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Influence of Peptide Supply and Cosubstrates on Phenylalanine Metabolism of *Lactobacillus sanfranciscensis* DSM20451^{T} and *Lactobacillus plantarum* TMW1.468

NICOLINE VERMEULEN,[†] MICHAEL G. GÄNZLE,[‡] AND RUDI F. VOGEL*,[†]

Technische Mikrobiologie, Technische Universität München, Weihenstephaner Steig 16, D-85350 Freising, Germany, and Department of Agricultural, Food and Nutritional Science, University of Alberta, 4-10 Agriculture/Forestry Centre, Edmonton, Alberta T6G 2P5, Canada

Bread spoilage is mainly due to the growth of filamentous fungi, and metabolites produced during sourdough fermentation by lactobacilli can inhibit fungal growth. One of these metabolites is phenyllactic acid (PLA), which is a catabolite from phenylalanine. In this work, the influence of peptide supply and cosubstrates was determined on PLA formation from phenylalanine by *Lactobacillus plantarum* TMW1.468 and *Lactobacillus sanfranciscensis* DSM20451^{T}. Transport of single amino acids is not efficient in lactobacilli, and only 1% of the offered phenylalanine was converted to PLA. PLA yields were increased 2−4-fold when peptides instead of single amino acids were used as a substrate. The accumulation of phenylalanine after peptide addition indicated that, after transport, transamination was the second limiting factor. In *L. plantarum* TMW1.468, PLA yields were increased from 5 to >30% upon the addition of α-ketoglutarate. In *L. sanfranciscensis* DSM20451, a combination of both citric acid and α-ketoglutarate increased PLA formation. The combined effect of citric acid and α-ketoglutarate can be attributed to changes in the NAD/NADH ratio.

KEYWORDS: ***Lactobacillus plantarum*; *Lactobacillus sanfranciscensis*; phenylalanine metabolism; phenyllactic acid; sourdough**

INTRODUCTION

Wheat sourdough fermentation by lactobacilli positively influences the overall bread quality, because it enhances flavor (*1−3*) and texture (*4*), delays staling (*5*), and prolongs shelf life (*6*). Bread spoilage is mainly due to the growth of filamentous fungi, and metabolites produced by lactobacilli can inhibit their growth. In addition to lactic and acetic acids, minor metabolites display antifungal activity (*7*). One of these minor metabolites is phenyllactic acid (PLA), which is a catabolite of phenylalanine. Mold growth was delayed by 5 days in wheat bread produced using sourdough started with a PLA-producing *Lactobacillus plantarum* strain in comparison to a bread produced using sourdough started with a nonphenyllactic acid-producing *Lactobacillus brevis* strain (*6*). PLA is produced by a wide range of *Lactobacillus* species, but its production is strain-dependent (*8−10*). PLA was formed by lactic acid bacteria growing in Man, Rogosa, and Sharp (MRS) broth in levels up to 99 mg L^{-1} (*10*).

Cells meet their nitrogen requirements mainly by oligopeptide transport followed by peptide hydrolysis (*11−14*). The preferential uptake of peptides suggests that peptides are more efficiently converted into metabolites than amino acids. However, all published studies on amino acid metabolism by lactobacilli have employed amino acids rather than peptides as substrates, and the possibility to increase amino acid turnover by an optimized supply of substrates remains to be elucidated. Amino acid catabolism in lactic acid bacteria (for a recent review, see ref *15*) starts from this intracellular pool of amino acids. It is initiated by a transamination reaction (*16−18*) where the α-amino group is tranferred to a keto acid acceptor by an aminotransferase. In lactococci, α-ketoglutarate is the main amino acceptor (*18*, *19*), which is then converted into its corresponding amino acid, glutamate. Other α-keto acids such as pyruvate and oxaloacetate may serve as amino acceptors as well (*20*). After removal of the amino group, the resulting α-keto acid can be metabolized by several enzymatic reactions, resulting in the corresponding aldehyde or alcohol, carboxylic acid, or hydroxy acid. The proportion of the metabolites formed is strain-dependent (*17*, *21*).

In case of phenylalanine degradation (a schematic overview is given in **Figure 1**), phenylpyruvate is generated after transamination. Phenylpyruvate can then be reduced by hydroxy acid dehydrogenases (*16*, *22*), which results in the production of PLA. When phenylpyruvate is decarboxylated, phenylacetaldehyde is formed, which in turn can be converted to phenylethanol or phenylacetate. When phenylpuryvate is not enzymatically converted, it may undergo a chemical oxidation to benzaldehyde (*23*).

* To whom correspondence should be addressed. Tel: + 49 8161 7135169. Fax: + 49 8161 713327. E-mail: rudi.vogel@wzw.tum.de.
[†] Technische Universität München.
[‡] University of Alberta.

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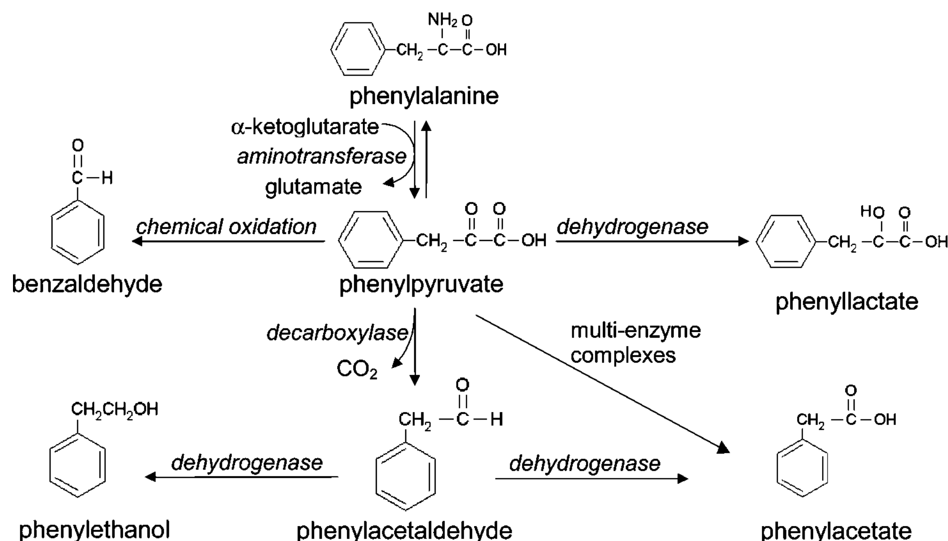

Figure 1. Pathways involved in phenylalanine metabolism in lactic acid bacteria. After ref *45*, modified.

In lactic acid bacteria, amino acid metabolism is limited by the availability of amino acceptors in the transamination reaction. It has been shown that the addition of α-ketoglutarate (*24*−*26*) or pyruvate (*27*) during cheese making enhances the amino acid degradation. The conversion of amino acids was increased by using a glutamate dehydrogenase (GDH) overproducing strain, which was able to produce extra α-ketoglutarate from glutamate under the reduction of NAD to NADH (*28*). Tanous et al. (*29*) have shown a positive correlation between the GDH activity in lactic acid bacteria and their ability to metabolize amino acids. Cometabolism of citric acid and glutamate resulted in higher amino acid conversion rates (*30*).

In *Lactococcus lactis*, some of the genes encoding enzymes involved in phenylalanine metabolism were identified. The expressions of the peptide transporter *opp*, several peptidases, and the aminotransferases *araT* and *bcaT* are under CodY repression (*31*, *32*). CodY senses the intracellular pool of branched chain amino acids (*33*, *34*); branched chain amino acids stimulate CodY's binding to the DNA, which leads to inhibition of the gene transcription and thus down-regulates peptide transport and hydrolysis and amino acid conversion. It is not known to which extent the regulation described for *Lactococcus* is valid for sourdough lactobacilli or how the formation of the intracellular amino acid pool, the transamination, the conversion of phenylpyruvate, and the regulation of these processes influence the formation of PLA during sourdough fermentation.

Many metabolic pathways are linked to cofactor availability, which is influenced by central carbon metabolism. Previous studies on amino acid metabolism and cofactor regeneration have focused on homofermentative lactobacilli. Under conditions prevailing in sourdough, *L. plantarum* uses the Embden−Meyerhoff pathway for sugar fermentation whereas *Lactobacillus sanfranciscensis* is obligatory heterofermentative as most major representatives of the microflora in traditional sourdoughs. In this group of organisms, the NADH-dependent enzymatic reactions are strongly influenced by citrate and fructose. These cosubstrates enable cofactor regeneration (*35*) and thus potentially increase amino acid turnover via an increased GDH activity.

Although the factors limiting amino acid metabolism in *L. lactis* are increasingly understood, the current knowledge does not enable the optimization of phenylalanine turnover by sourdough lactobacilli. It was therefore the aim of this work to determine the role of peptide supply and cosubstrates as rate-limiting factors in metabolite formation from phenylalanine in *L. plantarum* TMW1.468 and *L. sanfranciscensis* DSM20451^T. The influence of the substrate, the cofactor availability, and the presence of branched chain amino acids on PLA formation were studied, as well as the phenylpyruvate degydrogenase and GDH activities.

MATERIALS AND METHODS

Chemicals. All used chemicals were of analytical grade and, with few exceptions, were obtained from Merck (Darmstadt, Germany). Reference compounds for high-performance liquid chromatography (HPLC) analysis of phenyllactate, phenylacetate, and phenylethanol were obtained from Sigma-Aldrich (Munich, Germany), and acetonitrile was obtained from J. T. Baker (Griesheim, Germany). Peptides were purchased from Bachem (Weil am Rhein, Germany).

Strain and Growth Conditions. *L. sanfranciscensis* DSM20451^T (ATTC27651^T, TMW1.53) and *L. plantarum* TMW1.468 were grown at 30 °C in modified MRS medium (mMRS, composition per liter: 5 g of glucose × H_2O, 10 g of fructose, 10 g of maltose, 10 g of peptone from casein, 5 g of meat extract, 5 g of yeast extract, 4.0 g of KH_2PO_4, 2.6 g of K_2HPO_4 × $3H_2O$, 3.0 g of NH_4Cl, 1 g of Tween 80, 0.1 g of $MgSO_4$ × $7H_2O$, 0.05 g of $MnSO_4$ × H_2O, 0.5 g of l-Cys HCl × H_2O, 0.2 mg each of biotin, folic acid, nicotinic acid, pyridoxal phosphate, thiamin, riboflavin, cobalamin, and panthothenic acid; and 15 g of agar for solid media). The pH was adjusted to 6.2. In N-limited mMRS, peptone and meat extract were omitted and 5 g L^{-1} yeast extract was added as the sole source of complex nitrogen. The growth of the lactobacilli in N-limited medium was monitored by measuring the optical density (OD) at 590 nm every 20 min in a microtiterplate spectrophotometer (TECAN spectrafluor, Grödig, Austria).

Fermentation Conditions. After two sequential overnight fermentations, precultures were used to inoculate 1.2× concentrated N-limited mMRS at 2.5%. Stock solutions of peptides (phenylalanyl-leucine, FL; phenylalanyl-serine, FS; phenylalanyl-glutamate, FE; prolyl-phenylalanine, PF; leucyl-valine, LV; and leucyl-proline, LP; all obtained from Bachem), amino acid (phenylalanine, glutamate), α-ketoglutarate, citric acid, and fructose, or, when necessary, water were added in order to dilute the medium to 1.0× concentrations. The pH value of the stock solutions (50 mM) was adjusted to pH 6. The dipeptides FL and LP have a poor solubility; therefore, these peptides were solved directly in the 1.2× concentrated N-limited mMRS, which was subsequently sterilized by filtration and diluted to 1.0× concentrations. Fermentations were allowed to proceed for 72 h at 30 °C. Samples were taken after 0, 6, 24, and 72 h and immediately frozen.

Sourdough Fermentations. Wheat flour with an ash content of 0.55% was obtained at a local supermarket. To inoculate sourdoughs,

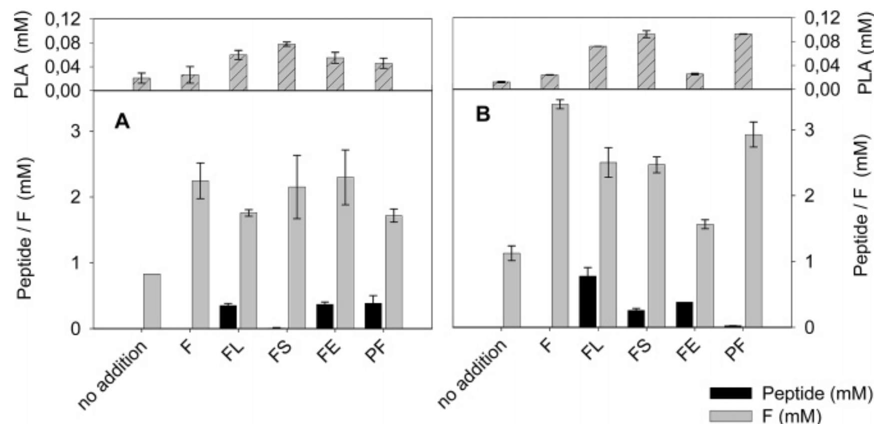

Figure 2. Peptide, F (lower panels), and PLA (upper panels) levels in nitrogen-limited mMRS medium fermented for 72 h with *L. sanfranciscensis* DSM20451^T (**A**) and *L. plantarum* TMW1.468 (**B**). Substrates were added to the medium to a concentration of 1.7 mM. Presented results are average values ± standard deviations of two independent fermentations.

5 mL of an overnight culture was washed twice with tap water. The pellet was resuspended in 5 mL of tap water, and this cell suspension was mixed with 10 g of flour and 5 mL of tap water containing the required addititives (F, FS, citric acid, α-ketoglutarate, and fructose, 5 mM, pH 7). Sourdoughs were incubated at 30 °C, and samples were taken after 0, 6, 24, and 48 h.

Concentrations of Organic Acids and Sugars in Sourdough and mMRS Samples. The concentrations of maltose, glucose, and fructose as well as the metabolites lactate, acetate, ethanol, PLA, phenylethanol, phenylacetate, and phenylacetaldehyde were determined by HPLC. Sourdough samples were diluted 1:1 with distilled water; medium samples were used undiluted. After overnight protein precipitation using 3.5% perchloric acid, the precipitates were removed by centrifugation (15000*g*, 20 min). The 20 μL samples were eluted from a Polyspher^R OA KC column (300 mm × 7.8 mm, Merck, 70 °C) with 5 mM H_2SO_4 in 5% acetonitrile at a flow of 0.4 mL min^{-1}. Except for phenyl group-containing metabolites, all compounds were detected using a refractive index detector. Phenyl group-containing metabolites were detected using a UV detector set at 210 nm, and their detection limit was <5 μM.

Concentrations of Phenylalanine and Phenylalanine-Containing Peptides in Sourdough and mMRS Samples. For determination of phenylalanine and phenylalanine-containing peptides, sample preparation was the same as used for the detection of sugars and organic acids. The 20 μL samples were injected on a C18 RP column (250 mm × 4.6 mm, 300 Å, 5 μm particle size, Phenomenex, Torrence, CA) coupled to a UV detector set to 210 and 257 nm; the flow was 1 mL min^{-1}. Solvents A and B consisted of 0.1% trifluoroacetic acid in water and acetonitrile, respectively. Before injection, the column was equilibrated with 2% B for 5 min. Samples were eluted with the following solvent gradient: 0−25 min, gradient from 2 to 20% B; 25−40 min, gradient from 20 to 80% B.

Measurement of GDH Activity in Cell Free Extract. GDH activity was measured essentially according to Tanous et al. (*29*). Cells were grown overnight in 50 mL of mMRS. The cells were washed twice with reaction buffer (50 mM MOPS, pH 9), the cell pellet was resuspended in 5 mL of reaction buffer, and cells were lysed using sonication. Cell debris was removed from the cell-free extract by centrifugation (6000*g*, 10 min). The reaction mixture for the measurement of the GDH activity consisted of cell-free extract, 5 mM glutamate, and 5 mM NADH or NADPH. The oxidation of NAD(P)H was measured by measuring the absorbance of the reaction mixture at 340 nm. As a control, cell-free extract was inactivated at 90 °C for 5 min and incubated in the presence of glutamate and NADPH or NADH. Furthermore, cell-free extract was incubated without glutamate but in the presence of NADH or NADPH. The protein concentration in the cell free extracts was measured using the BioRad Protein Assay (BioRad Laboratories GmbH, Munich, Germany) according to the manufacturers directions. Bovine serum albumin was used for the calibration curve.

Statistical Data Analysis. The results are mean values of two separate fermented medium or dough samples. Significant differences in PLA formation upon the addition of substrates and cosubstrates were evaluated using an on-line available *t*-test tool (calculators.stat.ucla.edu).

RESULTS

PLA Formation by *L. plantarum* TMW1.468 and *L. sanfranciscensis* DSM20451 from Different Substrates. In all fermentations, the growth of the inoculated strains to high cell counts and the absence of a metabolically relevant number of contaminations were verified by observation of a uniform colony morphology on agar plates and by measuring lactate, acetate, and ethanol levels in the doughs (data not shown). To determine whether amino acids or peptides are preferred substrates for amino acid metabolism, F was offered as a single amino acid or as part of a dipeptide. The concentrations of the peptides, F, and PLA after 72 h of fermentation are shown in **Figure 2**. For both strains, a 2−4-fold increase in PLA formation can be seen when F is offered as part of a peptide as compared to when F is offered as a single amino acid. Only when F is offered as part of FE, PLA formation is not increased in *L. plantarum* TMW1.468. In the case of *L. sanfranciscensis* DSM20451, a 0.0−0.38 mM concentration of the initial 1.7 mM concentration of the added dipeptides was recovered as dipeptides; thus, *L. sanfranciscensis* hydrolyzed 76−100% of the peptides. This caused an increase in phenylalanine concentration as compared to the fermentation without additional peptide: Phenylalanine levels were 0.88−1.46 mM higher, indicating that a substantial part of the phenylalanine is not further converted. *L. plantarum* hydrolyzed the dipeptide as well; after 72 h of fermentation, dipeptide concentrations ranged from 0.02 to 0.78 and phenylalanine levels increased from 0.44 to 1.38 mM. Phenylacetic acid, phenylethanol, and phenylacetaldehyde concentrations were below detection limits after fermentation (data not shown). Kinetic analysis of growth rate and substrate and PLA levels showed that peptide hydrolysis and PLA formation are ongoing when growth has ceased, indicating that amino acid conversion is not related to exponential growth but an ongoing process in stationary cells (data not shown). After 0 and 6 h, PLA levels were below detection limits. After 24 h, small amounts of PLA were formed, but after 72 h, PLA levels increased further, and the differences between strains and fermentation conditions were more pronounced and enabled statistical data analysis. For example, *L. sanfranciscensis* produced 0.048 mM PLA from FS and reached OD 0.75. After 72 h, the OD remained essentially constant at 0.75 but PLA levels increased to 0.078 mM (data not shown). Because of low amounts of amino

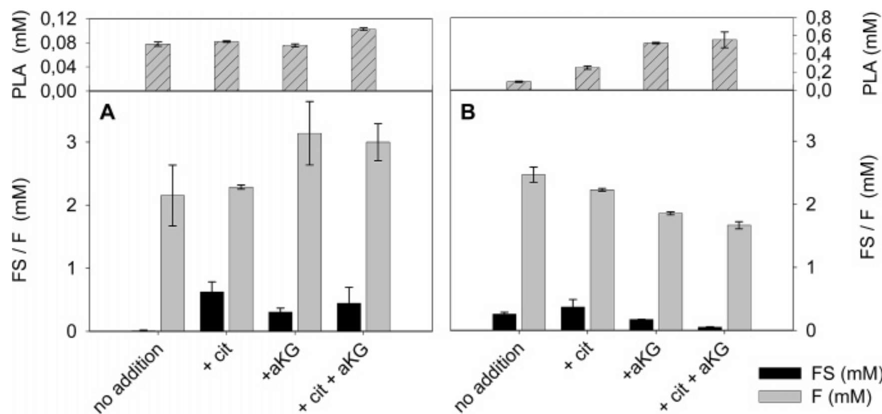

Figure 3. FS, F, and PLA levels in nitrogen-limited mMRS medium after 72 h of fermentation with *L. sanfranciscensis* DSM20451^T (**A**) and *L. plantarum* TMW1.468 (**B**) are shown. FS (1.7 mM) was added at the start of the fermentation, as were citrate (cit, 5 mM) and α-ketoglutarate (aKG, 5 mM). Presented results are average values ± standard deviations of two independent fermentations.

nitrogen available, only rather low ODs were reached when lactobacilli grew in N-limited medium.

Influence of α-Ketoglutarate on the PLA Formation by *L. plantarum* TMW1.468 and *L. sanfranciscensis* DSM20451. The accumulation of F indicates that peptide transport and hydrolysis are not limiting factors, whereas the transamination is a bottleneck. To investigate whether the PLA formation can be enhanced by stimulation of the transamination, α-ketoglutarate and/or citric acid were added in excess, and substrate utilization and PLA formation (**Figure 3**) were monitored. As a control, α-ketoglutarate was added to medium without an additional phenylalanine source but this addition did not enhance PLA formation significantly (data not shown). Independent of the supply with peptides and cosubstrates, *L. sanfranciscensis* and *L. plantarum* grew to an OD of 0.7−0.8 within 20 h and the cell density remained essentially unchanged during further incubation. The addition of α-ketoglutarate strongly enhanced PLA formation in *L. plantarum*, and the PLA yield increased from 5 to >30%. Corresponding to the increased PLA formation, decreased F levels were measured. The addition of citric acid did not enhance PLA formation further. Contrary, to stimulate PLA formation by *L. sanfranciscensis*, the presence of both α-ketoglutarate and citric acid was a prerequisite. The achieved yield was by far not as high as the yield reached by *L. plantarum*; only 6% from the initially offered FS was recovered as PLA. This combined effect of α-ketoglutarate and citric acid was also observed when FL was used as a substrate (data not shown). In addition to α-ketoglurarate, pyruvate may additionally act as an amino acceptor to form alanine. However, none of the fermentations with *L. sanfranciscensis* and *L. plantarum* provided evidence for alanine accumulation through the use of pyruvate as an amino acceptor.

GDH activity enables generation of α-ketoglutarate from glutamate. The measurement of GDH activities in cell-free extracts of both strains revealed that *L. plantarum* had no active GDH, whereas cell-free extracts from *L. sanfranciscensis* displayed both GDH−NAD and GDH−NADP activity, 1.8 and 19.5 nmol/(mg protein × min), respectively. In *L. sanfranciscensis*, α-ketoglutarate was exchanged by the single amino acid glutamate or by a glutamate-containing dipeptide. PLA levels in the medium were measured and are shown in **Figure 4**. The combined effect of α-ketoglutarate and citric acid on amino acid metabolism was also observed when α-ketoglutarate was replaced by glutamate, either as a single amino acid or as part of a dipeptide, indicating that glutamate may substitute α-ketoglutarate in GDH-positive strains. When glutamate was

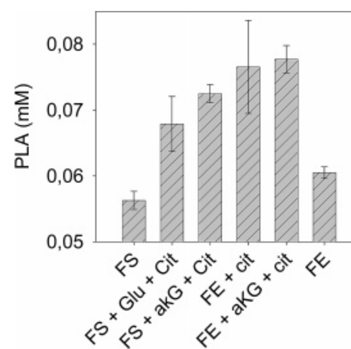

Figure 4. Influence of glutamate, α-ketoglutarate, and citrate on PLA formation by *L. sanfranciscensis* DSM20451^T. PLA levels were measured in nitrogen-limited mMRS medium containing either 1.7 mM FS or 1.7 mM FE and cosubstrates that were fermented for 72 h with *L. sanfranciscensis* DSM20451^T. Used cosubstrates were (glutamate, Glu; α-ketoglutarate, aKG; and citrate, cit) added to a concentration of 5 mM. Shown results are average values and standard deviations of two independent fermentations.

present, the addition of α-ketoglutarate did not increase PLA formation further.

Stimulation of PLA Formation in *L. sanfranciscensis* DSM20451. Citric acid uptake and conversion may have several effects on the cell. (i) Its transport into the cell influences the membrane potential, an effect that is also described for the uptake and subsequent decarboxylation of malic acid (*36*). (ii) The cometabolism of citric acid by *L. sanfranciscensis* results in the oxidation of one NADH to NAD; the same occurs when fructose is converted into mannitol (*35*). The combined effect of α-ketoglutarate and various cosubstrates on PLA formation was investigated in *L. sanfranciscensis*. The combination of citric acid and α-ketoglutarate did not influence the growth of *L. sanfranciscensis* (data not shown). Subsequently, citric acid was replaced by fructose or malic acid, and PLA formation was monitored in a buffer fermentation and in nitrogen-limited MRS medium with two different substrates (**Figure 5**). Malic acid did not enhance PLA formation, indicating that the membrane potential does not play a role in amino acid metabolism. With FL being the substrate, the addition of fructose resulted in significantly enhanced PLA formation ($P < 0.05$), although not as strong as observed upon the addition of citric acid.

Influence of Branched Chain Amino Acids on the PLA Formation by *L. sanfranciscensis* DSM20451. The influence of increased intracellular branched chain amino acids levels on PLA formation by *L. sanfranciscensis*, which harbors *codY*, was

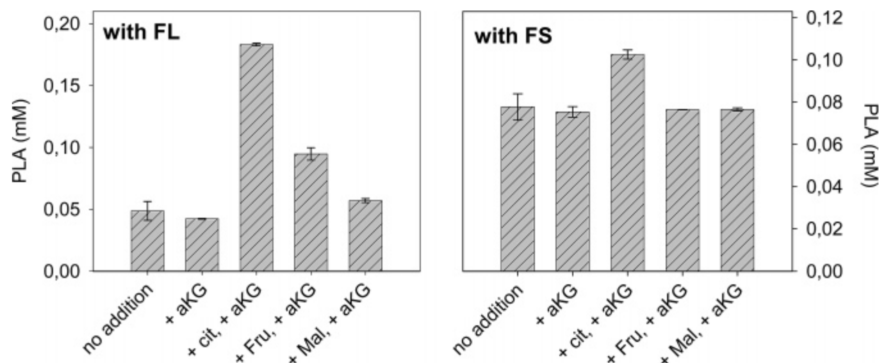

Figure 5. Influence of several cosubstrates on PLA formation by *L. sanfranciscensis* DSM20451^T. PLA levels were measured in nitrogen-limited medium containing either 1.7 mM FL (left panel) or 1.7 mM FS (right panel) that was fermented for 72 h with *L. sanfranciscensis* DSM20451^T. Additional substrates (fructose, Fru; α-ketoglutarate, aKG; citrate, cit; and malate, Mal) were added to a concentration of 5 mM. Presented results are average values ± standard deviations of two independent fermentations.

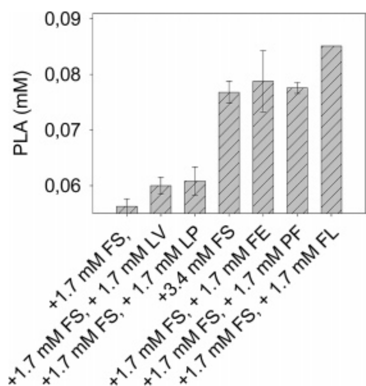

Figure 6. Influence of the presence of branched chain amino acids on PLA formation by *L. sanfranciscensis* DSM20451^T. PLA levels in nitrogen-limited medium contained additional peptides fermented for 72 h with *L. sanfranciscensis* DSM20451^T. Presented results are average values and standard deviations of two independent fermentations.

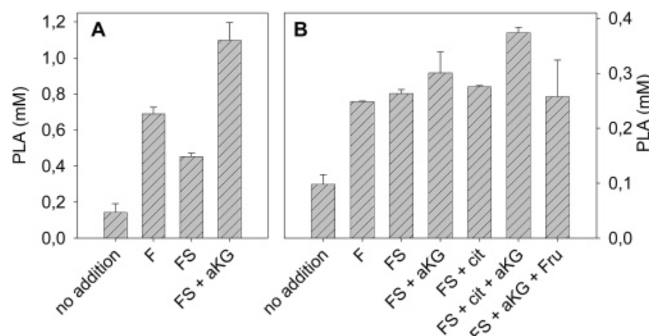

Figure 7. PLA levels in sourdough fermented for 48 h with *L. plantarum* TMW1.468 (**A**) and *L. sanfranciscensis* DSM20451^T (**B**). F, FS, α-ketoglutarate (aKG), citric acid (cit), and fructose (fru) were added to a concentration of 5 mM.

investigated. Various combinations of dipeptides were added to the medium. PLA levels after 72 h of fermentation are shown in **Figure 6**. Neither the addition of LV nor the addition of LP to FS-containing medium decreased the PLA formation. When 50% of FS was replaced by PF, FL, or FE, no significant change ($P > 0.05$) in PLA levels was measured.

PLA Formation by *L. sanfranciscensis* DSM20451 and *L. plantarum* TMW1.468 during Sourdough Fermentation. Sourdough fermentations were performed in order to examine whether the results obtained in medium can be transferred to PLA formation in sourdough. *L. plantarum* was grown in a standard dough and in doughs containing additional F, FS, and both α-ketoglutarate and FS. *L. sanfranciscensis* was grown in doughs with and without the addition of F, FS, and FS combined with citric acid, α-ketoglutarate, and fructose. The PLA levels reached after 48 h are shown in **Figure 7**. Proper growth of the strains and the absence of a metabolically relevant number of contaminations were verified by observation of a uniform colony morphology on agar plates (data not shown) and by measuring lactate, acetate, and ethanol levels in the doughs (data not shown). PLA is formed during dough fermentation, and its production was clearly increased when F or FS was added ($P < 0.1$). In contrast to N-limited medium, there was no significant difference detectable between the use of a dipeptide or the use of a single amino acid. PLA production by *L. plantarum* in dough was enhanced when α-ketoglutarate was added. When *L. sanfranciscensis* was used as a starter culture, the addition of α-ketoglutarate and citric acid was needed in order to increase PLA production from FS significantly ($P < 0.05$). This effect was not observed when fructose was added to the dough. A combined effect of citric acid and α-ketoglutarate but not of fructose and α-ketoglutarate was also observed when FL was added to the sourdough (data not shown).

DISCUSSION

The formation of minor metabolites is influenced by many metabolic pathways. Consequently, the formation of PLA from phenylalanine has been described as strain-dependent (*8*−*10*). In this work, distinct differences were observed in PLA formation and amino acid metabolism and its regulation in *L. sanfranciscensis* DSM20451 and *L. plantarum* TMW1.468. These differences between the two strains are partially explained by species specific metabolic differences; however, as only one strain was considered for each of the species, strain specific differences may additionally influence the effect of cofactors on amino acid metabolism.

Low intracellular amino acid levels are reported to limit amino acid metabolism, due to the poor transport of single amino acids; transamination of amino acids is more efficient when cells are permeabilized (*37*, *38*) or lysed (*39*, *40*). The peptides used in this study were all quantitatively hydrolyzed by the intracellular peptidases. Accumulated F was transported out of the cell, either actively or by facilitated diffusion, or it left the cell via passive diffusion as was shown for proline (*14*). In this work, it was shown that peptides stimulate PLA formation by lactobacilli growing in N-limited medium more than single amino acids. We therefore concluded that transport of single amino acids was not very efficient in lactobacilli, which resulted in low

intracellular amino acid concentrations and poor amino acid conversion. This was overcome when peptides instead of single amino acids were used as a substrate. In sourdough, however, the increase of PLA production is similar upon the addition of single amino acids and peptides. During sourdough fermentation, the expression of genes involved in peptide transport is lowered, due to the high levels of peptides present in the dough (*41*). This could explain why peptide addition does not increase PLA formation during dough fermentation.

Phenylalanine and PLA accumulated during fermentation whereas the intermediate metabolite phenylpyruvate remained below the detection level. Hence, the transaminase activity was lower than peptide transport, peptide hydrolysis, and phenyllactate dehydrogenase activity. Transamination is the second bottleneck in PLA formation identified in this work. In many other lactobacilli, transamination is the rate-limiting step in the formation of metabolites from amino acids. Because transamination can be stimulated using α-ketoglutarate, cofactor availability and not enzyme activity is the limiting factor (*24*−*26*). The addition of α-ketoglutarate was sufficient to stimulate PLA formation in *L. plantarum* TMW1.468. PLA formation in *L. sanfranciscensis* DSM20451 was only enhanced when citric acid was added. In the case of *L. sanfranciscensis* DSM20451, α-ketoglutarate could be replaced by glutamate, which can be explained by the GDH activity of this strain. In *L. plantarum* TMW1.468, the addition of FE instead of the other F-containing dipeptides to the medium did not result in enhanced PLA levels, which correlates with the absence of GDH activity. These observations are in agreement with Tanous et al. (*29*), who reported that GDH-positive strains degrade amino acids more efficiently in the presence of glutamate as do GDH-negative strains.

A combination of citric acid and α-ketoglutarate or glutamate was required for the stimulation of amino acid metabolism by *L. sanfranciscensis* DSM20451. This effect has previously been attributed to the production of additional α-ketoglutarate: Citric acid is transformed to oxaloacetate, which serves as an amino acceptor for the deamidation of glutamate resulting in the formation of α-ketoglutarate and aspartate (*30*). Generation of additional α-ketoglutarate upon the addition of citric acid does not explain the stimulatory effect observed in this work. (i) Citric acid was required for the efficient use of externally added α-ketoglutarate. (ii) Aspartate levels in sourdough were not elevated, when citric acid and α-ketoglutarate were added to the dough (data not shown). The stimulatory effect of citric acid cannot be attributed to the generation of a pmf because malate metabolism, which has a comparable effect on proton export as citric acid, failed to increase PLA production. However, fructose could replace citric acid for the increase of PLA production from FL, which linked the flux through the transaminase reaction to the availability of oxidized NAD in the GDH-positive strain *L. sanfranciscensis* DSM20451. Therefore, the NAD/NADH ratio in the cell appears to play a significant role in amino acid metabolism. The addition of fructose did not enhance PLA formation when FS was the substrate. Serine can be converted into pyruvate and thus influences the redox status of the cell as well. This pathway of serine degradation has been observed in *L. lactis* (*42*). Contrary to fructose, the combination of citric acid and α-ketoglutarate enhanced PLA formation from both FS and FL, indicating that the synergistic effect does not rely on changes in the NAD/NADH ratio only.

In *L. lactis*, aromatic aminotransferase activity is increased when branched chain amino acids are removed from the environment (*43*). Both in *Bacillus subtilis* and in *L. lactis*, the transcription repressor CodY senses intracellular levels of branched chain amino acids (*33*, *34*). *L. sanfranciscensis* DSM20451 harbors *codY* and the amino acid sequence of $\mathrm{CodY}_{L.sanfrancsicensis}$ differs from $\mathrm{CodY}_{L.lactis}$ in one amino acid (data not shown). CodY regulation in *L. lactis* has been studied in chemically defined medium (*31*, *32*). In this work, however, this medium could not be used since it does not support the growth of *L. sanfranciscensis* (*11*). The increase of peptide availability in sourdough has been shown to induce a CodY regulation-like change in gene expression of *L. sanfranciscensis* DSM20451 (*41*). We therefore assume that CodY-like expression regulation can also be observed in other growth media than chemically defined medium. The addition of leucine and valine as part of a peptide to the medium did not significantly change PLA formation nor did the addition of proline. Thus, although CodY regulation-like changes in gene expression cannot be excluded based on these data, CodY regulation does not negatively influence PLA formation by *L. sanfranciscensis* DSM20451.

In liquid medium, 45 mM PLA is required in order to inhibit mold growth (*44*). In sourdough fermentation, PLA levels did not exceed 1.2 mM. On the other hand, local concentrations in the diffusible space of doughs and breads may be higher, and antifungal activity of PLA in sourdough is believed to depend on synergistic effects with other antifungal compounds present in the dough (*7*). It is therefore anticipated that even the PLA levels measured here influence mold growth on baked goods. Increased transamination not only will enhance PLA levels but also will stimulate the degradation of other aromatic amino acids present in the environment, since the aminotranferases have a rather broad substrate specifity (*18*). This will cause a rise in other antifungal organic acids such as hydroxyphenyllactic acid, which originates from tyrosine.

The addition of α-ketoglutarate and citric acid influenced PLA formation in dough in the same way as was observed in nitrogen-limited medium. In sourdough, PLA levels are generally higher than in medium. This is attributable to generally higher substrate concentrations in dough resulting in higher final cell densities in sourdough as compared to nitrogen-limited medium. Specifically, peptide concentrations are much higher than in nitrogen-limited medium. This explains the absence of an increase in PLA formation when F was replaced by FS in dough systems because peptide transport systems are down-regulated during growth of *L. sanfranciscensis* DSM20451 in sourdough when the peptide availability increases (*41*).

Bottlenecks and regulation in PLA formation were found to differ in the investigated strains. Both transport and transamination are limiting factors in PLA formation by *L. sanfranciscensis* DSM20451 and *L. plantarum* TMW1.468. Transport can be optimized when phenylalanine is offered as part of a dipeptide. In *L. plantarum* TMW1.468, transamination can be stimulated by the addition of α-ketoglutarate. In *L. sanfranciscensis*, the addition of α-ketoglutarate is not sufficient, and citric acid has to be added as well. Bottlenecks in phenylalanine metabolism differ in the two considered strains; therefore, strategies for their alleviation must be adapted for the development of starter strains for specific applications.

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