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RESEARCH ARTICLE

Pivotal role of cheese salting method for the production of 3-methylbutanal by *Lactococcus lactis*

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The aldehyde 3-methylbutanal is a key aroma compound for many types of cheeses. Intracellular enzymes of cheese starter cultures are predominantly responsible for the conversion of leucine into 3-methylbutanal. Environmental conditions during cheese manufacturing also play a role in the production of 3-methylbutanal. Our work shows that salting has a detrimental effect on the capacity of Lactococcus lactis *subsp.* lactis *DS84445 to produce 3-methylbutanal. In that respect, a promising way to control the concentration of 3-methylbutanal is by using a cheese starter culture that produces the aimed level of 3-methylbutanal before salting starts and preferably when lactose is still available.*

Keywords Cheese ripening, Cheese salting, Lactococcus species, Flavour.

INTRODUCTION

The aldehyde 3-methylbutanal supports the aroma intensity and profile of many types of cheeses. Levels of 3-methylbutanal can vary up to 10-fold between different types of cheeses and depending on the concentration contribute to a chocolate and malty flavour profile (Engels *et al*. 1997; Ayad *et al*. 2003; Avsar *et al*. 2004; Carunchia Whetstine *et al*. 2006; Afzal *et al*. 2017). The production of 3-methylbutanal is predominantly the result of enzymatic catalysed breakdown of leucine by intracellular enzymes that are present in most lactic acid bacteria applied for cheese manufacturing (Atiles *et al*. 2000; Rijnen *et al*. 2003; Helinck *et al*. 2004). In particular, the production of 3-methylbutanal by *Lactococcus lactis* strains, which are used for the manufacturing of most types of (semi-)hard cheeses such as Gouda and Cheddar, was extensively studied. It was demonstrated that the amino group of leucine is first transferred by an aromatic and/or branched-chain aminotransferase to an α-ketoacid, preferably α-ketoglutarate, which results in the formation of α-ketoisocaproate (Yvon *et al*. 1997, 2000; Atiles *et al*. 2000; Rijnen *et al*. 2003). Subsequently, α-ketoisocaproate is decarboxylated by a decarboxylase to 3-methylbutanal (Ayad *et al*. 2001; de la Plaza *et al*. 2004a, 2004b; Smit *et al*. 2005). Previous researchers showed that aminotransferase and decarboxylase activities strongly depend on environmental conditions such as pH and sodium chloride concentration. For that matter, it is expected that the biochemical pathway leading to 3-methylbutanal production is mostly active in intact and metabolically active cells that maintain intracellular homeostasis (Weimer *et al*. 1997; Klein *et al*. 2001; Thage *et al*. 2004; Brandsma *et al*. 2012). For instance, in the presence of 4% w/v sodium chloride, the aromatic and branched-chain aminotransferase activities are reduced by, respectively, 40 and 18% (Yvon *et al*. 1997, 2000), and at a pH of 5.2, the activity of α-ketoisocaproate decarboxylase is about 50% lower compared with a pH in the range 6 to 7 (Smit *et al*. 2005). This suggests that apart from the capacity of Lactococcus lactis cells to catabolise leucine into 3-methylbutanal also cheese manufacturing conditions that affect the metabolic activity and/or integrity of cells might play a role in the 3-metylbutanal production. In this context, the salting method used to control lactic acid

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concentration and to stabilise the pH of the cheese curd is relevant. For instance, curd of brine-salted cheeses, such as Gouda and Edam, is washed to remove lactose which stops lactic acid production and stabilises the pH at around 5.2. On the contrary, the lactic acid production in dry-salted cheeses is controlled by adding sodium chloride to the curd which directly inhibits the starter culture and stabilises the pH also at a value of approximately 5.2. We hypothesise that the way to control the pH not only affects the composition of the curd, for example concentration of sodium chloride, lactose and lactic acid, but also affects production of flavour compounds such as 3-methylbutanal by the starter culture. In this work, we studied the effect of sodium chloride, complexity of the nitrogen source and lactose deprivation on the production of 3-methylbutanal by *L. lactis* subsp. *lactis* DS84445. This strain was selected for its capacity to produce relative high concentration of 3-methylbutanal in Gouda-type cheeses (Brandsma *et al*. 2018).

MATERIALS AND METHODS

Materials

Lactococcus lactis subsp. *lactis* DS84445 was isolated from an artisanal-produced dairy product and selected for its high capacity to produce 3-methylbutanal in milk (DSM, Delft, the Netherlands). A frozen cryovial with DS84445 stored in sterile skimmed milk (s-milk) (Tritium, Eindhoven, the Netherlands) was incubated at 30 °C for 18 h and used to inoculate (1% v/v) pre-cultures of s-milk or M17 broth (Oxoid, Hampshire, UK) supplemented with 10 g/L lactose (LM17). Viable cell counts were determined with M17 agar plates supplemented with 5 g/L lactose (LM17) or with β-glycerolphosphate-buffered milk agar (GMA). GMA plates were made of Bacto agar (BD, Frederick, MD, USA), β-glycerolphosphate (Sigma-Aldrich, Steinheim, Germany), skimmed milk (Promex, FrieslandCampina, the Netherlands) and bromocresol purple (Merck, Darmstadt, Germany). Other chemicals used in this study were obtained from Sigma-Aldrich.

Incubation experiments

Full-grown pre-cultures of strain DS84445 were used to inoculate (1% v/v) 20 mL s-milk with different concentrations of sodium chloride. Directly after inoculation, the s-milk was divided into two portions. One portion of 18 mL was transferred to 100-mL bottles, and the other portion of 2 mL was transferred to 10-mL air-tide sealed vials and both were incubated at 30 °C. In addition, the pH of the milk in the bottles was measured in line. After 18 h of incubation, microbial analysis of bottles was performed and air-tide sealed vials were stored at −40 °C until flavour analysis.

A buffered liquid medium (BLM) was used to mimic and study the 3-methylbutanal production during cheese manufacturing. The BLM is based on the chemically defined medium (CDM) described by Otto *et al*. (1983), whereby the following adaptions were made. The potassium phosphate buffer was replaced by a citrate phosphate buffer containing 0.05 M citric acid and 0.1 M disodium hydrogen phosphate. Amino acids with concentrations according to the original CDM were used (BLM + AA), or amino acids were replaced by 15 g/L pasteurised (70 °C for 10 min) nonhydrolysed bovine casein (BLM + CAS). In addition, 0% and 4% w/v sodium chloride was added after which the pH was adjusted to 5.2 with 5 M HCl. The buffer capacity was high enough to maintain the pH during all experiments at 5.2 ± 0.1. Depending on the experiment, 0 to 30 mM lactose was added. The BLM was inoculated with concentrated cell suspension to a final optical density of 0.50. Thereto, cells of strain DS84445 were harvested from a full-grown LM17 culture and concentrated by centrifugation at 13 100 *g* for 5 min at 4 °C and washed twice with citrate phosphate buffer. The citrate phosphate buffer was made of 0.1 M citric acid and 0.2 M disodium hydrogen phosphate with a pH adjusted to 7.0. Directly after inoculation, the BLM was divided into two portions. One portion of 15 mL was transferred to 15-mL conical tubes (Greiner, Frickenhausen, Germany) and the other portion of 2-mL was transferred to 10-mL air-tide sealed vials, and both were incubated for 18 h at 30 °C. In addition, the conical tubes were sampled directly after inoculation and after incubation, and cell counts were determined via selective plating or live/dead staining, while vials were stored at −40 °C until flavour analysis. For each experiment, noninoculated blanks were taken along and incubated under the same conditions. Presented data represent average of biological triplicates.

Cheese was made from batches of 5 L pasteurised full-fat milk (Albert Heijn, Zaandam, the Netherlands). Milk was heated in a water bath with an in-line pH measurement (Hettich, Tuttlingen, Germany), while gently stirring at 200 rpm. At a temperature of 31 °C, the milk was inoculated with 7×10^9 cells of DS84445 per mL. Milase premium 750 BF (DSM) (0.385 mL per 5 L milk) was added as coagulant, 25 min after inoculation, and after 1 min of additional stirring, the stirrer was removed. Chymosin time was about 30 min after which the gel was cut. Curd–whey mixture was slowly stirred for 10 min and then heated from 31 to 39 °C in 30 min while gently stirring every 10 min. The curd was cooked for 40 min at 39 °C, and then, the whey was drained at a pH of about 6.2. Both curd and whey were sampled for further analysis. The curd was kept at a temperature of 39 °C and divided in two equal batches. For each 15 min, the curd was turned, and whey was removed until pH of 5.7, which took approximately 3.5 h after inoculation. The curd was cut into dices of approximately 1 by 1 cm, and only to the dry-salted cheese, 22 g sodium chloride was added per 550 g curd. Salt was given 5 min to absorb in the curd dices. The cheese dices of non-salted cheese and dry-salted cheese each with a weight of

about 550 g were placed in cheese moulds and pressed (15 min at 0.2 bar, 15 min at 0.4 bar, 60 min at 0.8 bar and 660 min at 1.6 bar). Pressed cheeses were stored in vacuumised bags at 7 °C for 7 days. Curd and cheese samples were taken directly after slicing and after 1 and 7 days. Cheese samples were grated, and portions of 25 g were mixed with 50 mL demi-water and ground for 20 s at intensity 8000 followed by 10 s at intensity 13 500 with a Turax (Ultra Turrax T25, Staufen, Germany). The mixture was centrifuged (Eppendorf centrifuge 5810R, Hamburg, Germany) for 10 min at 10 000 rpm, and the clear supernatant was collected. Supernatant was filtered with a 0.2-μm pore size filter and transferred into HPLC vials. Additionally, 2 mL supernatant was added to 10 mL HS-GC vials and stored at −40 °C until flavour analysis.

Microbial analysis

Plate counting

Appropriate dilutions of samples taken from s-milk and BLM + CAS incubations were made in peptone physiological salt solution (Tritium) and streaked out on LM17 or GMA plates that were incubated in an anaerobic jar at 30 °C for, respectively, 3 days and 5 days.

Live-dead counting

The number of live and dead cells of samples taken from BLM + AA incubations was determined using LIVE/DEAD BacLight Bacterial Viability kit (Thermo Fisher Scientific, Waltham, MA, USA) and analysed using CytoFLEX Flow Cytometer (Beckman Coulter, San Jose, CA, USA). BLM + AA samples were prepared by diluting them 100 times with filtered 0.85% w/v sodium chloride solution, and 200 μL of each diluted sample was added to 96-well F-bottom microtiter plate (Greiner). Cells were stained by adding a mixture of 0.1 μL of SYTO 9 and 0.1 μL of propidium iodide (PI) to each 96 well, which was stored in the dark for 15 min at room temperature. Other cells were stained by diluting 199-μL cell suspension with 1 μL of a work stock solution made from 4.5 μL of SYTO 9, 4.5 μL of propidium iodide (PI) and 141 μL of demi-water. The green fluorescent SYTO 9 labelled the nuclei of live cells, while the red fluorescent PI labelled dead or dying cells which have a compromised cell wall membrane. Number of live and compromised cells were counted with the flow cytometer at 488 nm to a maximum of 10 000 events. Number of compromised cells were during all measurements <1% of number of live cells and not considered for the results section.

Chemical analysis

Volatile compounds

The 3-methylbutanal level was determined by headspace solid-phase microextraction gas chromatography–mass spectrometry using a Trace 1300 Gas Chromatograph with a TriPlus RSH autosampler and an ISQ QD mass spectrometer (Thermo Fisher Scientific). The air-tide sealed vials were kept at −1 °C during the total run. Samples were incubated at 60 °C for 20 min, followed by an extraction at 60 °C for 20 min with a divinylbenzene/carboxen/polymethylsiloxane fibre (DVB/Car/PDMS) (Supelco, Bellefonte, PA, USA). The volatiles were desorbed from the fibre to the injector for 2 min on a Stabilwax-DA-Crossbond-Carbowax-polyethylene-glycol column (30 m length, 0.25 mm ID, 0.5 μm df). The gas chromatograph was used in split mode at a ratio of 1:25 at 250 °C. The oven temperature was initially set at 35 °C for 2 min, gradually increased with 10 °C per min and was kept at 240 °C for 5 min. The total run time was 27.5 min. Helium was used as carrier gas with a constant flow of 1.2 mL per min. Mass spectral data were collected over a range of *m/z* 33-250 in full-scan mode with 3.0 scans per second. The obtained data were analysed using Chromeleon 7.2. Peak integration was done using ICIS algorithm, and NIST main library was used to match the mass spectral profiles with the profiles of NIST. Peak areas were calculated using the MS quantification peak area (highest *m/z* peak per compound) and subsequently corrected with blank's containing the same medium and incubated under the same conditions. Corrections were less than 3% of total peak areas of samples.

Sugars and organic acids

The concentration of sugars and organic acids was determined using UltiMate 3000 UHPLC (Thermo Fisher Scientific). The standards contain lactose, glucose, galactose, L-lactic acid, formic acid and acetic acid. The standard curves were obtained by preparing standard mixtures with the following concentrations: 1.25, 2.5, 5, 10, 20 and 40 g/L. All standard curves had $r^2 > 0.99$ and concentration of each compound was calculated by comparing its peak area against the standard curve.

Enzymatic analysis

Leucine and alanine aminotransferase activity

Strain DS84445 was grown in 20 mL LM17 and incubated at 30 °C. Cells were harvested after 18 h by centrifugation at 10 000 rpm for 10 min at 5 °C. Concentrated cells were washed three times with 50 mM sodium phosphate buffer (pH 7.0) and re-suspended in 3 mL of the same buffer. Then, 1 mL of the washed biomass was added to a 2-mL beat tube that contained 1–1.5 g zirconia beats. Cell-free extract (CFE) was prepared by beating the tubes at 4 m/s in a FastPrep®-24 (MP Biomedicals, Solon, OH, USA) for 30 s, followed directly by cooling on melting ice for 5 m. The beating and cooling steps were repeated four times. The cell debris and supernatant were separated by centrifuging at 10 000 rpm for 10 min at 5 °C. The supernatant was collected as CFE and kept on melting ice until enzyme

activity and protein concentration measurement. Aminotransferase activity measurement was previously described by Brandsma *et al*. (2012). In short, the reaction mixture contained 25 mM phosphate buffer (pH 7.5), 0.05 mM pyridoxal-5'-phosphate, 50 mM ammonium sulphate, 0.35 mM NADH, 10 mM α-ketoglutarate, 10 mM L-leucine or L-alanine as a substrate and 100 µL CFE in a final volume of 1 mL. Both leucine aminotransferase and alanine aminotransferase activities were determined by the maximum reduction rate of NADH measured by OD_{340} for 15 min at 30 °C. The protein concentrations of CFE were measured using Pierce BCA Protein Assay Kit (Thermo Fisher Scientific). The amount of protein was expressed in µg/mL and used to calculate the specific enzyme activities. The average leucine aminotransferase is 120 nmol/mg protein/min (sd = 0.015), and alanine aminotransferase activity was not detected.

Statistics

All experiments were carried out in triplicates, and each replicate was analysed once unless otherwise stated. The data were analysed using Analysis ToolPak in Excel. The t-test: paired two-sample for means was used to study the impact of 18 hours of incubation at the same experimental conditions, and t-test: two-sample assuming equal variances was used to study the impact of sodium chloride. The data were expressed as mean ± standard deviation (sd), and for measurements with different letters, the difference between the means is statistically ($P < 0.05$) significant.

RESULTS

Acidification of cheese milk and 3-methylbutanal production in milk

Lactococcus lactis subsp. *lactis* DS84445 contains the gene *ipdC* that codes for a protein belonging to the proteins of the α-ketoacid decarboxylase family, and according to Smit *et al*. (2005), these proteins are responsible for decarboxylation of α-ketoisocaproate to 3-methylbutanal. Indeed, strain DS84445 expresses an active α-ketoisocaproate decarboxylase protein, and when grown in milk, it produces relatively large amounts of 3-methylbutanal compared with semihard cheese starter cultures (Brandsma *et al*. 2018). Generally, cheeses contain sodium chloride concentrations in the moisture phase of about 4% w/v, and as expected, we observed a sharp decrease in the maximum acidification rate of strain DS84445 until 4% w/v in s-milk and simultaneously a steep reduction in the 3-methylbutanal content (Figure 1). This experiment demonstrates how salting affects the 3-methylbutanal production during cheesemaking.

Effect of salting regime on 3-methylbutanal production

The salting method strongly affects the salt distribution during the early cheese maturation phase. In dry-salted cheeses,

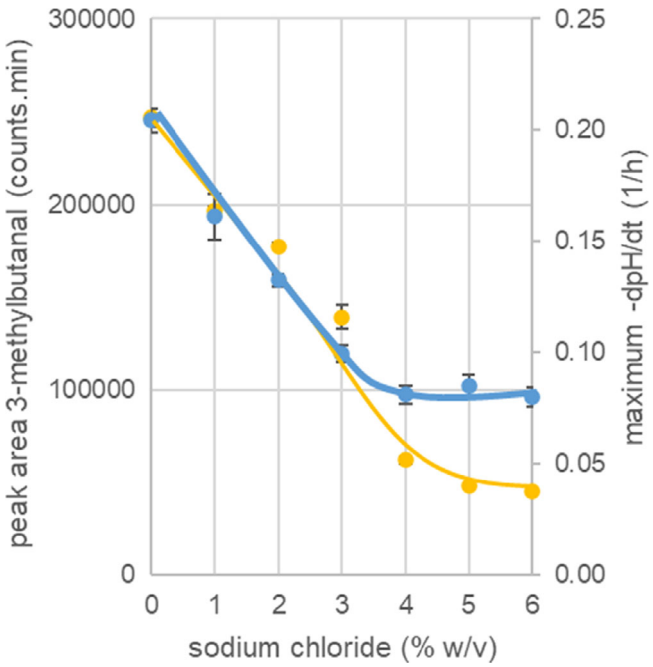

Figure 1 Maximum acidification rate (–dpH/dt) (blue) and 3-methylbutanal (orange) content in s-milk with various concentrations of sodium chloride acidified with *Lactococcus lactis* subsp. *lactis* DS84445 after 18 h of incubation at 30 °C. Average of 3 biological replicates.

the salt is fairly uniformly distributed throughout the whole cheese matrix, while in brined cheeses, such as Gouda, it takes 7 to 9 weeks before the salt is homogenously distributed throughout the cheese (Guinee 2004). A buffered liquid medium (BLM) was used to mimic early cheese maturation for studying the effect of sodium chloride on the 3-methylbutanal production. This is basically the chemically defined medium described by Otto *et al*. (1983) with a pH buffered at 5.2 in which the amino acids (BLM + AA) are replaced by nonhydrolysed bovine casein (BLM + CAS) (Smid *et al*. 1989). This particular pH value was chosen because it represents the prevailing pH at the start of cheese maturation. In line with milk incubations (Figure 1), the plate counts significantly ($P < 0.05$) decreased by approximately one order of magnitude in the presence of 4% w/v sodium chloride. This decrease is significantly larger compared with the effect observed in the absence of added sodium chloride (Figure 2a) and in addition sodium chloride almost completely suppressed the 3-methylbutanal production (Figure 2b).

Impact of amino acids during cheese maturation on the production of 3-methylbutanal

The concentration of free amino acids increases during cheese maturation as result of casein hydrolysis by the action of coagulant, indigenous milk enzymes, caseinolytic extracellular proteinases and proteolytic enzymes released

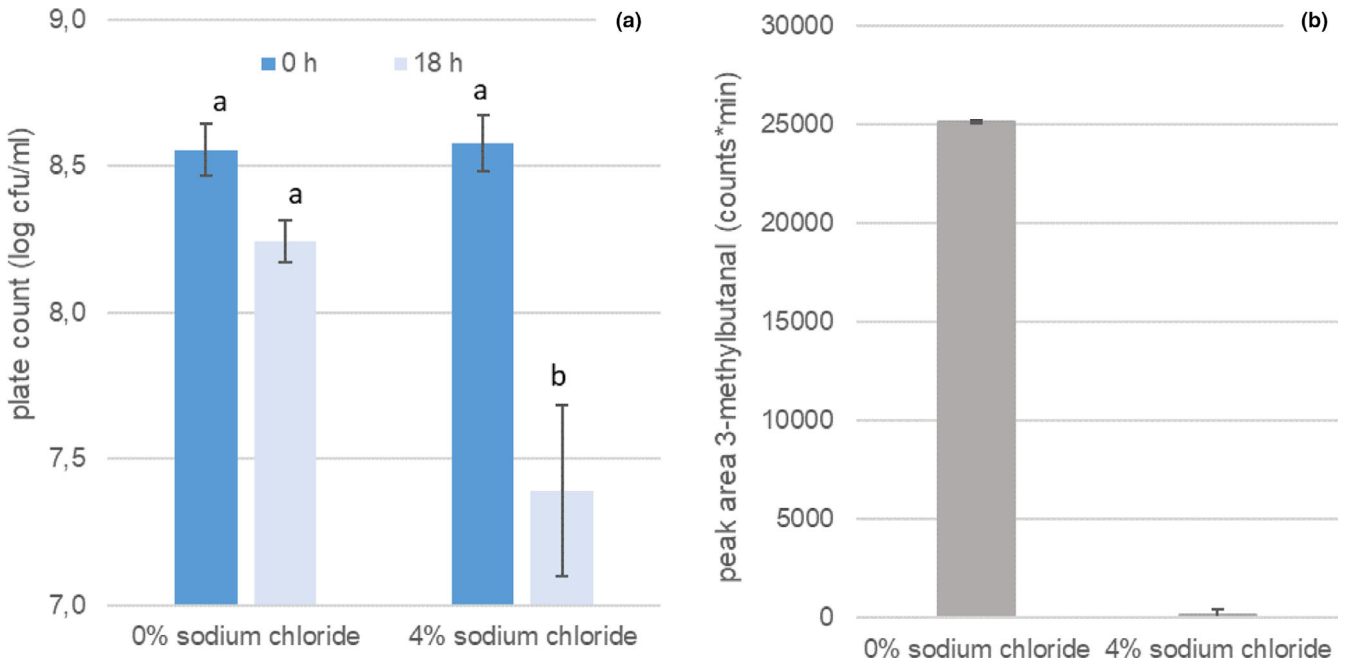

Figure 2 Plate counts of *Lactococcus lactis* subsp. *lactis* DS84445 (a) and 3-methylbutanal level (b) in BLM + CAS with 0 and 4% w/v sodium chloride after 18 h of incubation at 30 °C. Average of 3 biological replicates.

by lysed cells. Here, the effect of casein hydrolysis on 3-methylbutanal production without and with 4% w/v sodium chloride was tested in the buffered liquid medium in which the nonhydrolysed casein was completely replaced by amino acids. Cells incubated with amino acids (BLM + AA) produced about 3 times more 3-methylbutanal (Figure 3) in comparison with cells incubated with nonhydrolysed casein (BLM + CAS) (Figure 2). Surprisingly, sodium chloride stimulated the 3-methylbutanal production by strain DS84445 in incubation with amino acids (Figure 3). In contrast to cells incubated with nonhydrolysed casein, the average cell counts of strain DS84445 incubated with amino acids were not significantly ($P < 0.05$) affected by the presence of sodium chloride (0% w/v sodium chloride: 8.7 log cfu/mL, sd = 0.01, and 4% w/v sodium chloride: 8.6 log cfu/mL, sd = 0.01).

Salt induces osmotic stress leading to water efflux that can be restored by the uptake of amino acids and other osmolytes (Bremer and Krämer 2019). In our buffered liquid medium, free amino acids comprise the main organic nitrogen source, and elevated uptake of amino acids such as leucine to handle osmotic stress would explain an increased production of 3-methylbutanal. However, in cheese, a combination of intact and partly hydrolysed casein and free amino acids are present. Cheese was made to determine whether amino acids or nonhydrolysed casein should be added to the BLM to correctly predict the effect of sodium chloride on the production of 3-methylbutanal during the early maturation phase. Cheeses were made on pilot scale whereby salt was omitted to curd (0% w/v sodium chloride) and whereby curd was salted with 4% w/v sodium chloride (dry-salted cheese). The 3-methylbutanal concentration of the reference cheese declined during maturation at 7 °C (Figure 4). Adding sodium chloride reduces the 3-methylbutanal concentration even more, which is in line with the results obtained from the milk incubations (Figure 1) and the buffered liquid medium supplemented with nonhydrolysed casein (Figure 2). Irrespective of sodium chloride addition, when the curd is made and maturation phase starts, the 3-methylbutanal concentrations declined, meaning that the 3-methylbutanal production by strain DS84445 is lower than for instance enzymatic and/or chemical degradation in cheese.

As expected, salt strongly inhibited the reduction in residual sugar leading to less production of lactic acid (Figure 4), which is also reflected by about 2 times lower plate count after 7 days of maturation in salted cheese (3.3×10^9 cfu/g) compared to cheese without sodium chloride (7.2×10^9 cfu/g).

The effect of lactose and pyruvate on the production of 3-methylbutanal

The amount of residual lactose during cheese maturation phase strongly depends on the way of controlling the pH of the curd. The curd of brined cheeses is washed, and residual lactose is not detectable after brining (<48 h), while on the contrary, curd of dry-salted cheeses can still contain lactose after 3 weeks of maturation (Fox *et al*. 1990). Cells of strain DS84445 were incubated in BLM + CAS with different concentrations of lactose. With the availability of

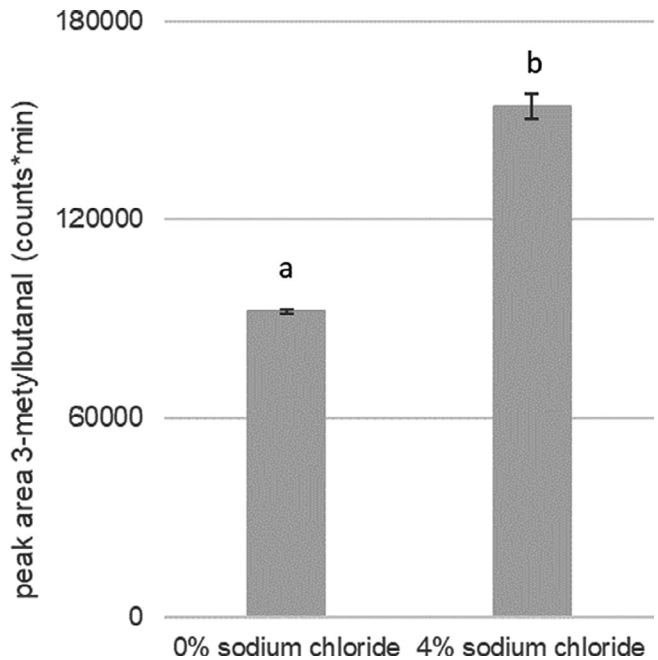

Figure 3 3-Methylbutanal production by *Lactococcus lactis* subsp. *lactis* DS84445 after 18 h of incubation at 30 °C in BLM + AA with 0% w/v and with 4% w/v sodium chloride. Average of 3 biological replicates.

lactose, irrespectively of the concentration, strain DS84445 produces about 4 times more 3-methylbutanal than without lactose, while the plate counts vary less than approximately 20% (Figure 5).

Lactose feeds into the glycolytic pathway that generates metabolic energy in the form of ATP and produces pyruvate. To regenerate NAD^+ from the reduced redox cofactor NADH, pyruvate is converted into lactic acid. Both net ATP generation and NAD^+ regeneration are essential for growth and maintenance but are not directly necessary to produce 3-methylbutanal. However, indirectly the NADH/NAD^+ balance can affect the 3-methylbutanal concentration. For instance, when lactose-deprived cells have a relative high concentration of NADH, 3-methylbutanal can be converted to 3-methylbutanol to restore the NADH/NAD^+ balance. Based on that hypothesis, supplementing lactose-deprived cells with pyruvate would reduce the need of cells to dehydrogenase 3-methylbutanal into 3-methylbutanol and regenerate NAD^+. This is, however, not observed (Figure 6). On the contrary, we observed that pyruvate-supplemented and sugar-deprived DS84445 cells produce 3 times more 3-methylbutanal, without significantly affecting the production of 3-methylbutanol (Figure 6). Pyruvate is easily taken up by lactococci (Zuljan *et al*. 2014) and strongly stimulates the conversion of leucine into 3-methylbutanal by strain DS84445.

DISCUSSION

Salt and salting method plays an important role in many types of cheeses. Salt supports the development of a natural cheese rind, it inhibits growth of spoilage and potential pathogenic bacteria, and it prevents post-acidification by the

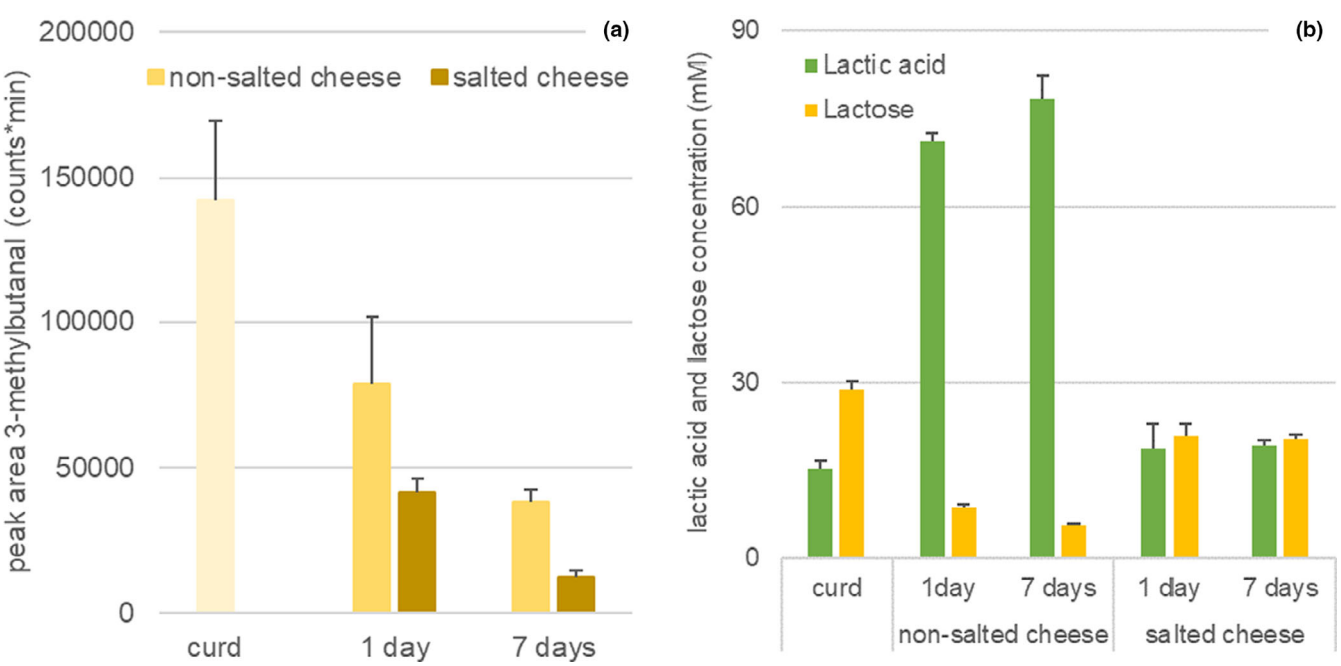

Figure 4 Nonsalted and salted pilot scale produced cheeses with *Lactococcus lactis* subsp. *lactis* DS84445. The 3-methylbutanal content (a) and lactose and lactic acid concentrations (b). Average of 2 biological replicates and 2 technical replicates.

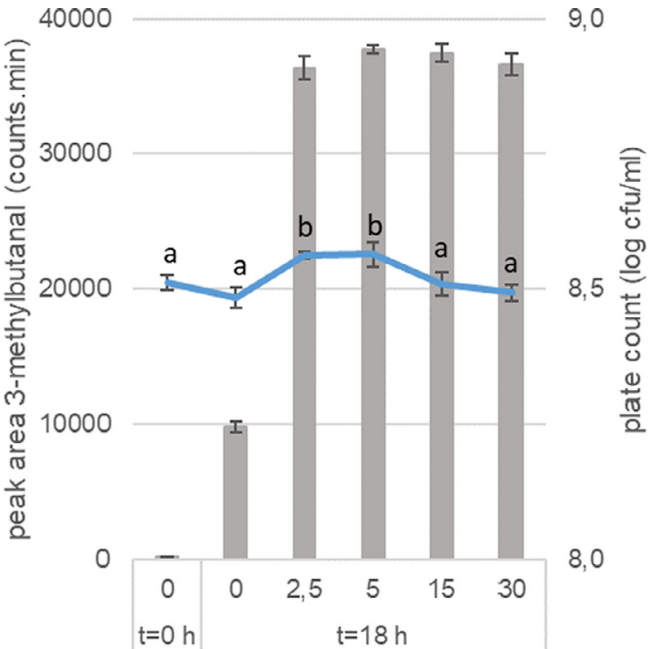

Figure 5 3-Methylbutanal levels (bars) and plate counts (line) of *Lactococcus lactis* subsp. *lactis* DS84445 incubated in BLM + CAS with different concentrations lactose (mM) directly after inoculation (t = 0 h) and after 18 h of incubation at 30 °C. Average of 3 biological replicates.

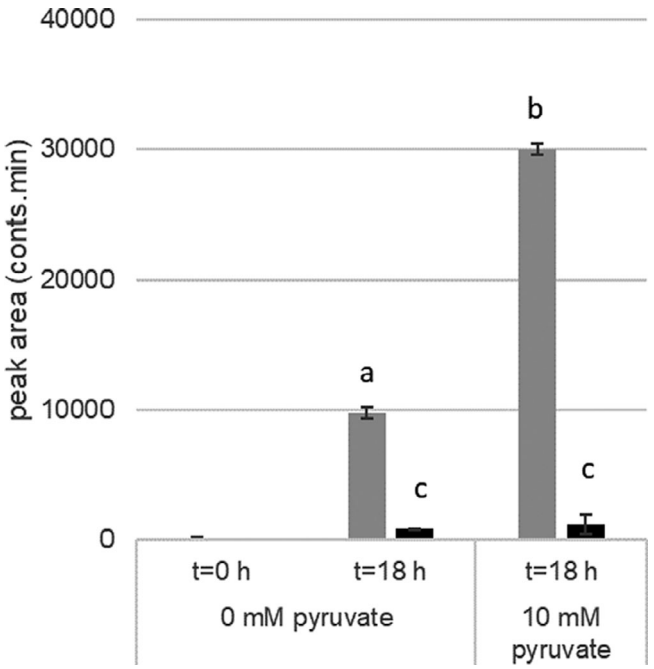

Figure 6 3-Methylbutanal (grey bars) and 3-methylbutanol (black bars) levels produced by *Lactococcus lactis* subsp. *lactis* DS84445 incubated in BLM + CAS with 0 and 10 mM pyruvate for 18 h at 30 °C. Average of 3 biological replicates.

starter culture. Moreover, it contributes to the overall flavour characteristics of the cheese (Guinee 2004). Our work showed that 4% w/v sodium chloride strongly inhibited the production of 3-methylbutanal by strain DS84445. Surprisingly, when casein is replaced by free amino acids as sole nitrogen source, sodium chloride actually stimulated the production of 3-methylbutanal. This might be explained by an increased uptake of leucine to counteract the osmotic pressure induced by salt. So far, the stimulatory effect of salts on the uptake of osmolytes to battle osmotic pressure is mainly described for the accumulation of glycine betaine and proline and to lesser extent with aspartate and glutamate by *L. lactis* (Molenaar *et al*. 1993). The uptake of leucine by *L. lactis* MG1363 is actually not stimulated by hyperosmotic conditions (Poolman and Heide 2000). Transport of leucine is facilitated by the branched-chain amino acid permease BcaP which is under control of the pleiotropic transcriptional repressor CodY (den Hengst *et al*. 2006). Intracellular accumulation of branched-chain amino acids is sensed by CodY, which downregulates the major components of the proteolytic system in *L. lactis* that are needed for the release of amino acids from casein (Guédon *et al*. 2001; Kok *et al*. 2017). Strain DS84445 contains both the genes BcaP and CodY, and thus, it would be expected that high intracellular levels of leucine would reduce its uptake in the absence and in the presence of sodium chloride comparable to MG1363. However, strain DS84445 has, in contrary to MG1363, an active α-ketoisocaproate decarboxylase that enables swift conversion of α-ketoisocaproate into 3-methylbutanal. Therefore, fast decarboxylation of α-ketoacids originating from branched-chain amino acids is expected to prevent the accumulation of branched-chain amino acids that would otherwise be sensed by CodY, resulting in a downregulation of the proteolysis capacity of cells. In that case, the possession of active decarboxylases could be a competitive advantage for *L. lactis* strains allowing fast growth in milk as it ensures a continuously upregulated proteolytic activity.

The results of our pilot scale produced cheese suggest that the concentration of free amino acids is not high enough to counteract the inhibitory effect of salt on the production 3-methylbutanal. We, furthermore, saw that the highest concentration of 3-methylbutanal was obtained in the curd and then during maturation gradually decreased, which is in line with observations made by Meng *et al*. (2021) for non-salted Raclette cheese. In addition, we saw that sodium chloride further reduced the 3-methylbutanal concentration in cheese. Consequently, stimulating the 3-methylbutanal production in cheese is most promising in the phase of the cheesemaking process when salt is not yet present. More precisely, 3-methylbutanal production is expected to occur mostly during curd production in case of dry-salted cheeses or during early cheese maturation phase in case of brined cheese.

So far, studies aiming to increase 3-methylbutanal concentration have predominantly focused on relieving intracellular rate-limiting bottlenecks in the metabolism of starter bacteria during cheese maturation. For instance, replenishing α-ketoglutarate via glutamate dehydrogenase (GDH) activity sustains the conversion of amino acids into α-keto acids and subsequent conversion into fusel aldehydes and fusel alcohols via the Ehrlich pathway. For that reason, *L. lactis* strains that possess GDH activity were isolated from natural sources or were enriched via GMO or non-GMO techniques with a GDH coding gene from *Peptostreptococcus asaccharolyticus* or plasmid from *L. lactis* (Ayad *et al*. 1999; Rijnen *et al*. 2000; Tanous *et al*. 2002; Gutiérrez-Méndez *et al*. 2008). In that context, also indirect pathways to produce α-ketoglutarate from citrate or pyruvate were studied (Tanous *et al*. 2005).

These strains with improved capability to generate α-ketoglutarate did enhance degradation of amino acids and/or production of flavour compounds but increased production of 3-methylbutanal or other branched-chain aldehydes were not reported. Also directly adding α-ketoglutarate to the cheese matrix increased flavour production; however, again only flavours originated from aromatic amino acids like benzaldehyde were increased while an increase in 3-methylbutanal level and other flavours originating from branched-chain amino acids were not reported (Yvon *et al*. 1998; Banks *et al*. 2001). In addition, our work points out that pyruvate supplementation strongly promotes the production of 3-methylbutanal by strain DS84445. Pyruvate can stimulate the conversion of leucine to 3-methylbutanal in several ways. Pyruvate can act as an acceptor of an amino group in the transamination of glutamate into α-ketoglutarate catalysed by alanine transaminase (Figure 7). However, strain DS84445 does not show alanine aminotransferase activity, nor does it contain the gene *alaC* coding for this protein. Alternatively, pyruvate can have a direct stimulatory effect when it acts as an additional ammonia acceptor next to α-ketoglutarate in the deamination of leucine catalysed by aromatic or branched-chain aminotransferases. Yvon *et al*. (2000) showed that the affinity of purified branched-chain aminotransferase for pyruvate is about 40-fold lower compared with α-ketoglutarate. Average 3-methylbutanal concentrations in 6-week-old Gouda types of cheeses are in the range of 0.1 to 0.2 µmol/kg, which increases about 150-fold to 25 µmol per kg cheese when strain DS84445 is used in addition to the starter culture (Van Leuven *et al*. 2008; Brandsma *et al*. 2018). At the same time, lactic acid concentrations in these types of cheeses are 28 to 44 mmol lactic acid per kg cheese (Jo *et al*. 2017). This shows that the glycolytic pathway produces at least 1000 times more pyruvate than the amount of α-ketoglutarate that is needed to produce organoleptically noticeable amounts of 3-methylbutanal in cheese. However, fast acidifying cells have a homolactic metabolism whereby more than 90% of the produced pyruvate is converted to lactate to balance NADH/NAD^+ ratio. However, when sugar becomes limiting, such as during the early cheese maturation phase, cells shift from homolactic to mixed-acid metabolism whereby under anaerobic conditions additional NAD^+ is produced (Cocaign-Bousquet *et al*. 1996). These conditions might also support re-directing pyruvate into leucine aminotransferase and eventually leading to higher 3-methylbutanal production.

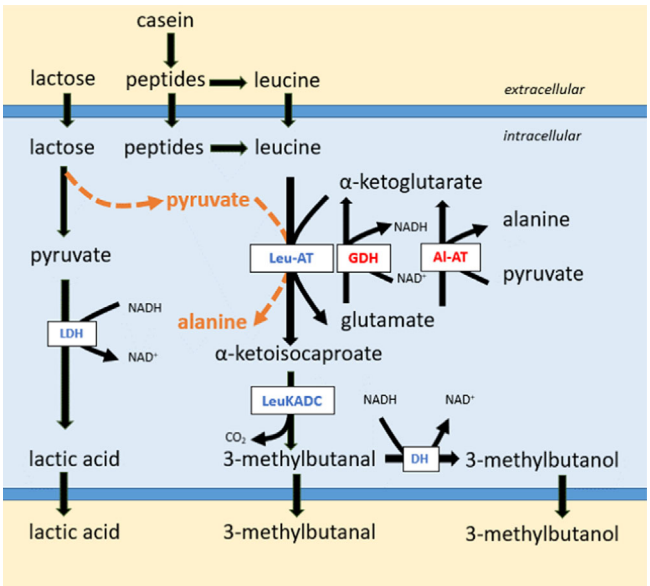

Figure 7 Schematic overview of leucine breakdown to volatile flavours in *Lactococcus lactis*. Blue- and red-depicted abbreviations in boxes are enzymes that are, respectively, present or not present in *L. lactis subsp. lactis* DS84445. Dotted orange arrow is the proposed use of pyruvate as ammonia acceptor by Leu-AT. Abbreviations are as follows: Leu-AT is leucine aminotransferase, LeuKADC is α-ketoisocaproate decarboxylase, DH is alcohol dehydrogenase, LDH is lactate dehydrogenase, GDH is glutamate dehydrogenase, and Al-AT is alanine aminotransferase.

So far, studies on the production of 3-methylbutanal during cheese maturation were mainly focusing on resolving rate-limiting bottlenecks within *L. lactis*. Our work showed that the salting method, especially supplementing salt has a detrimental effect on the capacity of *L. lactis* DS84445 to produce 3-methylbutanal. In that respect, the most promising way to manipulate the concentration of 3-methylbutanal in cheese is by using a specific strain that produces the aimed level of 3-methylbutanal before salting starts and preferably when lactose is still available.

AUTHOR CONTRIBUTIONS

Johannes B Brandsma: Conceptualization; Data curation; Formal analysis; Investigation; Methodology; Supervision; Validation; Visualization; Writing – original draft. **Judith**

Brinkman: Data curation; Formal analysis; Methodology. **Natassa Rustandi:** Data curation; Formal analysis; Methodology. **Judith C M Wolkers-Rooijackers:** Data curation; Formal analysis; Methodology. **Marcel H Zwietering:** Writing – review & editing. **Eddy J Smid:** Conceptualization; Writing – review & editing.

REFERENCES

Afzal M I, González Ariceaga C C, Boulahya K A, Jacquot M, Delaunay S and Cailliez-Grimal C (2017) Biosynthesis and role of 3-methylbutanal in cheese by lactic acid bacteria: major metabolic pathways, enzymes involved, and strategies for control. *Critical Reviews in Food Science and Nutrition* **57** 399–406.

Atiles M W, Dudley E G and Steele J L (2000) Gene cloning, sequencing, inactivation of the branched-chain aminotransferase of *Lactococcus lactis* LM0230. *Applied and Environmental Microbiology* **66** 2325–2329.

Avsar Y K, Karagul-Yuceer Y, Drake M A, Singh T K, Yoon Y and Cadwallader K R (2004) Characterization of nutty flavor in Cheddar cheese. *Journal of Dairy Science* **87** 1999–2010.

Ayad E H E, Verheul A, de Jong C, Wouters J T M and Smit G (1999) Flavour forming abilities and amino acid requirements of Lactococcus lactis strains isolated from artisanal and non-dairy origin. *International Dairy Journal* **9** 725–735.

Ayad E H E, Verheul A, Engels W J M, Wouters J T M and Smit G (2001) Enhanced flavour formation by combination of selected lactococci from industrial and artisanal origin with focus on completion of a metabolic pathway. *Journal of Applied Microbiology* **90** 59–67.

Ayad E H E, Verheul A, Bruinenberg P, Wouters J T M and Smit G (2003) Starter culture development for improving the flavour of Proosdij-type cheese. *International Dairy Journal* **13** 15–168.

Banks J M, Yvon M, Gripon J C, de la Fuente M A, Brechany E Y, Wiliams A G and Muir D D (2001) Enhancement of amino acid catabolism in Cheddar cheese using α-ketoglutarate: amino acid degradation in relation to volatile compounds and aroma character. *International Dairy Journal* **11** 235–243.

Brandsma J B, Kevelam J and Meijer W C (2018) Production of a roasted type of flavour in semi hard cheese. *Poster at 10th Cheese Symposium, 4-6 April, France*.

Brandsma J B, van de Kraats I, Abee T, Zwietering M H and Meijer W C (2012) Arginine metabolism in sugar deprived *Lactococcus lactis* enhances survival and cellular activity, while supporting flavour production. *Food Microbiology* **29** 27–32.

Bremer E and Krämer R (2019) Responses of microorganisms to osmotic stress. *Annual Review of Microbiology* **73** 313–334.

Carunchia Whetstine M E, Drake M A, Broadbent J R and McMahon D (2006) Enhanced nutty flavour formation in cheddar cheese made with malty *Lactococcus lactis* adjunct. *Journal of Dairy Science* **89** 3277–3284.

Cocaign-Bousquet M, Garrigues C, Loubiere P and Lindley N D (1996) Physiology of pyruvate metabolism in *Lactococcus lactis*. *Antoni Van Leeuwenhoek* **70** 253–267.

Den Hengst C D, Groeneveld M, Kuipers O P and Kok J (2006) Identification and functional characterization of the *Lactococcus lactis* CodY-regulated branched-chain amino acid permease BcaP (CtrA). *Journal of Bacteriology* **188** 3280–3289.

Engels W J M, Dekker R, de Jong C, Neeter R and Visser S A (1997) Comparative study of volatile compounds in the water-soluble fraction of various types of ripened cheese. *International Dairy Journal* **7** 255–263.

Fox P F, Lucey J A and Cogan T M (1990) Glycolysis and related reactions during cheese manufacture and ripening. *Food Science and Nutrition* **29** 237–253.

Guédon E, Serror P, Dusko Ehrlich S, Renault P and Delorme C (2001) Pleiotropic transcriptional repressor CodY senses the intracellular pool of branched-chain amino acids in *Lactococcus lactis*. *Molecular Microbiology* **40** 1227–1239.

Guinee T P (2004) Salting and the role of salt in cheese. *International Journal of Dairy Technology* **57** 99–109.

Gutiérrez-Méndez N, Velenzuela-Soto E, González-Córdova A F and Vallejo-Cordoba B (2008) α-Ketoglutarate biosynthesis in wild and industrial strains of *Lactococcus lactis*. *Letters in Applied Microbiology* **47** 202–207.

Helinck S, Le Bars D, Moreau D and Yvon M (2004) Ability of thermophilic lactic acid bacteria to produce aroma compounds from amino acids. *Applied and Environmental Microbiology* **70** 3855–3861.

Jo Y, Benoist D M, Ameerally A and Drake M A (2017) Sensory and chemical properties of Gouda cheese. *Journal of Dairy Science* **101** 1967–1989.

Klein N, Maillard M B, Thierry A and Lortal S (2001) Conversion of amino acids into aroma compounds by cell-free extracts of *Lactobacillus helveticus*. *Journal of Applied Microbiology* **91** 404–411.

Kok J, van Gijtenbeek L A, de Jong A, van der Meulen S B, Solopova A and Kuipers O P (2017) The Evolution of gene regulation research in *Lactococcus lactis*. *FEMS Microbiology Reviews* **41** 220–243.

Meng H Y, Piccand M, Fuschsmann P, Dubois S, Baumeyer A, Tena Stern M and von Ah U (2021) Formation of 3-methylbutanal and 3-methylbutan-1-ol recognized as malty during fermentation in Swiss Raclette-type cheese, reconstituted milk, and de Man, Rogosa, and Sharpe broth. *Journal of Agricultural and Food Chemistry* **69** 717–729.

Molenaar D, Hagting A, Alkema H, Driessen A J M and Konings W N (1993) Characteristics and osmoregulatory roles of uptake systems for proline and glycine betaine in *Lactococcus lactis*. *Journal of Bacteriology* **175** 5438–5444.

Otto R, Ten Brink B, Veldkamp H and Konings W N (1983) The relation between growth rate and electrochemical proton gradient on *Streptococcus cremoris*. *FEMS Microbiology Letters* **6** 69–74.

de la Plaza M, Fernández de Palencia P, Peláez C and Requena T (2004a) Biochemical and molecular characterization of α-ketoisovalerate decarboxylase, an enzyme involved in the formation of aldehydes from amino acids by *Lactococcus lactis*. *FEMS Microbiology* **238** 367–374.

de la Plaza M, Fernández de Palencia P, Peláez C and Requena T (2004b) Biochemical and molecular characterization of a-ketoisovalerate decarboxylase, an enzyme involved in the formation of aldehydes from amino acids by *Lactococcus lactis*. *FEMS Microbiology Letters* **238** 367–374.

Poolman B and van der Heide T (2000) Glycine betaine transport in *Lactococcus lactis* is osmotically regulated at the level of expression and translocation activity. *Journal of Bacteriology* **182** 203–206.

Rijnen L, Courtin P, Gripon J C and Yvon M (2000) Expression of a heterologous glutamate dehydrogenase gene in *Lactococcus lactis* highly improves the conversion of amino acids to aroma compounds. *Applied and Environmental Microbiology* **66** 1354–1359.

Rijnen L, Yvon M, van Kranenburg R, Courtin P, Verheul A, Chambellon E and Smit G (2003) Lactococcal aminotransferases AraT and BcaT are key enzymes for the formation of aroma compounds from amino acids in cheese. *International Dairy Journal* **13** 805–812.

Smid E J, Plapp R and Konings W N (1989) Peptide uptake is essential for growth of *Lactococcus lactis* on the milk protein casein. *Journal of Bacteriology* **171** 6135–6140.

Smit B A, Vlieg H, Van J E T, Engels W J M, Meijer L, Wouters J T M and Smit G (2005) Identification, cloning and characterization of a *Lactococcus lactis* branched-chain α-keto acid decarboxylase involved in flavor formation. *Applied and Environmental Microbiology* **71** 303–311.

Tanous C, Gori A, Rijnen L, Chambellon E and Yvon M (2005) Pathways for α-ketoglutarate formation by *Lactococcus lactis* and their role in amino acid catabolism. *International Dairy Journal* **15** 759–770.

Tanous C, Kieronczyk A, Helinck S, Chambellon E and Yvon M (2002) Glutamate dehydrogenase activity: a major criterion for the selection of flavour-producing lactic acid bacteria strains. *Antoni Van Leeuwenhoek* **82** 271–278.

Thage B V, Houlberg U and Ardö Y (2004) Amino acid transamination in permeabilised cells of *Lactobacillus helveticus, Lb. paracasei* and *Lb. danicus*. *Journal of Dairy Research* **71** 461–470.

Van Leuven I, Van Caelenberg T and Dirinck P (2008) Aroma characterisation of Gouda-type cheeses. *International Dairy Journal* **18** 790–800.

Weimer B, Dias B, Ummadi M, Broadbent J, Brennand C, Jaegi J, Johnson M, Milani F, Steel J and Sisson D (1997) Influence of NaCl and pH on intracellular enzymes that influence Cheddar cheese ripening. *Le Lait* **77** 383–398.

Yvon M, Berthelot S and Gripon J C (1998) Adding α-ketoglutarate to semi-hard cheese curd highly enhances the conversion of amino acids to aroma compounds. *International Dairy Journal* **8** 889–898.

Yvon M, Chambellon E, Bolotin A and Roudot-Algaron F (2000) Characterization and role of the branched-chain aminotransferase (BcaT) isolated from *Lactococcus lactis* subsp. *cremoris* NCDO 763. *Applied Environmental Microbiology* **66** 571–577.

Yvon M, Thirouin S, Rijnen L, Fromentier D and Gripon J C (1997) An aminotransferase from *Lactococcus lactis* initiates conversion of amino acids to cheese flavor compounds. *Applied and Environmental Microbiology* **63** 414–419.

Zuljan F A, Repizo G D, Alarcon S H and Magni C (2014) α-Acetolactate synthase of *Lactococcus lactis* contributes to pH homeostasis in acid stress conditions. *International Journal of Food Microbiology* **188** 99–107.

