Keto acid decarboxylase and keto acid dehydrogenase activity detected during the biosynthesis of flavor compound 3-methylbutanal by the nondairy adjunct culture *Lactococcus lactis* ssp. *lactis* F9

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ABSTRACT

3-Methylbutanal is one of the primary substances that contribute to the nutty flavor in cheese. *Lactococcus* strains have been shown to have higher aminotransferase and α -keto acid decarboxylase activities compared with other microbes, indicating that they might form a higher amount of 3-methylbutanal by decarboxylation. Several dairy lactococcal strains have been successfully applied as adjunct cultures to increase the 3-methylbutanal content of cheese. Moreover, compared with dairy cultures, the nondairy lactococci are generally metabolically more diverse with more active AA-converting enzymes. Therefore, it might be appropriate to use nondairy lactococcal strains as adjunct cultures to enrich the 3-methylbutanal content of cheese. This study thereby aimed to select a nondairy *Lactococcus* strain that is highly productive of 3-methylbutanal, identify its biosynthetic pathway, and apply it to cheese manufacture. Twenty wild nondairy lactococci isolated from 5 kinds of Chinese traditional fermented products were identified using 16S rRNA sequence analysis and were found to belong to *Lactococcus lactis* ssp. *lactis*. The nondairy strains were then screened in vitro for their production of 3-methylbutanal and whether they met the criteria to become an adjunct culture for cheese. The *L. lactis* ssp. *lactis* F9, isolated from sour bamboo shoot, was selected because of its higher 3-methylbutanal production, suitable autolysis rate, and lower acid production. The enzymes involved in the catabolic pathway of leucine were then evaluated.

Both α -keto acid decarboxylase (6.96 $\mu\text{mol/g}$ per minute) and α -keto acid dehydrogenase (30.06 $\mu\text{mol/g}$ per minute) activities were detected in nondairy *L. lactis* F9. Cheddar cheeses made with different F9 levels were ripened at 13°C and analyzed after 90 d by a combination of instrumental and sensory methods. The results showed that adding nondairy *L. lactis* F9 significantly increased 3-methylbutanal content and enhanced the nutty flavor of the cheese without impairing its textural properties. Thus, nondairy *L. lactis* F9 efficiently enhanced the biosynthesis of 3-methylbutanal in vitro and in manufactured cheese.

Key words: 3-methylbutanal, nondairy, biosynthesis pathway, adjunct culture

INTRODUCTION

Cheese flavor plays a critical role in determining cheese quality and consumer acceptance. Nutty flavor is an important flavor compound of many cheeses, such as Cheddar, Gouda, and Proosdij, and is considered desirable by consumers (Young et al., 2004; Zhang et al., 2011). However, this nutty flavor is generally found only in aged cheeses (Avsar et al., 2004), with a correspondingly increased cost of manufacture. Therefore, enhancing the nutty flavor in younger cheeses would help improve the efficiency of the cheese manufacturing industry.

Using descriptive sensory analysis coupled with quantitation of the volatile aroma components, Avsar et al. (2004) determined that 2/3-methylbutanal and 2-methylpropanal were the basic compounds producing the nutty flavor. Of these 3 compounds, 3-methylbutanal has been given most attention for 2 reasons. First, it is always present at a higher concentration or relative abundance than 2-methylbutanal and 2-methylpropanal

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because of the efficient microbial breakdown of leucine (Morales et al., 2003; Irigoyen et al., 2007), which is highly dependent on microbiota, growth, and culture conditions. Second, its average flavor threshold value is 0.06 $\mu\text{g/mL}$ (Sheldon et al., 1971), a much lower value than that of 2-methylbutanal and 2-methylpropanal. Therefore, enriching the content of 3-methylbutanal in cheese would help to enhance its nutty flavor.

3-Methylbutanal can be generated in cheese using 2 approaches. First, leucine can be catabolized by chemically degrading the aldehydes through the Strecker degradation (Smit et al., 2009). Second, and more important, 3-methylbutanal can be derived from leucine by the cheese microbiota, generally by 2 possible biosynthetic pathways: a direct pathway involving α -keto acid decarboxylase or an indirect pathway using α -keto acid dehydrogenase and aldehyde dehydrogenase (Afzal et al., 2017). Previous studies have revealed that either the direct or indirect pathway or both pathways could be present (Ward et al., 1999; Helinck et al., 2004; Afzal et al., 2012) and that these pathways were highly dependent on the strain and species (Afzal et al., 2017).

Lactococcus strains have been shown to have higher aminotransferase and α -keto acid decarboxylase activities compared with other microbes (de Palencia et al., 2006), indicating that they might form a higher amount of 3-methylbutanal by decarboxylation. Although the nonstarter lactic acid bacteria consist mainly of lactobacilli, lactococci are also part of this population (Gobbetti et al., 2015). Many *Lactococcus* strains have been used as adjunct cultures to improve the flavor, texture, or potential healthy features of cheese in previous studies (Garde et al., 2002, 2007; Nascimento et al., 2010). *Lactococcus lactis* ssp. *lactis* B851 and the malty *L. lactis* ATCC 29146 have been successfully applied as adjunct cultures to increase the 3-methylbutanal content of cheese (Ayad et al., 2003; Whetstone et al., 2006). In addition, compared with dairy cultures, nondairy lactococcal strains are generally found to be metabolically more diverse (Kranenburg et al., 2002), with more active AA-converting enzymes and less auxotrophic AA (Ayad et al., 1999). Cavanagh et al. (2014) have recently used nondairy lactococcal strains as adjunct cultures to enhance the proteolytic and sensory characteristics of a Gouda-type cheese. Therefore, it might be appropriate to use nondairy lactococcal strains as adjunct cultures to enrich the 3-methylbutanal content of cheese.

Many traditional fermented foods, which are a vital source of lactic acid bacteria, have been consumed for hundreds of years in the Yunnan province of China. The present study aimed to screen lactococcal strains from the traditional nondairy fermented products from the Yunnan province to select one that preferentially generates 3-methylbutanal. The activities of the enzymes

involved were determined to analyze the biosynthetic pathway of the strain. The strain was then added to cheese as an adjunct culture to assess the accumulation of 3-methylbutanal and the nutty flavor of the cheese. The study may contribute to developing an adjunct culture for producing cheeses with an intense nutty flavor or accelerating cheese ripening.

MATERIALS AND METHODS

Materials

Bulk raw cow milk for cheese manufacture was obtained from San Yuan Dairy Co. (Beijing, China) and skim milk powder was obtained from Nouriz Dairy Co. (Shanghai, China). Freeze-dried direct vat-set starter culture (R704, *L. lactis* ssp. *lactis*/*L. lactis* ssp. *cremoris*) was from Chr. Hansen (Hoersholm, Denmark). The 3-methylbutanal standard, alkane series (C6-C20), BSA, nicotinamide adenine dinucleotide hydrogen (NADH), NAD^+ , glutamic acid, 2-methyl-3-heptanone, butyric acid, and ethyl butyrate were purchased from Sigma-Aldrich (St. Louis, MO); a 96 T Glu assay kit was from Jianglai Biological (Shanghai, China); and rennet (Naturen Stamix 1150 NB) was from Chr. Hansen. All other chemicals were of analytical grade.

Screening of Adjunct Culture

Microorganisms. A total of 20 nondairy *Lactococcus* strains were isolated from 5 types of Chinese traditional fermented products (sour bamboo shoots, salted and dried turnip strips, pickled garlic, pickled mustard cabbage, and pickled radish chives) from Yunnan province, China, and stored at our laboratory (Key Laboratory of Functional Dairy, China Agricultural University, Beijing, China). At the same time, 10 *Lactococcus* strains isolated from raw milk and other dairy products from Yunnan province were included to determine their 3-methylbutanal production. The species of the strains were confirmed by genotypic characterization using 16S rRNA sequence analysis and PCR amplification. Bacterial genomic DNA was extracted using the phenol-chloroform method as described by Via and Falkinham (1995). The PCR amplification was performed according to a reported method (Wang et al., 2016), and the sequencing libraries were sequenced on the Illumina HiSeq platform (Illumina, San Diego, CA). Sequence similarity searches were performed by comparing the isolated sequences with the ones collected in GenBank using the BLAST search program of the National Center for Biotechnology Information (Bethesda, MD). The detailed sequence of each strain is shown in Supplemental Table S1 (<https://doi.org/>

10.3168/jds.2018-14760), and the basic information on the 30 isolated strains is shown in Table 1.

All isolated strains were routinely cultivated in M17 medium. After incubation overnight at 30°C, the early stationary phase cells were harvested by centrifugation at $8,000 \times g$ at 4°C for 10 min. The cells were then washed twice with 100 mM sodium phosphate buffer (pH 6.0) and resuspended in cryoprotectant (12 g of skim milk powder added to 90 mL of distilled water, mixed with 30 mL of glycerol immediately before use). Aliquots of these cell suspensions were quickly frozen in liquid nitrogen and then stored at -80°C. When the experiments were performed with growing cells, the cultures were harvested by centrifugation just before entering the stationary phase as described earlier.

Determination of 3-Methylbutanal Production. An AA culture medium was used to screen strains that preferentially generated 3-methylbutanal. The AA culture medium was prepared according to van de Bunt et al. (2014) with the following additions: 10 mM α -ketoglutarate, 0.2 mM NADH, 0.1 mM pyridoxal-5-phosphate, and 18 AA (10 mM). α -Ketoglutarate, NADH, and pyridoxal-5-phosphate were found to be essential for the conversion of L-leucine to α -ketoisocaproic acid by *Propionibacterium freudenreichii* (Thierry et al., 2002). Amino acid mixtures were used because the 3-methylpentanal not only was produced from a single AA but also could be generated upon incubation with several AA mixtures (van de Bunt et al., 2014). The lactococcal cells were washed with 100 mM sodium phosphate buffer (pH 6.0) and then added to the AA culture medium until a final optical density at 600 nm value of 20 was attained (van de Bunt et al., 2014). After incubation for 24 h at 30°C, the incubation was terminated by flash-freezing the vials in liquid nitrogen. The vials were then stored at -40°C before analysis for 3-methylbutanal. Mixtures without cells were incubated as the controls.

The 3-methylbutanal and other volatile compounds were measured in the headspace using a 50/30- μ m solid-phase microextraction divinylbenzene/carboxen/polydimethylsiloxane fiber (Supelco, Bellefonte, PA) coupled to a GC-mass spectrometer (6890N/5975C, Agilent Technologies Inc., Santa Clara, CA). After equilibration for 20 min at 55°C, the volatile compounds were concentrated onto the fiber by exposure to the headspace for 40 min. The fiber was then retracted into the needle and the compounds were desorbed from the syringe needle into the injector by using a thermal desorption system (250°C, 5 min, TDS3/TDSA2; Gerstel, Mülheim an der Ruhr, Germany) followed by cryofocusing at -110°C (cooled injection system CIS4; Gerstel) onto the top of the analytical column. The cold trap was rapidly heated to 250°C and the com-

Table 1. Basic information on 30 *Lactococcus lactis* ssp. *lactis* strains isolated from the Yunnan province, China

Strain designation	Isolation source	Location in Yunnan province
Nondairy		
A73	Sour bamboo shoot	Yuxi
A90	Pickled mustard cabbage	Yuxi
B22	Sour bamboo shoot	Deqing
B28	Pickled garlic	Deqing
B143	Pickled radish chives	Dali
C16	Pickled mustard cabbage	Dali
C21	Pickled mustard cabbage	Dali
D08	Pickled radish chives	Dali
D44	Pickled mustard cabbage	Dali
D64	Pickled radish chives	Dali
D96	Pickled garlic	Dali
D105	Pickled mustard cabbage	Dali
E12	Pickled garlic	Baoshan
E22	Sour bamboo shoot	Baoshan
E28	Pickled mustard cabbage	Dehong
E33	Pickled mustard cabbage	Shilin
E37	Pickled garlic	Dehong
E46	Sour bamboo shoot	Dehong
F9	Sour bamboo shoot	Jinghong
F16	Pickled garlic	Kunming
Dairy		
A29	Ewe milk	Shilin
A119	Raw milk	Kunming
B17	Butter	Deqing
B80	Yak butter	Deqing
C133	Dairy fan (Rushan)	Dali
C140	Whey	Dali
C178	Dairy fan (Rushan)	Dali
E52	Raw milk	Dehong
E91	Buffalo milk	Dehong
G20	Milk dregs	Dali

pounds were separated on a DB-wax capillary column (30.0 m $\times$ 320 μ m $\times$ 0.25 μ m; Agilent). The solid-phase microextraction fibers were introduced into the split injector at 250°C. The GC injection parameters were as follows: injection volume, 1 μ L; split, 10:1; helium flow rate, 1.2 mL/min; 40°C for 3 min, increased to 70°C at 3°C/min, isothermal for 2 min, then increased to 240°C at 10°C/min, and finally isothermal for 10 min. The MS parameters used for the analyses were as follows: temperature of transfer line (direct interface), 250°C; ion source, electron impact mode; electron energy, 70 eV; ion trap temperature, 230°C; quad temperature, 150°C; and full scanning mode (35–450 m/z). The volatile compounds were identified using the NIST2.0 mass spectral library (National Institute of Standards and Technology, Gaithersburg, MD) or from spectra published in the literature. To confirm the identity of each component, the linear retention index was calculated using the retention times of a homologous series of an alkane series and by comparing the retention index of authentic compounds analyzed under similar conditions (Alves Bezerra et al., 2016). The 3-methylbutanal was quantified by comparison with the standard curves. The standard curves were established using solutions of

a 3-methylbutanal standard at concentrations of 6.25, 12.5, 25, 50, 100, and 500 μM .

Evaluation of the Biological Characteristics

Assay of Phenotypic Properties. After recovery, 2% nondairy *Lactococcus* colony strains were inoculated into fresh M17 medium and cultured for 24 h to test their ability to grow at 13°C, or in the presence of 2 or 4% (wt/vol) NaCl, or at pH values of 5.0 or 5.5 separately as described by Parente et al. (1997).

Assay of Acid Production. After recovery, 2% nondairy *Lactococcus* colony strains were inoculated into 10% skim milk and then cultured at 30°C for 24 h. The pH was measured immediately after inoculation and after 24 h, and the decrease in pH after incubation was calculated.

Assay of Autolysis Rate. The autolysis of the strains was assessed as described by Piraino et al. (2008).

Determination of Enzyme Activity

The cell free extract (CFE) was prepared according to a modified method of Brandsma et al. (2008). After culture, the cells were harvested and washed twice in 100 mM PBS (pH 6.0) and then were resuspended in the same buffer. The cell suspensions were prepared by beating 4 times for 30 s. After beating, the cells were stored for 1 min on melting ice. After totally breaking up, the cell isolates were removed by centrifugation at $10,000 \times g$ at 4°C for 10 min. The CFE was defined as the supernatant after being filtered through a 0.22- μm membrane. This was stored at -20°C until use. The protein content of the CFE was measured using BSA as the standard (Bradford, 1976).

The leucine aminotransferase (**LeuAT**) activity was determined using the modified method of Hugo Peralta et al. (2014). An aliquot of 100 μL of CFE was incubated for 1 h at 30°C in a 250- μL reaction mixture containing 1 mM of leucine substrate, 0.05 mM of pyridoxal phosphate, 6 mM of α -ketoglutaric acid, and 100 mM of PBS (pH 6.0). The reaction was terminated by heating at 80°C for 15 min. The levels of glutamic acid produced in the transamination reaction were quantified using a Glu assay kit. The optical density was measured at 450 nm. One unit of LeuAT activity ($\mu\text{mol/g}$ per hour) was defined as the protein content (g) needed to produce 1 μmol of glutamic acid per hour of reaction.

The keto acid dehydrogenase (**KADH**) activity was determined as described by Danner et al. (1979) with some modifications. Ten microliters of CFE was incubated for 30 min at 30°C in a 250- μL reaction mixture

containing 2.5 mM of NAD^+ substrate, 7.5 mM of α -ketoisocaproate, 35 mM of MgCl_2 , 1 mM of thiamine pyrophosphate, 7.5 mM of coenzyme A, and 100 mM of PBS (pH 6.0). The optical density was measured at 340 nm. One unit of KADH activity (μmol of NADH/g per minute) was defined as the protein content (g) needed to produce 1 μmol of NADH/min of reaction.

The aldehyde dehydrogenase (**AldDH**) activity was determined as described by Afzal et al. (2012) with some modifications. Fifty microliters of CFE was incubated for 15 min at 30°C in a 250- μL reaction mixture containing 0.5 mM of NAD^+ substrate, 150 mM of 3-methylbutanal, and 100 mM of PBS (pH 7.4). The optical density was measured at 340 nm. One unit of AldDH activity (μmol NAD^+ /g per minute) was defined as the protein content (g) needed to produce 1 μmol of NAD^+ /min of reaction.

The glutamate dehydrogenase (**GDH**) activity was determined as described by Tanous et al. (2006). The hydroxyl acid dehydrogenase (**HADH**) activity and the alcohol dehydrogenase (**AlcDH**) activity were determined as described by Brandsma et al. (2008). The keto acid decarboxylase (**KADC**) activity was determined as described by Smit et al. (2005).

Manufacture of Cheese

Cheddar cheese was produced on 3 different days and in a randomized order as described by Hu et al. (2013) with some modifications. After pasteurization, a milk starter culture (10^8 cfu/mL) was added and then incubated at 32°C for 30 min. The adjunct *L. lactis* F9 strain was harvested and washed twice with 0.85% (wt/wt) saline solution and resuspended in the same solution. The cell suspension was diluted to set concentrations (10^5 , 10^6 , and 10^7 cfu/mL) and then added to the cheese milk with 0.05 g/kg (wt/wt) of rennet right before the renneting procedure. The cheeses were ripened at 13°C to shorten the maturation time (Law, 2001), and samples were collected after 1, 30, 60, and 90 d for further analyses. Cheese made with no adjunct culture was used as the control.

Analysis of Cheese

Cheese Basic Composition. Cheese samples were analyzed for water, fat, and protein content as described by Hu et al. (2013). The pH from a grated cheese sample was measured using a pH meter (Delta 320, Mettler Toledo Instruments, Shanghai, China) at 25°C.

3-Methylbutanal Content. The development and accumulation of 3-methylbutanal in cheese were determined during ripening. Ten grams of grated cheese

(accurately weighed) were transferred into the 40-mL headspace. One microliter of 0.816 mg/L internal standard 2-methyl-3-heptanone was added, and the bottle was quickly sealed. The qualitative analysis was then performed as described above. The semiquantitative analysis was calculated according to the internal standard method as follows: $C_i = C_{is} \times A_j/A_{is}$, where C_i and C_{is} are the concentrations of the flavor compounds and the internal standard 2-methyl-3-heptanone, respectively, and A_j and A_{is} are the peak areas of the flavor compounds and the internal standard, respectively.

Sensory Properties. After ripening for 90 d, the cheeses were sampled for sensory analysis. The cheese samples were presented as $2 \times 2 \times 2$ -cm cubes in lidded cups labeled with a random 2-digit code. A trained descriptive sensory panel ($n = 15$; 10 women, 5 men; ages 25–35 yr) with >100 h of experience analyzing cheese flavor evaluated the samples. Descriptive analysis using a 0- to 9-point universal intensity scale and an established cheese flavor sensory language (definitions and references) was conducted in exactly the same manner as described by Drake et al. (2001).

Statistical Analysis

All measurements were performed in triplicate and expressed as the mean and standard deviation. The data were analyzed by 1-way ANOVA (with Duncan's multiple range method) using SPSS software (version 22.0; IBM, Armonk, NY). The level for statistical significance was set at $P < 0.05$. The heat map of the flavor metabolites of strains was drawn using Heml (1.0.3.3-Heatmap Illustrator; Huazhong University of Science and Technology, Wuhan, China).

RESULTS AND DISCUSSION

Screening the 3-Methylbutanal Producing Strains

Though normally associated with a dairy environment, the *L. lactis* strains could also be isolated from a nondairy environment. Compared with dairy cultures, genome sequence analysis has revealed that nondairy lactococcal strains are generally metabolically more diverse (Kranenburg et al., 2002), with more active AA-converting enzymes and less auxotrophic AA (Ayad et al., 1999). Alemayehu et al. (2014) found that some plant-derived *L. lactis* strains could metabolize more carbohydrates than the dairy control strains. Moreover, Cavanagh et al. (2014) identified a bank of *L. lactis* strains isolated from different nondairy environments that possessed increased AA-converting enzyme activities compared with dairy strains. Moreover, they found that some strains performed as well as the dairy strains

in terms of their secondary proteolysis in a Gouda-type cheese. Therefore, we isolated lactococcal strains from nondairy environments in an effort to select one that preferentially generates 3-methylbutanal.

Based on 16S rRNA sequencing, all of the 20 nondairy and 10 dairy lactococcal strains were identified to belong to *L. lactis* ssp. *lactis*. The heat maps of the AA metabolite profiles of the 20 strains isolated from the nondairy fermented products and the 10 from the dairy products are shown in Figure 1A. The volatile flavor compounds included 9 aldehydes, 6 alcohols, 2 esters, 4 ketones, 4 alkanes, 2 ethers, 3 aromatic hydrocarbons, and 1 heterocyclic compound. Regardless of the origin of the strains, most could catabolize leucine and generate 2-methylbutanal, 3-methylbutanal, and 2-methylbutanol, but the concentrations varied significantly between the different strains ($P < 0.05$).

Figure 1B shows the specific 3-methylbutanal content of the 30 strains. Compared with dairy cultures, the ability of nondairy *L. lactis* to generate 3-methylbutanal seemed more dependent on the strains. In addition, the results indicated that nondairy *L. lactis* might have superior 3-methylbutanal generation ability over the dairy *L. lactis*. The nondairy *L. lactis* ssp. *lactis* B35, C177, D105, and F9 produced significantly higher amounts of 3-methylbutanal compared with the other strains at concentrations $>120 \mu\text{M}$ (Figure 1B; $P < 0.05$). Therefore, these 4 nondairy *L. lactis* were evaluated further as potential adjunct cultures for cheese.

Biological Characteristics of 3-Methylbutanal-Producing Strains

The criteria for an adjunct culture to be suitable for cheese making include the ability of the culture to (1) reach the desired counts during cheese ripening, (2) not cause defects in the cheese, and (3) impart desirable biochemical properties on the overall cheese quality through the introduction of selected enzyme activities (Crow et al., 2001; Cagno et al., 2011). Because of the huge differences between the dairy and nondairy environments, the nondairy strains might be difficult to adapt to the dairy environment, especially the hostile conditions of the cheese environment during ripening. Therefore, selecting a 3-methylbutanal-producing nondairy strain that can tolerate the cheese environment is crucial. Table 2 shows that all strains could grow in the presence of 2 and 4% (wt/vol) NaCl, at pH 5.0 and 5.5, and at 4 and 13°C. Because Cheddar cheese is usually ripened at pH 5.0 to 5.5, with 2 to 4% salt in moisture, and at 4 to 13°C (Azarnia et al., 2006; Murtaza et al., 2014), these results indicated that the 4 nondairy strains could survive in a normal cheese ripening environment. However, there were significant differences in

acid production and autolysis rates among the strains after incubation for 24 h at 30°C (Table 2). A strain with a poor ability to acidify and a suitable ability to lyse and release intracellular enzymes is a desirable trait for adjunct cultures (Ayad et al., 2004). According to a previous study (Ayad et al., 2004), the autolytic activity level of lactococci was assigned as high = 25 to 37%, fair = 15 to 24%, and poor = 1 to 14%. Therefore, the nondairy *L. lactis* ssp. *lactis* F9, isolated from sour bamboo shoot, proved to be the most viable adjunct

culture of the 4 selected strains because of its lower acid production and suitable autolysis rate.

Biosynthesis Pathways of 3-Methylbutanal in Nondairy *L. lactis* F9

Four strains with low 3-methylbutanal formation (nondairy *L. lactis* ssp. *lactis* A10, A81, D42, and E22) were chosen as references to determine the biosynthesis pathways for 3-methylbutanal in nondairy *L. lactis* ssp.

Figure 1. (A) Heat map of the AA metabolite profiles. (B) The production of 3-methylbutanal of 20 strains isolated from nondairy fermented products and 10 from dairy products. Flavor products are indicated by a gradient map representing the concentration (the darker the color, the higher the concentration) of different strains. Asterisk (*) indicates that the concentration was too low to be detected. Error bars represent the SD of triplicate experiments. Color version available online.

Table 2. Biological characteristics of 4 selected strains isolated from nondairy fermented products¹

Strain	Source	Growth						pH decrease	Autolysis (%)
		At 2% NaCl (wt/vol)	At 4% NaCl (wt/vol)	At pH 5.0	At pH 5.5	At 4°C	At 13°C		
B35	Pickled garlic	+	+	+	+	+	+	1.82 ± 0.03 ^c	13.94 ± 0.07 ^a
C177	Pickled mustard cabbage	+	+	+	+	+	+	1.75 ± 0.02 ^b	14.18 ± 0.05 ^b
D105	Pickled mustard cabbage	+	+	+	+	+	+	1.72 ± 0.02 ^b	13.91 ± 0.11 ^a
F9	Sour bamboo shoot	+	+	+	+	+	+	0.24 ± 0.01 ^a	15.27 ± 0.09 ^c

^{a-c}Different superscripts within a column indicate a significant difference between groups ($P < 0.05$).

¹Mean ± SD; n = 3.

²Plus sign (+) indicates that the strains could grow in the condition.

lactis F9. Figure 2A shows that all strains possessed LeuAT activity, but F9 was significantly higher than the others. Leucine aminotransferase is the key enzyme directly implicated in the first step in leucine catabolism by converting leucine into α -ketoisocaproate (Smit et al., 2004), which has been found to be present in most cheese-related lactic acid bacteria (Afzal et al., 2017). The GDH activity of nondairy *L. lactis* ssp. *lactis* F9, with a value of 25.52 $\mu\text{mol/g}$ per minute, was also significantly higher than that of the other strains (Figure 2B). This value was higher than those previously reported where the GDH activity varied from 18 to 20 $\mu\text{mol/g}$ per minute for some wild *L. lactis* strains (Amárita et al., 2006). Glutamic acid can be converted into the substrate α -ketoisocaproate by GDH, which favors the generation of 3-methylbutanal (Tanous et al., 2002). Therefore, the high LeuAT and GDH activities in nondairy *L. lactis* F9 indicated that it could efficiently utilize and resynthesize the substrate α -ketoisocaproate and lead to a high level of 3-methylbutanal, which agreed with the results shown in Figure 1.

To identify the biosynthesis pathway for 3-methylbutanal in nondairy *L. lactis* F9, the enzymes involved in both the direct and indirect pathways were evaluated. The direct pathway refers to the KADC pathway, where α -keto acids are directly biotransformed into aldehydes in a single step (Afzal et al., 2017). Keto acid decarboxylase has been revealed to be of prime importance for the generation of 3-methylbutanal in *L. lactis* because most of the aldehydes are formed through this pathway (Smit et al., 2004). The indirect pathway consists of the oxidative decarboxylation of α -ketoisocaproate to isovaleryl-CoA by the multienzyme complex KADH for the biosynthesis of 3-methylbutanal (Afzal et al., 2017). The multistep indirect KADH pathway has been rarely studied and not yet reported regarding *L. lactis* (Afzal et al., 2012, 2017). Figure 2C shows that only *L. lactis* F9 possessed detectable levels of KADC activity. The lack of KADC activity in other strains would probably be a result of the absence of the *KdcA* gene

(Smit et al., 2005). In addition, F9 exhibited KADH activity, with the KADH activity appearing to be much higher than that of KADC (30.06 vs. 6.96 $\mu\text{mol/g}$ per minute; Figure 2D; $P < 0.05$). To our knowledge, this is the first occasion that both KADC and KADH activity have been found in vitro in a *Lactococcus* strain. The results indicated that nondairy *L. lactis* F9 can biosynthesize 3-methylbutanal through both direct and indirect pathways. Afzal et al. (2012) also determined that both metabolic pathways were present in *Carnobacterium maltaromaticum*, where the KADC activity was higher than the KADH activity. The balance between the direct and indirect pathways may be affected by the extracellular redox potential (Afzal et al., 2012). Oxidative conditions are favored by *L. lactis* because it can stimulate KADC activity and contribute to the conversion of α -ketoisocaproate into 3-methylbutanal (Kieronczyk et al., 2006). As both pathways are available in nondairy *L. lactis* F9, it could function under various redox conditions, which would be greatly beneficial for catabolizing leucine.

Activity of HADH, AldDH, and AlcDH was exhibited by all the strains studied, although their values varied. The activities of all 3 enzymes in nondairy *L. lactis* F9 occurred at a very low level (Figure 3). A high HADH activity does not favor 3-methylbutanal production because it mainly functions to convert the α -ketoisocaproate substrate into α -hydroxyisocaproate (Smit et al., 2004). 3-Methylbutanal can also be further oxidized to 3-methylbutyrate by AldDH or reduced to 3-methylbutanol by AlcDH depending on the intracellular redox environment (Kieronczyk et al., 2006; Afzal et al., 2012). Therefore, the low levels of HADH, AldDH, and AlcDH activity in nondairy *L. lactis* F9 would benefit the accumulation of 3-methylbutanal.

Effects of Nondairy *L. lactis* F9 on Cheese

Composition. To evaluate the effect of adjunct *L. lactis* F9 on cheese, the starter culture of all groups

Figure 2. Enzyme activity of individual strains: (A) leucine aminotransferase activity (LeuAT); (B) glutamate dehydrogenase (GDH) activity; (C) ketoisocaproate decarboxylase (KADC) activity; (D) keto acid dehydrogenase (KADH) activity. Different letters (a–e) indicate a significant difference between groups ($P < 0.05$). ND = not detectable. Error bars represent the SD of triplicate experiments.

was maintained at the same content, whereas the level of adjunct culture was varied. Because of the huge differences between the dairy and nondairy environments, the nondairy adjunct *L. lactis* F9 was added at a higher level compared with the dairy strains in previous reports (Whetstine et al., 2006; van Hoorde et al., 2010). Table 3 shows that the fat, protein, and moisture contents and the pH of the cheeses were not significantly different ($P > 0.05$). Therefore, any differences observed in the flavor, sensory, and textural characteristics of the cheese could be attributed to the use of the adjunct culture *L. lactis* F9.

Flavor. Table 4 shows the development and accumulation of 3-methylbutanal in the Cheddar cheese during ripening. No 3-methylbutanal was detected in the control cheese during the 90 d of ripening. These results are consistent with previous studies showing that 3-methylbutanal was absent in young Cheddar cheeses (Avsar et al., 2004; Whetstine et al., 2006). In contrast, the 3-methylbutanal content of cheese made with the adjunct *L. lactis* F9 increased significantly during ripening ($P < 0.05$). The 3-methylbutanal appeared significantly earlier and at a higher level in cheeses as the level of *L. lactis* F9 increased ($P < 0.05$). These results

Table 3. Primary composition of Cheddar cheeses after 1 d of ripening¹

Group ²	Starter culture (cfu/mL of milk)	Adjunct culture (cfu/mL of milk)	Fat (%)	Protein (%)	Moisture (%)	pH
Control	$\sim 1 \times 10^{8a}$	0 ^a	30.15 ± 0.19^a	21.51 ± 0.05^a	41.50 ± 0.14^a	5.14 ± 0.02^a
F91	$\sim 1 \times 10^{8a}$	$\sim 1 \times 10^{5b}$	30.46 ± 0.20^a	20.96 ± 0.55^a	41.80 ± 0.89^a	5.15 ± 0.01^a
F92	$\sim 1 \times 10^{8a}$	$\sim 1 \times 10^{6c}$	29.01 ± 0.50^a	20.78 ± 0.31^a	41.01 ± 0.34^a	5.12 ± 0.03^a
F93	$\sim 1 \times 10^{8a}$	$\sim 1 \times 10^{7d}$	29.25 ± 0.35^a	21.00 ± 0.05^a	42.53 ± 0.16^a	5.13 ± 0.02^a

^{a–d}Different superscripts within a column indicate a significant difference between groups ($P < 0.05$).

¹Mean $\pm$ SD; n = 3.

²F91, F92, and F93 = addition of 10^5 , 10^6 , and 10^7 cfu/mL of adjunct culture F9, respectively.

Table 4. Development and accumulation of 3-methylbutanal ($\mu\text{g}/\text{kg}$) in Cheddar cheese during ripening¹

Group ²	Ripening time (d)			
	1	30	60	90
Control	— ³	—	—	—
F91	—	—	0.37 ± 0.06^a	0.76 ± 0.03^a
F92	—	—	0.42 ± 0.03^a	0.98 ± 0.04^b
F93	—	0.41 ± 0.03	0.61 ± 0.07^b	1.26 ± 0.06^c

^{a-c}Different superscripts within a column indicate a significant difference between groups ($P < 0.05$).

¹Mean $\pm$ SD; $n = 3$.

²F91, F92, and F93 = addition of 10^5 , 10^6 , and 10^7 cfu/mL of adjunct culture F9, respectively.

³Means not detectable.

have confirmed that *L. lactis* F9 produced a high level of enzymatic activity. Higher levels of added adjunct culture increased the rate of relative enzymatic activity in the cheese, leading to the increased production of 3-methylbutanal. The 3-methylbutanal concentration in Cheddar cheeses made with 10^5 cfu/mL of *L. lactis* F9 after 3 mo of ripening reached the level of those made with the same concentration of adjunct malty *L. lactis* ATCC 29146 aged at the same temperature for 4 mo (Whetstine et al., 2006). Therefore, adding the nondairy *L. lactis* F9 could also efficiently reduce the ripening time of Cheddar cheese. The 3-methylbutanal concentrations present in the current study and that of Whetstine et al. (2006) were lower than those reported by Avsar et al. (2004), probably due to a shorter ripening period. Furthermore, volatile compounds formed in a ripened cheese are highly complex and exposed to the mutual influence of several microorganisms. The 3-methylbutanal content was found to be significantly higher in ripening Grana Padano cheese with a more complex microbial composition (Lazzi et al., 2016). Nonstarter lactic acid bacteria *Lactobacillus paracasei* FMAC21 and *Pediococcus acidilactici* FMAC31 isolated

from Caciocavallo Palermitano cheeses showed a great ability to generate 3-methylbutanal in a cheese-based medium (Guarrasi et al., 2017). Therefore, some adventitious and viable nonstarter lactic acid bacteria may also contribute to the formation of 3-methylbutanal.

In accordance with the results of the 3-methylbutanal concentration, addition of adjunct *L. lactis* F9 significantly enhanced the intensity of nutty flavor in cheese ($P < 0.05$; Table 5). Moreover, those cheeses made with the adjunct *L. lactis* F9 showed superior sensory characteristics because they tasted nutty without any bitterness and acerbité after ripening 90 d (Table 5). There were no significant differences in cooked, umami, whey, milk fat, sour, and salty flavor between cheeses; however, the fruity flavor was higher in the cheeses made with *L. lactis* F9. This could probably be attributed to a high alcohol acyltransferase activity in *L. lactis* F9. Additionally, there were no significant differences in the textural properties between the cheeses (see Supplemental Table S2; <https://doi.org/10.3168/jds.2018-14760>), indicating that adding *L. lactis* F9 notably enhanced the generation of 3-methylbutanal and the corresponding nutty flavor of cheese without impairing its textural properties.

CONCLUSIONS

The present study selected a wild nondairy *L. lactis* ssp. *lactis* F9 from a sour bamboo shoot product that preferentially generated 3-methylbutanal. The in vitro activities of the enzymes involved in the biosynthesis pathway of 3-methylbutanal have also been investigated. The results showed that both KADC and KADH activities were detected in vitro in *L. lactis* F9, indicating that both the direct KADC and indirect KADH pathways were functional in *L. lactis* F9. As both pathways were available in nondairy *L. lactis* F9, it could function under various redox conditions, which would

Figure 3. Enzyme activity of individual strains: (A) hydroxy acid dehydrogenase (HADH) activity; (B) aldehyde dehydrogenase activity (AldDH) activity; (C) alcohol dehydrogenase (AlcDH) activity. Different letters (a–d) indicate a significant difference between groups ($P < 0.05$). Error bars represent the SD of triplicate experiments.

Table 5. Sensory evaluation of Cheddar cheese after ripening for 90 d^{1,2}

Attribute	Group			
	Control	F91	F92	F93
Aromatic				
Nutty	3.23 ± 0.41 ^a	3.55 ± 0.58 ^a	5.34 ± 0.82 ^b	7.18 ± 1.08 ^c
Cooked	3.88 ± 0.64 ^a	4.23 ± 0.71 ^a	4.25 ± 0.19 ^a	4.04 ± 0.60 ^a
Whey	5.08 ± 0.85 ^a	5.17 ± 1.11 ^a	4.46 ± 0.74 ^a	5.15 ± 0.85 ^a
Milk fat	5.10 ± 1.38 ^a	5.20 ± 0.92 ^a	5.30 ± 0.87 ^a	5.58 ± 1.30 ^a
Fruity	1.73 ± 0.32 ^a	1.83 ± 0.45 ^a	2.17 ± 0.48 ^{ab}	2.75 ± 0.42 ^b
Taste				
Umami	3.64 ± 0.26 ^a	3.59 ± 0.18 ^a	3.68 ± 0.53 ^a	3.45 ± 0.46 ^a
Sour	3.73 ± 0.30 ^a	4.08 ± 0.24 ^a	3.63 ± 0.26 ^a	3.93 ± 0.16 ^a
Salty	3.42 ± 0.48 ^a	4.00 ± 0.41 ^a	3.75 ± 0.22 ^a	3.50 ± 0.60 ^a
Bitter	4.62 ± 0.64 ^a	4.23 ± 0.55 ^a	4.46 ± 0.75 ^a	4.08 ± 0.07 ^a
Sweet	1.33 ± 0.41 ^a	1.83 ± 0.67 ^a	1.42 ± 0.21 ^a	1.58 ± 0.19 ^a

^{a-c}Different superscripts within a row indicate a significant difference between groups ($P < 0.05$).

¹Mean ± SD; n = 3.

²F91, F92, and F93 = cheeses with addition of 10⁵, 10⁶, and 10⁷ cfu/mL of adjunct culture F9, respectively.

be greatly beneficial for catabolizing leucine and cheese aroma development. This is the first time that the co-existence of both pathways has been demonstrated in a *Lactococcus* strain. Adding nondairy *L. lactis* F9 as an adjunct culture during the manufacture of Cheddar cheese significantly increased its 3-methylbutanal content and nutty flavor without causing dramatic changes in its basic composition and textural properties. This study has improved the understanding of the biosynthesis pathway of 3-methylbutanal in a specific strain and could thus contribute to developing an adjunct culture for producing cheese with an intense nutty flavor and reducing its ripening time.

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