Influence of Cultivation Conditions on the Growth of *Lactobacillus acidophilus*, *Bifidobacterium* sp., and *Streptococcus thermophilus*, and on the Production of Organic Acids in Fermented Milks

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Abstract

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The parameters influencing the formation of organic acids and the ratio between the optical isomers of lactic acid were evaluated. Five different factors were tested, namely the form of starter, inoculum, temperature of fermentation, time of fermentation, and enhanced non-fat dry matter or addition of whey protein concentrate. Out of them, optimal conditions were chosen for the preparation of fermented milk beverage with ABT culture (*Lactobacillus acidophilus*, *Bifidobacterium* sp., *Streptococcus thermophilus*) with a lowered content of D(–)-lactic acid. The inoculum of bifidobacteria had the only significant effect on the ratio between lactic acid isomers. When 1% v/w used, the ratio of D(–)-lactic acid to L(+)-lactic acid was 0.05. When 5% v/w used, the ratio was 0.02. The addition of dried skimmed milk (max. effect at 12% w/w) enhanced the growth of bifidobacteria, while whey protein concentrate was effective for the growth of lactobacilli. The optimal temperature and time of cultivation were 37°C and 17 ± 0.5 h, respectively.

Keywords: lactic acid bacteria; ABT fermented milk; L(+)-lactic acid; D(–)-lactic acid

Lactic acid is the major final product of fermentative metabolism of lactic acid bacteria. It can be formed from carbohydrates by two different types of fermentative pathway. Several species of lactic acid bacteria produced mainly lactic acid with trace amounts of acetic acid, formic acid, and ethanol by homofermentative metabolic pathway. On the contrary, some species have heterofermentative metabolism and produce one molecule of lactic acid, ethanol, and CO_2 from one molecule of hexose. This heterofermentative metabolic pathway can be found in *Leuconostoc* and some species of *Lactobacillus* (Alur 2000). The production of acetic acid, ethanol, and formic acid as the main products can be observed during the fermentation of carbohydrates with hexose limitation (Kandler & Weiss 2010). Lactic acid exists in two enantiomeric forms, due to its asymmetric carbon C2. The enantiomers have identical chemical and physical properties except for the ability to rotate polarised light. D(–)-Isomer rotates the light in the clockwise direction and L(+)-isomer in the counterclockwise direction (Ewaschuk *et al*. 2005). The presence of an isomer-specific enzyme is the deciding factor for the production of the definite lactic acid isomer. L(+)-Lactic acid is produced by L-lactic acid dehydrogenase (L-LDH), a group by enzymes that is found in bacteria, plants, and animal. D(–)-Lactic acid is produced by the group of D-lactic acid dehydrogenase which is structurally unrelated to L-LDH. Lactic acid bacteria can be

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Table 1. Bacterial starter cultures and their production of optical isomers of lactic acid (Krieg *et al.* 2010)

Species	Isomer	Species	Isomer
Lactobacillus helveticus	DL	*Lactobacillus fermentum*	DL
Lactobacillus delbrueckii subsp. *lactis*	D	*Leuconostoc lactis*	D
Lactobacillus delbrueckii subsp. *bulgaricus*	D	*Leuconostoc mesenteroides* subsp. *cremoris*	D
Lactobacillus casei	L	*Lactococcus lactis* subsp. *cremoris*	L
Lactobacillus paracasei subsp. *paracasei*	L	*Lactococcus lactis* subsp. *lactis*	L
Lactobacillus rhamnosus	L	*Enterococcus faecalis*	L
Lactobacillus plantarum	DL	*Enterococcus faecium*	L
Lactobacillus acidophilus	DL	*Streptococcus thermophilus*	L

sorted out according to the presence of the specific enzyme (Jin *et al.* 2009). The bacteria of the genus *Lactococcus* produce only L(+)-lactic acid, those of genus *Leuconostoc* produced only D(–)-lactic acid, some lactobacilli produce only L(+)-lactic acid (e.g. *Lb. casei* subsp. c*asei*), some produce both isomers of lactic acid (e.g. *Lb. brevis*), and some lactobacilli produce only D(–)-lactic acid (e.g. *Lb. delbrueckii* subsp*. lactis*). A few species (*Lb. curvatus, Lb. sakei*) produce an enzyme racemase. This enzyme converts L(+)-lactic acid to D(–)-lactic acid (Axcelsson 2004). The starter bacteria and their production of lactic acid isomer can be seen in Table 1 (Krieg *et al.* 2010).

Lactic acid is normally present in the blood of mammals due to the activity of gastrointestinal (GIT) microflora (Ewaschuk *et al.* 2005) or due to glycogen cleavage (Gleeson & Dalessio 1990). Increased amount of D(–)-lactic acid in blood serum, ≥ 3 mmol/l, can cause D-lactic acidosis. This metabolic disease occurs more often in humans suffering from short-bowel syndrome (Bongaerts *et al.* 1997; Uribarri *et al.* 1998; Ewaschuk *et al.* 2005). The patients with D(–)-lactic acid acidosis can exhibit neurological dysfunctions characterised by ataxia, slurred speech, and confusion. Hallucination, sleepiness, clumsiness, lethargy, and dizziness can occur as well (Ewaschuk *et al.* 2005). The main aim of this work was to evaluate the parameters influencing the formation of organic acids and the ratio of optical isomers of lactic acid during the fermentation of milk, and to design milk beverage fermented with ABT culture (*Lactobacillus acidophilus*, *Bifidobacterium* sp., *Streptococcus thermophilus*) with a lowered content of D(–)-lactic acid.

MATERIAL AND METHODS

Materials. UHT skimmed milk (Mlékárna Hlinsko s.r.o., Czech Republic) was used as the basic medium for the fermentation with bacterial starters. This medium was supplemented with dried skimmed milk (VENTUS-ALIANCE, Prague, Czech Republic) or commercial whey protein concentrate (Volac, Hertfordshire, UK) for some samples. *Lactobacillus acidophilus* CCDM 151, *Streptococcus thermophilus* CCDM 144, and *Bifidobacterium* sp. CCDM 94 were used as liquid or frozen commercial starters in milk-based medium (Laktoflora®, MILCOM a.s., Prague, Czech Republic). Liquid starters were stored at 4–6°C, freeze-shocked starters at –43°C and these were thawed at ambient temperature before use.

Preparation of the media. The media were prepared as 100 ml portions of UHT milk filled in sterile bottles with the addition of 2% v/v yeast extract (YE) for separate cultivation of lactobacilli and bifidobacteria. Dried skimmed milk or whey protein concentrate were added to the media to determine the influence of proteins. These media were pasteurised in a water bath at 85°C/10 minutes.

Influence of the starter forms. The prepared milk media (with the addition of 2% v/v YE for lactobacilli and bifidobacteria) were inoculated with liquid or frozen commercial starters with inoculum 1% v/v and fermented at 37°C/17 ± 0.5 hours.

Influence of the inoculum. Inocula 0.1, 0.5, 1, 2.5, and 5% v/v of liquid starters were used in this part of experiment the respective. Milk samples (with the addition of 2% v/v YE for lactobacilli and bifidobacteria) were fermented at 37°C/17 ± 0.5 h after inoculation.

Influence of temperature and time of fermentation. The effects of the temperature and time of fermentation were determined at 30, 37, and 43°C for 13 ± 0.5, 15 ± 0.5, 17 ± 0.5, 19 ± 0.5, and 21 ± 0.5 hours. The prepared milk media (with the addition of 2% v/v YE for lactobacilli and bifidobacteria) were inoculated with 1% v/v starters and fermentation was carried out at each temperature for each time period. Fermentation was stopped by rapid cooling to 4–6°C.

Influence of proteins content. The milk media (with the addition of 2% v/v YE for lactobacilli and bifidobacteria) enriched with non-fat dry matter or whey protein were used to determine the influence of the protein content. 6, 12, or 18% w/w of dried skimmed milk was added to the basic milk media to achieve 5.3, 7.5 or 9.6% content of proteins, respectively. 6, 8, 10, or 12% w/w of whey protein concentrate were added to the milk media to achieve 8.0, 9.6, 11.2, or 12.8% content of proteins, respectively. The prepared media were inoculated with 1% v/v of liquid starters and fermentation was carried out at 37°C for 17 ± 0.5 hours.

Preparation of the ABT fermented milk with a low content of D(–)-lactic acid. Fermented milk beverage with ABT culture was prepared from milk medium with the addition of 6% w/w dried skimmed milk, without the addition of yeast extract. This medium was inoculated with liquid starters, namely 2.5% v/v of *Bifidobacteria* sp. CCDM 94, 1% v/v of *Streptococcus thermophilus* CCDM 144, and 2.5% of *Lactobacillus acidophilus* CCDM 151. Fermentation was carried out at 37°C for 17 ± 0.5 hours.

Analysis of all samples. The densities of the starter microorganisms, pH, and concentration of L(+)-lactic, D(–)-lactic, acetic, and formic acids were evaluated. All samples were prepared and measured three times. *Lactobacillus acidophilus* was determined according to IDF Standard 149A (1997) using diagnostic medium MRS (pH 5.7). The cultivation was carried out at 37°C/3 days anaerobically. *Streptococcus thermophilus* was determined according to International Standard ISO 7889 (2003) on medium M 17 at 37°C/2 days. Bifidobacteria were determined according to International Standard ISO 29981 (2010) using medium TOS with the addition of mupirocin at 37°C/3 days anaerobically. The contents of lactic acid isomers were determined by enzymatic kit K-DLATE (Megazyme International Ireland, Bray, Ireland) and total amount of organic acids (lactic, acetic, and formic acids) was determined by isotachophoresis IONOSEP 2003 (RECMAN, Ostrava, Czech Republic) according to the application list No. 47 (RECMAN 2008).

Statistical analysis. Statistical analysis was performed using MS Excel 2007 (Microsoft Corporation, Redmond, USA). The results are presented as the arithmetic means of three parallel samples. The outliers were removed from the obtained data by Grubbs' test and the results were evaluated by ANOVA test on the level of significance $P(\alpha) = 0.05$.

RESULTS AND DISCUSSION

Form of starters and inoculum size. Obvious difference between the frozen and liquid starters can be seen for all strains tested (Table 2). The liquid starters provided a higher cell density and a higher amount of organic acids and especially with *Bifidobacterium* sp. CCDM 94 it resulted in a fermentation profile closer to the theoretical ratio of lactic acid to acetic acid, which is 0.67 (White 2007). *Lb. acidophilus* is supposed to form racemic mixture of optical isomers of lactic acid (Kandler & Weiss 2010) and the results obtained matched this premise more closely with the liquid starter.

The higher was the inoculum (maximally 2.5% v/v) the higher density (Table 3) and the lower ratio of D(–)-lactic acid/L(+)-lactic acid (Table 3) were achieved with bifidobacteria and lactobacilli while streptococci were unaffected. The inoculum of bifidobacteria or lactobacilli had a significant effect on the pH value and ratio of organic acids. Higher inoculum of bifidobacteria or lactobacilli led to abundant production of organic acids, lower pH, and more favourable fermentation profiles, that means a lower ratio of D(–)-lactic acid/L(+)-lactic acid and a lower ratio of lactic acid/acetic acid. Acetic acid provides specific flavour to the product. When 1% v/v

Table 2. Influence of the form of starter on formation of organic acids (n = 3)

Type of culture		log CFU/ml	D/L[1]	Lactic/acetic[2]	Total amount of organic acids[3]	pH
Bifidobacterium sp. CCDM 94	frozen	7.55 ± 0.07	0.015 ± 0.001	75.81 ± 0.99	61.74 ± 1.03	5.25 ± 0.00
	liquid	8.57 ± 0.06	0.054 ± 0.008	1.67 ± 0.05	107.10 ± 0.84	4.42 ± 0.02
Streptococcus thermophilus CCDM 144	frozen	7.93 ± 0.04	0.013 ± 0.001	43.62 ± 3.68	96.20 ± 1.54	4.36 ± 0.03
	liquid	8.51 ± 0.09	0.012 ± 0.002	111.02 ± 2.30	133.50 ± 0.17	4.26 ± 0.01
Lactobacillus acidophilus CCDM 151	frozen	7.97 ± 0.01	0.013 ± 0.001	16.74 ± 0.45	41.09 ± 1.34	5.71 ± 0.01
	liquid	8.65 ± 0.03	0.319 ± 0.058	11.89 ± 0.23	79.47 ± 1.55	4.04 ± 0.03

[1]ratio D(–)-lactic acid/L(+)-lactic acid; [2]ratio lactic acid/acetic acid; [3]amount of acetic acid, lactic acid and formic acid (mg/100 g)

Table 3. Influence of the starter inoculum on formation of organic acids (n = 3)

Culture	Inoculum (%)	log CFU/ml	D/L[1]	Lactic/acetic[2]	Total amount of organic acids[3]	pH
Bifidobacterium sp. CCDM 94	0.1	6.12 ± 0.06	0.187 ± 0.046	7.82 ± 0.44	29.53 ± 1.42	6.46 ± 0.03
	0.5	7.76 ± 0.53	0.283 ± 0.165	1.16 ± 0.01	29.09 ± 0.05	6.16 ± 0.02
	1.0	8.57 ± 0.06	0.054 ± 0.008	1.67 ± 0.05	107.10 ± 0.84	4.42 ± 0.02
	2.5	9.17 ± 0.04	0.038 ± 0.004	1.19 ± 0.01	74.84 ± 0.36	4.82 ± 0.01
	5	8.97 ± 0.01	0.023 ± 0.003	1.27 ± 0.02	125.47 ± 0.20	4.47 ± 0.01
Streptococcus thermophilus CCDM 144	0.1	7.93 ± 0.01	0.013 ± 0.001	98.16 ± 0.72	121.68 ± 0.54	4.44 ± 0.02
	0.5	8.58 ± 0.03	0.012 ± 0.001	96.35 ± 1.31	123.27 ± 0.33	4.28 ± 0.01
	1.0	8.51 ± 0.09	0.012 ± 0.002	111.02 ± 2.30	133.50 ± 0.17	4.26 ± 0.01
	2.5	9.36 ± 0.05	0.013 ± 0.001	91.82 ± 0.97	130.58 ± 0.94	4.22 ± 0.01
	5	8.22 ± 0.10	0.015 ± 0.002	100.09 ± 0.10	129.05 ± 0.42	4.36 ± 0.02
Lactobacillus acidophilus CCDM 151	0.1	8.81 ± 0.01	0.514 ± 0.016	9.79 ± 0.13	126.08 ± 1.29	4.07 ± 0.02
	0.5	8.05 ± 0.04	0.342 ± 0.023	22.76 ± 2.39	36.74 ± 0.20	4.05 ± 0.02
	1.0	8.65 ± 0.03	0.319 ± 0.058	11.89 ± 0.23	79.47 ± 1.55	4.04 ± 0.03
	2.5	9.41 ± 0.05	0.179 ± 0.020	29.45 ± 0.54	32.16 ± 0.13	4.01 ± 0.02
	5.0	9.03 ± 0.04	0.177 ± 0.039	35.89 ± 0.50	77.08 ± 0.36	4.95 ± 0.02

[1]ratio D(–)-lactic acid/L(+)-lactic acid; [2]ratio lactic acid/acetic acid; [3]amount of acetic acid, lactic acid and formic acid (mg/100 g)

of bifidobacteria was used, the ratio of D(–)-lactic acid to L(+)-lactic acid was 0.05. When 5% v/v used, the ratio was 0.02.

Temperature and time of cultivation. Optimal temperature for cultivation was 37°C. Under this condition maximal density was reached after fermentation for 17 ± 0.5 hours. However, the metabolic activity of bifidobacteria continued and pH below 4.6 was not achieved within 21 ± 0.5 h (Table 4). Slower acidification by bifidobacteria need not be a problem in a fermented dairy product with mixed culture. *Str. thermophilus* grew well and reached the desired pH even after fermentation at 37°C for 13 ± 0.5 h (Table 4). The results obtained with *Lactobacillus acidophilus* CCDM 151 can be seen in Table 4. Fermentation at 37°C for 17 ± 0.5 h seems to be the optimal choice for the mixture of all strains tested.

The fermentation profiles were slightly affected by temperature. Optimal conditions at 37°C for 17 ± 0.5 h led to a lower lactic acid/acetic acid ratio in bifidobacteria and substantially higher lactic acid/acetic acid ratio in streptococci and lactobacilli.

Content of proteins. The addition of dried skimmed milk or whey protein concentrate as sources of proteins led to a higher production of organic acids due to the buffering capacity of proteins (Tables 5 and 6). Opposite effects were described by Němečková *et al.* (2011) for vegetable substrates with lower contents of proteins in comparison with milk. Significantly lower amounts of acids were formed in vegetable substrates.

The growth of *Bifidobacterium* sp. CCDM 94 was supported particularly by the addition of dried skimmed milk up to 12 g/100 g milk and only slightly by the addition of whey protein concentrate due to the buffering effect of proteins and promoting effect of other substances present in skimmed milk, e.g. oligosaccharides. On the contrary, *Lb. acidophilus* CCDM 151 grew better in the presence of whey protein concentrate than in the samples containing dried skimmed milk, probably due to its proteolytic features and utilisation of amino-acids as described by Christensen *et al.* (1999). The growth of *Str. thermophilus* CCDM 144 was unaffected by the addition of protein sources.

The only effect on the fermentation profiles in connection with protein sources was that of *Str. thermophilus* CCDM 144. The addition of whey protein concentrate decreased the lactic acid/acetic acid ratio.

ABT fermented milk. Milk with the addition of 6% w/w of dried skimmed milk was inoculated with 2.5% v/v *Bifidobacterium* sp. CCDM 94, 1% v/v *Str. thermophilus* CCDM 144, and 2.5% v/v *Lb. acidophilus* CCDM 151, and the fermented at 37°C/17 ± 0.5 hours. The yeast extract used in the previous test as a source of amino acids, short peptides, B-complex vitamins, carbon-sources and other compounds which stimulate

Table 4. The influence of temperature and cultivation time of *Bifidobacterium* sp. CCDM 94, *Streptococcus thermophilus* CCDM 144, and *Lactobacillus acidophilus* CCDM 151 on formation of organic acids (n = 3)

	Cultivation time (h)	Temperature (°C)	log CFU/ml	D/L[1]	Lactic/acetic[2]	Total amount of organic acids[3]	pH
Bifidobacterium sp. CCDM 94	13 ± 0.5	30	7.63 ± 0.03	0.084 ± 0.006	2.09 ± 0.006	50.55 ± 0.92	6.05 ± 0.02
		37	7.53 ± 0.03	0.071 ± 0.007	2.18 ± 0.01	61.26 ± 0.31	5.25 ± 0.03
		43	8.34 ± 0.06	0.041 ± 0.002	1.33 ± 0.13	59.21 ± 2.41	4.67 ± 0.02
	15 ± 0.5	30	7.38 ± 0.08	0.319 ± 0.029	1.66 ± 0.02	32.91 ± 0.41	6.35 ± 0.02
		37	8.15 ± 0.14	0.080 ± 0.002	2.53 ± 0.04	47.45 ± 0.36	5.46 ± 0.03
		43	8.49 ± 0.05	0.038 ± 0.001	1.45 ± 0.02	84.66 ± 0.55	4.74 ± 0.02
	17 ± 0.5	30	7.46 ± 0.06	0.029 ± 0.001	3.61 ± 0.07	28.77 ± 0.32	5.17 ± 0.00
		37	8.57 ± 0.06	0.054 ± 0.008	1.67 ± 0.05	107.10 ± 0.84	4.42 ± 0.02
		43	7.90 ± 0.01	0.022 ± 0.001	2.49 ± 0.05	33.62 ± 0.18	4.55 ± 0.01
	19 ± 0.5	30	7.60 ± 0.04	0.247 ± 0.007	3.81 ± 0.13	17.65 ± 0.29	6.23 ± 0.02
		37	8.15 ± 0.11	0.104 ± 0.005	3.76 ± 0.13	17.39 ± 0.30	5.60 ± 0.01
		43	7.74 ± 0.04	0.088 ± 0.003	14.15 ± 0.46	34.87 ± 1.05	5.33 ± 0.02
	21 ± 0.5	30	7.43 ± 0.07	0.946 ± 0.047	1.33 ± 0.02	43.42 ± 0.17	6.18 ± 0.01
		37	8.29 ± 0.05	0.051 ± 0.005	1.36 ± 0.02	112.60 ± 1.19	4.46 ± 0.01
		43	6.45 ± 0.05	0.015 ± 0.001	6.61 ± 0.06	122.24 ± 0.15	4.35 ± 0.02
Streptococcus thermophilus CCDM 144	13 ± 0.5	30	8.52 ± 0.05	0.009 ± 0.001	54.77 ± 3.27	80.43 ± 0.32	4.83 ± 0.02
		37	8.32 ± 0.13	0.013 ± 0.001	155.99 ± 0.85	99.80 ± 0.44	4.27 ± 0.01
		43	7.94 ± 0.02	0.049 ± 0.004	82.20 ± 3.55	95.11 ± 0.64	4.24 ± 0.02
	15 ± 0.5	30	8.20 ± 0.13	0.015 ± 0.001	86.29 ± 0.06	87.26 ± 0.51	4.36 ± 0.00
		37	8.05 ± 0.08	0.008 ± 0.001	312.16 ± 10.12	105.98 ± 0.44	4.22 ± 0.02
		43	7.57 ± 0.04	0.011 ± 0.001	54.23 ± 2.56	106.71 ± 0.17	6.27 ± 0.00
	17 ± 0.5	30	8.60 ± 0.06	0.022 ± 0.002	105.34 ± 2.59	69.80 ± 0.28	4.25 ± 0.01
		37	8.51 ± 0.09	0.012 ± 0.002	111.02 ± 2.30	133.50 ± 0.17	4.26 ± 0.01
		43	7.62 ± 0.04	0.021 ± 0.001	110.36 ± 2.50	79.75 ± 0.58	4.18 ± 0.01
	19 ± 0.5	30	7.65 ± 0.04	0.029 ± 0.003	29.16 ± 0.28	54.51 ± 0.74	4.45 ± 0.02
		37	7.92 ± 0.01	0.671 ± 0.035	55.89 ± 0.64	88.41 ± 0.47	4.39 ± 0.01
		43	7.87 ± 0.02	0.012 ± 0.001	75.64 ± 0.95	99.86 ± 0.04	4.16 ± 0.01
	21 ± 0.5	30	7.74 ± 0.04	0.035 ± 0.001	67.91 ± 1.24	120.74 ± 0.14	4.43 ± 0.03
		37	8.44 ± 0.03	0.012 ± 0.001	316.14 ± 9.61	105.31 ± 0.67	4.18 ± 0.01
		43	7.55 ± 0.04	0.009 ± 0.001	54.91 ± 0.77	88.86 ± 0.41	4.16 ± 0.01
Lactobacillus acidophilus CCDM 151	13 ± 0.5	30	8.53 ± 0.50	0.069 ± 0.004	8.27 ± 0.07	48.54 ± 0.26	5.64 ± 0.02
		37	8.61 ± 0.04	0.098 ± 0.010	34.47 ± 1.37	34.74 ± 0.23	5.53 ± 0.00
		43	8.49 ± 0.03	0.055 ± 0.004	29.71 ± 1.69	48.20 ± 0.65	4.00 ± 0.02
	15 ± 0.5	30	96.91 ± 0.02	0.025 ± 0.001	30.70 ± 0.43	45.27 ± 0.14	6.27 ± 0.00
		37	7.89 ± 0.03	0.011 ± 0.001	13.94 ± 0.09	88.46 ± 0.16	5.46 ± 0.02
		43	8.33 ± 0.11	0.007 ± 0.001	15.89 ± 0.17	72.83 ± 0.80	5.24 ± 0.01
	17 ± 0.5	30	8.03 ± 0.04	0.331± 0.018	9.68 ± 0.06	54.36 ± 0.26	5.20 ± 0.01
		37	8.65 ± 0.02	0.319 ± 0.058	11.89 ± 0.23	79.47 ± 1.55	4.04 ± 0.03
		43	8.56 ± 0.04	0.715 ± 0.022	10.34 ± 0.07	152.65 ± 0.42	3.99 ± 0.01
	19 ± 0.5	30	7.34 ± 0.08	0.070 ± 0.004	20.30 ± 1.30	28.40 ± 0.41	5.89 ± 0.01
		37	8.43 ± 0.06	0.010 ± 0.001	24.50 ± 0.53	26.92 ± 0.19	5.20 ± 0.02
		43	8.23 ± 0.09	0.007 ± 0.001	26.14 ± 1.11	37.58 ± 0.76	5.00 ± 0.02
	21 ± 0.5	30	6.22 ± 0.12	0.480 ± 0.014	24.76 ± 0.16	25.05 ± 0.05	6.27 ± 0.02
		37	8.85 ± 0.02	0.181 ± 0.002	38.54 ± 0.35	33.62 ± 0.37	4.73 ± 0.02
		43	9.36 ± 0.10	0.172 ± 0.004	25.36 ± 0.27	43.42 ± 0.36	3.95 ± 0.00

[1]ratio D(–)-lactic acid/L(+)-lactic acid; [2]ratio lactic acid/acetic acid; [3]amount of acetic acid, lactic acid and formic acid (mg/100 g)

Table 5. The influence of addition of dried skimmed milk as a source of proteins on formation of organic acids (n = 3)

Culture	Addition of dried milk (%)	log CFU/ml	D/L[1]	Lactic/acetic[2]	Total amount of organic acids[3]	pH
Bifidobacterium sp. CCDM 94	0	8.57 ± 0.06	0.054 ± 0.008	1.67 ± 0.05	107.10 ± 0.84	4.42 ± 0.02
	6	9.71 ± 0.02	0.013 ± 0.003	1.49 ± 0.02	167.65 ± 0.50	4.47 ± 0.02
	12	10.32 ± 0.08	0.016 ± 0.002	2.93 ± 0.01	244.87 ± 1.23	4.55 ± 0.02
	18	9.58 ± 0.04	0.020 ± 0.003	1.75 ± 0.01	369.10 ± 1.45	4.72 ± 0.01
Streptococcus thermophilus CCDM 144	0	8.51 ± 0.09	0.012 ± 0.002	111.02 ± 2.30	133.50 ± 0.17	4.26 ± 0.01
	6	8.28 ± 0.09	0.036 ± 0.003	182.88 ± 1.58	153.99 ± 0.46	4.47 ± 0.03
	12	8.56 ± 0.04	0.008 ± 0.001	112.99 ± 1.86	157.05 ± 1.70	4.45 ± 0.02
	18	9.08 ± 0.08	0.021 ± 0.001	179.94 ± 0.63	416.10 ± 0.90	4.52 ± 0.02
Lactobacillus acidophilus CCDM 151	0	8.65 ± 0.03	0.319 ± 0.058	11.89 ± 0.23	79.47 ± 1.55	4.04 ± 0.03
	6	8.75 ± 0.02	0.046 ± 0.003	80.79 ± 0.66	115.84 ± 1.48	5.02 ± 0.03
	12	9.66 ± 0.04	0.157 ± 0.013	30.78 ± 0.21	211.27 ± 0.72	4.83 ± 0.02
	18	8.68 ± 0.02	0.173 ± 0.010	60.82 ± 0.19	202.15 ± 0.44	5.05 ± 0.03

[1]ratio D(–)-lactic acid/L(+)-lactic acid; [2]ratio lactic acid/acetic acid; [3]amount of acetic acid, lactic acid and formic acid (mg/100 g)

the growth of bifidobacteria (Russell *et al.* 2011) and lactobacilli (Altaf *et al.* 2005) was not necessary in this case. The reason was the interaction between the starter bacteria. Streptococci lowered the redox potential (Brasca *et a*l. 2007), which supported the growth of bifibobacteria (Bolbuc *et al.* 2006) and lactobacilli. On the contrary, lactobacilli released amino acids from milk proteins (Lane & Fox 1996) which stimulated the growth of streptococci (Ashraf & Shah 2011) and bifidobacteria (Bolbuc *et al.* 2006). The conditions used provided satisfactory density and pH (Table 7). The ratio D(–)-lactic acid/L(+)-lactic acid was 0.28 and total amount of organic acids was 180 g/100 g.

CONCLUSION

The influence of the cultivation conditions on the quality of milk fermented by *Lactobacillus acidophilus* CCDM 151, *Streptococcus thermophilus* CCDM 144,

Table 6. The influence of addition of whey protein concentrate (WPC) as a source of proteins on formation of organic acids (n = 3)

Culture	Addition WPC (%)	log CFU/ml	D/L[1]	Lactic/acetic[2]	Total amount of organic acids[3]	pH
Bifidobacterium sp. CCDM 94	0	8.57 ± 0.06	0.054 ± 0.008	1.67 ± 0.05	107.10 ± 0.84	4.42 ± 0.02
	6	9.10 ± 0.07	0.018 ± 0.002	1.06 ± 0.00	145.91 ± 0.19	4.45 ± 0.02
	8	9.20 ± 0.06	0.009 ± 0.002	1.23 ± 0.00	135.52 ± 0.38	4.53 ± 0.04
	10	9.34 ± 0.06	0.008 ± 0.001	1.02 ± 0.00	165.73 ± 0.26	4.38 ± 0.01
	12	9.19 ± 0.09	0.009 ± 0.002	1.10 ± 0.01	189.13 ± 0.91	4.57 ± 0.00
Streptococcus thermophilus CCDM 144	0	8.51 ± 0.09	0.012 ± 0.002	111.02 ± 2.30	133.50 ± 0.17	4.26 ± 0.01
	6	8.36 ± 0.02	0.005 ± 0.001	95.89 ± 2.86	118.97 ± 2.07	4.50 ± 0.01
	8	8.39 ± 0.04	0.004 ± 0.001	64.93 ± 0.18	115.09 ± 0.09	4.40 ± 0.01
	10	8.46 ± 0.03	0.003 ± 0.001	59.30 ± 0.31	124.58 ± 0.08	4.42 ± 0.02
	12	8.72 ± 0.03	0.004 ± 0.001	40.08 ± 0.95	119.27 ± 1.95	4.50 ± 0.02
Lactobacillus acidophilus CCDM 151	0	8.65 ± 0.03	0.319 ± 0.058	11.89 ± 0.23	79.47 ± 1.55	4.04 ± 0.03
	6	10.12 ± 0.11	0.452 ± 0.030	36.17 ± 0.33	308.65 ± 1.27	3.86 ± 0.01
	8	10.09 ± 0.09	0.446 ± 0.017	61.29 ± 0.54	249.59 ± 0.66	3.85 ± 0.02
	10	9.69 ± 0.05	0.494 ± 0.014	60.80 ± 0.74	239.24 ± 2.20	3.91 ± 0.01
	12	9.77 ± 0.03	0.498 ± 0.024	55.46 ± 0.06	256.65 ± 1.30	4.00 ± 0.01

[1]ratio D(–)-lactic acid/L(+)-lactic acid; [2]ratio lactic acid/acetic acid; [3]amount of acetic acid, lactic acid and formic acid (mg/100 g)

Table 7. The parameters of ABT fermented milk (n = 3)

Culture	log CFU/ml	D/L[1]	Lactic/acetic[2]	Total amount of organic acids[3]	pH
Bifidobacterium sp. CCDM 94	8.63 ± 0.04				
Streptococcus thermophilus CCDM 144	8.78 ± 0.02	0.028 ± 0.001	3.51 ± 0.07	179.87 ± 1.37	4.06 ± 0.05
Lactobacillus acidophilus CCDM 151	8.32 ± 0.03				

[1]ratio D(–)-lactic acid/L(+)-lactic acid; [2]ratio lactic acid/acetic acid; [3]amount of acetic acid, lactic acid and formic acid

and *Bifidobacterium* sp. CCDM 94 was evaluated. The form of starter, inoculum size, temperature and time of cultivation, and addition of dried skimmed milk or whey protein concentrate were tested. The optimal conditions for fermented milk beverage with ABT culture were chosen according to the results obtained. These conditions implied the addition of 6% of dried skimmed milk to the basic milk medium, inoculum of 2.5% v/v of *Lb. acidophilus* CCDM 151, 2.5% v/v of *Bifidobacterium* sp. CCDM 94, and 1% v/v of *Str. thermophilus* CCDM 144, and fermentation at 37°C/17 ± 0.5 hours. ABT fermented milk had a low ratio of D(–)-lactic acid to L(+)-lactic acid (0.025 ± 0.001), sufficient density of the starter bacteria (8.63 ± 0.04 log CFU/ml of bifidobacteria, 8.78 ± 0.02 log CFU/ml of streptococci and 8.32 ± 0.03 log CFU/ml of lactobacilli), and a low pH (4.06 ± 0.05). Further studies should be done to verify the palatability and texture of ABT fermented milk before possible commercial applications.

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