J. Dairy Sci. 99:1755–1761
http://dx.doi.org/10.3168/jds.2015-10549

Aldehyde dehydrogenase activity in *Lactococcus chungangensis*: Application in cream cheese to reduce aldehyde in alcohol metabolism

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ABSTRACT

Previous studies have shown that the metabolic capability of colonic microflora may be at least as high as that of the liver or higher than that of the whole human body. Aldehyde dehydrogenase (ALDH) is an enzyme produced by these bacteria that can metabolize acetaldehyde, produce from ethanol to acetate. *Lactococcus* species, which is commonly used as a starter in dairy products, was recently found to possess the ALDH gene, and the activity of this enzyme was determined. In this study, the ALDH activity of *Lactococcus chungangensis* CAU 28^T and 11 other type strains in the genus *Lactococcus* was studied. Only 5 species, 3 of dairy origin (*Lactococcus lactis* ssp. *lactis* KCTC 3769^T, *Lactococcus lactis* ssp. *cremoris* KCCM 40699^T, and *Lactococcus raffinolactis* DSM 20443^T) and 2 of non-dairy origin (*Lactococcus fujiensis* NJ317^T and *L. chungangensis* CAU 28^T), showed ALDH activity and possessed a gene encoding ALDH. All of these strains were capable of making cream cheese. Among the strains, *L. chungangensis* produced cream cheese that contained the highest level of ALDH and was found to reduce the level of acetaldehyde in the serum of mice. These results predict a promising role for *L. chungangensis* CAU28^T to be used in cheese that can be developed as functional food.

Key words: *Lactococcus*, alcohol metabolism, acetaldehyde, aldehyde dehydrogenase

INTRODUCTION

Lactococcus is well known and widely used in fermentation of foods such as cheese, yogurt, kefir, and butter. *Lactococcus* species are wildly used as starter in the production of cheese such as Cheddar (Madkor et al., 2000) and Parmesan (Fenster et al., 2003). Mixed-strain mesophilic cultures mainly consist of *Lactococcus lactis* ssp. *lactis* and *L. lactis* ssp. *cremoris*, which provide the primary acid-producing function during fermentation and also contribute to cheese ripening through production of enzymes related to proteolysis and conversion of AA into flavor compounds (Fox and Wallace, 1997). *Lactococcus raffinolactis* has also been found to play an important role in production of Moroccan soft white cheese, a traditional dairy product from Morocco (Ouadghiri et al., 2005). Lactococci have several other metabolic properties; mesophilic cultures are famously used in the production of Cheddar, Gouda, Edam, blue, Camembert, and cream cheese (Beresford et al., 2001). Cream cheese is a soft, fresh, acid-coagulated cheese produced by acidification by *Lactococcus,* used as a mesophilic lactic acid starter (Guinee et al., 1993). This type of cheese is available worldwide and is very popular in North America. It is used in several kinds of desserts, such as cheesecake, in salad dressings, and as a spread on bagels or bread. Moreover, the market share of this cheese increased from 7 to 17% between 2001 and 2005 (Bourroul, 2006). Because it is simple to manufacture and because it requires no ripening step and is processed using only whole milk enriched with cream and starter, cream cheese has been the object of much research (Buriti et al., 2007; Coutouly et al., 2014; Silva et al., 2014).

Acetaldehyde is a product of the metabolism of ethanol by many intracolonic microbes and also by the colonic mucosal cells (Salaspuro, 1998). Indeed, many aerobic bacteria representing the normal flora of the human large intestine possess significant cytosolic $NADP^+$- and NAD^+-dependent aldehyde dehydrogenase activity (Nosova et al., 1996). In accordance with their aldehyde dehydrogenase activity, these bacteria metabolize acetaldehyde to acetate. Aldehyde dehydrogenases (**ALDH**) are the group of enzymes catalyzing the conversion of aldehyde to corresponding acids by means of a virtually irreversible, NAD^+-dependent reaction and are present in a wide range of organisms, from bacteria to humans (Yoshida et al., 1998).

In recent decades, consumers have begun to demand foods that contribute directly to their health. Today, food not only satisfies hunger and provides necessary nutrients, but also prevents nutrition-related disease

Received October 21, 2015.
Accepted November 15, 2015.
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and improves the physical and mental well-being of humans (Roberfroid, 2002; Menrad, 2003). Cheese is a fermented food that is produced by microorganisms, such as lactic acid bacteria, which play a vital role in producing the physiochemical, sensory, nutritional, preservability, and safety characteristics of the final product, and which also act as probiotics for human health. The ALDH activity of lactococci, especially *Lactococcus chungangensis*, in cheese has only recently been shown; cheese can be a source of ALDH, which can decrease aldehyde levels in the human body, indicating that it shows potential to become a new choice as a functional food.

Lactococcus chungangensis CAU 28^T is a strain of *Lactococcus* spp. that is of nondairy origin; it was isolated from activated sludge (Cho et al., 2008). Previous studies have shown that it exhibits activity of several beneficial enzymes, such as aldehyde dehydrogenase (Konkit et al., 2014, 2015). The objective of the present study was to evaluate the potential ALDH activity of *L. chungangensis* in both cheese and in vivo compared with other lactococci cultures, such as *L. lactis* ssp. *lactis*, *L. lactis* ssp. *cremoris*, and *L. raffinolactis,* which are routinely used in the dairy industry.

MATERIALS AND METHODS

Bacterial Strains Cultured

Lactococcus chungangensis CAU 28^T (= KCTC 13185^T), *L. lactis* ssp. *lactis* KCTC 3769^T, *L. lactis* ssp. *cremoris* KCCM 40699^T, *L. lactis* ssp. *hordiniae* KCTC 3768^T, *L. lactis* ssp. *tructae* NBRC 110453^T, *L. raffinolactis* DSM 20443^T, *Lactococcus garvieae* KCTC 3772^T, *Lactococcus piscium* DSM 6634^T, *Lactococcus plantarum* DSM 20686^T, *Lactococcus taiwanensis* NBRC 109049^T, *Lactococcus formosensis* NBRC 109475^T, and *Lactococcus fujiensis* DSM 27937^T were cultured in tryptic soy broth (Becton, Dickinson and Company, East Rutherford, NJ) at 30°C for 24 h. Strains were obtained from the Korean Collection for Type Cultures (KCTC; Taejon, South Korea), the Korean Culture Center of Microorganisms (KCCM; Seoul, South Korea), the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSM; Braunschweig, Germany), the National Biological Resource Center (NBRC; Tokyo, Japan), and the Culture Collection, University of Göteborg (CCUG; Göteborg, Sweden).

ALDH Gene and Primer Design

Genomic DNA was then extracted using an i-genomic BYF DNA Extraction Mini Kit (iNtRON Biotechnology, Seoul, Korea). Primers for amplification of the genes encoding ALDH were designed using PRIMER3 software (http://bioinfo.ut.ee/primer3-0.4.0/), based on the gene sequence of *L. chungangensis* CAU 28^T. The primer sequences were as follows: forward = 5′-GTTGCCATCATTGACACTTGGT-3′, and reverse = 5′-AGGAATGTTCCAAGCACCAC-3′. The total volume of the PCR reaction mixture was 20 μL, and consisted of 5 U of Taq DNA polymerase (Beams Biotechnology, Seongnam, South Korea), 2.0 μL of 10 × Taq buffer, 1 μL of deoxynucleotide triphosphates (dNTP) mixture, 1 μL of each primer, and 3 μL of genomic DNA as the template. Amplifications were performed in a TProfessional Thermocycler (Biometra GmbH, Göttingen, Germany) as follows: an initial cycle of 95°C for 5 min, 30 cycles of denaturation at 95°C for 1 min, annealing at 55°C for 1 min, and extension at 72°C for 1 min, and a final extension at 72°C for 10 min. After PCR amplification, 3 μL of each PCR product were run on a 1.2% Seakem LE agarose gel (FMC Bioproducts, Rockland, ME) and visualized with a Gel DOC XR$^+$ Imaging system (Bio-Rad Laboratories, Hercules, CA). The PCR products were sequenced using a BigDye Terminator Cycle Sequencing kit and an automatic DNA sequencer (model 3730; Applied Biosystems, Foster City, CA).

Cream Cheese Production

Pasteurized milk (Pasteur Milk Co., Ltd., Seoul, South Korea) from a local market was heated at 68°C for 30 min, then cooled, and inoculated with a 5% (vol/vol) *Lactococcus* starter and incubated for 48 h (acidified milk). This mixture was stirred and heated at 70°C for 5 min, then the whey was separated using a cloth bag, and the curd was set and drained of water by adding 0.5% salt. Finally, the cream cheese samples were freeze-dried and stored in the dark at 4°C until further testing.

In Vitro Assay of ALDH Activity

A modified version of a previously published method (Tottmar et al., 1973) was used to identify ALDH activity. Briefly, ALDH activity was determined enzymatically using a reaction mixture containing 1.0 *M* Tris/HCl buffer (pH 8.0), 20 m*M* NAD^+, 3.0 *M* KCl, 0.33 *M* 2-mercaptoethanol, and 1.0 *M* acetaldehyde in a final volume of 3.0 mL. The ALDH activity of baker's yeast, *Saccharomyces cerevisiae* (Sigma-Aldrich, Madison, WI), was used for calibration. The ALDH activity of the bacterial strains was determined using 1 mL of 1 × 10^6 cells of each *Lactococcus* strain, in triplicate; cells were added to the reaction mixture and incubated at 30°C for 5 min. The absorbance was measured at 340

nm and compared with a standard curve to determine ALDH levels. For cream cheese samples, 10 mg/mL of each cream cheese sample was added and the reaction mixture was incubated at 30°C for 5 min. The absorbance was measured at 340 nm and compared with a standard curve.

In Vivo Assay of ALDH Activity

Animal Administrations. Because levels of ALDH activity in cream cheese were higher than those in cell culture, cream cheese was used to determine the effects of ALDH activity on acetaldehyde levels in mouse blood. Seven-week-old male Imprinting Control Region (**ICR**) mice were purchased from Samtako (Samtako, Osan, South Korea). For 1 wk before the experiments, mice were acclimatized in cages at 22°C under a 12-h day/night cycle; these conditions were maintained throughout the experiment. The mice were divided into 7 groups (n = 5 for each group) based on cream cheese sample treatment, as follows: (1) normal (untreated) group; (2) control (ethanol) group; (3) cream cheese made with *L. chungangensis* CAU 28^T (100 mg/kg) group; (4) cream cheese made with *L. fujiensis* DSM 27937^T (100 mg/kg) group; (5) cream cheese made with *L. lactis* ssp. *cremoris* KCCM 40699^T (100 mg/kg) group; (6) cream cheese made with *L. lactis* ssp. *lactis* KCTC 3769^T (100 mg/kg) group; (7) cream cheese made with *L. raffinolactis* DSM 20443^T (100 mg/kg) group. Prior to the test, mice were fasted from food for 18 h. Ethanol or the cream cheese sample was orally administered. Mice were gavaged with 22% ethanol (2 g of ethanol/kg of BW).

Determination of Blood Alcohol and Acetaldehyde Concentrations in Mice. Whole-blood samples were collected from the tail vein of the experimental ICR mice using a serum separation tube at 1, 3, 5, and 7 h after the acetaldehyde challenge. After incubation at 4°C for 10 min, the blood samples were centrifuged at 1,500 × *g* for 15 min at 4°C. The supernatant, which consisted of serum, was used in the analysis of blood alcohol and acetaldehyde levels. The reaction mixture contained 0.3 *M* KH_2PO_4 buffer (pH 9.0), 49 m*M* NAD^+, and a serum sample in a final volume of 3.0 mL. The reaction mixture was incubated at 20°C for 5 min. The absorbance was measured at 340 nm using a spectrophotometer (A1). After the measurement, 50 μL of ALDH was added and incubated at 20°C for 5 min. The absorbance was measured at 340 nm using a spectrophotometer (A2). The blood acetaldehyde concentration was calculated as a percentage using the following equation:

$$\text{Concentration} = 0.7158/3.6 \times \Delta A,$$

where ΔA = sample (A2 – A1) – blank (A2 – A1).

Statistical Analysis

All measurements are expressed as the mean ± standard deviation and were performed in triplicate. Statistical analyses of the differences between samples were performed using one-way ANOVA, followed by a post-hoc multiple comparison using the Duncan and *t*-tests in the Predictive Analytics Software (PASW) statistics package for Windows program (Microsoft Corp., Redmond, WA). Differences of $P < 0.01$ were considered to be statistically significant.

RESULTS

Detection of Genes Encoding ALDH

The ALDH enzymes showed a broad range of substrate specificity for aliphatic and aromatic aldehydes, and ALDH enzymes have been identified in animals, fungi, and bacteria. The effect of cream cheese produced using *Lactococcus* strains on metabolism of alcohol to aldehyde was determined by measuring the generation of reduced NADH (Figure 1). A PCR, using newly designed ALDH primers, was used to identify the presence of genes encoding ALDH in all 9 species and 4 subspecies of *Lactococcus.* Only 5 strains—*L. chungangensis* CAU 28^T, *L. fujiensis* DSM 27937^T, *L. lactis* ssp. *cremoris* KCCM 40699^T, *L. lactis* ssp. *lactis* KCTC 3769^T, and *L. raffinolactis* DSM 20443^T—were found to

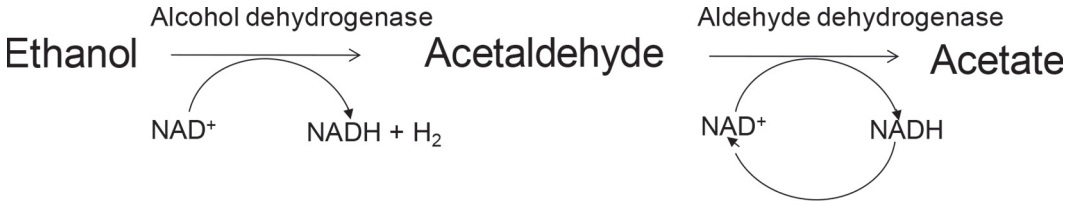

Figure 1. The chemical reaction of alcohol (ethanol) and aldehyde metabolism.

possess an ALDH gene, indicated by a strong band in the gel (953 bp in size; Figure 2).

Cream Cheese Production

Cream cheese was made using *Lactococcus* spp., such as *L. chungangensis* CAU 28^T, *L. fujiensis* DSM 27937^T, *L. lactis* ssp. *cremoris* KCCM 40699^T, *L. lactis* ssp. *lactis* KCTC 3769^T, and *L. raffinolactis* DSM 20443^T. All strains were found to be suitable as starter cultures, and caused coagulation and acidification of the milk mixture during the cheese manufacturing process. All cream cheeses had a smooth, soft, mild, rich, and creamy white texture.

In Vitro Assay of ALDH Activity

The ALDH activity of cells grown in tryptic soy broth was determined by measuring the generation of reduced NADH. *Lactococcus chungangensis* CAU 28^T showed the highest ALDH activity of all *Lactococcus* strains, at 45.9 nmol/min per gram; *L. lactis* ssp. *cremoris* KCCM 40699^T, *L. lactis* ssp. *lactis* KCTC 3769^T, *L. fujiensis* DSM 27937^T, and *L. raffinolactis* DSM 20443^T showed ALDH activity levels of 40.8, 37.9, 33.4, and 30.2 nmol/min per gram, respectively (Figure 3A).

In vitro studies were performed to evaluate the effect of cream cheese made using *Lactococcus* strains on 1 of the 2 key chemical reactions of alcohol metabolism by ALDH. *Lactococcus chungangensis* CAU 28^T showed the highest level of ALDH activity, at 74.2 nmol/min per gram, followed by *L. lactis* ssp. *cremoris* KCCM 40699^T, *L. lactis* ssp. *lactis* KCTC 3769^T, *L. raffinolactis* DSM 20443^T, and *L. fujiensis* DSM 27937^T, which showed ALDH activities of 54.0, 53.6, 48.6, and 47.4 nmol/min per gram, respectively (Figure 3B).

In Vivo ALDH Activity

Because *L. chungangensis* CAU 28^T showed higher ALDH activity in cheese than in cell culture, cheese was used to determine the effect of ALDH from *L. chungangensis* CAU 28^T on blood aldehyde levels. In vivo studies were used to determine the acetaldehyde concentrations in the blood of mice treated with cream cheese to confirm the present of acetaldehyde, the by-product of ethanol metabolism on hangover phase (Verster and Penning, 2010). After 5 h, a marked difference ($P = 0.01$) was observed in acetaldehyde concentrations (Figure 4). The blood acetaldehyde concentration decreased in all mice treated with cheese made with all 5 strains of *Lactococcus* spp. Cheese made using *L. chungangensis* CAU 28^T produced the largest decrease in the concentration of aldehyde in mouse blood, with an aldehyde level of 494.3 nmol/mL, followed by *L. lactis* ssp. *lactis* KCTC 3769^T, *L. lactis* ssp. *cremoris* KCCM 40699^T, *L. fujiensis* DSM 27937^T, and *L. raffinolactis* DSM 20443^T, which produced blood acetaldehyde concentrations of 504.6, 522.5, 551.2, 559.5 nmol/mL, respectively.

DISCUSSION

Lactococcus species are lactic acid bacteria that are commonly used as starters in cheese manufacturing; they produce acid and contribute to the ripening process. The main role of starter bacteria is to provide a suitable environment for the bacteria involved in cheese production, in terms of redox potential, pH, and moisture content (Beresford et al., 2001). *Lactococcus lactis* ssp. *lactis* and *L. lactis ssp. cremoris* are used for the production of many kinds of dairy-based fermented milk products, such as cheese, sour cream, and lactic

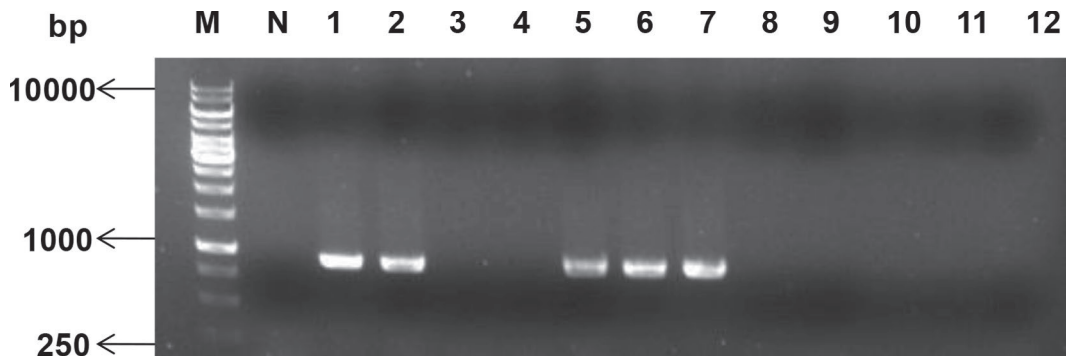

Figure 2. Gel electrophoresis of PCR amplification of the gene for aldehyde dehydrogenase in all *Lactococcus* species. M = marker; N = negative control; 1 = *Lactococcus chungangensis* CAU 28^T; 2 = *Lactococcus lactis* ssp. *lactis* KCTC 3769^T; 3 = *Lactococcus formosensis* NBRC 109475^T; 4 = *Lactococcus plantarum* DSM 20686^T; 5 = *Lactococcus fujiensis* DSM 27937^T; 6 = *Lactococcus raffinolactis* DSM 20443^T; 7 = *Lactococcus lactis* ssp. *cremoris* KCCM 40699^T; 8 = *Lactococcus lactis* ssp. *hordiniae* KCTC 3768^T; 9 = *Lactococcus lactis* ssp. *tractae* NBRC 110453^T; 10 = *Lactococcus taiwanensis* NBRC 109049^T; 11 = *Lactcooccus garvieae* KCTC 3772^T; 12 = *Lactococcus piscium* DSM 6634^T.

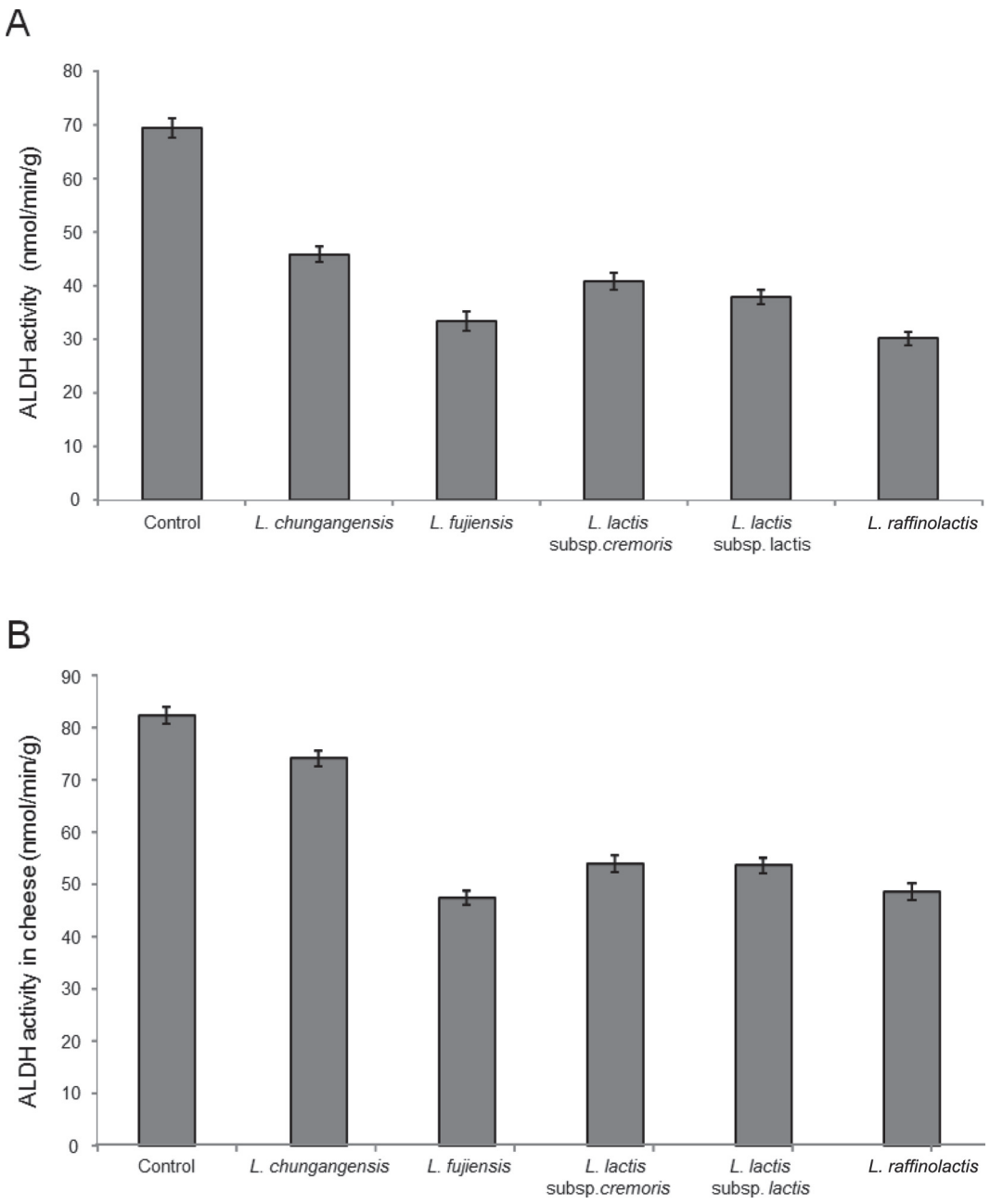

Figure 3. Aldehyde dehydrogenase activity of 5 strains of *Lactococcus*. (A) Aldehyde dehydrogenase (ALDH) activity of cell culture of *Lactococcus* spp.; (B) ALDH activity in cheese made with *Lactococcus* spp.

casein (Klijn et al., 1995). Lactic acid bacteria and bifidobacteria have been extensively studied and widely employed in the field of probiotics, they are also normal components of the intestinal microbiota and have a long tradition of safe application within the food industry (Kociubinski and Salminen, 2006). Extensive research and development activity into probiotics has resulted in a large number of new specialty dairy products, such as drinking yogurt, fermented drinks, sour cream, butter, cream, and cream cheese (Szakály et al., 2007).

Acetaldehydes are unique toxicants that are metabolically detoxified by in vivo oxidation (to acids) and reduced to alcohols (O'Brien et al., 2005). Aldehyde dehydrogenase is the primary enzyme in this process and is involved not only in the formation of acetaldehyde from glucose (Lees and Jago, 1978), but also in a

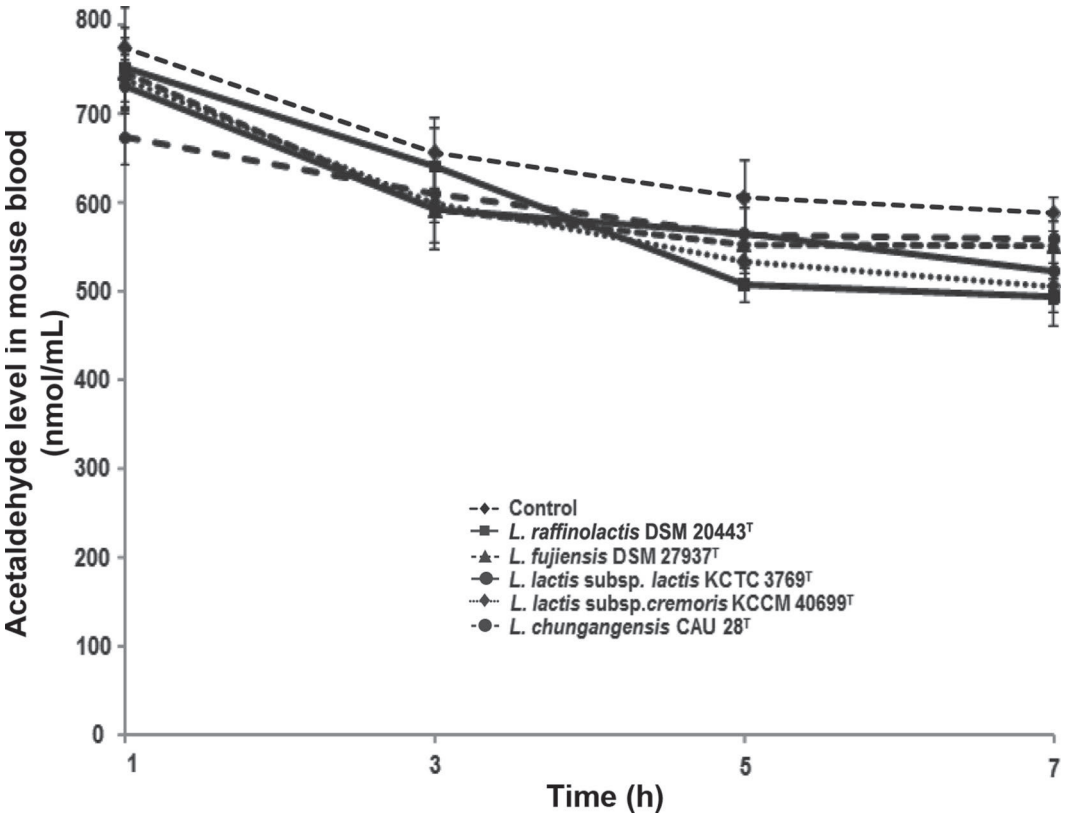

Figure 4. Effects of cream cheese on acetaldehyde concentrations in the blood of mice. Mice were orally administered 22% ethanol (2 g/kg of BW). Blood alcohol and acetaldehyde concentration were measured at 1, 3, 5, and 7 h after ethanol administration. The results are expressed as the mean ± SD from 5 independent experiments.

variety of biological processes, such as stress responses, in a broad range of bacteria, plant, and animal species (Singh et al., 2013). Aldehyde dehydrogenase has been shown to be related to oxidative stress-associated diseases such as atherosclerosis, cancer, diabetes, and chronic alcohol exposure (Ouadghiri et al., 2005; Jacobs and Marnett, 2010; Yin et al., 2011).

Moreover, ALDH catalyzes the conversion of aldehydes to their corresponding acids by means of a virtually irreversible $NAD(P)^+$-dependent reaction. The ALDH enzymes have been identified in a wide range of organisms, from bacteria to humans (Yoshida et al., 1998); most, if not all, members of the ALDH family are likely present in other mammals. The protein, gene, and cDNA sequences of these enzymes have been reported for more than 50 species of animals, fungi, and bacteria (Kedishvili et al., 1992; Chambliss et al., 1995). In addition, ALDH is the primary enzyme involved in the elimination of alcohol from the body and usually reduces alcohol levels following consumption of alcohol (Zakhari, 2006). Ethanol has been shown to be of clinical significance in gastric metabolism (Julkunen et al., 1985), and the presence of a bacteriocolonic pathway for ethanol oxidation has been reported (Jokelainen et al., 1996a; Salaspuro, 1998). Via this pathway, intracolonic ethanol is first oxidized by bacterial alcohol dehydrogenase to acetaldehyde (Jokelainen et al., 1996a,b). Acetaldehyde is then oxidized either by colonic mucosal cells or bacterial aldehyde dehydrogenase to acetate (Matysiak-Budnik et al., 1996).

The current study found that cream cheese samples produced with *L. chungangensis* CAU 28^T showed the highest ALDH activity (74.2 nmol/min per gram) compared with those produced with other *Lactococcus* spp. The aldehyde concentration in the blood of mice was decreased to 494.3 nmol/mL by application of cream cheese samples produced with *L. chungangensis* CAU 28^T, corresponding to a high level of ADH activity. Cell cultures and cheese samples produced using *L. chungangensis* CAU 28^T were found to decrease aldehyde levels, indicating that cheese made using a starter culture containing *L. chungangensis* CAU 28^T has potential as a functional food to increase the activity of aldehyde dehydrogenase and to reduce acetaldehyde levels in the blood.

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