Vicinal Diketone Formation in Yogurt: ^{13}C Precursors and Effect of Branched-Chain Amino Acids

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Addition of branched-chain amino acids (BCAA) or an inhibitor of the BCAA biochemical pathways during fermentation of milk with a lac$^-$ mutant of *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptococcus thermophilus* strongly influenced the formation of two aroma-impact compounds, 2,3-butanedione and 2,3-pentanedione, as well as their direct precursors 2-acetolactate and 2-acetohydroxybutyrate. This suggests a connection between vicinal diketone formation and BCAA biosynthesis in yogurt bacteria. A recently developed static-and-trapped headspace technique combined with gas chromatography−mass spectrometry demonstrated incorporation of ^{13}C from [U-$^{13}C_6$]-D-glucose and [U-$^{13}C_4$]-L-threonine into both vicinal diketones. For 2,3-butanedione, glucose is the major precursor via pyruvate and activated acetaldehyde. For 2,3-pentanedione, L-threonine is a precursor via 2-ketobutyrate, but glucose is the major contributor via activated acetaldehyde and, possibly, also via 2-ketobutyrate, which is a degradation product of 3-methylaspartate, an intermediate in glutamate synthesis.

Keywords: *Yogurt; volatile; aroma; diacetyl; 2,3-butanedione; 2,3-pentanedione; branched-chain amino acids; formation; fermentation; Streptococcus thermophilus; Lactobacillus bulgaricus; 3-methylaspartate*

INTRODUCTION

The vicinal diketones, 2,3-butanedione and 2,3-pentanedione, play an important role in the aroma of many fermented foods and beverages. In yogurt and fermented milks they belong to the key aroma compounds (Imhof et al., 1995; Ott et al., 1997), whereas in alcoholic beverages, for example, wine and beer, they contribute, above a certain concentration, to off-flavors (Hugenholtz, 1993; Blomqvist et al., 1991).

Diacetyl biosynthesis in Gram-positive bacteria was focused on citrate-utilizing mesophilic lactic acid bacteria. Condensation of acetyl coenzyme A and activated acetaldehyde catalyzed by diacetyl synthase was shown to yield directly diacetyl (Kaneko et al., 1990; Speckman and Collins, 1968). More probable, however, is that condensation of pyruvate and activated acetaldehyde by 2-acetolactate synthase yields diacetyl via 2-acetolactate (Ramos et al., 1994) (Figure 1). Indeed, during mild yogurt fermentation, the precursors of diketones, 2-acetolactate and 2-acetohydroxybutyrate, were shown to accumulate before being converted to the respective diketones, 2,3-butanedione (diacetyl) and 2,3-pentanedione, during storage at 4 °C (Ott et al., 1999).

During glucose catabolism 2,3-butanedione serves as an electron acceptor and can be reduced to 2,3-butanediol via acetoin (Hugenholtz and Starrenburg, 1992; Snoep et al., 1992). This pathway was shown to be important in the removal of toxic amounts of pyruvate and in the maintenance of pH homeostasis (Tsau et al., 1992). In addition to its role in catabolism, 2-acetolactate is also an intermediate of the anabolic pathway leading to the branched-chain amino acids (BCAA) valine and leucine (Figure 1). Their formation is regulated by feedback inhibition in *Escherichia coli* (Umbarger, 1987). To our knowledge, the influence of BCAA on the formation of vicinal diketones and their precursor 2-acetohydroxy acids in yogurt has not been reported in the literature.

Lactobacillus delbrueckii subsp. *bulgaricus* and *Streptococcus thermophilus* are associated in the fermentation of milk for the production of yogurts. *L. bulgaricus* is mainly responsible for the production of the flavor compounds and the sharp acidity of the yogurt. As consumers prefer mild products, mutants of *L. bulgaricus*, unable to ferment lactose (lac$^-$), have been developed. In mixed milk culture, such mutants are dependent on *S. thermophilus* for their energy supply, leading to a low metabolic activity. The result is the production of mild, less acidic yogurt. In such mild product, the biosynthesis of 2-acetohydroxy acids was increased compared to that in acidic yogurts. These two precursors are then converted to the two diketones during storage of the products (Ott et al., 1999).

The aims of this study were (1) to elucidate the role of BCAA in milk fermented with a lac$^-$ mutant of *L. bulgaricus* associated with *S. thermophilus* and (2) to identify major anabolic pathways of diketone formation on the basis of metabolic pathways established in other microorganisms as summarized in Figure 1 and by using ^{13}C-labeled precursors. All analyses were performed using a new static-and-trapped headspace (S&T-HS) and gas chromatography−mass spectrometry (GC-MS) techniques.

MATERIALS AND METHODS

Chemicals. Methanol and 2,3-pentanedione were from Merck (Les Acacias, Switzerland); 2,3-butanedione, D-(+)-glucose, L-isoleucine, L-leucine, L-valine, sodium pyruvate,

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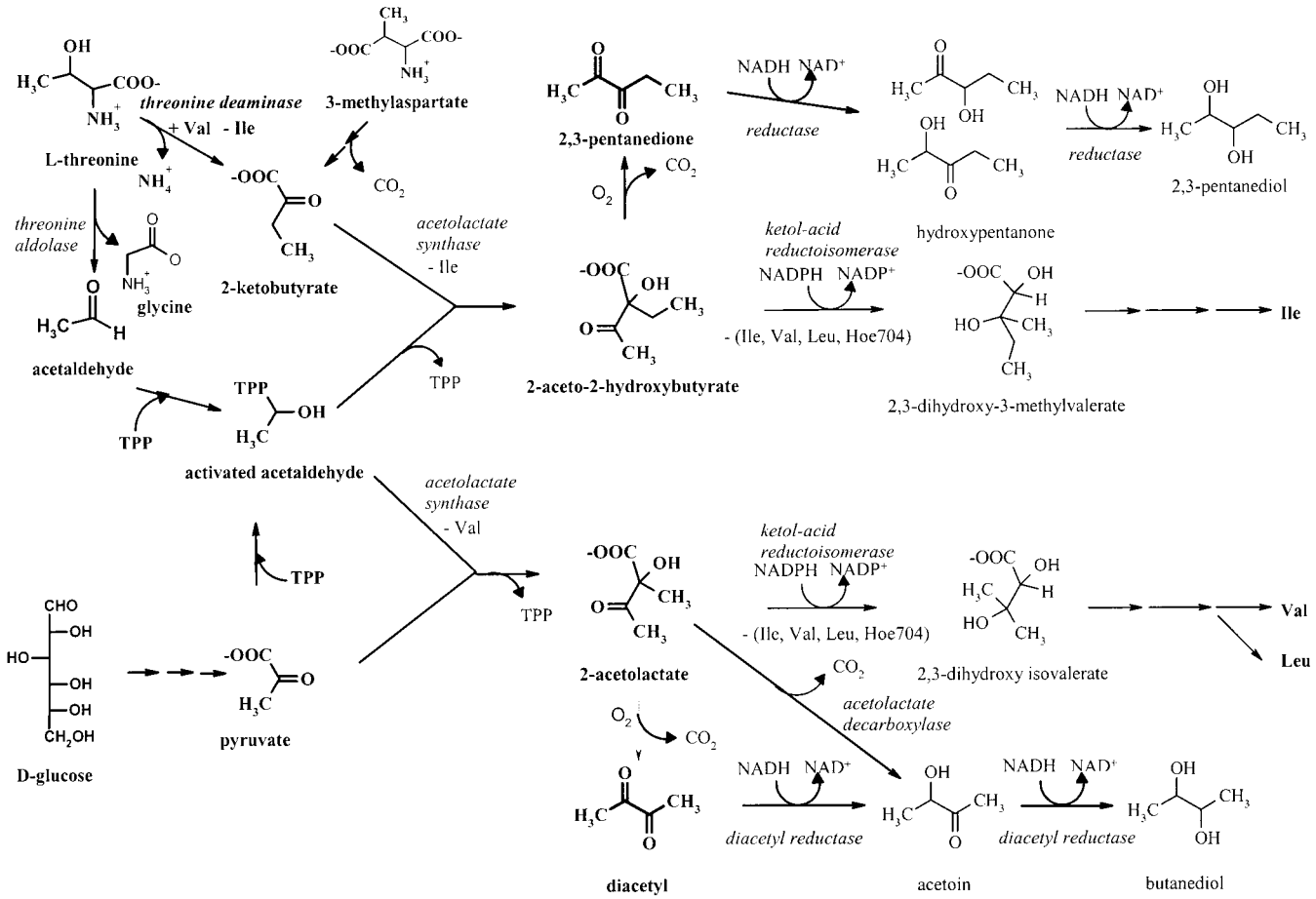

Figure 1. Proposed synthetic overview of the pathways involved in the biosynthesis of BCAA and vicinal diketones. The activation/induction (+) or reduction/repression (−) of enzymes by amino acids or other compounds is indicated. TPP, thiamine pyrophosphate.

sodium 2-ketobutyrate, and $FeCl_3$ (purum) were from Fluka AG (Buchs, Switzerland). They were of analytical grade if not otherwise stated. The acetolactate reductoisomerase inhibitor 2-methylphosphinoyl-2-hydroxyacetic acid (Hoe 704) was a gift from AgrEvo (AgrEvo GmbH, Frankfurt, Germany). [U-$^{13}C_6$]-D-Glucose (^{13}C 99%+) (U indicates uniformly labeled compound throughout this paper), [U-$^{13}C_4$]-L-threonine (^{13}C 97−98%), and sodium [2,3-$^{13}C_2$]pyruvate (^{13}C 99%) were from Cambridge Isotope Laboratories (CIL, Inc., Andover, MA).

Preparation of Fermented Samples. Full-fat cow's milk supplemented with 2.5% (w/w) of skimmed milk powder (Berneralpen Milchgesellschaft, Konolfingen, Switzerland) and sterilized (98 °C, 15 min) was used throughout this study. Fermentations were carried out in 2 L glass bottles containing 1 L of milk in order to favor oxidative decarboxylation of 2-acetohydroxyacids into 2,3-butanedione and 2,3-pentanedione by the headspace oxygen. Decarboxylation by acetolactate decarboxylase directly leads to acetoin. Fermentations with labeled precursors were carried out in 10 mL glass tubes containing 5 mL of sterilized (98 °C, 15 min) reconstituted skimmed milk (10%, w/w). The amino acids were added before sterilization of the milk. Labeled and unlabeled precursors as well as inhibitor (Hoe 704) were filter sterilized and added to the milk.

The bacterial strains used were from the Nestlé Culture Collection (NCC). The starters were prepared in MRS medium (DeMann et al., 1960) for *L. delbrueckii* subsp. *bulgaricus* and in the Hogg and Jago medium (1970) containing 1% lactose for *S. thermophilus*. The different strains were inoculated after careful washing with physiological sodium chloride solution to avoid carryover, at 2% for *L. bulgaricus* YL30 and its lac$^-$ mutant LB52, at 0.5% for *S. thermophilus* YS4 and YS7, and at 1% for the lac$^-$ mutant of *S. thermophilus* S97A1 (inocula ~10^8 cfu/mL for both).

Samples were incubated for 4.5 h, except those containing 1 g/L isoleucine, which were incubated for 6.5 h to allow the same pH to be reached because isoleucine was shown to be an inhibitor at high concentration (Bracquart and Lorient, 1979). Before analysis, all samples were stored in the dark at 4 °C for 1 or 14 days (storage conditions for yogurt).

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

GC and MS Analysis. Volatiles of the vapor phase in equilibrium with the yogurt inside the headspace cell were collected on Tenax traps and injected onto the GC column using a thermal desorber according to a previously reported procedure [procedure 1c of Ott et al. (1997)]. 2,3-Butanedione, 2,3-pentanedione, 2-acetolactate, 2-acetohydroxybutyrate, acetoin, and hydroxypentanone were quantified as previously described (Ott et al., 1999). 2-Acetolactate and 2-acetohydroxybutyrate were transformed to 2,3-butanedione and 2,3-pentanedione, respectively, by heating for 1 h at 80 °C under strong agitation and quantified as the corresponding diketones. Acetoin and hydroxypentanone were oxidized to the corresponding diketones using solid $FeCl_3$. Five grams of sample was diluted with 45 g of distilled water, and 2 g of $FeCl_3$ was added. This solution was incubated at 80 °C for 1 h under strong agitation in a hermetically closed jar. The solution was cooled to 4 °C, and the contents of 2,3-butanedione and 2,3-pentanedione were determined according to procedure 1 of a previous paper (Ott et al., 1999).

The amount of acetoin was calculated from the difference of total 2,3-butanedione after oxidation and the amount of 2,3-butanedione formed from 2-acetolactate by oxidative decarboxylation. Hydroxypentanone was quantified similarly. The

concentration of each compound was determined twice for each sample and presented as the mean of both values.

Volatiles were analyzed using an HP 5973 MS detector (Hewlett-Packard, Avondale, PA) in the SIM mode focusing on the following fragments (ions): 2,3-butanedione, *m/z* 86, 87, 88, 89, 90; 2,3-pentanedione, *m/z* 100, 101, 102, 103, 104, 105.

MS conditions were the following: dwell time, 40 ms; MS source temperature, 230 °C; MS quadrupole, 106 °C; interface temperature, 220 °C; EI, 70 eV.

RESULTS AND DISCUSSION

Influence of BCAA and Reductoisomerase Inhibitor on Vicinal Diketone Formation. The direct precursors of the two vicinal diketones, 2,3-butanedione (diacetyl) and 2,3-pentanedione, that is, 2-acetolactate and 2-acetohydroxybutyrate, have also been shown to be the precursors of BCAA in *Lactococcus lactis* (Goupil-Feuillerat et al., 1997), the biosynthesis of which can be strictly controlled by feedback inhibition in *E. coli* (Umbarger, 1987) (Figure 1). In lactic acid bacteria the essential requirement (auxotrophy) of these amino acids is strain specific (Chopin, 1993). For *L. delbrueckii*, valine and leucine are essential, whereas isoleucine can be nonessential in some strains (Ledesma et al., 1977; Guirard, 1974). For *S. thermophilus* the BCAA are not essential but stimulate growth in milk, except isoleucine, which inhibits growth at high concentrations (Bracquart and Lorient, 1979).

Our previous experiments showed that yogurt fermented with a lac$^-$ mutant of *L. delbrueckii* subsp. *bulgaricus* (LB52) and two strains of *S. thermophilus* (YS4 and YS7) contained ~3 times more 2,3-butanedione and 2,3-pentanedione after 14 days of storage at 4 °C than a yogurt fermented with the wild type *L. bulgaricus* (YL30) and the same strains of *S. thermophilus* (Ott et al., 1999). This lac$^-$ mutant has no β-galactosidase activity and is metabolically poorly active in the presence of *S. thermophilus* in milk.

Quantification of Free Amino Acids. The concentration of free amino acids was determined in milk used for yogurt preparation and in the two types of yogurt (Table 1). In milk, the concentration of free amino acids is low or even below the limit of detection for BCAA (Table 1). The low metabolic activity of the lac$^-$ mutant of *L. bulgaricus* (LB52) is responsible for the depletion of the amino acids except alanine and glutamine. Glycine and threonine concentrations do not change. By its high proteolytic activity, the wild type of *L. bulgaricus* (YL30) supplies peptides from the degradation of milk proteins for growth of both partners (Thomas and Pritchard, 1987). In milk fermented in the presence of this bacteria, the concentration of half of the amino acids increased, among them the BCAA. Only three were depleted, glutamic acid, lysine, and histidine (Table 1). When glucose was added during fermentation with the lac$^-$ mutants, to mimic a "lac$^+$" behavior, a similar amino acid profile was obtained (Table 1). The comparison to reported results (Sieber et al., 1996) shows a few differences, which can be attributed to the strain-specific nitrogen metabolism. This difference in the concentration of BCAA may be responsible for the higher concentration of vicinal diketones observed in fermented products in the presence of the lac$^-$ mutant (Figure 1).

Influence of BCAA on Vicinal Diketones. To test the influence of BCAA on vicinal diketone formation, yogurt was fermented in the presence of the lac$^-$ mutant of *L. bulgaricus* (LB52), two strains of *S. thermophilus* (YS4 and YS7), and increasing concentrations of these amino acids.

Table 1. Concentration (Milligrams per Kilogram) of Free Amino Acids in Milk (Fortified with 2.5% w/w Skim Milk Powder) Used for Yogurt Preparation and Yogurts Fermented with *S. thermophilus* (YS4 and YS7) and *L. bulgaricus* either the Wild Type (YL30: lac$^+$) or the lac$^-$ Mutant (LB52: lac$^-$) in the Absence or Presence of 1% of D-Glucose

amino acid	milk	LB52: lac$^-$	YL30: lac$^+$	LB52: lac$^-$ + 1% glucose	literature[a]
Ala	3.4	46.8	39.6	35.5	30
Arg	nd[b]	nd	7.5	3.2	16
Asn	nd	nd	8.8	1.4	8
Asp	4.1	nd	30.3	5.4	22
Cys	na[c]	na	na	na	na
Gln	nd	40.0	10.5	2.7	20
Glu	64.6	nd	4.8	7.0	59
Gly	7.6	5.9	6.9	4.9	8
His	2.2	nd	nd	7.6	16
Ile	**nd**	**nd**	**3.2**	**3.4**	**8**
Leu	**1.4**	**nd**	**13.6**	**12.2**	**24**
Lys	10.9	nd	nd	1.9	32
Met	nd	nd	nd	0.3	3
Phe	nd	nd	7.0	5.9	11
Pro	77.5	nd	33.6	43.0	63
Ser	nd	nd	19.9	15.7	20
Thr	23.7	23.0	25.5	23.0	7
Trp	nd	nd	nd	2.3	1
Tyr	nd	nd	nd	3.2	6
Val	**nd**	**nd**	**10.8**	**11.0**	**15**

[a] Literature values are given for yogurt (Sieber et al., 1996). [b] nd, not detected. [c] na, not analyzed.

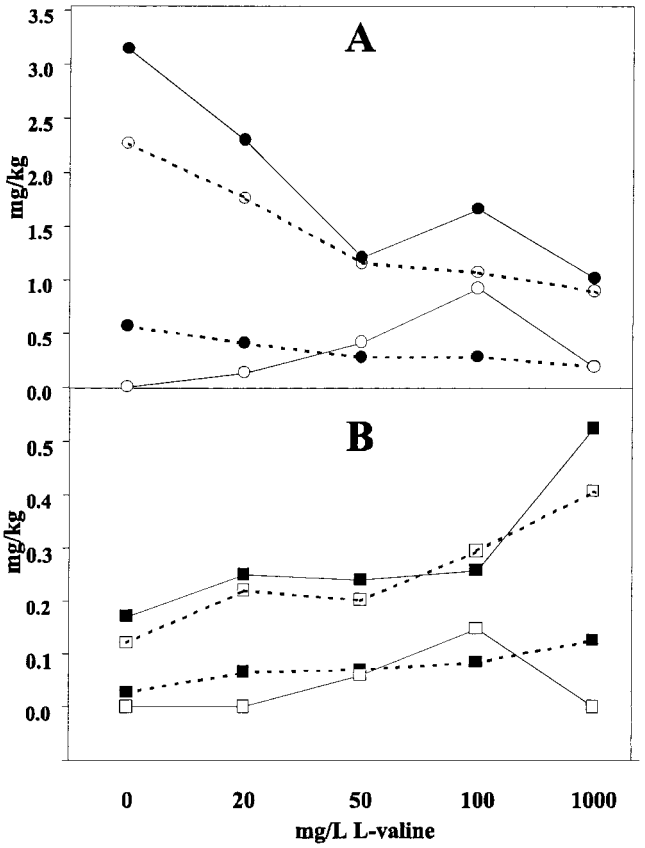

Figure 2. Concentrations of 2,3-butanedione (●) and 2-acetolactate (○) (A) and 2,3-pentanedione (■) and 2-acetohydroxybutyrate (□) (B) in milk fermented with *L. bulgaricus* (LB52) and *S. thermophilus* (YS4, YS7) in the presence of increasing amounts of L-valine after 1 day (···) and 2 weeks (—) of storage.

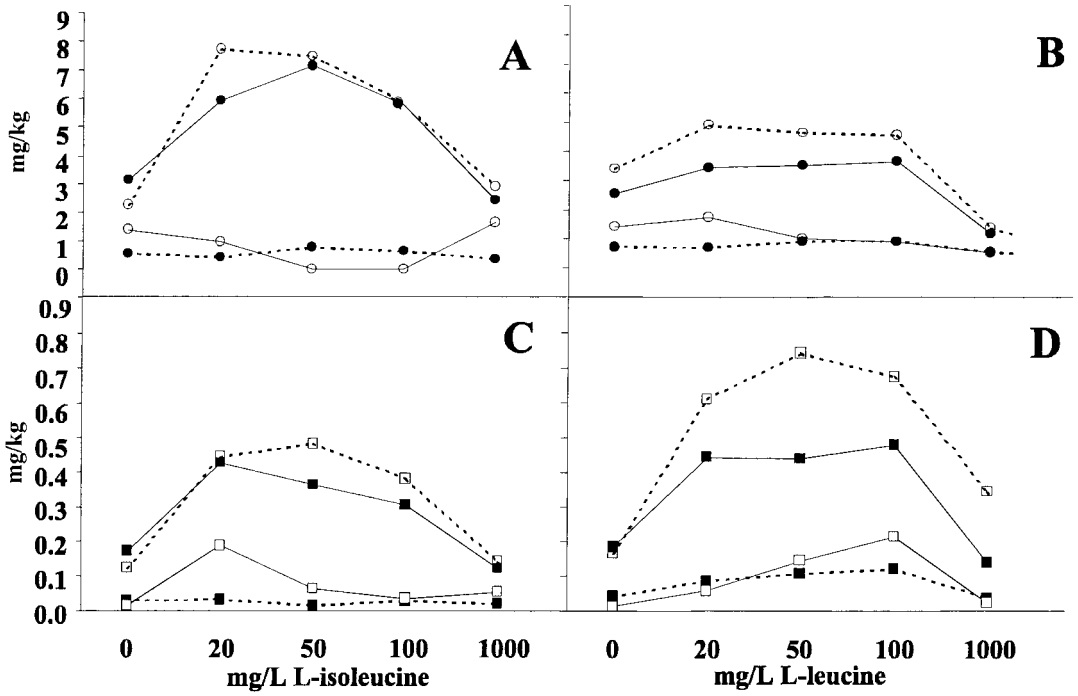

Figure 3. Concentrations of 2,3-butanedione (●) and 2-acetolactate (○) (A, B) and 2,3-pentanedione (■) and 2-acetohydroxybutyrate (□) (C, D) in milk fermented with *L. bulgaricus* (LB52) and *S. thermophilus* (YS4, YS7) in the presence of increasing amounts of the two BCAA L-isoleucine (A, C) and L-leucine (B, D) after 1 day (···) and 2 weeks (—) of storage.

In the presence of increasing amounts of valine during fermentation, the concentration of the vicinal diketones and their precursors was shown to decrease for 2,3-butanedione and to increase for 2,3-pentanedione. In both cases, the concentration of diketones was lower than that of their precursors at 1 day postfermentation. Two weeks after fermentation, the precursors were converted to their respective diketones by oxidative decarboxylation (Figure 2).

For 2,3-butanedione, the rate of this conversion seems to diminish when valine increases, as the concentration of 2-acetolactate increased up to 100 mg/kg valine. The general decrease of 2,3-butanedione in the presence of valine should be attributed to an inhibition of 2-acetolactate synthase by valine as described for *E. coli* (Umbarger et al., 1987; Vyazmensky et al., 1996) or activation of acetolactate decarboxylase (Phalip et al., 1994); however, the concentration of acetoin stayed constant at ∼12 mg/kg.

For 2,3-pentanedione, a steady increase in its concentration was observed in the presence of increasing concentration of valine. Here, too, the conversion of the precursor to the diketone diminished when valine increased. The general increase of 2,3-pentanedione in the presence of valine could be attributed to a stimulation of threonine deaminase, as was observed for *E. coli* (Eisenstein, 1991), which catalyzes the degradation of L-threonine into 2-ketobutyrate, a direct precursor of 2-acetohydroxy butyrate. L-Valine is also known to inhibit the enzyme ketol−acid reductoisomerase in *E. coli* (Umbarger, 1987), which catalyzes the transformation of 2-acetohydroxybutyrate into dihydroxy methylvalerate (Figure 1).

In the presence of increasing amounts of leucine or isoleucine during fermentation, the concentration of the vicinal diketones and their precursors started first to increase up to 20−50 mg/kg of added free BCAA. Above these concentrations formation of vicinal diketones decreased progressively to a basal level (Figure 3). Here, too, storage at 4 °C for 2 weeks after fermentation led the precursors to be mostly converted into their corresponding vicinal diketones by oxidative decarboxylation (Figure 3).

The increase of vicinal diketone production could be attributed to an inhibition of ketol−acid reductoisomerase at low concentration of amino acid as this enzyme was found to be controlled by all three BCAA in *S. typhimurium* (Umbarger, 1987). At higher amino acid concentration, threonine deaminase and acetolactate synthase could be inhibited, as shown for isoleucine in *E. coli* (Eisenstein, 1991, 1995; De Felice et al., 1982).

Leucine and isoleucine should also play a role in acetolactate decarboxylase or diacetyl reductase as acetoin concentration was shown to increase (acetoin data not shown). At low concentration of isoleucine acetoin production is depressed to half the basal level of 5 mg/kg, concomitantly with the large increase of 2,3-butanedione concentration (Figure 3A). With increasing isoleucine concentration, acetoin production came back to the basal level of 15 mg/kg. For leucine the conversion to acetoin seems to be immediate as its concentration increased steadily from 15 to 30 mg/kg at 1 mg/kg leucine. The concentration of 2,3-butanedione was shown to stay almost constant when leucine concentration varied (Figure 3B). At all concentrations of valine used, acetoin production was depressed to ∼11 mg/kg.

Influence of Reductoisomerase Inhibitor (Hoe 704). The influence of BCAA on vicinal diketone formation suggested the effect of an inhibitor of BCAA synthesis on diketones should be studied. The herbicide 2-methylphosphinoyl-2-hydroxyacetate (Hoe 704) is a strong competitive inhibitor of 2-acetolactate reductoisomerase. It stops growth of plants by inhibition of BCAA biosynthesis with accumulation of large amounts of 2-acetolactate and acetoin (Schultz et al., 1988).

Yogurts were fermented with *L. bulgaricus* and *S. thermophilus* in the absence or presence of Hoe 704. Table 2 shows that for the yogurt fermented with the

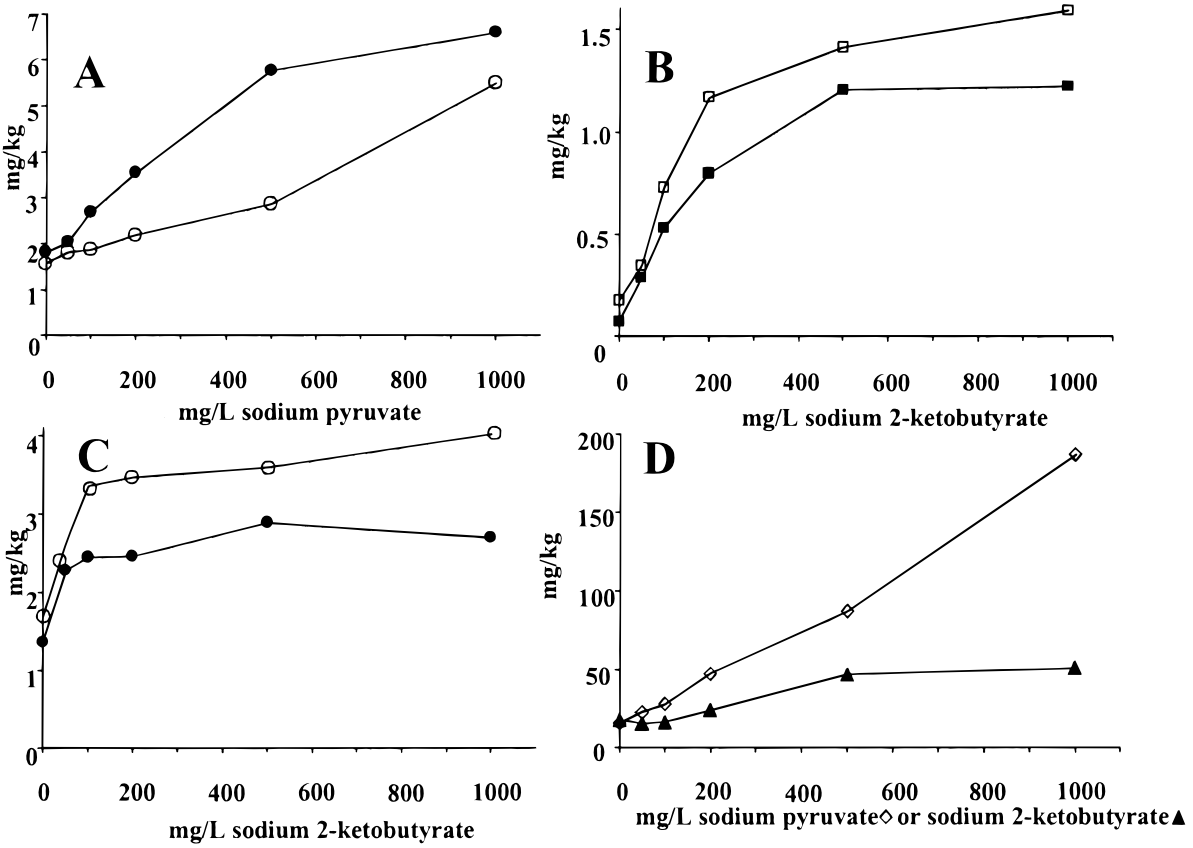

Figure 4. Concentrations of 2,3-butanedione (●) and 2-acetolactate (○) (A, C), 2,3-pentanedione (■) and 2-acetohydroxybutyrate (□) (B), and acetoin (◇, ▲) (D) in milk fermented with *L. bulgaricus* LB52 and *S. thermophilus* (YS4, YS7) in the presence of increasing amounts of pyruvate (A, D) or 2-ketobutyrate (B−D) after storage at 4 °C for 1 day.

Table 2. Concentration (Milligrams per Kilogram) of Precursors, Vicinal Diketones, and Reduction Products in Yogurts Fermented with *S. thermophilus* (YS4 and YS7) and *L. bulgaricus* either the lac⁻ Mutant (LB52: lac⁻) or the Wild Type (YL30: lac⁺) in the Absence or Presence of Hoe 704 (10 mg/kg) after Storage at 4 °C for 1 Day

	LB52: lac⁻		YL30: lac⁺	
	− Hoe 704	+ Hoe 704	− Hoe 704	+ Hoe 704
2-acetolactate	1.31	4.12	0.12	0.21
2,3-butanedione	2.15	5.52	1.28	0.92
acetoin	13.85	25.90	22.26	22.60
2-acetohydroxy-butyrate	0.06	2.30	nd[a]	nd
2,3-pentanedione	0.16	2.69	0.16	1.10
hydroxypentanone (both isomers)	0.10	nd	0.33	0.41

[a] nd, not detected.

lac⁻ mutant of *L. bulgaricus* (LB52), the precursor, diketone, and reduction product concentrations increased in the presence of Hoe 704. For the 2,3-butanedione series an accumulation of 2−3-fold can be observed, whereas for 2,3-pentanedione the accumulation reaches 10−30-fold (Table 2). The same quantification was done for yogurt fermented with the wild type *L. bulgaricus* (YL30), for which no significant differences were observed (Table 2). In the presence of the wild type *L. bulgaricus* (YL30) a fully active metabolism generates BCAA. These amino acids presumably modulate the metabolic pathways of diketones (Figure 1), and Hoe 704 has limited effect. On the contrary, in the presence of the lac⁻ mutant of *L. bulgaricus* (LB52), the reduced metabolic activity does not generate enough BCAA. In these conditions, no modulation of diketone formation occurs and inhibition of reductoisomerase by Hoe 704 increased the production of diketones (Figure 1).

These results suggest a connection between the anabolic pathway to BCAA biosynthesis and formation of vicinal diketones at the level of 2-acetohydroxyacids.

Influence of Intermediates in Vicinal Diketone Formation. Two intermediate metabolites in the vicinal diketone formation were first studied: pyruvate, available from glycolysis, and 2-ketobutyrate from deamination of threonine (Figure 1).

In the presence of increasing amounts of sodium pyruvate during milk fermentation with the lac⁻ mutant of *L. bulgaricus* (LB52) and *S. thermophilus* (YS4 and YS7), the concentration of 2,3-butanedione and its direct precursor 2-acetolactate steadily increased up to 1 g/L added sodium pyruvate (Figure 4A). Almost no effect was observed on 2,3-pentanedione and its precursor (data not shown). These results show that pyruvate readily entered into the cell and served as a precursor for 2-acetolactate (Tsau et al., 1992). In the presence of increasing amounts of 2-ketobutyrate, the concentrations of 2,3-pentanedione and its direct precursor 2-acetohydroxybutyrate increased up to 10-fold above 600 mg/L added 2-ketobutyrate (Figure 4B). 2,3-Butanedione and its precursor increased 2-fold (Figure 4C), in contrast to observations in *E. coli* and *S. thyphimurium* for which 2-acetolactate synthesis is inhibited by 2-ketobutyrate (De Felice et al., 1998; Shaw and Berg, 1998). At this level it is not possible to tell if this increased production of 2,3-butanedione in the presence of 2-ketobutyrate is due to a precursor effect or a stimulatory/

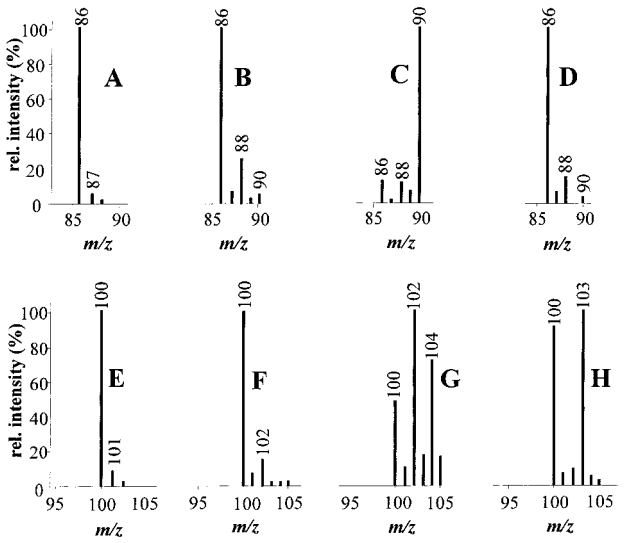

Figure 5. Mass spectra of 2,3-butanedione (A−D) and 2,3-pentanedione (E−H) produced in milk containing 0.5% glucose fermented with lac⁻ mutants of *L. bulgaricus* (LB52) and *S. thermophilus* (S97A1) (A, E), supplemented with sodium [2,3-$^{13}C_2$]pyruvate (B, F), [U-$^{13}C_6$]-D-glucose (C, G) or [U-$^{13}C_4$]-L-threonine (D, H).

inhibition effect of enzymatic pathways around 2,3-butanedione.

Acetoin concentration was also quantified in the different fermented products. In the presence of increasing amounts of sodium pyruvate, a linear increase of acetoin up to 200 mg/kg at 1 g/L was observed (Figure 4D). In these conditions only 6 mg/kg of 2,3-butanedione could be detected. It is known that pyruvate and 2,3-butanedione are toxic for the cells (Tsau et al., 1992; Jay, 1982). These results suggest that almost half of the input sodium pyruvate is detoxified to acetoin via 2-acetolactate during the fermentation time.

Addition of 2-ketobutyrate led to a slight increase of acetoin production, which confirm the tendency of this compound to have an influence on the metabolism of 2,3-butanedione as seen above.

Incorporation of ^{13}C from Different Precursors into Vicinal Diketones. Three ^{13}C-labeled molecules (pyruvate, glucose, and threonine) were used to get a clear picture of the precursors of vicinal diketones and to exclude any effect on enzymatic activities. As glucose was one of the ^{13}C-labeled molecules and the fermentations were done in milk, it was important to avoid interference from lactose. Two β-galactosidase-negative mutants (lac⁻), unable to degrade lactose, were used in all tests (*L. bulgaricus* LB52 and *S. thermophilus* S97A1), and milk was supplemented with ^{13}C-labeled or unlabeled glucose.

Incorporation of ^{13}C into 2,3-Butanedione. Both microorganisms were grown in milk supplemented with glucose in the presence of sodium [2,3-$^{13}C_2$]pyruvate. One day postfermentation, 2,3-butanedione formation was analyzed by GC-MS. In the absence of labeled pyruvate, 2,3-butanedione presented a molecular ion at *m/z* 86 (Figure 5A). In the presence of sodium [2,3-$^{13}C_2$]-pyruvate two molecular ions appeared at *m/z* 88 and

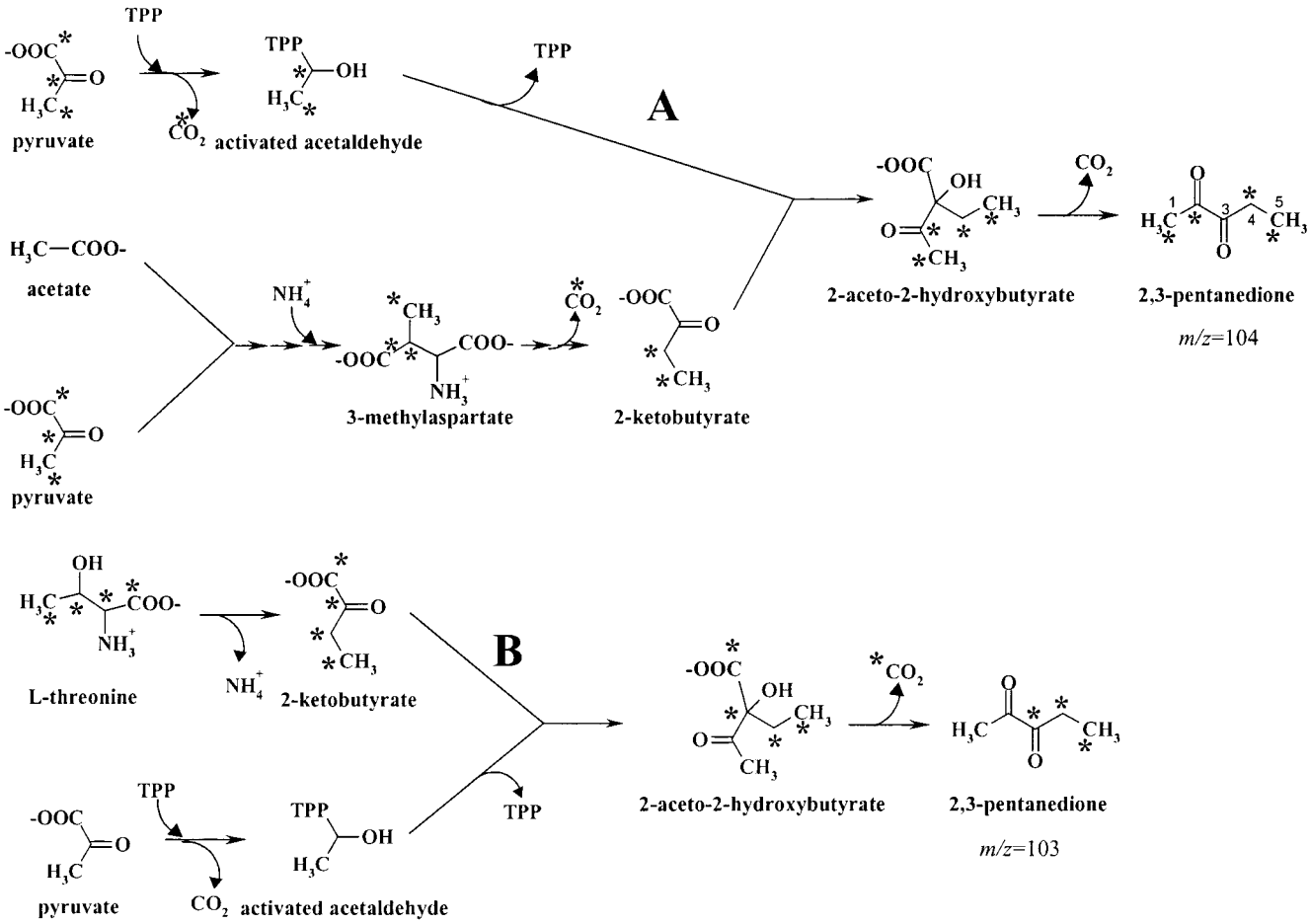

Figure 6. Proposed metabolic pathways for 2,3-pentanedione biosynthesis from [U-$^{13}C_6$]-D-glucose (A) and [U-$^{13}C_4$]-L-threonine (B) (*, ^{13}C; see text for details).

90 (18 and 1.7% of total 2,3-butanedione, respectively) representing incorporation into 2,3-butanedione of one molecule and two molecules of pyruvate, respectively (Figure 5B).

In the presence of [U-$^{13}C_6$]-D-glucose, almost 80% of 2,3-butanedione appeared as a molecular ion at *m*/*z* 90 (Figure 5C) in which all carbons were ^{13}C. These results confirm that glucose is the major precursor of 2,3-butanedione in the lac$^-$ mutants used in this study. The two molecular ions *m*/*z* 86 and 88 (8 and 10% of total 2,3-butanedione, respectively) could be explained by incorporation of internal reserves as found in *L. lactis* subsp. *lactis* (Ramos et al., 1994). They could also occur from a different metabolic pathway suggested in Figure 1.

Effectively, in the presence of [U-$^{13}C_4$]-L-threonine, 10% of total 2,3-butanedione appeared as a molecular ion *m*/*z* 88 (Figure 5D). This indicates that threonine could also be a precursor of 2,3-butanedione via activated acetaldehyde as suggested for *L. lactis* subsp. *lactis* (Ramos et al., 1994).

Incorporation of ^{13}C into 2,3-Pentanedione. 2,3-Pentanedione was analyzed by GC-MS**,** using the same conditions as for 2,3-butanedione, 1 day postfermentation. In the absence of any ^{13}C-labeled precursor, 2,3-pentanedione appeared as a molecular ion at *m*/*z* 100 (Figure 5E). In the presence of sodium [2,3-$^{13}C_2$]-pyruvate a molecular ion appeared at *m*/*z* 102 (Figure 5F) (11% of total 2,3-pentanedione), indicating the possible incorporation of one molecule of pyruvate into 2,3-pentanedione via activated acetaldehyde (Figure 1).

This observation was confirmed when [U-$^{13}C_6$]-D-glucose was added to the milk during fermentation. In these conditions, several new molecular ions appeared (Figure 5G). The molecular ion at *m*/*z* 102 (39% of total 2,3-pentanedione) had acquired two ^{13}C resulting from the incorporation of one molecule of pyruvate via activated acetaldehyde. The molecular ion at *m*/*z* 104 (28% of total 2,3-pentanedione) cannot be explained directly from metabolic pathways suggested in Figure 1. The full mass spectra of 2,3-pentanedione depicted in Figure 5G showed two ions at *m*/*z* 45 and 59 (not shown), due to the classical fragmentation of vicinal diketones between both keto groups. This implies that each resulting acetyl and propionyl fragment contained two ^{13}C carbons. As the bond breakage between carbons 3 and 4 yielded almost only an ion at *m*/*z* 73, it suggests that the keto carbon of the propionyl fragment, originating from the molecule containing two or four ^{13}C, was not labeled. The presence of ^{13}C in positions 1, 2, 4, and 5 of 2,3-pentanedione agreed with the biological pathway starting with uniformly labeled pyruvate coming from [U-$^{13}C_6$]-D-glucose and 2-ketobutyrate labeled on positions 3 and 4 (Figure 6A). To get such a label on 2-ketobutyrate it was shown in *Acetobacter suboxydans* that glutamate is synthesized from pyruvate and acetate via 3-methylaspartate (Maragoudakis et al., 1966). This molecule was shown in *E. coli* to be converted to 2-ketobutyrate (Abramsky et al., 1965; Phillips et al., 1972; LeMaster and Cronan, 1982). The result is a 2-ketobutyrate labeled on positions 3 and 4 (Figure 6A).

Finally, when [U-$^{13}C_4$]-L-threonine was added during fermentation, only one new molecular ion appeared at *m*/*z* 103, which should occur from a condensation of uniformly labeled 2-ketobutyrate and unlabeled activated acetaldehyde, leading after decarboxylation to 2,3-pentanedione labeled on three carbons (Figure 6B).

CONCLUSIONS

In cow's milk, the reduced metabolic activity of the lac$^-$ mutant of *L. bulgaricus* as a partner of *S. thermophilus* led to a reduction of almost all free amino acids compared to fermentation with the wild type. This depletion in free amino acids was shown to be linked to an increase in the production of vicinal diketones (Ott et al., 1999). When the milk was supplemented with BCAA (valine, leucine, and isoleucine), fermentation with the lac$^-$ mutant of *L. bulgaricus* resulted in increased production of diketones and their derivatives. The selective inhibition of reductoisomerase, by Hoe 704, also led to an accumulation of diketones. These results strongly suggest an interconnection between the metabolism of BCAA and vicinal diketones in lactic acid bacteria.

The association of ^{13}C-labeled glucose and threonine with S&T-HS GC allowed incorporation of ^{13}C into vicinal diketones to be demonstrated. Glucose is the major precursor for 2,3-butanedione. For 2,3-pentanedione, threonine was shown to be a precursor, as well as glucose via activated acetaldehyde. An unexpected labeling pattern allows us to propose another metabolic pathway for 2,3-pentanedione via 3-methylaspartate, an intermediate in glutamate synthesis.

To our knowledge, these results clarify for the first time the metabolic pathway of diketone formation in the *L. bulgaricus*−*S. thermophilus* association in milk fermentation.

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