Origin of Acetaldehyde during Milk Fermentation Using ^{13}C-Labeled Precursors

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Acetaldehyde formation by *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptococcus thermophilus* during fermentation of cow's milk was investigated using ^{13}C-labeled glucose, L-threonine, and pyruvate with a recent static-and-trapped-headspace technique that does not require derivatization of acetaldehyde prior to gas chromatography−mass spectrometry. Over 90% and almost 100% of acetaldehyde originated from glucose during fermentation by *L. delbrueckii* subsp. *bulgaricus* and *S. thermophilus*, respectively, taking into account both singly and doubly labeled acetaldehyde. As both microorganisms showed threonine aldolase activity and formed labeled acetaldehyde from ^{13}C-labeled threonine during the fermentation of milk, this amino acid should also contribute to the acetaldehyde produced.

Keywords: *Acetaldehyde; aroma; milk; fermentation; yogurt; precursor; threonine; threonine aldolase; pyruvate; glucose; stable isotope; ^{13}C; Streptococcus thermophilus; Lactobacillus bulgaricus*

INTRODUCTION

Acetaldehyde is one of the key aroma compounds in yogurt (Imhof, 1994; Ott et al., 1997) and is responsible for its fresh-fruity note. It is formed during milk fermentation by microorganisms and in yogurt is the predominant synthesized volatile compound (Görner et al., 1968), with concentrations in the range of 5−21 mg/L in the final product (Kneifel et al., 1992; Ott et al., 1999). In other fermented dairy products, such as cheese, butter, or buttermilk, only small amounts of acetaldehyde are necessary for the formation of their typical flavors. Above a certain concentration it contributes in these products to a green yogurt-like note, which is considered as an off-flavor (Lindsay and Day, 1965; Lindsay et al., 1967; Keenan and Bills, 1968; Sandine et al., 1972; Raya et al., 1986a). Understanding acetaldehyde biosynthesis and how to control it, would, therefore, be beneficial for many dairy products.

In yeast, acetaldehyde can be directly formed from pyruvate by the action of pyruvate decarboxylase (EC 4.1.1.1) (Pronk et al., 1996). However, the same organism can also form acetaldehyde as a byproduct of glycine biosynthesis from threonine by threonine aldolase (Monschau et al., 1997).

The production of acetaldehyde in milk by lactic acid bacteria seems to be strain dependent. *Lactobacillus delbrueckii* subsp. *bulgaricus* has been reported by some authors to be a greater acetaldehyde producer than *Streptococcus thermophilus*, whereas other authors have reported the contrary (Pette and Lolkema, 1950; Shankar, 1977). Although the biosynthesis of acetaldehyde itself remains unclear, three main metabolic pathways have been shown to lead to its formation (Figure 1).

Glucose, through the glycolytic pathway, was shown to be a source of acetaldehyde in group N streptococci (Lees and Jago, 1976a). Addition of uniformly labeled ^{14}C glucose to Cheddar cheese slurry led to the labeling of a number of compounds, among them acetaldehyde, diacetyl, and formic and acetic acids (Lin et al., 1979).

The degradation product of DNA, 2-deoxyribose-5-phosphate, was shown to be a precursor of acetaldehyde in the presence of deoxyriboaldolase (EC 4.1.2.4). This enzyme was detected in some strains of *L. bulgaricus* and *S. thermophilus* (Lees and Jago, 1977; Raya et al., 1986a). The contribution of this metabolic route should be rather small, as DNA degradation itself during the exponential growth of bacteria must be low.

Threonine aldolase (EC 2.1.2.1), found in most group N streptococci as well as *S. thermophilus* and *L. bulgaricus*, contributes to the formation of acetaldehyde from L-threonine (Lees and Jago, 1976b; Raya et al., 1986b). From ^{14}C uniformly labeled L-threonine, only 2% of acetaldehyde was found to be labeled in a mixed culture of *S. thermophilus* and *L. bulgaricus* (Wilkins et al., 1986a). In a similar type of mixed culture Marranzini et al. (1989) and Schmidt et al. (1989) showed that threonine stimulates, whereas glycine inhibits, the formation of acetaldehyde. *S. thermophilus* was shown to be more sensitive to glycine inhibition than *L. bulgaricus* (Wilkins et al., 1986b).

The aim of this paper was to determine the importance of the glucose and threonine pathways leading to acetaldehyde in single and mixed cultures of *S. thermophilus* and *L. bulgaricus* in cow's milk. To evaluate the contribution of glucose to acetaldehyde formation, it was necessary to prevent any contribution of lactose. For this reason, β-galactosidase-deficient mutants (lac$^-$) of *S. thermophilus* and *L. bulgaricus* were used throughout this study. Instead of tedious derivatization methods, the acetaldehyde production pathway was investigated by a combination of ^{13}C-labeled precursors and detection of their labeled volatile metabolites using a recently developed trapping on Tenax: static-and-trapped headspace (S&T-HS) followed by gas chromatography−mass spectrometry (GC-MS) analysis.

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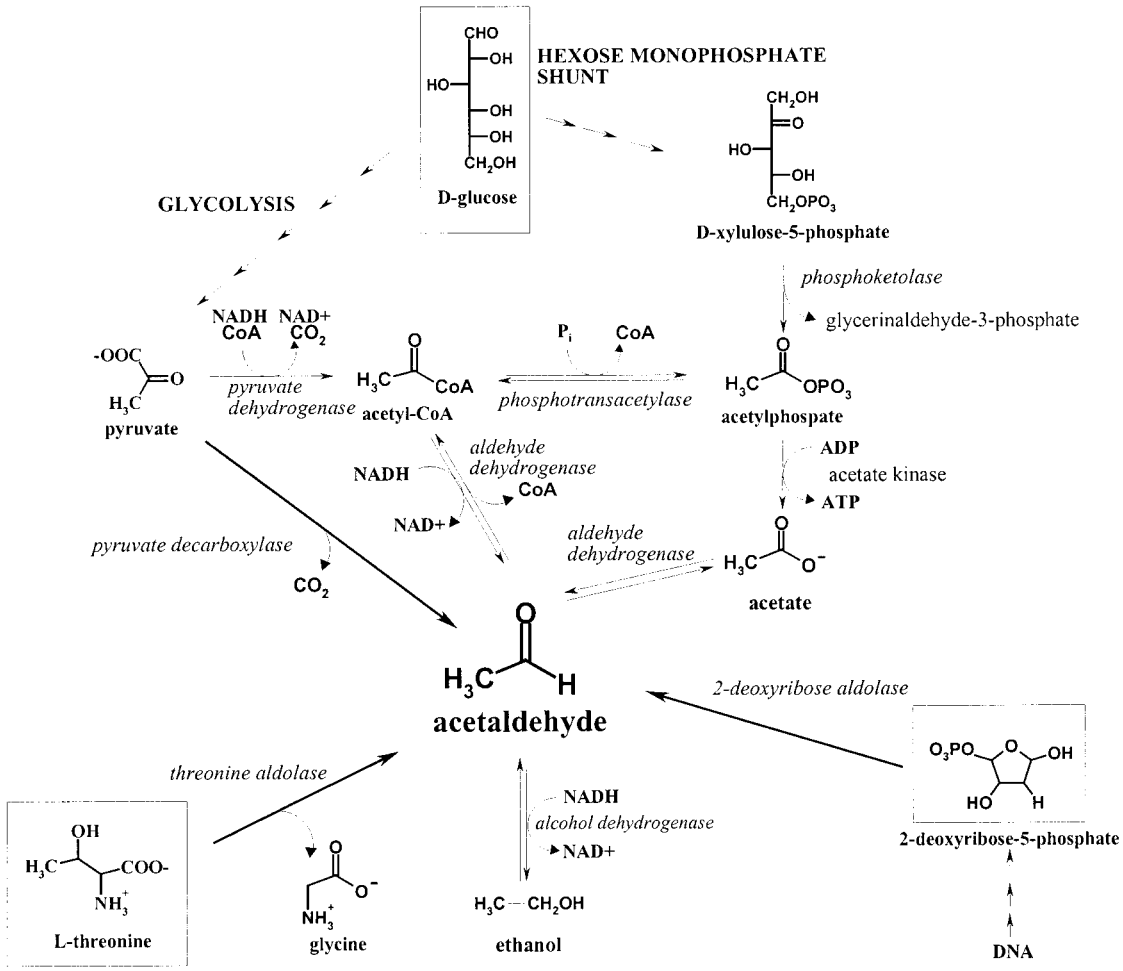

Figure 1. Overview of the different possible metabolic pathways leading to acetaldehyde [according to Raya et al. (1986a) and Gonzalez et al. (1994)].

MATERIALS AND METHODS

Chemicals. All chemicals used were of analytical grade from Fluka (Fluka AG, Buchs, Switzerland) unless otherwise mentioned. Labeled acetaldehyde U-$^{13}C_2$ (^{13}C 99%), D-glucose U-$^{13}C_6$ (^{13}C 99%+), L-threonine U-$^{13}C_4$ (^{13}C 97−98%) (U, uniformly labeled compound), and sodium pyruvate 2,3-$^{13}C_2$ (^{13}C 99%) were purchased from Cambridge Isotope Laboratories (CIL, Inc., Andover, MA).

Preparation of Fermented Milk Samples. The following microorganisms from the Nestlé culture collection (NCC) were used: *Streptococcus thermophilus* S97A1 (NCC 1029) and *Lactobacillus delbrueckii* ssp. *bulgaricus* LB52 (NCC 499), both β-galactosidase negative (lac$^-$). They were propagated in reconstituted skimmed milk powder containing 10 g/L yeast extract (Difco, Detroit, MI) and 10 g/L D-(+)-glucose.

Milk was reconstituted by dissolving 100 g of skimmed milk powder (Berneralpen Milchgesellschaft, Konolfingen, Switzerland) in 1 L of distilled water and sterilized at 98 °C for 35 min. Threonine, glucose, or sodium pyruvate was added as sterile filtered aqueous solutions (0.45 μm). Fermentation was carried out in 150 mL sterile glass jars. Samples containing labeled compounds were fermented in sterile closed glass tubes of 10 mL at 41 °C for 6 h (5 g/L glucose) and 8 h (5 and 10 g/L glucose). Starters were grown to late logarithmic phase in MRS medium (DeMann et al., 1960) for *L. bulgaricus* and in the Hogg and Jago (1970) medium containing 10 g/L glucose for *S. thermophilus*. Cells were washed once with physiological salt solution and resuspended in the initial volume. Inoculation of milk with the cell suspension was 1 and 2% for *S. thermophilus* and *L. bulgaricus*, respectively.

Free amino acids were quantified according to the method of Schuster (1988) by derivatization using *o*-phthalaldehyde−3-mercaptopropionic acid (OPA−3-MPA) for primary amino acids and 9-fluorenylmethylchloroformate (FMOC) for secondary amino acids.

Threonine Aldolase Assay. Bacteria were grown at 41 °C to late logarithmic phase either in MRS (Difco) or in Hogg and Jago (1970) medium containing 10 g/L of glucose. Cells were harvested by centrifugation at 1800*g* for 10 min, washed three times with sodium phosphate buffer (100 mM, pH 7), and frozen at −20 °C until use. One volume of cell pellet was mixed with 1 volume of acid-washed glass beads (425−600 μm, Sigma) and 1 volume of homogenization buffer [100 mM sodium phosphate buffer, pH 7, 1 mM DTT (Merck), 20 μM pyridoxal phosphate (Fluka)]. The cells were disrupted on a Heidolph vortex mixer (Verrerie Carouge, Carouge, Switzerland) for 90 s at 4 °C. The lysates were diluted 3−4 times with the homogenization buffer, centrifuged twice at 15000*g* for 5 min at 4 °C, and immediately used for enzymatic assays.

Reactions were carried out in 5 mL of buffer (100 mM sodium phosphate buffer, pH 7, containing 80 mM threonine and 30 μM pyridoxal phosphate), containing 100 μL of crude extract and 50 μL of a 2-propanol solution in water (1 mg/mL) as internal standard, incubated for 1 h at 41 or 36 °C, and cooled on ice.

Acetaldehyde was quantified by headspace gas chromatography, using a calibration curve of acetaldehyde in enzyme assay buffer containing 2-propanol as internal standard. Conditions of analysis were the same as previously described [procedure 2 of Ott et al. (1999)]. Protein concentration was determined with the Bio-Rad test (Bio-Rad, Glattbrugg, Switzerland) using bovine serum albumin (Sigma) as standard.

Gas Chromatography—Mass Spectrometry (GC-MS) Analysis. Aliquots (2 g) of fermented milk were analyzed using a headspace cell and thermal desorber according to a reported procedure [procedure 1c of Ott et al. (1997)].

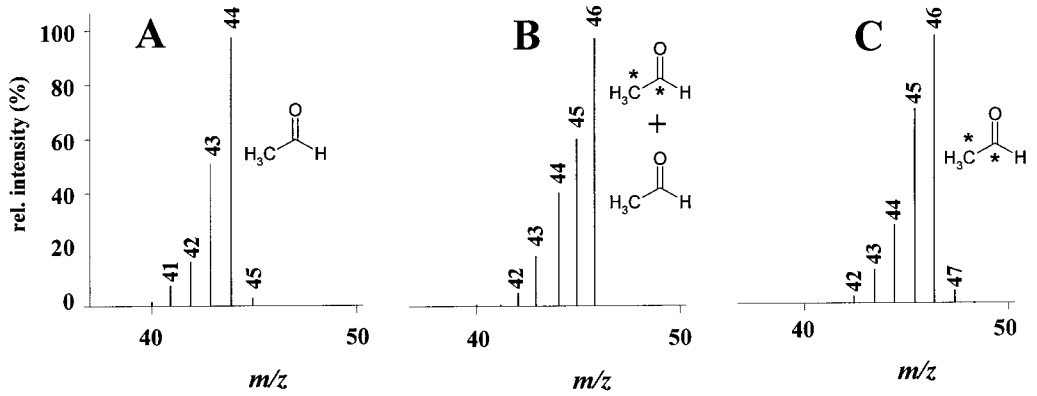

Figure 2. Mass spectra of (A) unlabeled, (B) a mixture of unlabeled (40%) and ^{13}C-double-labeled (60%), and (C) pure ^{13}C-double-labeled acetaldehyde.

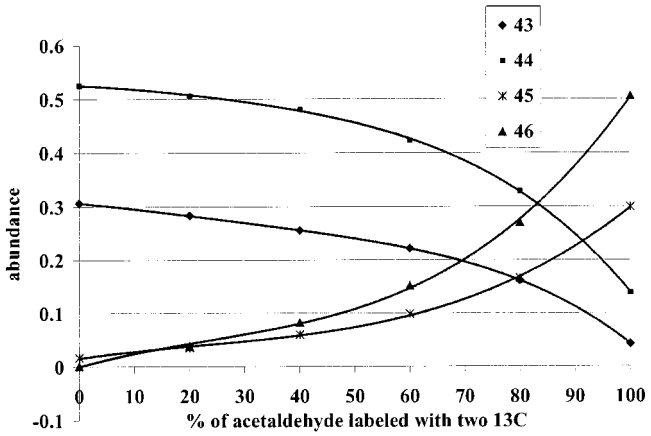

Figure 3. Abundances of ions 43−46 in a mixture of unlabeled and double-labeled acetaldehyde.

Volatiles trapped on Tenax were separated using an HP 6890 GC (Hewlett-Packard, Avondale, PA) equipped with a DB-Wax column (J&W Scientific, Folsom, CA) (60 m length, 0.53 mm i.d., 1.00 μm phase thickness). The carrier gas was helium. The column was kept at 20 °C for 5 min, increased at 4 °C/min to 200 °C, and maintained for 10 min. The column outlet was split and connected to an FID and an HP 5973 MS detector (Hewlett-Packard). Acetaldehyde was analyzed in the SIM mode focusing on the ions with *m/z* from 39 to 47. Labeling of all other molecules was followed in the full-scan mode. MS conditions were the following: dwell time (SIM), 40 ms; MS source temperature, 230 °C; MS quadrupole, 106 °C; interface temperature, 220 °C; EI, 70 eV.

Labeling Ratio of Acetaldehyde. A calibration curve was constructed by injecting standards containing known ratios of unlabeled and uniformly labeled acetaldehyde. Mass spectra of unlabeled acetaldehyde, labeled, and a mixture of both are shown in Figure 2. For each standard solution, detector counts of masses 39−47 were normalized to 100% of the sum of counts. Plotting relative abundances of ions 43−46 as a function of the percentage of labeled acetaldehyde gave rise to curved calibration lines (Figure 3) due to the normalization (see Results and Discussion).

The ratio of doubly labeled acetaldehyde after fermentation was determined from these curves. As some labeling experiments gave a higher abundance at *m/z* 45 than expected from the corresponding *m/z* 46, the excess was attributed to formation of singly labeled acetaldehyde.

RESULTS AND DISCUSSION

Validation of Acetaldehyde Labeling Ratio Determination. Acetaldehyde (radioactive) labeling determination as described by Wilkins et al. (1986a) consisted of derivatization, separation, and subsequent counting of the radioactivity found in the derivative. Drawbacks lie in the manipulation of radioactive material. We used S&T-HS sampling, which allowed trapping, GC separation, and MS analysis of acetaldehyde without any tedious derivatization. In addition, the use of compounds with stable instead of radioactive isotopes was possible. To our knowledge the combination of a headspace technique with stable isotopes for metabolism studies has not yet been reported in the literature. The mixture of unlabeled and ^{13}C-labeled acetaldehyde was directly sampled on Tenax and subsequently desorbed and injected into a GC-MS. Low breakthrough volumes of acetaldehyde in Tenax (Maier and Fieber, 1988) were overcome by the use of labeled acetaldehyde as internal standard as both isotopomers were assumed to be trapped in the same proportion. Coeluted GC peaks of labeled and unlabeled acetaldehyde were analyzed and quantified by MS in the SIM mode.

Because of the possible formation of monolabeled acetaldehyde, which would significantly alter abundances of *m/z* 42−46, the quantitation could not be based on the calibration curve of a single ion. Therefore, a less common calibration procedure was chosen, taking simultaneously into account the four most abundant masses of the molecular ion cluster (43−46) as shown in Figure 3. For each standard solution, counts measured for masses 41−47 were normalized to reduce possible experimental variations of absolute values: $a_i^x = c_i^x/\sum_i c_i^x$ (c_i^x is counts for the ion i in the spectrum of the standard containing a fraction x of labeled acetaldehyde).

Counts measured for a given $m/z = i$ must be $c_i^x = c_i^0(1 - x) + c_i^* x$ (c_i^0 and c_i^* are the counts for the ion i in the nonlabeled and labeled acetaldehyde, respectively).

The normalized abundances of a given ion become

$$a_i^x = \frac{c_i^0(1 - x) + c_i^* x}{(1 - x)\sum_i c_i^0 + x\sum_i c_i^*} \qquad (1)$$

Such an equation justifies the curvature of calibration lines shown in Figure 3. Deviations of experimental normalized abundances did not differ by more than 3.3% from those calculated with eq 1 from the pure labeled and nonlabeled compounds.

These curves were used to determine ratios of doubly labeled acetaldehyde from fermentation media spiked with a labeled precursor. In the case of experiments yielding solely a double labeling, abundances of all ions

of the 43−46 series corresponded exactly to those found in Figure 3 for a given percentage of acetaldehyde enrichment. When the abundance of *m/z* 45 was higher than the contribution of the $[M - 1]^+$ resulting from the doubly labeled acetaldehyde, the difference was attributed to formation of a monolabeled isotopomer.

Incorporation of ^{13}C in Acetaldehyde from D-Glucose U-$^{13}C_6$. The two strains used (*L. bulgaricus* LB52 and *S. thermophilus* S97A1) are β-galactosidase negative (lac$^-$). They are unable to degrade lactose and depend entirely on the presence of glucose for growth. Both microorganisms were grown in milk in the presence of 5−10 g/L of uniformly ^{13}C-labeled glucose. After 8 h of fermentation in the presence of ^{13}C-glucose (10 g/L), most, if not all, of the acetaldehyde was labeled (Figure 4). For *L. bulgaricus* alone, 90% of acetaldehyde was found to be labeled on both carbons, whereas for *S. thermophilus* only 60% of acetaldehyde had both carbons labeled and 40% was labeled only on one carbon. The formation of this high amount of monolabeled acetaldehyde cannot yet be explained. Nevertheless, glucose turns out to be the main precursor for acetaldehyde synthesis.

In the presence of sodium 2,3-$^{13}C_2$ pyruvate only 5−10% of acetaldehyde was shown to be labeled (Figure 4, glc/pyr*). This small incorporation of ^{13}C should be due to competition between the labeled pyruvate, which crosses the cell membrane, and the unlabeled pyruvate generated during glycolysis within the cell. About half of the labeled acetaldehyde was found to be only monolabeled. Pyruvate has been shown to be able to cross the cell membrane in *Lactobacillus plantarum* (Tsau et al., 1992).

Incorporation of ^{13}C into Acetaldehyde from L-Threonine U-$^{13}C_4$. Uniformly ^{13}C-labeled threonine (100 mg/L) was added to milk fermented with the two lac$^-$ strains used above. After 6 h of fermentation, incorporation of ^{13}C in acetaldehyde was observed (Figure 4, glc/thr*). With *S. thermophilus* 90% of the 10 mg/L acetaldehyde produced was labeled. Formation of monolabeled acetaldehyde was negligible (Figure 4, glc/thr*). With *L. bulgaricus*, the presence of threonine seems to reduce growth behavior. In these conditions, 60% of the 3.8 mg/L acetaldehyde produced was labeled. In mixed culture, production of labeled acetaldehyde was similar to that observed with the pure culture of *S. thermophilus* but with the production of some monolabeled isotopomer. These results indicate that threonine is one of the precursors for the synthesis of acetaldehyde in yogurt bacteria.

Influence of Threonine Metabolism on Incorporation of ^{13}C into Acetaldehyde from D-Glucose U$^{13}C_6$. In the presence of increasing concentrations of threonine added prior to fermentation, the concentration of acetaldehyde doubles for both microorganisms at 300 mg/L L-threonine (data not shown). During fermentation with *S. thermophilus* in the presence of labeled D-glucose U$^{13}C_6$, the addition of 100 mg/L L-threonine (Figure 4, glc*/thr) led to an increase in the production of acetaldehyde and a reduction of the labeled fraction compared to fermentation without added theronine (Figure 4, glc*). Under these conditions, the fraction labeled by the D-glucose U$^{13}C_6$ is roughly comparable to the unlabeled fraction in the presence of glucose and L-threonine U$^{13}C_4$ (Figure 4, glc*/thr and glc/thr*). Increasing amounts of L-threonine in the presence of glucose U$^{13}C_6$ led to a continuous increase of acetaldehyde produced (Figure 5A) and a decrease of the labeled fraction (Figure 5B). These results indicate that L-threonine at high concentration is the major precursor of acetaldehyde, by inhibiting the contribution of glucose. However, such a high concentration of free L-threonine does not reflect the normal situation in cow's milk, which contains ~1 mg/L of this amino acid in the free form and ~1.5 g/L as bound protein available only upon proteolysis. This approximate symmetry between the labeling patterns of glc*/thr and glc/thr* in *S. thermophilus* (Figure 4) is also observed in *L. bulgaricus* and in the mixed culture, with a larger variability associated with living organisms (Figures 4 and 5).

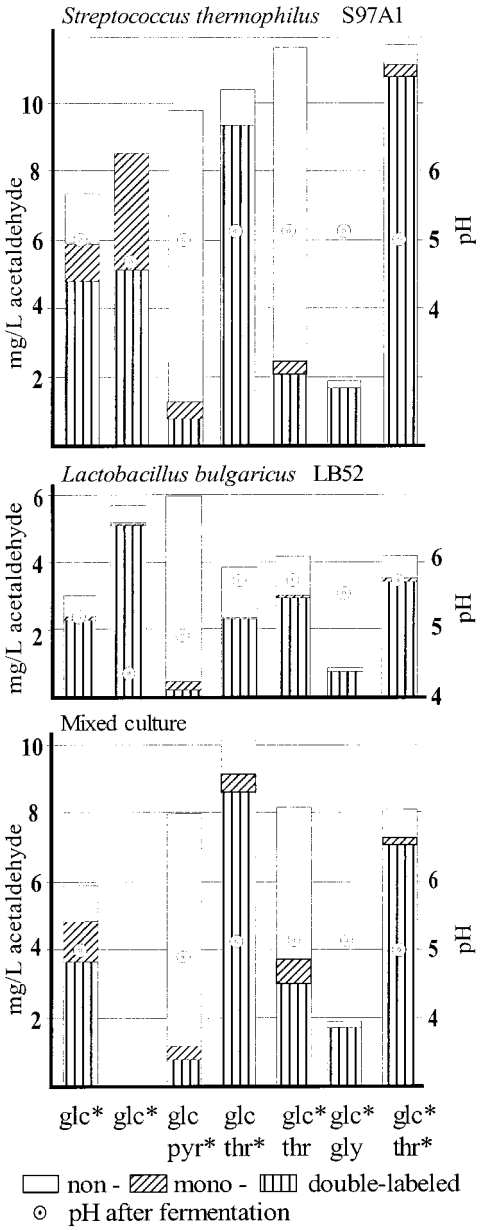

Figure 4. Production of acetaldehyde and proportion of unlabeled, monolabeled, and double-labeled molecules during fermentation (6 h) with *S. thermophilus* (S97A1), *L. bulgaricus* (LB52), and mix of both microorganisms of milk supplemented with glc***** (5 or 10 g/L of labeled D-glucose U-$^{13}C_6$), glc/pyr***** (5 g/L D-glucose and 1 g/L labeled sodium pyruvate 2,3-$^{13}C_2$), glc/thr***** (5 g/L D-glucose and 100 mg/L labeled L-threonine U-$^{13}C_4$), glc*/thr (5 g/L labeled D-glucose U-$^{13}C_6$ and 100 mg/L L-threonine), glc*/gly (5 g/L labeled D-glucose U-$^{13}C_6$ and 2 g/L glycine), glc*/thr (5 g/L labeled D-glucose U-$^{13}C_6$ and 100 mg/L labeled L-threonine U-$^{13}C_4$).

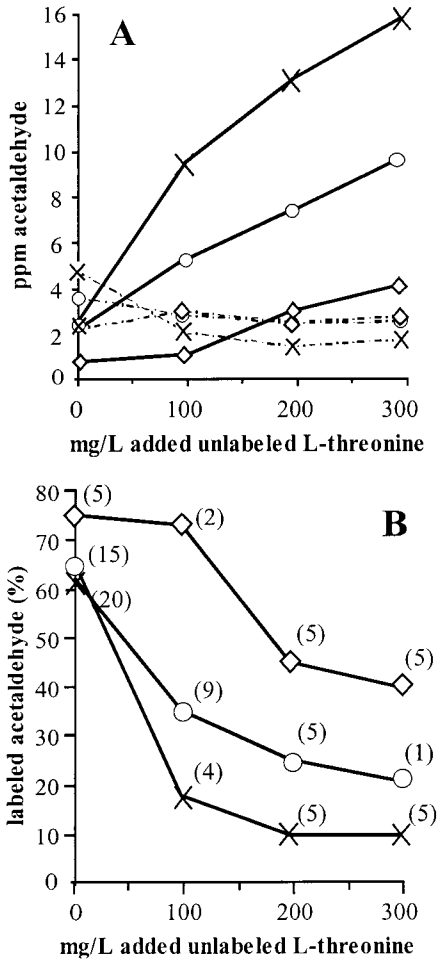

Figure 5. (A) Production of unlabeled (—) and labeled (-·-) acetaldehyde during fermentation (6 h) with *S. thermophilus* (S97A1) (×) and *L. bulgaricus* (LB52) (◇) and a mix of both microorganisms (○) in milk supplemented with 5 g/L labeled D-glucose $U^{13}C_6$ and increasing amounts of unlabeled L-threonine. (B) Percentage of double-labeled acetaldehyde (the percentage of monolabeled acetaldehyde is indicated in parentheses on curve points).

Table 1. Activity of Threonine Aldolase in Crude Extracts of β-Galactosidase Negative (lac⁻) Strains of *L. delbrueckii* Subsp. *bulgaricus* LB52 and *S. thermophilus* S97A1 Expressed as Nanomoles of Acetaldehyde Formed per Minute and per Milligram of Protein[a]

	specific activity	
microorganism	36 °C	41 °C
L. bulgaricus	3.2 [0.2]	5.0 [0.7]
S. thermophilus	27.8 [1.4]	43.0 [8.2]

[a] Standard deviations for three measurements are indicated in brackets.

Nevertheless, threonine aldolase, which can convert threonine to acetaldehyde, was quantified in crude extracts prepared from the two lac⁻ mutants of *L. bulgaricus* and *S. thermophilus*. The result showed that at 36 and 41 °C, threonine aldolase activity was found in both organisms and was higher for *S. thermophilus* than for *L. bulgaricus* (Table 1). This is in agreement with the findings of Wilkins et al. (1986b) but not with those of Lees and Jago (1976b), who showed the opposite results. However, their enzyme activities were measured for cells incubated for 18 h. This long growth period could influence the enzymatic activity of the cells, especially in the case of the rapid growing streptoccoci. Threonine is converted by threonine aldolase to acetaldehyde and glycine, which was shown to be an inhibitor of the reaction (Schmidt et al., 1989; Marranzini et al., 1989). Glycine was used in an attempt to minimize the contribution of threonine aldolase to acetaldehyde production, but in the presence of glycine (2 g/L) during milk fermentation by *S. thermophilus* and *L. bulgaricus*, the production of acetaldehyde, which is almost entirely double labeled, fell below 2 mg/L (Figure 4). The small amount of acetaldehyde produced in the presence of excess glycine could be explained by a displacement of the equilibrium of the reaction toward L-threonine. Glycine seems also to inhibit the metabolic pathway responsible for the production of monolabeled acetaldehyde in milk fermented with *S. thermophilus*. The small amount of double-labeled acetaldehyde should arise entirely from glucose.

In the presence of both labeled precursors (glucose and L-threonine), most of the acetaldehyde produced was doubly labeled (Figure 4). Under these conditions, threonine was already shown to be the major contributor to the biosynthesis of acetaldehyde (Figures 4 and 5). This explains why acetaldehyde was found to be almost entirely doubly labeled for *S. thermophilus*. The monolabeled form should then occur through an unknown metabolic pathway originating from glucose in *S. thermophilus*.

The labeling experiments with ^{13}C-labeled glucose, pyruvate, or threonine showed incorporation of ^{13}C into acetaldehyde and also into diacetyl, acetoin, and 2,3-pentanedione, which has been presented elsewhere (Ott et al., 2000). Acetone, ethanol, and acetate showed no significant modification of their mass spectra, suggesting that there is no significant production of these metabolites during milk fermentation with *L. bulgaricus* and *S. thermophilus*.

Conclusions. The metabolism of acetaldehyde in fermented dairy products can be studied by associating S&T-HS and GC-MS with stable isotopomers of precursors and β-galactosidase-deficient mutants (lac⁻) of lactic acid bacteria. Single or mixed cultures of lac⁻ mutants of *L. delbrueckii* subsp. *bulgaricus* and *S. thermophilus* were used to ferment milk in the presence of ^{13}C-labeled D-glucose, pyruvate, and L-threonine. Glucose was shown to be the main precursor of acetaldehyde, accounting for >90% of labeled acetaldehyde for *L. bulgaricus* and close to 100% for *S. thermophilus*. In the latter, 40% of the acetaldehyde was labeled on only one carbon. The occurrence of such a high amount of monolabeled acetaldehyde cannot actually be explained. In the presence of artificially high concentrations of L-threonine, more acetaldehyde was produced than with glucose, with even a repression of the glucose metabolic pathway. The consequence was that, in the presence of both labeled precursors, the majority of acetaldehyde produced was doubly labeled. Threonine aldolase activity was found in both microorganisms. If acetaldehyde is produced mainly through the glycolytic pathway in yogurt bacteria, threonine should contribute only to a small extent.

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