 ± 0.02 f	1.05 ± 0.01 b	0.10 ± 0.03 f	1.48 ± 0.02 a
Δ9t, 11c-18:2	Not Found	0.05 ± 0.01 f	0.09 ± 0.01 e	0.06 ± 0.01 f	0.29 ± 0.06 d	0.06 ± 0.01 f	0.48 ± 0.05 c	0.11 ± 0.01 e	0.92 ± 0.02 b	0.15 ± 0.01 e	1.39 ± 0.1 a
Δ10c,12t-18:2	Not Found	0.08 ± 0.02 f	0.17 ± 0.01 e	0.19 ± 0.02 e	0.31 ± 0.02 d	0.05 ± 0.01 g	0.35 ± 0.03 c	0.09 ± 0.01 f	0.68 ± 0.03 b	0.10 ± 0.05 f	1.18 ± 0.01 a
Δ8,9,11,10,12c-c18:2	Not Found	0.03 ± 0.01 f	0.05 ± 0.02 f	0.06 ± 0.02 f	0.15 ± 0.01 d	0.04 ± 0.01 f	0.27 ± 0.02 c	0.05 ± 0.01	0.51 ± 0.01 b	0.09 ± 0.06 e	1.10 ± 0.02 a
Δ8,9,11,10,12t-t18:2	Not Found	0.04 ± 0.01 f	0.07 ± 0.01 e	0.08 ± 0.01 e	0.12 ± 0.01 d	0.05 ± 0.01 f	0.21 ± 0.01 c	0.07 ± 0.02 e	0.38 ± 0.01 b	0.13 ± 0.02 d	0.82 ± 0.04 a
C18:3	0.25 ± 0.02	0.58 ± 0.02 a	0.41 ± 0.01 c	0.56 ± 0.1 a	0.39 ± 0.03 c	0.53 ± 0.03 a	0.31 ± 0.02 d	0.49 ± 0.04 b	0.24 ± 0.02 e	0.25 ± 0.01 e	0.15 ± 0.01 f
Σ CLA	-	1.18 ± 0.02 f	1.46 ± 0.04 e	1.36 ± 0.09 e	2.73 ± 0.03 d	1.10 ± 0.01 f	4.44 ± 0.11 c	1.18 ± 0.06 f	6.24 ± 0.15 b	1.20 ± 0.03 f	9.57 ± 0.19 a

In rows of this table, if means are bearing a different letter, it reveals a statistically significant variation ($p < 0.05$).

GC-MS analysis showed that after the end of ripening (90-Days), the content of CLA in the control, T₁, T₂, T₃, and T₄ was 1.46, 2.18, 4.44, 6.24, and 9.75 mg/100 g ($p < 0.05$), respectively. Due to the conversion of LA to CLA during the ripening phase, concentrations of LA in 90-Days T₁, T₂, T₃, and T₄ were considerably lower than the values recorded at 0-Day ($p < 0.05$). At the end of ripening, concentrations of LA in the control, T₁, T₂, T₃, and T₄ were 1.87, 2.18, 1.34, 0.98, and 1.11 mg/100 g only, respectively. Before ripening, the LA content of the control, T₁, T₂, T₃, and T₄ were 2.56, 5.52, 5.98, 8.25, and 11.49 mg/100 g, respectively. Conversion of linoleic acid to CLA was largely dependent upon the concentration of linoleic acid in the substrate. The fatty acid profile of fermented milk and probiotic cheese was used as an indication of lipolysis and flavor production [35,36]. In the control (at 0-Day), concentrations of $\Delta 9c,11t-18:2$, $\Delta 10t,12c-18:2$, $\Delta 9c,11c-18:2$, $\Delta 9t,11c-18:2$, $\Delta 10c,12t-18:2$, $\Delta 8,9,11,10,12c-c18:2$, and $\Delta 8,9,11,10,12t-t18:2$ were 0.23, 0.10, 0.09, 0.05, 0.08, 0.03, and 0.04 mg/100g, respectively. In the control (after 90-Days), concentrations of $\Delta 9c,11t-18:2$, $\Delta 10t,12c-18:2$, $\Delta 9c,11c-18:2$, $\Delta 9t,11c-18:2$, $\Delta 10c,12t-18:2$, $\Delta 8,9,11,10,12c-c18:2$, and $\Delta 8,9,11,10,12t-t18:2$ were 0.37, 0.16, 0.14, 0.09, 0.17, 0.05, and 0.07 mg/100g, respectively. In T₄ (at 0-Day), concentrations of $\Delta 9c,11t-18:2$, $\Delta 10t,12c-18:2$, $\Delta 9c,11c-18:2$, $\Delta 9t,11c-18:2$, $\Delta 10c,12t-18:2$, $\Delta 8,9,11,10,12c-c18:2$, and $\Delta 8,9,11,10,12t-t18:2$ were 0.26, 0.12, 0.10, 0.15, 0.16, 0.09, and 0.13 mg/100g, respectively. In T₄ (after 90-Days), concentrations of $\Delta 9c,11t-18:2$, $\Delta 10t,12c-18:2$, $\Delta 9c,11c-18:2$, $\Delta 9t,11c-18:2$, $\Delta 10c,12t-18:2$, $\Delta 8,9,11,10,12c-c18:2$, and $\Delta 8,9,11,10,12t-t18:2$ were 1.91, 1.54, 1.48, 1.39, 1.18, 1.10, and 0.82 mg/100 g, respectively.

Cheese produced using *Bifidobacterium lactis* and *Lactobacillus casei* was analyzed for CLA content for 60 days of ripening, and after 15 days, *cis*-9, *trans*-11-C18:2, CLA; *trans*-10, *cis*-12-C18:2, CLA; and *trans*-9, *trans*-12-C18:2, CLA were found, and all the determination intervals till 60 days of ripening showed an increasing trend (1.8–7.9 times higher) [37]. Phillips et al. [33] produced cheese by blending cow and buffalo milk using *Lactobacillus casei* and *Lactobacillus acidophilus* to adjudge the production of CLA during the ripening phase of 90 days. The results revealed that concentrations of CLA increased during the whole ripening phase, and the rise in CLA was due to the conversion of LA to CLA. The literature suggests that certain bacteria have the ability to efficiently produce CLA from LA. In the current investigation, it was established that *Lp. plantarum* has the ability to convert LA to CLA. Ahmed et al. [30] reported that increasing LA in Gouda cheese considerably increased the production of CLA in mature cheese.

3.3. Organic Acids

The production of organic acids in cheese ripening has driven a considerable volume of research, which has found that the sensorial and nutritional characteristics of ripened cheese largely depend upon lipolysis, and the metabolic activities of bacterial cultures are also estimated by the extent of organic acids produced [38].

In the current study, concentrations of BA, CA, AA, and LCA in the control and experimental samples increased during the entire ripening phase of 90 days but to different extents (Figure 2). During cheese ripening, the production of organic acids was dependent upon the concentration of LCA present in cheese matrix and *Lactiplantibacillus plantarum* (*Lp. plantarum*). The control sample had 2.56% LA of milk fat own origin with no addition of *Lp. plantarum*, and analysis intervals of 0, 45, and 90 days revealed significantly lesser values of BA, CA, AA, and LCA than all experimental samples. At the end of the ripening phase (90 days), the concentrations of BA, CA, AA, and LA in the control were 1076, 494, 417, and 16,118 ppm, respectively ($p < 0.05$). T₁ showed significantly higher concentrations of BA, CA, AA, and LCA than the control at all three determination intervals. T₁ had 2.52% LCA and 10^8 /g *Lp. plantarum*, and the concentrations of BA, CA, AA, and LA after 90 days of ripening were 1472, 1220, 481, and 16,891 ppm, respectively. A similar trend was observed after 60 days of analysis of organic acids. HPLC analysis showed that treatments that had higher extents of LA in the presence of *Lp. plantarum* yielded more BA, CA, AA, and LA ($p < 0.05$) after 45 and 90 days of the ripening phase. After 90 days of ripening, the

amounts of BA in T₂, T₃, and T₄ were 1792, 2875, and 3015 ppm ($p < 0.05$), respectively. At the end of the ripening phase (90 days), the amounts of CA in T₂, T₃, and T₄ were 1475, 1579, and 1743 ppm ($p < 0.05$), respectively. At the end of the ripening phase (90 days), the amounts of AA in T₂, T₃, and T₄ were 537, 849, and 1375 ppm ($p < 0.05$), respectively.

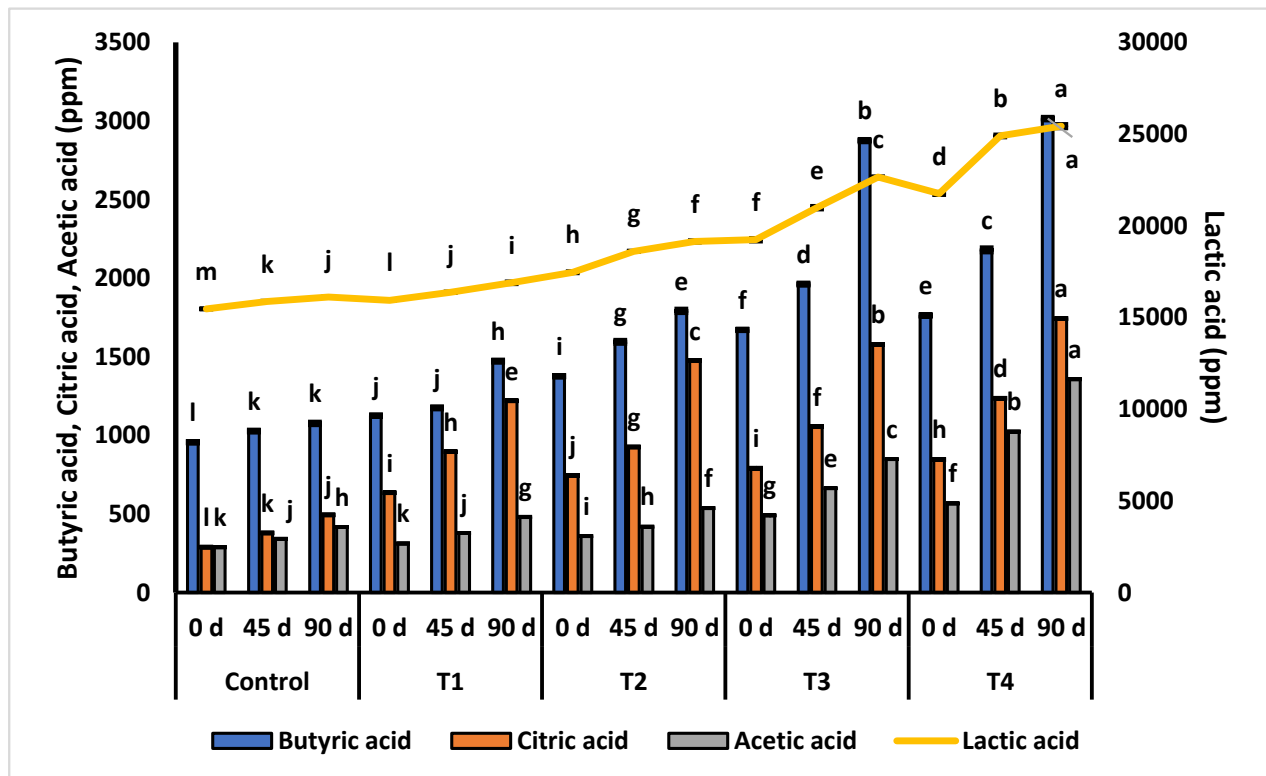


Figure 2. Effect of *Lp. plantarum* and safflower oil on the organic acids of cheddar cheese.

Lp. plantarum is capable of producing organic acids in LA-enriched cheddar cheese. *Lp. plantarum* is a technologically significant lactic acid bacterium that is highly prevalent in fermented foods of vegetable and animal origins, and it can tolerate low pH values and relatively high NaCl contents [39]. In cheese, acetic acid contributes to the development of flavor, and microbial activities lead to the production of acetic acid from lactose and citrate. Heterofermentative lactobacilli can also produce citric acid from lactose and amino acid [40]. Bacterial activities, fat hydrolysis, and added acids may lead to the generation of organic acids throughout ripening. A mechanism for the generation of organic acids in dairy products has been defined at a symposium on flavor in dairy foods and meat: oxidation converts LA to hydroperoxides which are transformed into ketones, aldehydes, and organic acids [37]. Ullah et al. [29] produced cheddar cheese using traditional cheddar, chia oil, and milk fat blends; the effect was estimated on the production of organic acids till 120 days. From the results, it was concluded that cheese samples having chia oil had higher amounts of organic acids than cheese produced from 100% milk fat.

3.4. Lipolysis

In cheese matrix, the hydrolysis of fat can take place due to several reasons, such as the presence of indigenous and bacterial lipases, moisture contents, metal ions, etc., and it leads to the liberation of fatty acids from triglyceride molecules—and these are then referred to as FFA. In almost all ripened cheeses, hydrolysis takes place; however, the degree of lipolysis may vary from one variety of cheese to another. In certain hard Italian varieties, excessive lipolysis takes place and higher amounts of FFA are produced [41]. FFAs play an imperative part in the expansion of flavors in ripened cheese. In this research study, the FFA content of the freshly prepared control and CLA-enriched cheddar cheese was not different

($p > 0.05$) (Table 3). At the end of ripening, the highest estimated FFA content was in T₄ followed by T₃ and T₂, which indicated that *Lactiplantibacillus plantarum* (*Lp. plantarum*) did not play any significant role in the hydrolysis of fat molecules. The production of FFA was mainly dependent upon the ripening period and the presence of safflower oil from 3 to 9% (used as a source of LA). The ripening period and concentration of safflower oil played their roles in the production of FFA: 90-day-old samples had more FFA than 45-day-old samples. Similarly, cheese samples having more safflower oil generated more FFA during the ripening period of 90 days. Higher FFA in cheddar cheese containing vegetable oil is supported by the literature.

Table 3. Effect of *Lp. plantarum* and safflower oil on lipolysis of cheddar cheese.

Treatments	Ripening Days	FFA%	Cholesterol (mg/100 g)	POV (MeqO ₂ /kg)
Control	0	0.08 ± 0.01 ^h	165 ± 1.27 ^a	0.24 ± 0.02 ^e
	45	0.13 ± 0.02 ^g	155 ± 1.14 ^b	0.25 ± 0.01 ^e
	90	0.17 ± 0.01 ^f	121 ± 0.87 ^c	0.45 ± 0.05 ^d
T ₁	0	0.08 ± 0.02 ^h	161 ± 1.26 ^a	0.25 ± 0.03 ^e
	45	0.14 ± 0.03 ^f	144 ± 1.49 ^b	0.28 ± 0.02 ^e
	90	0.21 ± 0.01 ^d	119 ± 0.45 ^c	0.47 ± 0.06 ^d
T ₂	0	0.09 ± 0.01 ^h	158 ± 0.88 ^b	0.22 ± 0.02 ^e
	45	0.19 ± 0.04 ^e	139 ± 0.78 ^d	0.30 ± 0.05 ^e
	90	0.25 ± 0.02 ^c	122 ± 0.26 ^e	0.64 ± 0.07 ^c
T ₃	0	0.08 ± 0.01 ^h	155 ± 0.96 ^b	0.26 ± 0.04 ^e
	45	0.22 ± 0.01 ^d	125 ± 1.32 ^e	0.29 ± 0.02 ^e
	90	0.29 ± 0.02 ^b	114 ± 0.66 ^f	0.72 ± 0.01 ^b
T ₄	0	0.08 ± 0.01 ^h	152 ± 1.19 ^b	0.23 ± 0.01 ^e
	45	0.26 ± 0.02 ^c	121 ± 1.55 ^e	0.31 ± 0.02 ^e
	90	0.37 ± 0.01 ^a	78 ± 1.31 ^g	0.85 ± 0.03 ^a

In columns of this table, if means are bearing a different letter, it reveals a statistically significant variation ($p < 0.05$).

In the present study, a change in the amount of cholesterol was used as an indication of lipolysis. The addition of safflower oil and ripening both affected the cholesterol content of cheese samples. At 0-Days, the lowest cholesterol was found in T₄, followed by T₃ and T₂, while T₁ and the control almost had the same level of cholesterol. During ripening, a decline in cholesterol was dependent upon the ripening period, and non-significant differences in the cholesterol content of the control and T₁ showed that *Lp. plantarum* did not affect cholesterol concentration during the ripening phase of cheese.

Dairy products enriched with unsaturated fatty acids are more exposed to lipid oxidation. Therefore, in this investigation, the POV of all types of cheese samples were analyzed during the whole ripening period at pre-set intervals. Several methods of lipid oxidation have been discovered, such as the induction period by Rancimat; however, accelerated methods of lipid oxidation are not traditionally used for dairy products as these methods were established to determine the oxidative stability of plant-based oils. The peroxide value of the control and CLA-enriched cheddar samples increased in the ripening phase. The POV of all types of cheese samples was within the permissible limits of the EU. Oxidative stability (induction period) of CLA isomers was in the order of t,t -CLA > c,t -CLA > c,c -CLA [12].

3.5. Sensory Evaluation

The color, flavor, and texture of the control, T₁, T₂, T₃, and T₄ were not significantly different from each other. *Lactiplantibacillus plantarum* (*Lp. plantarum*) and safflower oil from 3 to 9% concentrations did not affect the sensory properties of cheese samples (Figure 3). After 90 days of ripening, the flavor scores of the control, T₁, T₂, T₃, and T₄ were 91, 90, 88, 91, and 92% of the total score (9), respectively. Sensory observation is a straightforward parameter to judge customer likes and dislikes.

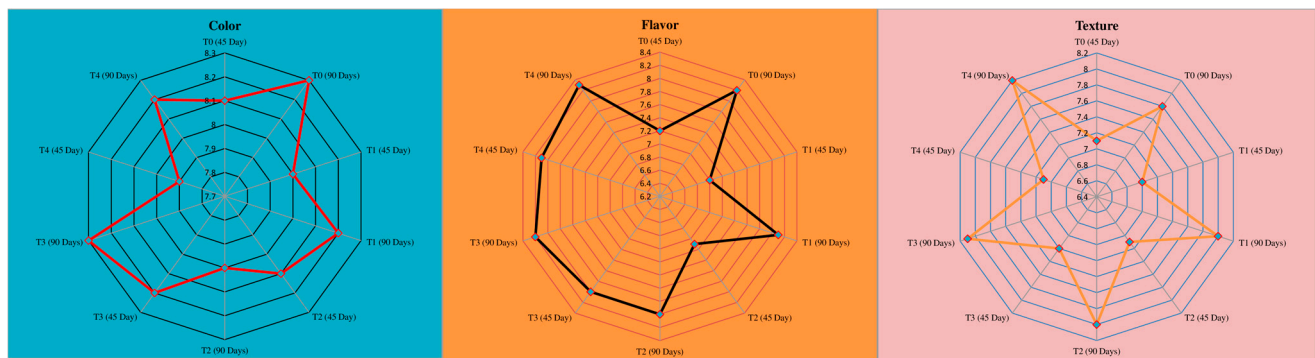


Figure 3. Effect of *Lp. plantarum* and safflower oil on sensory characteristics (color, flavor, texture) of cheddar cheese.

4. Conclusions

The addition of *Lactiplantibacillus plantarum* and safflower oil from 3 to 9% concentrations did not affect cheddar cheese composition. *Lactiplantibacillus plantarum* efficiently converted LA to CLA isomers. In T₃ and T₄ (6 and 9% safflower, respectively, and starter culture of *L. lactis* ssp. *Lactis* (*L. lactis* ssp. *Lactis*) and *L. lactis* ssp. *Cremoris* and *Lp. plantarum* 10⁸/mL), CLA was 6.24 and 9.75 mg/100 g. To prevent metabolic disease, CLA should be taken on a regular basis. The peroxide value of CLA-enriched cheese was within the allowable limits of EU. Overall, *Lp. plantarum* and safflower oil from 6 to 9% concentrations can be used to produce CLA-enriched cheddar cheese. Further research work should be conducted on the application of *Lactiplantibacillus plantarum* in different foods.

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Abbreviations

Lactiplantibacillus plantarum (*Lp. plantarum*); acetic acid (AA); butyric acid (BA); citric acid (CA); completely randomized design (CRD); conjugated linoleic acid (CLA); Duncan's multiple range (DMR); fatty acid methyl ester(s) (FAME); flame ionization detector (FID); gas chromatography–mass spectrometry (GC-MS); high performance liquid chromatography (HPLC); hydrogen chloride (HCl); internal diameter (id); lactic acid (LCA); linoleic acid (LA); nitric acid (HNO₃); free fatty acids (FFA); peroxide value (POV); polymerase chain reaction (PCR); two-way analysis of variance (two-way ANOVA)

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