Effect of fatty acids on the β -oxidation system and thioesterase of *Lactococcus lactis* subspecies *lactis*

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ABSTRACT

The influence of fatty acids on the β -oxidation system and thioesterase of *Lactococcus lactis* was investigated in this study. The results showed that fatty acids (C8:0–C16:0) significantly inhibited the growth of *Lactococcus lactis*, and laurate (C12:0) had the highest bactericidal effects. We detected the maximum activity of β -oxidation at different incubation times (8, 12, and 18 h) to be 6.460, 7.751, and 8.203, respectively, and the maximum activity of thioesterase at different incubation times (8, 12, and 18 h) to be 19.498, 27.180, and 12.800, respectively. Fatty acids were seen to induce the β -oxidation system and activity of thioesterase; decanoic acid (C10:0) and palmitic acid (C16:0) were also seen to induce the β -oxidation system of *Lactococcus lactis*, but the induced ability was significantly different. Octanoic acid (C8:0) and palmitic acid (C16:0) were seen to induce thioesterase activity in *Lactococcus lactis*. When 1 mM palmitic acid (C16:0) was added to M17 broth, the activity of thioesterase increased 5-fold after 2 min; however, adding octanoic acid (C8:0) changed the activity little. Evidence showed that the ability to induce the β -oxidation system and thioesterase activity was related to the fatty acids' chain lengths.

Key words: fatty acid, β -oxidation system, thioesterase, *Lactococcus lactis* ssp. *lactis*

INTRODUCTION

Free fatty acids released as a result of milk fat lipolysis are important flavors in food, but they must be counter-balanced with other flavor compounds to develop a pleasing aroma (Bosset and Gauch, 1993; Fox et al., 1995). Free fatty acids are substrates of enzymatic reactions yielding flavors (Marilley, 2004) and can be metabolized to produce many flavor compounds, such as methyl ketones, lactones, esters, and secondary alcohols (Fox and Wallace, 1997).

Methyl ketones are important flavor compounds, particularly in cheese (Urbach, 1997). In cheese made from pasteurized milk, lactic acid bacteria are a major source of lipolytic enzymes (Boumerdassi et al., 1997). Cheese made with a starter system containing a blend of 2 commercial *Lactococcus lactis* strains with the highest levels of 2-pentanone and 2-heptanone highlighted the effect of starter *Lc. lactis* on methyl ketones (Hannon et al., 2007). Therefore, *Lc. lactis* has a positive effect on methyl ketones in most dairy products.

Methyl ketones are formed from incomplete β -oxidation; the steps include β -oxidation of the released FFA to β -ketoacyl-CoA, which are then deacylated into β -ketoacids under the action of the thioesterases, and then the keto acids are decarboxylated to methyl ketones (Engelvin et al., 2000; Town et al., 2000). Many studies have researched the β -oxidation of fungi such as *Aspergillus niger* (Baltazar et al., 1999), *Penicillium roqueforti* (Chalier and Crouzet, 1998), *Sporidiobolus* spp. (Feron et al., 2005), and *Yarrowia lipolytica* (Groguein, 2004). Studies on bacteria were less frequent, however, with research available for strains such as *Staphylococcus carnosus* (Engelvin et al., 2000; Groguein, 2004) and *Caulobacter crescentus* (O'Connell, 1986); no information about *Lc. lactis* was found, however. *Lactococcus lactis* is widely used in food industry for production of fermented milk products (Sirén et al., 2009) such as fresh and semi-hard cheeses and sour milks (Samaržija et al., 2001). Therefore, it is very important to study the role of *Lc. lactis* in the production of methyl ketones.

In the present study, we attempted to prove that *Lc. lactis* had the ability to generate methyl ketone by measuring β -oxidation capacity and thioesterase activity; this process could be induced by FFA of different chain lengths. The results of this research will provide a pre-theoretical basis for the future regulation of a *Lc. lactis*-methyl ketone generation system.

MATERIALS AND METHODS

Chemicals and Culture Media

Coenzyme A (CoA; trilithium salt) and palmitoyl-CoA were purchased from Sigma Chemical Co.

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(Shanghai, China); 5,5'-dithio-bis(2-nitrobenzoic acid; **DNTB**), HEPES, phenylmethylsulfonyl fluoride (**PMSF**), EDTA, and NAD^+ were purchased from Aladdin Chemistry Co. (Shanghai, China). Enhanced bicinchoninic acid (**BCA**) Protein Assay Kit was purchased from Beyotime Institute of Biotechnology (Jiangsu, China).

Microorganism

Lactococcus lactis ssp. *lactis* was grown in M17 broth medium (Hope Bio-Technology Co., Qingdao, China) at 37°C. Cell density was measured by using a spectrophotometer (TU-1800 Pgeneral Instrument Co. Ltd., Beijing, China) at 600 nm.

Volatile Compounds Analysis

Cream was obtained from unpasteurized bovine milk by centrifuging at $6,500 \times g$. Pasteurized cream was fermented by *Lactococcus lactis* ssp. *lactis* at 37°C and considered as fermented cream. Twenty grams of fermented cream was placed in a 100-mL vial and exposed to the headspace for 90 min at 70°C. All extractions were carried out using a solid-phase extraction (SPME) fiber (Supelco, Bellefonte, PA). The trapped compounds were injected onto the column of an HP 5890 Gas Chromatograph (Hewlett-Packard, Palo Alto, CA) equipped with DB-5 capillary column (60 m $\times$ 0.25 mm $\times$ 0.25 mm film thickness). Volatile compounds were separated under the following conditions: carrier gas was helium at 1 mL/min, and initial temperature was 60°C held for 3 min, heated to 150°C at 6°C/min, followed by heating to 230°C at 4°C/min. The GC column was connected without splitting to the ion source of a mass spectrometer. Ionization was performed by electron impact at 70 eV. Quantification was performed by integrating the peak areas of total ion chromatograms by the GC-MS software.

FFA as Growth Factors on *Lc. lactis*

Hexanoic acid (C6:0), octanoic acid (C8:0), decanoic acid (C10:0), lauric acid (C12:0), myristic acid (C14:0), and palmitic acid (C16:0; purity 97%, Aladdin Chemistry Co.) were used as FFA supplements in M17 broth media for growth tests; FA stock solutions (0.25 M) were made in absolute ethanol and further sterilized by membrane filtration (0.22 μm). Fatty acid stock solutions were added to the fresh sterile M17 medium to a final amount of 1 mM. After addition of inoculum (4.0% vol/vol), culture was mixed and incubated at 37°C. Growth tests were performed as optical density

value by a spectrophotometer (TU-1800 General Instrument Co. Ltd.) at 600 nm.

Preparation of Cell-Free Extracts

Cell-free extracts (**CFE**) were performed as described by Engelvin et al. (2000) with modifications. Cells were harvested at $5,000 \times g$ for 10 min at 4°C, washed with $\text{K}_2\text{HPO}_4/\text{KH}_2\text{PO}_4$ (100 mM, pH 7.5), and sonicated in HEPES buffer (20 mM, pH 7.5) containing 1 mM EDTA and 1 mM PMSF using a JY92-II (Ningbo Scientz Biotechnology Co. Ltd., Ningbo, China) ultrasonic homogenizer (30 cycles of 10 s on and 10 s off at 300 W). Unbroken bacteria were removed by centrifugation at $5,000 \times g$ for 10 min at 4°C. The supernatant fraction was designated crude CFE and was used immediately or stored at -80°C . The amount of protein in the CFE was determined by Enhanced BCA Protein Assay Kit.

β -Oxidation Assays

Assay of β -oxidation was completed by measuring the 3-hydroxyacyl-CoA dehydrogenase (EC 1.1.1.35) activity, which was determined using acyl-CoA as substrate. The assay mixture contained 72 nmol of CoA, 1.7 μmol of NAD^+ , 72 nmol of palmitoyl-CoA, 280 μg of CFE protein, and $\text{K}_2\text{HPO}_4/\text{KH}_2\text{PO}_4$ (300 mM, pH 8.0) in a final volume of 1 mL. The reduction of NAD was measured by its absorption at 340 nm against a control sample without substrates. The β -oxidation activity was expressed as nanomoles of NAD^+ reduced per milligram of protein per minute [$\epsilon_{\text{NAD}}(340) = 6,220 \text{ M}^{-1} \text{ cm}^{-1}$; Engelvin et al., 2000].

Thioesterase Activity

Thioesterase activity (EC 3.1.2.2) was measured by the release of coenzyme A (CoASH), which was assayed continuously by its reaction with DTNB. The assay contained, in a final volume of 1 mL, $\text{K}_2\text{HPO}_4/\text{KH}_2\text{PO}_4$ (300 mM, pH 8.0), 250 nmol of DTNB, 72 nmol of palmitoyl-CoA, and 280 μg of CFE protein. Activity was measured by following the increase in absorbance at 412 nm against a control without substrates [$\epsilon_{\text{DTNB}}(412) = 13,600 \text{ M}^{-1} \text{ cm}^{-1}$]. Activity was expressed as nanomoles of palmitoyl-CoA deacylated per milligram of protein per minute (Engelvin et al., 2000).

Statistical Analysis

All statistical treatments were carried out using the SPSS program for Windows (SPSS 17.0, SPSS Inc., Chicago, IL).

Figure 1. Growth curve of *Lactococcus lactis* for cells incubated at different times. Optical density (OD) at 600 nm value (●), Log₁₀cfu/mL value (×).

RESULTS AND DISCUSSION

Growth Curve of *Lc. lactis* ssp. *lactis*

Figure 1 indicates the growth curve of *Lc. lactis* on M17 medium at different incubation times. As shown in Figure 1, the lag phase was from 0 to 2 h, exponential phase was from 2 to 8 h, and stationary phase was from 8 to 24 h. This was similar to earlier findings for *Lc. lactis* UQ2 growing in supplemented whey, which entered the stationary phase after 8 h. In contrast, growth of *Lc. lactis* UQ2 in skim milk showed the beginning of the stationary phase after 6 h (García-Parra et al., 2011). Growth of *Lc. lactis* ssp. *lactis* ATCC 29146 and *Lc. lactis* IPLA 947 showed the beginning of the stationary phase after 12 h (Cárcoba et al., 2000; Valbuena et al., 2005). Cells of *Lc. lactis* ssp. *lactis* ATCC11454 entered the stationary phase after 5 or 10 h (Liu et al., 2005; Nagayasu et al., 2007). The different behaviors of growth characteristics could be caused by differences of species, broth medium nutrients, or incubation time.

Growth data of the bacteria *Lc. lactis* could be tracked by viable cell counts, optical density, and acidification rate (Ayala-Hernández et al., 2008). Optical density showed not only living bacterial biomass, which were able to multiply, but also partially autolysed or dead biomass; cell count method showed only viable

cells (Kabanova et al., 2009). Growth curve measured by optical density was in agreement with the results of cell counts measured, indicating that optical density could be used for the study of the bacterial growth in this experiment (Figure 1). According to the growth curve, this paper assayed the β -oxidation and thioesterase activity of *Lc. lactis* on incubation time at 8 h (late-exponential), 12 h (early-stationary), and 18 h (late-stationary phase).

Assessment of Volatile Compounds

Methyl ketones have typical odors and low perception thresholds (Curioni and Bosset, 2002). These ketones are important in the aroma compounds of most dairy products: 2-heptanone has a musty, sweet, moldy, varnish odor; 2-nonanone has a floral, fruity, peach odor (Frank, 2003); 2-undecanone has floral and musty notes (Delgado, 2011); and 2-tridecanone has a goat-like odor (Curioni and Bosset, 2002). Compared with cream, fermented cream made with *Lc. lactis* ssp. *lactis* had higher levels of 2-heptanone, 2-nonanone, 2-undecanone, and 2-tridecanone (Table 1). The results highlight the positive effect of *Lc. lactis* ssp. *lactis* on methyl ketones. Thus, *Lc. lactis* ssp. *lactis* may have the capacity to synthesize these compounds related to the β -oxidation pathway.

Figure 2. Progress curves observed in assays of β -oxidation at incubation times: 8 h (●), 12 h (○), and 18 h (Δ). OD = optical density.

β -Oxidation Enzyme Activity Assays

Figure 2 indicates a lag phase in β -oxidation assays (0–6 min). These results were consistent with *Corynebacterium* sp. strain 7E1C (Easterby, 1981; Broadway et al., 1992). Assay of β -oxidation was completed by detecting the NAD⁺ reduction formed at the third stage of β -oxidation; therefore, it needed a lag phase to accumulate enough free intermediate concentrations before the next phase of the reaction could be carried out (Broadway et al., 1992). In addition, different growth phases need different amounts of time to accumulate enough free intermediates, so they performed differently in the β -oxidation analysis (Figure 2).

The maximum β -oxidation activity of *Lc. lactis* in the late-exponential, early-stationary, and late-stationary phases appeared at 8, 12, and 14 min; maximum activity of β -oxidation was 6.46, 7.751, and 8.203 nmol/mg per minute, respectively. The maximum β -oxidation activity of *Lc. lactis* was higher than that of *Escherichia coli*, which was grown on amino acid and glucose (Weeks et al., 1969), and *Staphylococcus carnosus* (Engelvin et al., 2000).

Thioesterase Activity Assays

Thioesterase has been referred to in the literature as deacylase, acyl-CoA hydrolase, acyl-CoA thioester hydrolase, and acyl-CoA thioesterase (Hunt and Alexson, 2002). The acyl-CoA thioesterases are a group of enzymes that catalyze the hydrolysis of acyl-CoA to the FFA and CoA; these enzymes are localized in the cytosol (Hunt and Alexson, 2002) and they regulate concentrations of FA and acyl-CoA due to hydrolysis of acyl-CoA (Veronika et al., 2011).

Thioesterase activity assay curves had no lag period (as shown in Figure 3); the thioesterase activity had the effect of rapidly decreasing the acyl-CoA substrate concentration so that assays were only linear for 3 to 4 min (Broadway et al., 1992). Maximum activity of

Table 1. Methyl ketones analysis of fermenting cream

Item	Ketone	Peak area ¹
Cream	2-Heptanone	0.39
	2-Nonanone	0.83
	2-Undecanone	1.05
	2-Tridecanone	0.44
Fermented cream	2-Heptanone	0.72
	2-Nonanone	1.88
	2-Undecanone	1.47
	2-Tridecanone	1.13

¹Peak area (%) = (compound_{peak area}/total compound_{peak area})

Figure 3. Progress curves observed in assays of thioesterase activity at incubation times: 8 h (●), 12 h (○), and 18 h (△). OD = optical density.

thioesterase with different incubation times (8, 12, and 18 h) were 19.498, 27.180, and 12.800, respectively. In different incubation times, the maximum activity of thioesterase appears at 2 or 4 min (Figure 4); this is similar to the measurement time of *Campylobacter jejuni* (0–5 min; Yokoyama et al., 2009). After 4 min, thioesterase activity rapidly decreased; a feedback inhibitor for thioesterase activity might have occurred, as other studies have shown that CoA was a feedback inhibitor for thioesterase separated from mouse liver, pig heart (Hunt and Alexson, 2002), and *Escherichia coli* (Zhuang et al., 2008).

FFA As Growth Factors on *Lc. lactis*

Lactococcus lactis were inhibited by FA from C8:0 to C16:0, and laurate (12:0) had the highest bactericidal effect (Table 2). These results were not completely consistent with the report of *Lactobacillus leichmanii*, in which growth was inhibited by FA from 8:0 to 12:0 (de Kairúz et al., 1983). The bactericidal effect increased significantly when laurate concentration was increased from 0.25 to 0.5 mM, but increased slowly when laurate concentration was increased from 0.5 to 2 mM (Table 3). These results were consistent with the report of *Lactobacillus delbrueckii* (Partanen et al., 2001).

Effect of FFA on the β -Oxidation System

The β -oxidation system of *Lc. lactis* can be induced (Figure 5) as reported previously: β -oxidation enzymes of *Aspergillus niger* were induced by growth on triolein (Baltazar et al., 1999) and those of *E. coli* were induced by long-chain FA (Campbell et al., 2003). In the current study, decanoic acid (C10:0) and palmitic acid (C16:0) did induce the β -oxidation system of *Lc. lactis* (Figure 5), but the results were different. The ability of FA to induce the enzymes of β -oxidation were a func-

Table 2. Effects of even-numbered FA on the growth of *Lactococcus lactis*

Variable	Turbidity value ¹
Control	1.425 $\pm$ 0.015 ^a
Hexanoic acid (C6:0)	1.383 $\pm$ 0.013 ^a
Octanoic acid (C8:0)	1.287 $\pm$ 0.045 ^b
Decanoic acid (C10:0)	0.946 $\pm$ 0.013 ^c
Lauric acid (C12:0)	0.032 $\pm$ 0.008 ^d
Myristic acid (C14:0)	0.359 $\pm$ 0.030 ^e
Palmitic acid (C16:0)	0.767 $\pm$ 0.055 ^d

^{a–f}Values with different superscripts in the same row differ significantly ($P < 0.05$).

¹Optical density at 600 nm; values expressed as means $\pm$ standard deviation.

Table 3. Effects of laurate content on the growth of *Lactococcus lactis*

Laurate content (mM)	Turbidity value ¹
Control (0)	1.425 ± 0.015 ^a
0.25	0.447 ± 0.071 ^b
0.5	0.065 ± 0.004 ^c
0.75	0.068 ± 0.007 ^c
1	0.071 ± 0.007 ^c
2	0.045 ± 0.021 ^c

^{a-c}Values with different superscripts in the same row differ significantly (*P* < 0.05).

¹Optical density at 600 nm.

tion of chain length (Weeks et al., 1969). The ability of *Lactobacillus* organisms to withstand the toxic effect of FA could be attributed to the presence of FA-oxidation activity in the cell. In this case, as *Lc. lactis* is gram-positive and lacks a lipopolysaccharide layer or outer membrane, FA oxidation appears to be the principal mechanism of resistance (de Kairúz et al., 1983).

Effect of FFA on the Activity of Thioesterase

Octanoic acid (C8:0) and palmitic acid (C16:0) can induce thioesterase activity of *Lc. lactis* (Table 4). When we added 1 mM palmitic acid (C16:0) into M17 broth, the activity of thioesterase increased 5-fold after

Table 4. Effects of FFA on the activity of thioesterase

Fatty acid	Assay time of thioesterase (min)	Multiple ¹
Octanoic acid (C8:0)	2	1.06
	4	1.07
Palmitic acid (C16:0)	2	5
	4	2.67

¹Enzyme activity (FFA)/enzyme activity (Control).

2 min; however, little change of octanoic acid (C8:0) was noted. We speculate that long-chain FA can induce thioesterase activity, and chain length may be related to FA-induced thioesterase ability. Of course, these findings require further data for confirmation. After induction, thioesterase activity of *Lc. lactis* was still less than that of *Campylobacter jejuni* (Yokoyama et al., 2009) and *Saccharomyces cerevisiae* (Astari et al., 2009).

CONCLUSIONS

Lactococcus lactis had an effective β-oxidation system and a higher thioesterase activity. The β-oxidation system and thioesterase activity had been induced by FA, and the different induction abilities were due to

Figure 4. Thioesterase activity at different incubation times: 8 h (●), 12 h (○), and 18 h (△).

Figure 5. Effects of FFA on the activity of β -oxidation: decanoic acid (C10:0; ○), palmitic acid (C16:0; ▲), and control without fatty acid (●).

different chain lengths. The results showed that FA (C8:0–C16:0) can significantly inhibit the growth of *Lc. lactis*, and laurate (12:0) had the highest bactericidal effect. Thus, we could use FA (such as palmitic acid) to regulate the β -oxidation and thioesterase enzyme system and subsequently regulate the production of methyl ketone. These findings have important implications for the flavor of the fat-rich food using *Lc. lactis* as starter, such as cheese, sour cream, and natural butter flavor. These findings also have important quality and economic implications for the food industry. The mechanism of FA on the β -oxidation system of *Lc. lactis* and the influence of FA- β -oxidation on methyl ketones should be studied further.

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