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Identification and Quantification of Key Volatile Flavor Compounds Employing Different Adjunct Starter Cultures in Reduced-fat Cheddar Cheeses by Using GC and GC-MS

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Abstract

Background and Objective: Reduced fat cheese often exhibits poor sensory quality due to the reduction of fat, which plays a critical role in flavor and texture. Therefore, there is the challenge to produce a reduced fat cheese with improved sensory attributes and texture that is also comparable to its full-fat counterpart. The main objective of this research was to investigate the effect of different adjunct starter cultures of *Streptococcus thermophilus*, *Lactococcus helvetius* and *Lactococcus casei*, alone or mixed, on the sensory prperties and volatile flavor compounds of reduced-fat cheddar cheeses (formulated with xanthan gum) and compare them with full-fat cheddar cheese during 75-day ripening.

Material and Methods: Eight treatments according to completely randomized design with a control cheddar cheese (no adjunct starter cultures) were designed. Extraction of the volatiles was carried out using headspace solid phase microextraction. Identification and quantification of volatile flavor compounds were done by gas chromatography-Mass spectrophotometry and gas chromatography, respectively. The sensory analyses were carried out by the 5-point hedonic scale, using trained panelists.

Results and Conclusion: Among all the flavor compounds, more than 98% of the headspace volatile flavor compounds belonged to aldehydes, ketons, esters, alcohols, and acids. As compared to non-inoculated full-fat control, the use of *Streptococcus thermophilus*, *Lactococcus casei* and *Lactococcus helveticus* as adjunct starter cultures in the reduced fat cheddar cheese formulation increased the amount of volatile flavor compounds and enhanced the sensory attributes. The combination of this mixed culture with the reduced fat cheddar cheese containing xanthan gum as fat replacer is a viable alternative to improve the quality characteristics of reduced-fat cheddar cheese.

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1. Introduction

During cheese making, changes in the milk's principal constituents as a method of preservation take place before, after and during ripening or maturation. The added curd contains microorganisms and enzymes that will induce biological, biochemical and chemical changes during the cheese process thereafter. Particular biochemical metabolisms of cheese microbiota induce changes in flavor and texture during ripening, creating the characteristic flavor (taste and odor), texture, and (in most cases) appearance of individual varieties [1]. Nonetheless, increased awareness of people on fitness has led to an increased demand for low-calorie foods, in particular for reduced fat cheeses. Reduced fat cheeses have poor texture and flavor as compared to full fat ones; however, consumers expect to have all the characteristics of full-fat cheese in its reduced-fat counterpart. Various techniques used for improvement reduced fat cheeses structure include using fat replacers and special starter cultures. Fat replacers are ingredients reducing fat content with a substantial decrease of caloric value, and at the same time, providing rheological properties of fat [2]. In order to improve flavor development in reduced fat cheeses, the use of adjunct starter culture is recommended. Adjunct cultures have ability to improve the flavor of reduced fat cheeses by

increasing proteolysis, particularly the activity of amino peptidase, which has the properties of reducing bitterness and increasing the concentrations of desirable flavor peptides and precursors of flavor volatiles [3]. One of the basic functional requirements induced by starter cultures in fermented milks is their ability to produce aroma compounds. The production of high perceived quality fermented milks can be achieved by qualitative and quantitative analyses of volatile flavor compounds [4]. Solid Phase Micro Extraction (SPME) has received much attention in the literature for the analysis of food volatile compounds including dairy [5] and other fermented products [6]. Headspace solid phase microextraction (HS-SPME) is a potent extraction due to its fast, cheap and solvent free extraction method. SPME has been used normally in combination with Gas Chromatography (GC), and Gas Chromatography–Mass Spectrometry (GC-MS), and successfully employed to a wide variety of compounds.

Fabre et al. reported that SPME analysis followed be GC-MS allowed better discrimination of flavor release from different milk protein mixtures [7]. The objective of the present study was identification and quantification of key volatile flavor compounds (forming throughout the ripening of reduced fat cheddar cheeses made with different adjunct starter cultures) by using GC-MS and GC and compare them with full-fat cheddar cheese.

2. Materials and Methods

2.1. Materials

Fresh milk was collected in the morning from a farm located in University Putra Malaysia just before starting the trial. Direct-to-vat frozen starter cultures: (R-704 *Lactococcus (L.)lactis*, spp *cremories* and *Lactococcus lactis* spp *lactis*, LH-B02 *Lactobacillus helveticus*, *L. casei*-01 (*Lactobacillus casei*), ST-B01 *Streptococcus (S.)thermophilus* and standard cheese rennet (single-strength fermentation-derived chymosin) were kindly donated by Chr. Hansen (Milwaukee, WI). Xanthan gum (food grade) was purchased from V.I.S. Foodtech Ingredient Supplies (Kuala Lumpur, Malaysia). The standards of flavor compounds were purchased from Merck (Darmstadt, Germany). The SPME manual holder and fibers coated with 74 µm Carboxen polydimethy-lsiloxane (CAR-PDMS), glass vial, butyl septa and open center aluminum seal were obtained from Supelco (Bellefonte, PA, USA).

2.2. Preparation of cheddar cheese samples

In an earlier study, it was found that it is possible to produce a reduced-fat cheddar cheese with textural properties similar to those of the full-fat cheddar cheese [8]. The results obtained by full factorial multiple optimization revealed that the reduced fat cheddar cheese containing 0.045% (w w^{-1}) xanthan gum and 2% (w w^{-1}) fat provided the higher textural desirability (90.06%). Therefore, reduced fat cheddar cheeses were made using pasteurized milks (2% w w^{-1} fat) enriched with 0.045 (%w w^{-1}) xanthan gum in order to find best textural properties [8]. Cheddar cheese samples were prepared according to the method described by Awad et al. Single and mix adjunct starter cultures (*L. helveticus*, *L. casei* and *S. thermophilus*) were added at the same time with usual starter culture to the cheese milks, as shown in Table 1. The prepared cheeses were ripened at 12ºC and were taken for analysis at 1, 15, 30, 45, 60 and 75 days after production. The control full fat cheddar cheese was made from whole milk (3.5% w w^{-1} fat) without xanthan gum and adjunct starter cultures [9].

2.3. Gas chromatographic conditions (GC and GC-MS)

The initial identification and confirmation of the volatile compounds were performed using a Hewlett-Packard 6890N GC system (Agilent Technologies, Wilmington, DE, USA) equipped with Time-of-Flight Mass Spectrometer (TOFMS) (Pegasus III, LECO Corp., and St. Joseph, MI, USA). After confirmation analysis, the volatile compounds were quantified using a Hewlett-Packard 6890 GC device equipped with a flame ionization detector (FID). This was followed by separation of the compounds on a HP- Innowax (60 m×0.25 mm× 0.25 µm) capillary column (J and W Scientific, Folsom, CA, USA). In order to minimize peak broadening of the samples, the GC injection port was equipped with a 0.75 mm i.d. liner. For GC-FID consideration, desorption was carried out in the splitless mode for 10 min at 270°C.

The oven temperature was maintained at 40°C for 10 min, and then raised at a rate of 4°C min^{-1} up to 90°C. Then it was ramped to 240°C at 6°C min^{-1} and held for 10 min. Helium gas was used as a carrier maintained at a flow rate of 1.4 ml min^{-1} and the detector temperature was set at 300°C. Both the GC-MS and GC-FID were identical with respect to the injector at 270°C and detector temperature as well as oven program. Also the mass spectra in the electron impact (EI) mode were acquired at 70 eV. The ChromaTOF software (version 2.4, LECO Corporation) was used to process the data obtained from the GC-TOFMS. Furthermore, the volatile flavor compounds from the samples were identified by analogy of mass spectra, followed by matching of mass spectra fragment with the NIST library (version 2.0) and additional standards.

2.4. HS-SPME Procedure

Extraction of volatiles was carried out using a solvent less extraction technique [10]. The cheese samples ripened at 12°C for 75 days were assayed for aroma volatiles using SPME GC/MS analysis. The grated cheddar cheese samples were placed into a 20 mL glass vial. The extraction procedure was done by HS-SPME started with

the capping of the vials with 20 mm diameter butyl septum, sealed with 20 mm open center headspace aluminum seals. The volatile extraction was conducted by injecting a 75 mm carboxen-polydimethylsiloxane fiber into the headspace of vial containing 3 g grated cheese sample for 30 min at 40°C. Following the sampling procedure, the SPME fiber was instantly inserted into the GC injector, and the fiber was desorbed thermally. The fiber was positioned at 3.0 scale units in each run. The method is suitable for isolation of volatiles from the sample matrix, and has been used in various configurations for characterization of cheese samples.

2.5. Sensory evaluation

Organoleptic assessment of the cheeses, during the ripening period, was carried out according to Sipahioglu et al. with slight modifications in the procedure [11]. The study consisted of 15 trained panelists familiar with cheddar cheese. Experimental cheeses (Table 1) were subjected to evaluation for body, texture and flavor by the panelists employing a non-structured 10-point scale, with 1 being poor and 10 excellent. Overall acceptability was evaluated in the same manner employing a non-structured 1-9-point scale, with 1 being 'extremely dislike' and 9 representing 'extremely like'. The cheese blocks were cut into slices (1×1×1cm). The cheese pieces were presented in identical plastic sample cups sealed with plastic lids and recognized by a random 3-digit number. The coded samples were randomly presented. Water and unsalted crackers were offered without limit to the panelists during testing for cleaning their palates between the samples.

2.6. Statistical analysis

The data obtained from the measurements were subjected to univariate one way analysis of variance (ANOVA) to determine the significant differences among the samples, and the values were compared using the Tukey's test defined at $p \leq 0.05$. All measurements were carried out in triplicate and reported as the mean ± SD. The data analysis was performed using MINITAB 14 (MINITAB Inc., State College, PA and USA).

Table 1. Control and reduced fat cheddar cheeses with various combinations of adjunct starter culture[1]

Code of the adjunct starter culture	Adjunct starter culture	Concentration (g kg^{-1})	Concentration (CFU ml^{-1})
N	Non-inoculated (Full fat control cheddar cheese)	-	-
H	*L. helveticus*	0.0228	10^8
C	*L. casei*	0.0228	10^8
T	*S. thermophilus*	0.0228	10^8
H+C	*L. helveticus+L. casei*	0.0114	10^4+10^4
T+H	*S. thermophilus+L. helveticus*	0.0114	10^4+10^4
T+C	*S. thermophilus+L. casei*	0.0114	10^4+10^4
T+C+H	*S. thermophilus+L. casei+ L. helveticus*	0.0760	$10^{26}+10^{26}+10^{26}$

* Non-inoculated with adjunct starter culture and prepared with cheese milk (3.5% fat).

[1]All reduced fat and full-fat cheeses were inoculated with 0.015 gkg^{-1} direct-to-vat frozen commercial mesophilic lactic cultures (R-704), $p \leq 0.05$, n=3.

3. Results and Discussion

3.1. Identification of volatile flavor compounds

The results given in Table 2 indicate that 38 volatile compounds were extracted by HS-SPME, and >98% of headspace volatile flavor compounds quantified by GC-FID were 2-butanone (32.934%), ethyl butyrate (12.638%), 2,3-butanedione (10.582%), 3-hydroxy-2-butanone (9.676%), ethyl acetate (9.212%), 2,3-butandiol (6.285%), acetaldehyde (5.136%), acetone (2.698%), 2-heptanone (2.378%), ethanol (1.812%), octanoic acid (1.034%), hexanoic acid (1.738%), 2-nonanone (1.205%) and 1-hexanol (0.945%). Consequently, the corresponding flavor compounds were chosen as the representative of the volatile flavor compounds released into the headspace of matured cheese samples. These compounds have also been reported previously as the main volatile flavor compounds of matured cheese products [12]. Primary degradation pathways of milk constituents in cheese curd, including glycolysis of lactose and citrate, lipolysis of milk lipids, and proteolysis of caseins leads to the formation of a whole range of precursors of flavor compounds. Only some of the compounds formed by glycolysis, lipolysis, and proteolysis directly contribute to cheese flavor (like short chain fatty acids, acetaldehyde, diacetyl, peptide, and amino acids). Primary degradation of major caseins has major consequences for the cheese texture. These changes are followed and/or overlapped by a concerted series of secondary catabolic reactions, which are responsible for the unique aroma profile of a particular variety or type of cheese [13]. Ketones are intermediate compounds, and are the common aroma constituents of most dairy products [12]. The principal flavor compounds produced from the metabolism of citrate are acetate, diacetyl, acetoin, and 2, 3-butanediol [13]. The production of methyl ketones involves oxidation of fatty acids to β-ketoacids, which are then decarboxylated to corresponding methyl ketones [14], as a result of the lypolytic action of microflora in the cheese [15]. Esters are formed via the esterification of alcohols and free fatty acids [13]. Alcohols found in cheese

are usually derived directly from lactose or citrate fermentation [16]; however, they may also form through acetaldehyde reduction [17] and amino acid metabolism [12]. Aldehydes are characterized as intermediate and unstable compounds, which can be reduced to alcohols or oxidized to acids, and tend to appear at low concentration in the volatile fraction of most cheeses [18]. Free fatty acids are essential components of the aroma of various cheese types and derived from limited lipolysis of short chain fatty acids in cheeses [19].

3.2. Quantification of ketones

According to Table 2, six ketones were indentified in the non-inoculated full-fat and inoculated reduced fat cheddar cheeses with different adjunct starter cultures, representing almost 59.45% of the total volatile compounds.

Maximum concentration of 2,3-butanedione (common name diacetyl, $C_4H_6O_2$) was quantified as 4.90 mg kg^{-1} (Fig. 1-a). Diacetyl concentration decreased significantly ($p\leq0.05$) during the ripening period. *L. helveticus* inoculated samples resulted in significantly ($p\leq0.05$) higher diacetyl concentration, followed by *S. thermophilus* and the mixed culture of *S. thermophilus* and *L. helveticus*. Lower diacetyl content was detected in the non-inoculated samples (Fig. 1-a).

Maximum quantified concentration of 3-hydroxybutanone (common name acetoin, $C_4H_8O_2$) was 91.00 mg kg^{-1}. Acetoin concentration significantly ($p\leq0.05$) increased during the first 15 days of storage, and then decreased. Acetoin concentration was significantly ($p\leq0.05$) higher when *L. helveticus* was inoculated, followed by non-inoculated control cheese (full-fat) (Fig. 1-b).

Table 2. Linear retention indices for HP-Innowax column, similarity and FID peak area (%) of the representative volatile flavor compounds in the cheese samples (ND= not detected)

No.	Compound	Formula	LRI	Similarity	FID peak area%
1	Carbon dioxide	CO_2	921	932	ND
2	Carbonyl sulfide	COS	926	876	ND
3	Acetaldehyde	C_2H_4O	954	979	5.136
4	Acetone	C_3H_6O	1142	835	2.698
5	Ethyl acetate	$C_4H_8O_2$	1373	971	9.212
6	2-Butanone	C_4H_8O	1440	966	32.934
7	Ethanol	C_2H_6O	1673	976	1.812
8	1,2-Ethandiol,Diacetate	$C_2H_6O_2$	1741	879	ND
9	2,3-Butandione	$C_4H_6O_2$	1969	902	10.582
10	Hexan-2-one	$C_6H_{12}O$	1989	984	0.036
11	2-Methylpropan-1-o1	$C_4H_{10}O$	2062	921	ND
12	Pentan-2-o1	$C_6H_{12}NO_2$	2110	987	0.016
13	1-Methoxy-2-propanol	$C_4H_{10}O_2$	2179	879	ND
14	3-Methyl butan-1-o1	$C_5H_{12}O$	2319	982	ND
15	Ethyl butyrate	$C_6H_{12}O_2$	2490	926	12.638
16	Hexadecanoic acid, ethyl ester	$C_{16}H_{32}O_2$	2508	932	ND
17	Unknown 1	–	2519	889	ND
18	2-Heptanone	$C_7H_{14}O$	2545	966	2.378
19	Hexan-1-o1	C_6H_{14}	2565	941	ND
20	Heptanoic acid, ethyl ester	$C_7H_{14}O_2$	2605	894	0.019
21	Dodecanoic acid, ethyl ester	$C_{12}H_{24}O_2$	2628	903	ND
22	1-Propanol,2-methyl-	$C_5H_{12}O$	2667	996	0.036
23	1-Butanol,3-methyl-(s)	$C_5H_{12}O$	2681	853	0.005
24	Octanal	$C_8H_{16}O$	2698	964	ND
25	3-Hydroxy-2-butanone	$C_4H_8O_2$	2706	912	9.676
26	Propionic acid-2-hydroxy-,ethyl ester	$C_5H_{10}O_3$	2712	929	ND
27	1-Hexanol	$C_6H_{14}O$	2716	889	0.945
28	2-nonanone	$C_9H_{18}O$	2721	897	1.205
29	Nonanal	$C_9H_{18}O$	2726	987	ND
30	Ethanol,2-butoxy	$C_6H_{14}O_2$	2731	939	0.063
31	Acetic acid	$C_2H_4O_2$	2739	994	0.073
32	Propionic acid,2-methyl-	$C_4H_8O_2$	2757	881	ND
33	2,3-butandiol	$C_4H_{10}O_2$	2785	990	6.285
34	Butanoic acid	$C_4H_8O_2$	2813	944	0.033
36	Phenyl ethyl alcohol	$C_7H_{10}O$	2847	983	0.009
37	Heptanoic acid	$C_7H_{14}O_2$	2882	976	ND
38	Octanoic acid	$C_8H_{16}O_2$	2935	985	1.034

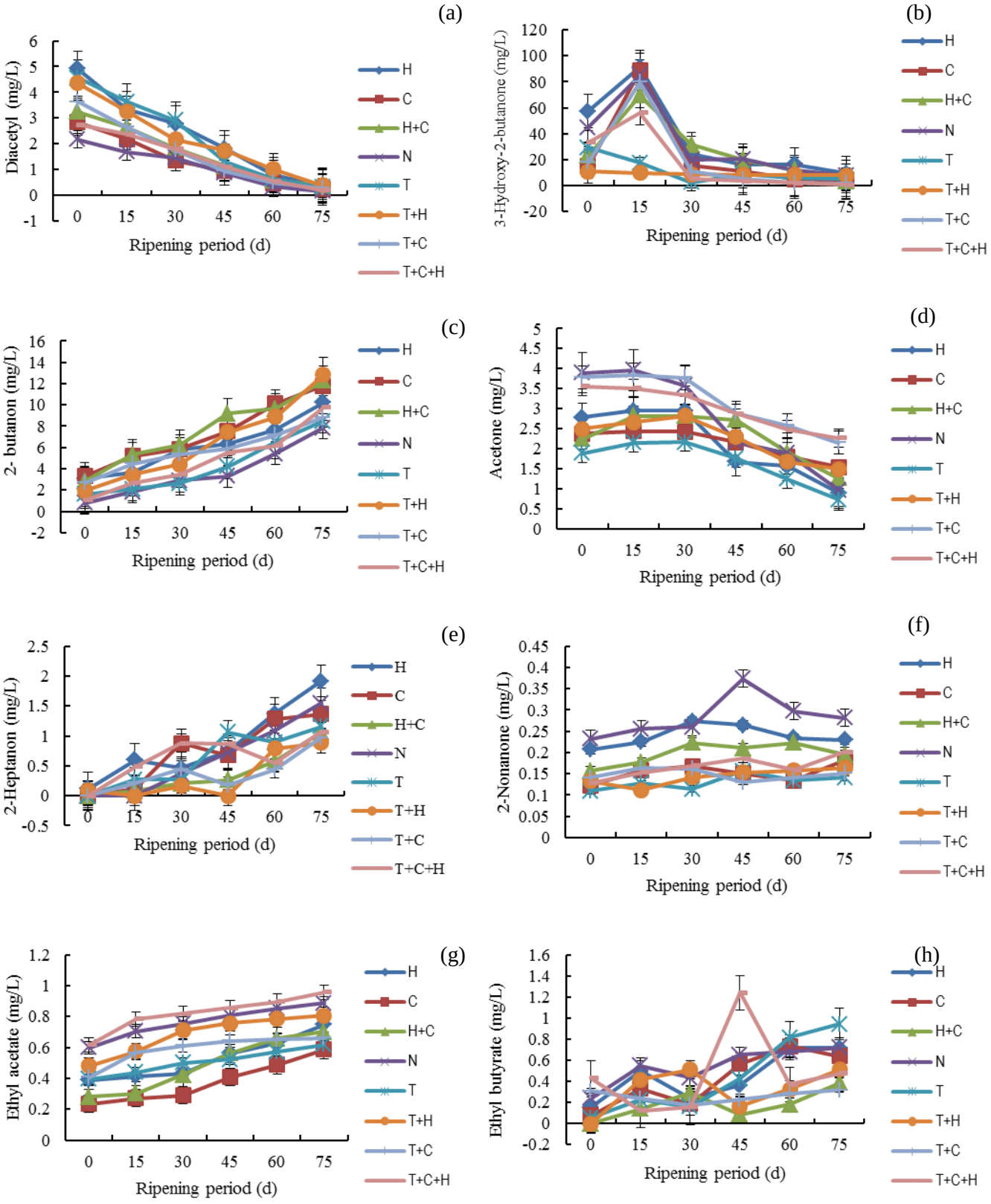

Fig. 1. Changes in volatile compounds (ketones, methy ketones and esters) during 75 days of ripening ($p \leq 0.05$, n=3) in non-inoculated full fat cheddar cheeses (N= No adjunct starter culture) and reduced fat cheddar cheeses inoculated with adjunct starter cultures (H= *L. helveticus*, C= *L. casei*, T= *S. thermophilus*, H+C= *L. helveticus*+*L. casei*, T+H= *L. thermophilus*+*L. helveticus*, T+C= *L. thermophilus*+*L. casei*, T+C+H= *L. thermophilus*+*L. casei*+*L. helveticus*).

Diacetyl is usually produced in small amounts but acetoin is generally produced in much higher concentrations (10 to 50 fold higher than diacetyl concentration) [13]. Diacetyl transformation into more reduced compounds progresses throughout ripening due to the enzymatic activities of microorganisms [20]. After ripening, diacetyl (Fig 1-a) in cheddar cheese is too low to participate in flavor because this compound is converted into acetoin, and then into 2, 3-butanediol (Fig. 2-a) and 2-butanone (Fig 1-c) [21]. Several reports stated the decrease of diacetyl during cheese ripening [22]. Strain type has a marked effect on diacetyl production and transformation. Thermophilic starter cultures are able to produce high levels of diacetyl alone or mixed with other lactic acid strains [23].

Acetoin is an important flavor compound in several cheeses such as cheddar or gouda [24]. The results of current research corroborate the results of previous research that acetoin concentration increased during the first 15-30 days of ripening and then subsequently decreased [18,19,25].

Butan-2-one (common name 2-butanone, C_4H_8O) was the most abundant compound (32.93%) found in the volatile fraction in all the cheddar cheese samples. The 2-butanone concentration increased significantly ($p\leq0.05$) with storage time. The ripened cheese samples inoculated with the mixed culture of *S. thermophilus* and *L. helveticus* obtained significantly ($p\leq0.05$) higher 2-butanone values (12.822 mg kg^{-1}). Lower 2-butanone content was detected in the non-inoculated samples (Fig. 1-c).

Pronan-2-one (common name acetone, C_3H_6O) was quantified in maximum concentration in the non-inoculated fresh cheese (3.896 mg kg^{-1}). Acetone content significantly ($p\leq0.05$) increased at day 15, and then decreased with ripening time. The samples inoculated with mixed cultures of *S. thermophilus* with *L. helveticus* and *L. casei* showed the highest acetone content (2.266 mg kg^{-1}) at the end of the ripening period (Fig. 1-d).

Maximum quantified concentration of Hepta-2-one (common name 2-heptanone, $C_7H_{14}O$) was 1.91 mg kg^{-1} in the ripened cheese sample inoculated with *L. helveticus.* Concentration of 2-heptanone increased significantly ($p\leq0.05$) with ripening time (Fig. 1-e).

Octan-2-one (common name 2-nonanone, $C_9H_{18}O$) was quantified in maximum concentration of 0.28 mg kg^{-1} in the non-inoculated ripened cheese samples followed by the samples inoculated with *L. helveticus.* 2-nonanone content was increased in all the cheese samples in contrast to the initial concentration (Fig. 1-f).

Among the ketones identified in this study, 2-butanone was the most abundant probably due to the transformation of diacetyl to these methyl ketones [26]. During the cheese ripening period, the concentration of 2-butanone increased [17] mainly due to the addition of thermophilic starter alone or mixed with other strains [23]. In contrast, during this period, acetone content decreased according to what reported for other ripened cheeses [25]. Other ketones, like 2-heptanone and 2-nonanone, increased as well during the storage period [5]. The use of *L. helveticus* as starter culture resulted in higher methyl ketone production than other strains during cheese ripening, like 2-heptanone [17; 23] and 2-nonanone, probably due to the proteolytic system of this strain, improving the flavor profile of cheddar cheese [16].

3. 3. Quantification of esters

According Table 2, two esters were detected in the cheddar cheese samples, representing 21.84% of the total volatile compounds.

Ethyl butanoate (common name ethyl butyrate, $C_6H_{12}O_2$) was quantified in maximum concentration of 0.94 mg kg^{-1} in the ripened reduced fat cheeses contains single culture of *St. thermophilus;* then the concentration of this ester was significantly ($p\leq0.05$) higher in the non-inoculated samples (Fig. 1-g). Ethyl butyrate increased significantly ($p\leq0.05$) with the storage ripening time.

The second ester, ethyl acetate (common name ethyl ester, $C_4H_8O_2$), was quantified in maximum concentration of 0.95 mg kg^{-1} in the ripened samples inoculated with mixed cultures of *S. thermophilus, L. helveticus* and *L. casei* (Fig. 1-h). Ethyl ester concentration significantly ($p\leq0.05$) increased with ripening time.

The relatively higher content of ethyl ester in the mixed cultures probably could be due to the ability of lactic strains to produce ethanol, generally associated with the esters' content [27].

The esters at low levels contribute to a positive overall cheese flavor; however, high levels can lead to a fruity sweet flavor defects in cheeses [27]. Previous researchers have reported that ethyl butyrate increased with ripening period. Although high amounts of ethyl butyrate in cheeses are associated with flavor defect, ethyl butyrate is formed by the esterification of fatty acids with ethanol [18].

3.4. Quantification of alcohols

As shown in Table 2, three alcohols were identified in the cheddar cheese samples, representing 9.04% of the total volatile compounds.

Butane-2, 3-diol (common name 2, 3-butanediol, $C_4H_{10}O_2$) was the most abundant alcohol, and quantified in maximum concentration of 1.023 mg kg^{-1} in the matured reduced fat cheddar cheeses containing single culture of *St. thermophilus*. The non-inoculated matured cheese samples presented lower values of 2,3-butanediol (Fig. 2-a). 2,3-butanedoil concentration increased significantly ($p\leq0.05$) with ripening time. 2,3-butanediol in cheeses is the result of 3-hydroxy-2-butanone reduction by starter bacteria (Fig. 1-b); besides, it has been reported that *S. thermophilus* inoculation increases the amount of this alcohol in cheddar cheeses [16].

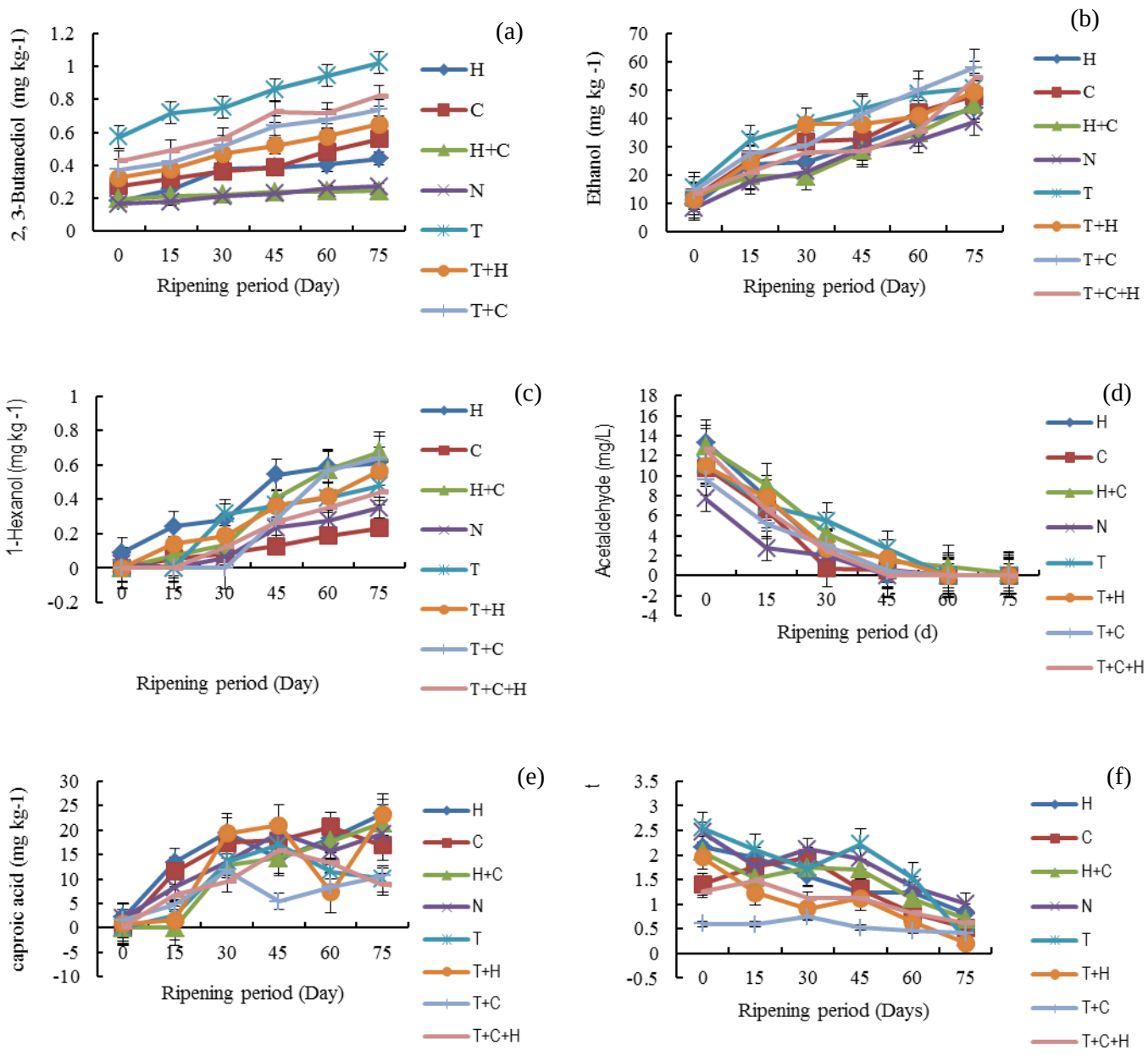

Fig. 2. Changes in volatile compounds (alcohols, aldehydes and fatty acids) during 75 days of ripening ($p \leq 0.05$, n=3) in non-inoculated full fat cheddar cheese (N= No adjunct starter culture) and fat-reduced-fat Cheddar cheeses inoculated with adjuncts starter cultures (H= *L. helveticus*, C= *L. casei*, T= *S. thermophilus*, H+C= *L. helveticus*+*L. casei*, T+H= *L. thermophilus*+*L. helveticus*, T+C= *L. thermophilus*+*L. casei*, T+C+H= *L. thermophilus*+*L. casei*+*L. helveticus*).

Ethanol (C_2H_6O) concentration in the ripened cheese samples inoculated with mixed cultures of *S. thermophilus* and *L. casei* was significantly ($p \leq 0.05$) higher (57.92 mg kg^{-1}) than in the non-inoculated samples (Fig. 2-b). The concentration of ethanol exhibited a significant ($p \leq 0.05$) increase in all batches during the ripening time. Pervious researchers have reported that ethanol concentration significantly increased with ripening time due to the presence of adjunct starter cultures and ability of them to metabolize lactose [22].

Hexan-1-ol (common name 1-hexanol, $C_6H_{14}O$) was quantified in maximum concentration (0.67 mg kg^{-1}) in the matured samples inoculated with mixed cultures of *L. helveticus* and *L. casei*, and the lowest concentration (0.35 mg kg^{-1}) was detected in the non-inoculated ripened samples (Fig. 2-c). 1-hexanol concentration increased significantly ($p \leq 0.05$) with ripening time. A similar behavior has been reported during cheese ripening, and higher level of 1-hexanol was observed in cheeses containing *L. helveticus* [17].

3.5. Quantification of aldehyde and free fatty acids

Ethanal (common name acetaldehyde, C_2H_4O) was the only identified aldehyde in maximum concentration of 13.30 mg kg^{-1} in the fresh samples inoculated with *L. helveticus*. The concentration of acetaldehyde in the fresh full-fat control cheeses was significantly ($p \leq 0.05$) lower (7.67 mg kg^{-1}) than in the reduced fat cheeses that contained adjunct starter cultures (Fig. 2-d). During the cheddar cheese ripening, acetaldehyde concentration decreased significantly ($p \leq 0.05$), which could be explained in relation to its change to corresponding alcohol.

Two free fatty acids were identified as volatile compounds formed during the cheddar cheese ripening. Hexanoic acid (common name caproic acid, $C_6H_{12}O_2$) quantified in maximum concentration of 23.300 mg kg^{-1} in the ripened cheeses inoculated with *L. helveticus* (Fig. 2-e). The concentration of caproic acid significantly ($p \leq 0.05$) increased with ripening time. This result may be explained by the higher ability of *L. helveticus* to produce short-chain fatty acids from the degradation of lactose and amino acids as well as oxidation of ketones, esters and aldehydes [28]. A best cheddar flavor was related to 20-25 mg kg^{-1} of caproic acid content [28]. The concentration of this free fatty acid presented some fluctuation during the cheeses' ripening time, but with a tendency to increase with storage time [15].

Maximum quantified concentration of octanoic acid (common name caprylic acid, $C_8H_{16}O_2$) was 2.54 mg kg^{-1} in the fresh cheeses inoculated with *S.thermophilus* (Fig. 2-f). During ripening, caprylic acid concentration significantly ($p \leq 0.05$) decreased probably due to the formation of ethyl esters [29].

3.6. Sensory evaluation

For cheddar cheeses' body and texture, there was a significant ($p \leq 0.05$) difference between the different adjunct starter cultures inoculated. At the end of ripening period, the cheese samples inoculated with mixed culture *S. thermophilus+L. casei+L. helveticus* obtained higher scores, followed by the *S. thermophilus* and *S. thermophilus +L. helveticus* culture inoculated samples. Lower scores were detected in the non-inoculated and *L. helveticus* samples. Regarding to ripening period, significantly ($p \leq 0.05$) higher scores were obtained at the end of the storage period (day 75), with lower scores at the beginning of the ripening stage (days 1 to 30) (Table 3).

After the 75-day ripening, the flavor scores obtained for the reduced-fat cheddar cheeses inoculated with *S. thermophilus+L. casei+L. helveticus* were significantly ($p \leq 0.05$) higher than those for the rest of the treatments. Lower flavor scores were achieved for the non-inoculated samples. The flavor was significantly ($p \leq 0.05$) more appreciated at the end of the ripening period (Table 3).

Finally, the overall acceptability for the ripened cheese samples was significantly ($p \leq 0.05$) higher in the samples inoculated with *S. thermophilus+L. casei+L. helveticus* and *S. thermophilus+L. helveticus* mixed cultures. Lower acceptability scores were obtained in the non-inoculated samples. For the storage period, after 75 days of ripening, the overall acceptability was significantly ($p \leq 0.05$) higher than at the beginning of the cheese ripening (Table 3).

Table 3. Results of sensory evaluation of full-fat and reduced fat cheeses

Attribute	Time (days)	N	H	C	T	H+C	T+H	T+C	T+C+H
Body and texture[1]	1	6.12±0.93$^{C, c}$	6.81±0.57$^{B, a}$	6.62± 0.26$^{B, b}$	6.73±0.79$^{B, c}$	6.11± 0.40$^{C, c}$	6.71±0.32$^{B, c}$	6.15± 0.52$^{C, c}$	7.01±0.41$^{A, c}$
	15	6.38±0.37$^{E, c}$	6.12±0.21$^{C, b}$	6.30±0.45$^{C, b}$	7.48±0.53^{A,b}	6.21±0.62$^{C, c}$	6.91±0.48$^{B, c}$	6.26±0.36$^{C, b}$	7.53±0.19$^{A, c}$
	30	5.81±0.40$^{E, c}$	5.77±0.30$^{C, c}$	6.60±0.26$^{B, b}$	7.21±0.24$^{A, c}$	6.56±0.46$^{B, c}$	7.00±0.44$^{A, c}$	6.66±0.50$^{B, a}$	7.31±0.29$^{A, c}$
	45	6.39±0.57$^{E, b}$	6.16±0.48$^{E, b}$	7.11±0.38$^{C, a}$	7.33±0.42$^{B, b}$	7.18±0.72$^{C, b}$	7.93±0.33$^{B, b}$	6.31±0.29$^{D, b}$	8.35±0.46$^{A, b}$
	60	6.27±0.55$^{E, b}$	6.55±0.29$^{C, b}$	7.51±0.51$^{B, a}$	7.35±0.46$^{B, b}$	7.71±0.83$^{B, b}$	7.87±0.61$^{B, b}$	6.35±0.58$^{C, b}$	8.28±0.29$^{A, b}$
	75	6.57±0.72$^{D, a}$	6.90±0.40$^{C, a}$	7.57±0.55$^{B, a}$	8.21±0.35$^{A, a}$	7.81±0.51$^{B, a}$	8.18±0.30$^{A, a}$	6.83±0.25$^{C, a}$	8.66±0.16$^{A, a}$
Flavor[1]	1	6.18±0.33$^{D, b}$	6.73±0.34$^{C, ab}$	7.06±0.89$^{B, a}$	7.61±0.87$^{B, c}$	7.13±0.80$^{B, c}$	8.12±0.73$^{A, b}$	7.21±0.58$^{B, b}$	8.21±0.44$^{A, b}$
	15	6.25±0.65$^{C, ab}$	6.82±0.44$^{C, ab}$	7.10±0.28$^{B, a}$	8.13±0.58$^{A, b}$	7.23±0.39$^{B, c}$	8.61±0.90$^{A, ab}$	7.34±0.12$^{B, b}$	8.43±0.37$^{A, b}$
	30	6.55±0.47$^{C, a}$	7.16±0.38$^{B, a}$	6.11±0.82$^{C, b}$	8.23±0.93$^{A, ab}$	7.81±0.30$^{AB, c}$	8.63±0.72$^{A, ab}$	7.12±0.34$^{B, b}$	8.75±0.30$^{A, b}$
	45	6.61±0.56$^{C, a}$	6.32±0.58$^{C, b}$	7.13±0.38$^{B, a}$	8.31±0.18$^{A, a}$	8.11±0.32$^{A, b}$	8.75±0.30$^{A, a}$	7.61±0.26$^{B, a}$	8.55±0.51$^{A, b}$
	60	6.56±0.44$^{C, a}$	6.81±0.53$^{C, a}$	7.35±0.31$^{B, a}$	8.35±0.58$^{A, a}$	8.12±0.44$^{A, b}$	8.88±0.20$^{A, a}$	7.69±0.16$^{B, a}$	8.90±0.38$^{A, ab}$
	75	6.76±0.37$^{D, a}$	6.91±0.25$^{D, a}$	7.37±0.57$^{C, a}$	8.66±0.53$^{B, a}$	8.74±0.51$^{AB, a}$	8.95±0.30$^{A, a}$	7.68±0.57$^{C, a}$	9.37±0.52$^{A, a}$
Overall acceptability[2]	1	4.24±0.72$^{B, d}$	4.50±0.24$^{AB, c}$	4.11±0.53$^{B, c}$	4.97±0.53$^{A, d}$	5.03±0.35$^{A, c}$	4.94±0.70$^{A, d}$	4.13±0.19$^{B, c}$	5.13±0.91$^{A, d}$
	15	4.84±0.65$^{C, c}$	5.16±0.15$^{B, b}$	4.16±0.41$^{C, c}$	5.18±0.17$^{B, c}$	6.14±0.85$^{A, b}$	5.23±0.79$^{B, c}$	4.21±0.44$^{C, bc}$	6.18±0.53$^{A, c}$
	30	4.96±0.82$^{C, b}$	5.51±0.25$^{B, ab}$	5.18±0.68$^{B, b}$	5.21±0.45$^{B, bc}$	6.21±0.57$^{A, b}$	5.81±0.33$^{AB, c}$	4.56±0.72$^{C, b}$	6.23±0.59$^{A, c}$
	45	5.08±0.44$^{C, a}$	5.76±0.51$^{C, a}$	5.40±0.40$^{C, b}$	5.33±0.72$^{C, b}$	6.81±0.44^{A,ab}	6.33±0.24$^{B, b}$	5.19±0.41$^{C, b}$	6.84±0.33$^{A, b}$
	60	5.16±0.43$^{E, a}$	5.75±0.16$^{D, a}$	5.68±0.45$^{D, b}$	6.00±0.12$^{C, a}$	7.04±0.40$^{A, a}$	6.58±0.37$^{B, b}$	5.53±0.30$^{D, a}$	7.54±0.58$^{A, a}$
	75	5.33±0.61$^{D, a}$	5.77±0.50$^{C, a}$	5.92±0.15$^{C, a}$	6.23±0.63$^{B, a}$	7.14±0.43$^{A, a}$	7.37±0.44$^{A, a}$	5.86±0.50$^{C, a}$	7.71±0.48$^{A, a}$

[1]Scale for body, texture and flavor: 1 = poor to 10 = excellent.
[2]Scale for overall acceptability: 1: dislike, 5: like moderately, 9: like extremely
[3]Code for starter cultures: N= No adjunct starter culture, H= *L. helveticus*, C= *L. casei*, T= *S. thermophilus*, H+C= *L. helveticus+L. casei*, T+H= *S. thermophilus+L. helveticus*, T+C= *S. thermophilus+L. casei*, T+C+H= *S. thermophilus+L. casei+L. helveticus*.
$^{A, B, C, D, E}$ Means with same superscript in same row are not significantly different ($p \leq 0.05$, n=15) for the adjunct starter culture employed for each sensory attribute evaluated.
$^{a, b, c, d}$ Means with same superscript in same column are not significantly different ($p \leq 0.05$, n=15) for the adjunct starter culture employed for each sensory attribute evaluated.

In general, the mixed adjunct starter culture of *S. thermophilus+L. casei+L. helveticus* inoculated in reduced-fat cheddar cheese resulted in higher sensory scores related to texture, flavor and overall acceptability. In contrast, the non-inoculated full-fat samples obtained lower scores. This means that the inoculation of mixed starter cultures improved the sensory attributes in the reduced-fat cheddar cheese samples formulated with xanthan gum during at least 75 days of ripening. Although the body and texture of cheeses containing protein- and carbohydrate-based fat replacers were improved during the maturation time [30], aroma, flavor and texture were associated strongly with starter type and to a starter adjunct [31]. During the post-manufacture period, the rapid release of intracellular enzymes due to autolysis of lactic acid bacteria in the cheese matrix plays a role in the acceleration of cheese ripening [32]. The use of adjunct cultures improved acceptance and flavor scores in the reduced fat cheddar cheeses as compared to the full-fat samples. Nonetheless, texture of cheddar cheese samples inoculated with *L. helveticus* at the experimental conditions employed resulted in lower scores, similar to the full-fat control, probably due the greater rate of proteolysis provoked by this strain [33]. The improvement in sensory body and texture in the cheese samples inoculated with *S. thermophilus* could be related to the capacity of certain capsule-forming non-ropy exopolysaccharides-producer adjunct strains of *S. thermophilus* to hold or absorb water, improving texture in reduced-fat cheddar cheese [8,34].

4. Conclusion

The HS-SPME method supplied a useful and potent tool for the extraction and determination of headspace volatile compounds of cheddar cheese. The results showed that type of starter culture had a marked influence on the amount of volatile compounds generated during the reduced fat cheddar cheese ripening. At the experimental conditions employed, the use of adjunct cultures enhanced the production the extracted and identified volatile compounds. In comparison to the non-inoculated full-fat cheddar cheese, diacetyl and acetoin increased when employing *L. helveticus*; however, alcohols like 2,3-butanediol and ethanol increased when *S. thermophilus* was employed as starter culture. As compared to the non-inoculated full-fat control, the use of *S. thermophilus+L. casei+L. helveticus* as adjunct starter culture for cheddar cheese during a 75-day ripening period increased the amount of volatile compounds, which, in turn, enhanced the sensory attributes. In conclusion, the use of this multi-strain mixed adjunct starter culture in reduced-fat cheddar cheese containing xanthan gum as fat replacer is a viable alternative to improve sensory characteristics of reduced-fat cheddar cheese.

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6. Conflict of Interest

The authors declare that there is no conflict of interest.

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