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ORIGINAL PAPER

Influence of low pH on the metabolic activity of *Lactobacillus buchneri* and *Lactobacillus parabuchneri* strains in Tilsit-type model cheese

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Abstract Round-shaped and uniformly distributed eyes are important quality features for several Swiss semi-hard cheese varieties such as Tilsit. Recently, the growth of histamine-producing strains of *Lactobacillus parabuchneri* has been associated with cheese defects, such as crack formation and burning taste. In this paper, the influence of pH on the metabolic activity of added strains of *Lactobacillus buchneri* and *L. parabuchneri*, which possess various CO_2-producing activities, was studied in Tilsit-type model cheeses. Two different pH values were obtained by modifying the cheese-making process (curd washing and type of ripening). Lactate, free amino acids, free short-chain fatty acids and 1,2-propanediol were determined in the ripened cheeses. In the acidic cheeses (average pH= 5.40), significantly more 1,2-propanediol was produced, presumably from lactate. *Lactobacillus parabuchneri* FAM 21731, a histamine-producing strain, produced small amounts of 1,2-propanediol (0.2 mmol.kg^{-1}) and high amounts of histamine (3.3 mmol.kg^{-1}). Ornithine was produced by all the studied strains, with the highest amount of 9.0 mmol.kg^{-1} produced by *L. parabuchneri* FAM 21835 in the acidic cheeses. Standard cheese making representing the high pH group (curd washed and smear ripened, average pH=5.70), and the addition of a glutamate decarboxylase-positive *L. buchneri*, resulted in higher amounts of γ-aminobutyric acid (GABA) (8.1 compared to 3.3 mmol.kg^{-1} in the control standard cheeses). Irrespective of the strain, the GABA level was much higher in all the acidic cheeses than in the standard cheeses (14.7

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compared to 4.6 mmol.kg^{-1}, respectively). The study clearly demonstrates the importance of the cheese pH in the metabolic activity of the added strains during cheese ripening.

Keywords Cheese · Obligately heterofermentative lactobacilli · Adjunct culture · Carbohydrate catabolism

1 Introduction

The number, size, roundness and uniform distribution of eyes are important quality features for several Swiss semi-hard cheese varieties, such as Appenzeller and Tilsit. If eye formation is unsatisfactory, the cheese is downgraded. The production of CO_2 by non-starter lactic acid bacteria (NSLAB) is an important contributor to eye formation. The NSLAB in cheese mainly consist of mesophilic lactobacilli. Based on their carbohydrate catabolism, they are subdivided into obligate homofermenters, facultative heterofermenters and obligate heterofermenters. Typically, facultatively heterofermentative lactobacilli predominate. Obligately heterofermentative lactobacilli (OHL) may also be present, especially during the later stages of cheese ripening. According to Coton et al. (2008), *Lactobacillus brevis*, *Lactobacillus fermentum* and *Lactobacillus parabuchneri* are the major OHL present in cheese. Park and Oh (2006) isolated various strains of *Lactobacillus buchneri*, another OHL, from naturally aged cheese.

Lactobacillus parabuchneri and *L. buchneri* are of interest because they possess various catabolic activities producing CO_2. First, hexoses are degraded by the phosphoketolase pathway, yielding lactate, ethanol/acetate and CO_2. Second, both species possess the arginine deiminase (ADI, EC 3.5.3.6) pathway by which arginine is converted into ornithine, with the concomitant release of CO_2 and ammonia (Manca de Nadra et al. 1988). Histidine decarboxylase (HDC, EC 4.1.1.22) and glutamate decarboxylase (GAD, EC 4.1.1.15) activity was detected in some strains of *L. parabuchneri* and *L. buchneri*, respectively (Fröhlich-Wyder et al. 2013; Cho et al. 2007; Sumner et al. 1985). HDC removes the carboxyl group from histidine to yield histamine, and GAD removes the carboxyl group from glutamate to yield γ-aminobutyric acid (GABA). Finally, both species produce 1,2-propanediol and acetic acid from lactate, which is probably linked to the formation of CO_2 (Oude Elferink et al. 2001).

During cheese making, lactose is rapidly metabolised by the starter culture to lactate (McSweeney 2011), so carbohydrate catabolism by OHL does not essentially contribute to CO_2 formation in cheese; other substrates must be available. During cheese ripening, free amino acids accumulate due to the proteolysis of caseins. Both lactate and amino acids are potential substrates for gas formation in *L. parabuchneri* and *L. buchneri*. In a previous study, when used as adjuncts for cheese making, we observed that both species metabolised arginine to ornithine and produced 1,2-propanediol (Fröhlich-Wyder et al. 2013). Furthermore, HDC-positive *L. parabuchneri* and GAD-positive *L. buchneri* produced histamine and GABA, respectively, in cheese. That study clearly showed that these CO_2-producing pathways from *L. parabuchneri* and *L. buchneri* were active in a cheese environment.

Both the ADI pathway and the formation of 1,2-propanediol were reported to be regulated by environmental factors. Manca de Nadra et al. (1986) demonstrated that arginine stimulated the enzymes of the ADI pathway in *L. buchneri* NCDO 110. Other studies confirmed that low pH activated the production of 1,2-propanediol (Oude Elferink et al. 2001) and that the formation of biogenic amines, such as histamine and GABA, depended on external factors, including the pH, salt concentration and temperature (Linares et al. 2011).

In this work, we studied the influence of pH on gas production by a thermophilic starter culture and an adjunct culture (*L. parabuchneri* and *L. buchneri*) in Tilsit-type model cheeses. Furthermore, we investigated the metabolic pathways involved in the formation of CO_2 in *L. buchneri* and *L. parabuchneri* during cheese ripening.

2 Materials and methods

2.1 Adjunct cultures and physiological testing

The adjuncts used in this study are listed in Table 1. They were cultured in MRS broth at 30 °C (de Man et al. 1960). To assess the formation of GABA, the adjunct cultures were grown in MRS medium, supplemented with 1 % monosodium L-glutamate (pH 6.2). For the assessment of the formation of histamine, the adjunct cultures were grown in MRS broth, supplemented with 0.2 % L-histidine (pH 6.2). To detect the formation of ornithine from L-arginine, the adjunct cultures were grown in MAM medium (pH 5.8). To assess the formation of 1,2-propanediol, the adjunct cultures were cultivated in MRS-MOD medium (modified MRS medium, pH 3.8), as described by Oude Elferink et al. (2001). The formation of GABA, histamine, ornithine and 1,2-propanediol was verified by high-performance thin-layer chromatography on cellulose plates (Merck, Darmstadt, Germany) by comparing their migration distances to those of standard compounds. The methods used have been previously described in detail (Fröhlich-Wyder et al. 2013).

Table 1 Adjunct cultures used in this study and their biochemical characterisation

Adjuncts	D-Xyl[a]	1,2-PD	HIST	GABA	Orn	Source or reference
L. buchneri FAM 22050	+	+	−	+	+	Isolated strain from the silage inoculant Lalsil Fresh LB (Lallemand S. A., Saint Simon, France) with the active ingredient *L. buchneri* NCIMB 40788
L. parabuchneri FAM 21731	−	+	+	−	+	Agroscope culture collection, isolated from a Swiss Emmental cheese
L. parabuchneri FAM 21835	−	+	−	−	+	Agroscope culture collection, isolated from a Swiss Tilsit cheese

1,2-PD 1,2-propanediol, *HIST* histamine, *GABA* γ-aminobutyric acid, *Orn* ornithine

[a] Production of acid from D-xylose

2.2 Cheese making

To produce different pH conditions, half the cheeses were produced and ripened according to the standard protocol, including curd washing and smear ripening (standard cheeses). For the other half, the curd washing step was omitted, and the cheeses were ripened in a plastic film (acidic cheeses). The Tilsit-type model cheeses of 30 cm in diameter were manufactured in a pilot plant of the Agroscope Research Station at Liebefeld, comprising eight vats. Four standard and four acidic cheeses were produced each from 70 L of the same batch of pasteurised full-fat (37 $g.kg^{-1}$) cow's milk. To each vat containing the 70 L of milk, 5 L of water and 0.2 % starter culture MK 401 (Agroscope, Liebefeld, Switzerland) containing various strains of *Lactobacillus delbrueckii* subsp. *lactis*, *Streptococcus thermophilus* and *Lactococcus lactis* subsp. *lactis* and 0.01 % adjunct culture (strain *L. buchneri* FAM 22050, *L. parabuchneri* FAM 21731 or *L. parabuchneri* FAM 21835 pre-cultured in MRS medium) were added. The milk was then pre-ripened at 31–32 °C for 15 min. The standard and acidic control cheeses were made without the addition of any adjunct culture. For coagulation, 10 mL of rennet (Winkler GR orange, Winkler AG, Konolfingen, Switzerland) was diluted in 1 L of water and added to the milk, which was then incubated at 32 °C for 35 min. According to the manufacturer's instructions, one part of rennet Winkler GR orange clots 9000 parts of non-heated full-fat cow's milk (pH 6.65 at 32 °C) within 30 min, which is equivalent to 194 $IMCU.mL^{-1}$. The coagulum was cut into cubes of about 10 mm using knives with vertical wires. Thereafter, for the standard cheeses, 20 L of water was added to the curd–whey mixture. This step was omitted in the acidic cheeses. The curd–whey mixture was then cooked at 44 °C for 20 min, followed by a final stirring (43 °C, 20 min). To remove the whey, the curds were transferred into perforated moulds and pressed for 7.5 h. The cheeses were then immersed in brine solution for 16 h at 11–13 °C. The four standard cheeses were finally smear ripened at 14–15 °C and 90–96% relative humidity for 90 days. During the first 10 days of ripening, the cheeses were smeared daily with the brine solution, which had previously been inoculated with a mixture of *Brevibacterium linens*, *Arthrobacter* ssp. and *Debaryomyces hansenii* (OMK 702, Agroscope, Liebefeld, Switzerland). Thereafter, the brine solution was applied twice a week. The four acidic cheeses were wrapped under vacuum (−980 mbar) in a PA/PE 20/70 plastic film (CO_2 permeability at 23 °C and 75% humidity 146 $mL.(m^2.day.bar)^{-1}$; Inauen Maschinen AG, Herisau, Switzerland) and ripened, together with the standard cheeses, at a temperature of 14–15 °C. The experiment was replicated on the second day (N=2 runs), yielding a total of 16 cheeses. The design is shown in Table 2.

2.3 Cheese sampling

Cheese samples were taken aseptically from cheeses after 1 and 90 days of ripening with a cheese trier. The rind (thickness, 5 mm) was discarded, and the remaining cheese sample was ground and mixed.

Table 2 Experimental design of the Tilsit-type model cheese production (Standard pH level includes curd washing and smear ripening; acidic pH level includes no curd washing and film ripening)

Cheese no.	Vat	Day	Adjunct	pH level
1+14	1+6	1+2	–	Acidic
2+13	2+5	1+2	–	Standard
3+16	3+8	1+2	*L. buchneri* FAM 21731	Acidic
4+15	4+7	1+2	*L. buchneri* FAM 21731	Standard
5+10	5+2	1+2	*L. parabuchneri* FAM 21835	Acidic
6+9	6+1	1+2	*L. parabuchneri* FAM 21835	Standard
7+12	7+4	1+2	*L. buchneri* FAM 22050	Acidic
8+11	8+3	1+2	*L. buchneri* FAM 22050	Standard

2.4 Microbiological analyses

To enumerate OHL, the OH medium described by Isolini et al. (1990) and the method described by Fröhlich-Wyder et al. (2013) were used.

2.5 Chemical and biochemical analyses

The pH value of the cheeses was determined with a Metrohm pH-metre 605 equipped with a spear-tip pH electrode (Metrohm AG, Herisau, Switzerland). Free amino acids and histamine were determined with a high-performance liquid chromatograph (HPLC). A gas chromatograph was used to analyse the free short-chain fatty acids and the amount of 1,2-propanediol. These methods were described in detail by Fröhlich-Wyder et al. (2013).

L-, D-lactate and citrate were quantified enzymatically according to the instruction protocol on the kit (R-Biopharm, Darmstadt, Germany).

2.6 Sensory analysis

The sensory analysis was performed by a panel of seven experts according to the Agroscope standard protocol. The procedure was described by Guggisberg et al. (2013).

2.7 Statistical analysis

A factorial design with two factors (adjunct and pH level; treated as categorical variables) on four levels (one control and three adjuncts from Table 1) and two levels (standard pH and acidic pH) was applied (Table 2). The experiment was replicated on the second day (N=2 runs, treated as random categorical variable). Statistical analysis of the instrumental data was carried out using the method of analysis of variance with the general linear model (GLM). The software used was SYSTAT 13 (Systat Software, Inc., Chicago IL, USA). Significant differences between the various levels within a factor were accepted at $P \leq 0.05$.

The analysis of covariance was performed with GLM, and the results are given as squared multiple R (coefficient of determination). It explains how well the model fits the data.

3 Results and discussion

3.1 Biochemical properties of the adjunct cultures

The results of the physiological testing of the three adjunct cultures used in this study are listed in Table 1. All the selected strains showed the capacity to produce 1,2-propanediol and ornithine. One of the two strains of *L. parabuchneri* additionally produced histamine (FAM 21731), whereas the selected strain of *L. buchneri* (FAM 22050) isolated from Lalsil fresh LB produced GABA.

3.2 pH values in the cheeses

As expected, the different cheese-making steps in the group of standard cheeses (curd washing and smear ripening) and group of acidic cheeses (no curd washing and ripening in a plastic film) led to significant differences in the pH values and lactate content (Table 3). The lack of a curd washing step had a considerable effect on the amount of lactate in the fresh cheese. As the lactose was not diluted, the lactate fermentation was much more intensive. The ripening in the plastic film and, thus, the absence of lactate-degrading surface flora maintained the pH of the acidic cheeses at a low level during ripening. These cheeses contained about twice as much lactate as that of the standard cheeses at 90 days of ripening. Accordingly, the mean pH value of the acidic cheeses was significantly lower than that of the standard cheeses throughout the whole ripening period (5.40 and 5.70, respectively, on day 90) (Table 3).

There was a noticeable difference in the lactate content between the acidic cheeses on day 1 and day 90. In contrast to expectations, the acidic cheeses contained more lactate on day 90 than on day 1. This finding was likely due to the loss of water during the brine salting and ripening (Table 3). Additionally, as the lactose was not diluted in the vat, the LAB of the starter probably could not degrade all the galactose before brining and thus continued to produce lactic acid during the first days of ripening.

3.3 Fermentation of lactate to 1,2-propanediol

The amount of 1,2-propanediol measured in the standard cheeses was very low (0.29 mmol.kg^{-1}) and comparable to the concentration previously found in other cheeses made with the same adjuncts (Fröhlich-Wyder et al. 2013). However, in the acidic cheeses, the degradation of lactate to 1,2-propanediol was significantly higher (3.12 mmol.kg^{-1}; $P \leq 0.001$). Remarkable differences were observed between the three adjuncts (Table 4): Although *L. buchneri* FAM 22050 and, particularly, *L. parabuchneri* FAM 21835 produced high amounts of 1,2-propanediol in the acidic cheeses, *L. parabuchneri* FAM 21731 did not (Fig. 1). Given the low amount of 1,2-

Table 3 Content of water, lactate, share of L(+)-lactate and pH value in Tilsit-type model cheeses grouped according to their pH level

Factor (N=8)	Level	Cheeses 1 day				Cheeses 90 days			
		Lactate (mmol.kg^{-1})	% L(+)-lactate	pH	Water (mg.kg^{-1})	Lactate (mmol.kg^{-1})	% L(+)-lactate	pH	Water (mg.kg^{-1})
Cheese pH	Standard[a]	137.2	53.4	5.08	448.3	88.0	50.4	5.70	398.8
	Acidic[b]	151.7	53.9	4.97	434.4	178.1	48.8	5.40	399.2
GLM (*P*-value)	Cheese pH	**	n.s.	***	***	***	**	***	n.s.
	Day/Run	n.s.	**	n.s.	**	n.s.	n.s.	n.s.	***

n.s. not significant

**$P \leq 0.01$; ***$P \leq 0.001$

[a] Standard = curd washed smear ripened Tilsit-type model cheeses

[b] Acidic = film ripened Tilsit-type model cheeses produced without curd washing

propanediol produced by *L. parabuchneri* FAM 21731 and assuming that the fermentation of lactate by *L. buchneri* and *L. parabuchneri* to 1,2-propanediol contributed to pH homeostasis, it appears that *L. parabuchneri* FAM 21731 used an alternative pathway to control acidic stress.

Acetic acid is an end product of various fermentative pathways, such as the fermentation of citrate by NSLAB or the fermentation of lactate by propionibacteria or clostridia. Moreover, acetic acid is formed during the degradation of lactate to 1,2-propanediol by *L. buchneri* or *L. parabuchneri*. According to Oude Elferink et al. (2001), 1,2-propanediol and acetic acid are produced in equimolar amounts. Figure 2 shows that there was a high correlation in the acidic cheeses between the content of acetic acid and 1,2-propanediol (r=0.994, with P<0.001). Therefore, most of the acetic acid likely originated from the degradation of lactate by *L. buchneri* or *L. parabuchneri*. However, it can be concluded from Fig. 2 that approximately 4–5 mmol of the acetic acid in the acidic cheeses seem to originate from metabolic pathways other than lactate degradation. Based on the analysis of the volatile fatty acids, the fermentation of citrate by NSLAB or lactate by propionibacteria or clostridia was not responsible for the additional acetic acid (data not shown). In the standard cheeses, the correlation between the acetic acid and 1,2-propanediol content was weak but still significant (r=0.793, with P=0.019; Fig. 2). In these cheeses, up to 14 mmol of the acetic acid could not be attributed to the formation of 1,2-propanediol by NSLAB nor to the metabolic activity of propionibacteria and clostridia (data not shown). Possible explanations for the excess acetic acid could be the degradation of selected amino acids (Yvon et al. 2011; Skeie et al. 2008).

1,2-Propanediol has a slightly sweet taste. The measured amounts of 1,2-propandiol were all well below the taste threshold of 13.9 mmol.L^{-1}, indicated by Hufnagel and Hofmann (2008) for wine. All the same, there was a significant, negative correlation between the sensorial attributes ‘bitterness’ and ‘sweetness’ in the model Tilsit-type cheeses (r=−0.809; P<0.001; Table 5).

Table 4 Content of lactate, 1,2-propanediol (1,2-PD), acetic acid (C2), soluble nitrogen at pH 4.6 (SN) and non-protein nitrogen (NPN), as well as the pH value of the Tilsit-type model cheeses produced with various adjuncts after 90 days of ripening. No adjuncts were added to the control cheese

Factor	Level	lactate ($mmol.kg^{-1}$)	1,2-PD ($mmol.kg^{-1}$)	C2 ($mmol.kg^{-1}$)	SN ($g.kg^{-1}$)	NPN ($g.kg^{-1}$)	pH
Adjunct (N=4)	Control	139.8	0.04	5.2	9.2	6.0	5.44
	Lpbu FAM 21731	133.4	0.22	5.5	9.7	6.1	5.65
	Lpbu FAM 21835	125.3	3.96	11.9	9.8	6.2	5.58
	Lbu FAM 22050	133.8	2.60	7.7	9.5	6.0	5.56
Cheese pH (N=8)	Standard[a]	88.0	0.29	7.4	10.9	6.5	5.70
	Acidic[b]	178.1	3.12	7.7	8.2	5.6	5.40
GLM (*P*-value)	Adjunct	*	***	***	*	n.s.	***
	Cheese pH	***	***	n.s.	***	***	***
	Day/Run	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.

Lpbu Lactobacillus parabuchneri, *Lbu Lactobacillus buchneri*, *n.s.* not significant

$*P \leq 0.05$; $***P \leq 0.001$

[a] Standard = curd washed smear ripened Tilsit-type model cheeses

[b] Acidic = film ripened Tilsit-type model cheeses produced without curd washing

3.4 Fermentation of arginine to ornithine

As previously shown by Fröhlich-Wyder et al. (2013), all the investigated strains had strong ADI activity. Independently of the pH value, almost all the

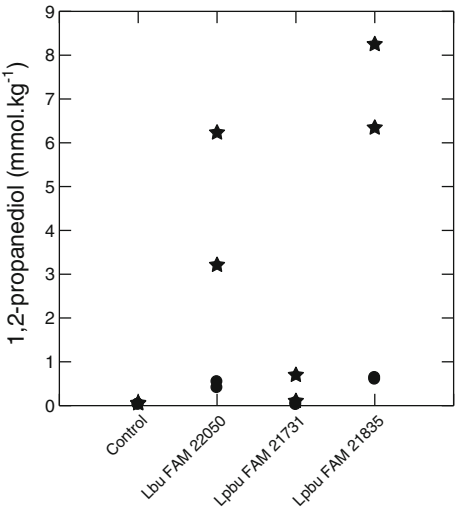

Fig. 1 Content of 1,2-propanediol in the 90-day aged Tilsit-type model standard cheeses (*black circle*) and acidic cheeses (*black star*). Both groups of cheeses included cheeses without adjuncts (control) and with adjuncts of *Lactobacillus parabuchneri* FAM 21731 (Lpbu FAM 21731), *L. parabuchneri* FAM 21835 (Lpbu FAM 21835) or *L. buchneri* FAM 22050 (Lbu FAM 22050)

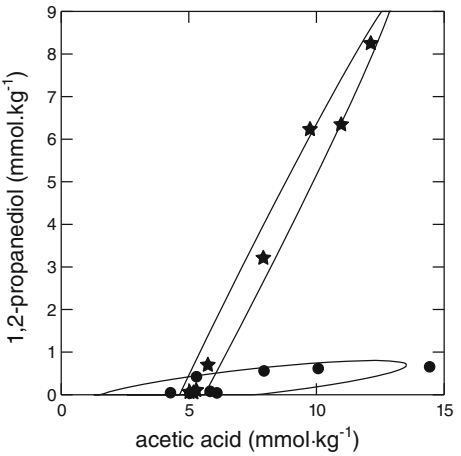

Fig. 2 Relationship between the content of 1,2-propanediol and acetic acid in 90-day aged Tilsit-type model standard cheeses (*black circle*) and acidic cheeses (*black star*). Both groups of cheeses included cheeses without adjuncts (control) and with adjuncts of *Lactobacillus parabuchneri* FAM 21731, *L. parabuchneri* FAM 21835 or *L. buchneri* FAM 22050

available arginine was metabolised to ornithine, an indicator of an active ADI pathway (Table 6). In addition, in the control cheeses, elevated concentrations of ornithine (4.01 mmol.kg^{-1}, on average) were observed. This was probably due to the lactobacilli present in the starter culture using the same pathway.

Table 5 Sensory evaluation of the Tilsit-type model cheeses produced with various adjuncts after 90 days of ripening. No adjuncts were added to the control cheese. The score given was from one for the lowest to five for the highest intensity of the assessed attribute

Factor	Level	bitter	sour	sweet	salty	Flavour quality	Texture elasticity	Eye quantity	Eye quality
Adjunct (N=4)	Control	1.75	2.93	1.89	2.36	3.29	3.25	2.79	2.64
	Lpbu FAM 21731	2.36	3.00	1.50	2.43	1.79	3.25	3.60	2.64
	Lpbu FAM 21835	1.89	3.00	2.00	2.39	3.11	3.32	2.97	2.64
	Lbu FAM 22050	2.04	2.93	1.82	2.36	3.36	3.43	3.57	2.64
Cheese pH (N=8)	Standard[a]	2.07	2.56	1.80	2.59	3.27	4.45	2.71	4.11
	Acidic[b]	1.95	3.38	1.80	2.18	2.50	2.18	3.77	1.18
GLM (*P*-value)	Adjunct	*	n.s.	*	n.s.	***	n.s.	*	n.s.
	Cheese pH	n.s.	***	n.s.	***	***	***	***	***
	Day/Run	n.s.	**	n.s.	n.s.	*	n.s.	n.s.	n.s.

Lpbu Lactobacillus parabuchneri, *Lbu Lactobacillus buchneri*, *n.s.* not significant

*$P \leq 0.05$; **$P \leq 0.01$; ***$P \leq 0.001$

[a] Standard = curd washed smear ripened Tilsit-type model cheeses

[b] Acidic = film ripened Tilsit-type model cheeses produced without curd washing

The enzymes involved in the ADI pathway appear to be inherently acid tolerant, displaying activity at pH 3.1, and even lower in some species (Cotter and Hill 2003). In acidic environments, arginine consumption leads to an increase in pH because ammonia is produced by the ADI pathway (Araque et al. 2013). In the present study, the low pH conditions of the acidic cheeses seemed to have only a slight influence on the formation of ornithine. However, there was one exception (Fig. 3): *L. parabuchneri* FAM 21835 produced significantly more ornithine in the acidic cheeses as compared to the standard cheeses (P=0.046). During lactate fermentation under acid stress, it also produced considerably more 1,2-propanediol than the other two adjuncts (Fig. 1). The aforementioned may be due to *L. parabuchneri* FAM 21835 neutralising the cell environment only by the metabolism of lactate and arginine. In contrast, the two other adjuncts possess additionally amino acid decarboxylation activities, enabling them to respond to acid conditions by the formation of biogenic amines and by proton uptake (De Angelis and Gobbetti 2004; Molenaar et al. 1993). Linares et al. (2012) suggested that the formation of biogenic amines by the decarboxylation of amino acids might be a system for neutralisation of low extracellular pH, thereby increasing survival under acidic stress conditions.

3.5 Formation of GABA

Lactobacillus buchneri FAM 22050 showed GAD activity during laboratory characterisation, showing that it has the ability to decarboxylase glutamic acid to GABA (Table 1). As expected, in the standard pH group, the cheeses with this adjunct

Table 6 Content of arginine (Arg), citrulline (Cit), ornithine (Orn), glutamic acid (Glu), γ-aminobutyric acid (GABA), histidine (His) and histamine (HIST) (mmol.kg−1) in Tilsit-type model cheeses produced with various adjuncts after 90 days of ripening. No adjuncts were added to the control cheese

Factor	Level	Arg	Cit	Orn	Glu	GABA	His	HIST
Adjunct (N=4)	Control	0.27	3.73	4.01	7.47	9.50	3.08	0.03
	Lpbu FAM 21731	0.05	0.10	7.67	6.92	10.17	0.21	3.28
	Lpbu FAM 21835	0.06	0.15	9.00	9.07	7.87	3.42	0.00
	Lbu FAM 22050	0.07	0.10	8.03	4.80	11.04	3.33	0.02
Cheese pH (N=8)	Standard[a]	0.12	1.02	6.74	11.78	4.62	3.18	0.91
	Acidic[b]	0.11	1.02	7.61	2.36	14.67	1.85	0.76
GLM (P-value)	Adjunct	***	***	***	n.s.	n.s.	***	***
	Cheese pH	n.s.	n.s.	**	***	***	**	n.s.
	Day/Run	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.

Lpbu Lactobacillus parabuchneri, *Lbu Lactobacillus buchneri*, *n.s.* not significant

**$P \leq 0.01$; ***$P \leq 0.001$

[a] Standard = curd washed smear ripened Tilsit-type model cheeses

[b] Acidic = film ripened Tilsit-type model cheeses produced without curd washing

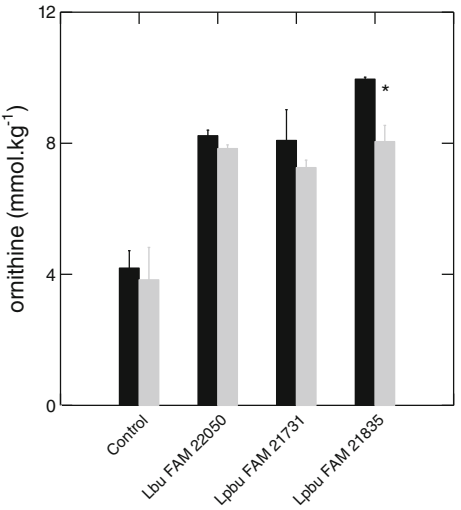

Fig. 3 Determination of ornithine in 90-day aged Tilsit-type model standard cheeses (*grey bar*) and acidic cheeses (*black bar*). Both groups of cheeses included cheeses without adjuncts (control) and with adjuncts of *Lactobacillus parabuchneri* FAM 21731 (Lpbu FAM 21731), *L. parabuchneri* FAM 21835 (Lpbu FAM 21835) or *L. buchneri* FAM 22050 (Lbu FAM 22050). The *error bars* indicate the standard error (N=2). *$P \leq$ 0.05

contained more GABA than the cheeses with the other adjuncts and the control. In the acidic cheeses, the control and the cheeses made with the adjuncts had comparable amounts of GABA. The optimum pH for decarboxylases is around 5.0. As shown in Fig. 4, there was a very significant interaction ($P<0.001$) between the strain-specific capability of GABA formation and the pH level of

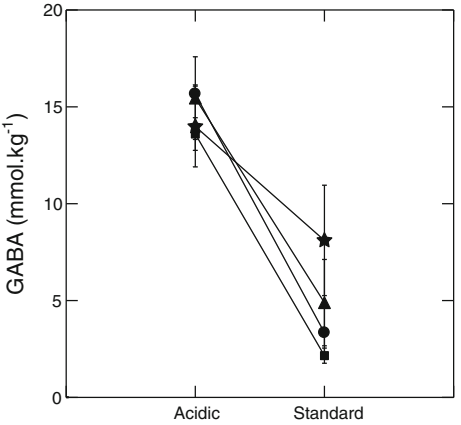

Fig. 4 Determination of γ-aminobutyric acid (GABA) in the standard and acidic Tilsit-type model cheeses. Both groups of cheeses included cheeses with adjuncts of *Lactobacillus buchneri* FAM 22050 (*black star*), *L. parabuchneri* FAM 21731 (*black triangle*), *L. parabuchneri* FAM 21835 (*black square*) and control cheeses without an adjunct (*black circle*). The error bars indicate the standard error (N=2).

the cheeses, revealing that the starter culture also possessed GAD activity. A previous study demonstrated that the same starter culture produced considerable amounts of GABA under similar acidic conditions (Bisig et al. 2014). Various species of LAB possess GAD activity, as demonstrated by several studies (e.g. Dhakal et al. 2012). It seems that the GAD activity of the applied starter was even more sensitive than *L. buchneri* FAM 22050 to acidic stress. A possible explanation could be that *L. buchneri* FAM 22050 is able to ferment lactate and arginine, both of which are also involved in its pH homeostasis (Cotter and Hill 2003). In contrast, the LAB present in the starter culture fermented only arginine, to some extent, but not lactate. Thus, the decarboxylation of glutamic acid was probably the main type of metabolism used by the starter culture to achieve pH homeostasis, whereas *L. buchneri* FAM 22050 combined several metabolic activities to do so (De Angelis and Gobbetti 2004).

3.6 Formation of histamine

Lactobacillus parabuchneri FAM 21731 showed HDC activity in laboratory analyses (Table 1) and converted histidine in cheese to histamine (Table 6). The decarboxylation of histidine is considered to contribute to pH homeostasis and the control of acidic stress (Linares et al. 2012; Molenaar et al. 1993). Moreover, acidic pH conditions induce structural changes in HDC that are necessary for its activity (Coton et al. 1998; Linares et al. 2011); HDC is inactive at alkaline to neutral pH. As expected, only *L. parabuchneri* FAM 21731 produced histamine in the cheeses. Histamine formation tends to be higher in standard cheeses than in the acidic ones (Table 6). This finding can be explained by the different availability of histidine in the standard and acidic cheeses. In the standard cheeses, the pH throughout the whole ripening period was closer to the pH optimum of most bacterial proteinases and peptidases, the latter being responsible for the release of free amino acids (Upadhyay et al. 2004). As a result, more intensive proteolysis was observed in the standard cheeses than in the acidic cheeses. The latter is the most likely explanation for the higher soluble nitrogen and non-protein nitrogen and thus the histidine content in the standard cheeses (Table 4).

In the studies of Bisig et al. (2014) and Fröhlich-Wyder et al. (2013), *L. parabuchneri* FAM 21731 played an important role in CO_2 production and in a strong burning taste detected in the cheeses. CO_2 originates from the decarboxylation of histidine, and the burning taste is associated with the inflammation of mucosa in the mouth by histamine (Bachmann et al. 2011).

3.7 Influence on pH in cheese

As shown in the previous sections, the different pH values in the two groups of Tilsit-type model cheeses had a strong effect on the metabolism of the investigated *L. buchneri* and *L. parabuchneri* strains responding to acidic stress. Previous studies showed that these pathways—fermentation of lactate and of arginine, decarboxylation of amino acids—contributed to the neutralisation of the surrounding matrix and thus, to some extent, influenced the development of

the pH during cheese ripening (Araque et al. 2013; Cotter and Hill 2003; De Angelis and Gobbetti 2004; Dhakal et al. 2012; Fröhlich-Wyder et al. 2013). In the present study, the increases in pH throughout the whole ripening time were dependent on the applied strains (Table 4 and Fig. 5). Compared to the control, the HDC-positive strain *L. parabuchneri* FAM 21731 had the most potent effect on pH, both in the acidic and standard cheeses, and was able to raise the pH by about 0.2 units. Although histamine production was less pronounced in the acidic cheeses (see section 3.6) in the present study, the results confirm the important role of histamine production in the neutralisation of the surrounding cheese body. The other two strains, *L. parabuchneri* FAM 21835 and *L. buchneri* FAM 22050, also had a significant alkalising effect, with FAM 22050 being the less potent. An analysis of covariance showed that the increase in pH could be explained by the degradation of lactate and the production of histamine and ornithine (R^2=0.966, with P<0.001).

3.8 CO_2 formation in the cheese

As reported elsewhere, the ADI metabolism, degradation of lactate to 1,2-propanediol and decarboxylation of amino acids by *L. buchneri* and *L. parabuchneri* all contribute to the production of CO_2 and thus influence eye formation and overall cheese quality (Fröhlich-Wyder et al. 2013; Bisig et al. 2014). In the present work, the lower pH value in the acidic cheeses promoted the degradation of lactate to 1,2-propanediol and of glutamic acid to GABA, both of which affected the production of CO_2 and eye formation. The production of CO_2 can be estimated by the quantity and types of metabolites generated in the abovementioned pathways. During the ADI metabolism, CO_2 and ornithine are released in equimolar amounts (Manca de Nadra et al. 1986). Lactate degradation results in equimolar amounts of 1,2-propanediol, acetic acid and CO_2 (Oude Elferink et al. 2001), and the decarboxylation of amino acids yields equimolar amounts of the corresponding amine and CO_2 (e.g. Park and Oh 2006). The data presented in Fig. 6 reveal that the amount of CO_2 produced in the acidic cheeses was twice as high as that produced in the standard cheeses.

In terms of cheese quality, the pH of the cheese plays an important role not only in the development of the texture and flavour during ripening but also in the solubility of CO_2. Figure 7a of the cross-sections of the standard cheeses shows that they exhibited mostly round-shaped eyes. It also shows that the Tilsit-type model cheeses with the addition of an adjunct culture tended to have more eyes than the control cheeses (nos. 2 and 13). In contrast to the standard cheeses, the acidic cheeses exhibited mainly splits and cracks and no round-shaped eyes (Fig. 7b). The pH of a cheese has an important influence on the texture of the cheese body and the solubility of CO_2 (Upadhyay et al. 2004; Jakobsen and Jensen 2009). A cheese with a low pH is associated with the formation of a brittle texture and reduced solubility of CO_2 in the cheese body. Therefore, it is not surprising that the eye formation in the acidic cheeses was defective. These observations reflect the results of the sensorial evaluation of the attributes eye quantity and quality (Table 5).

The sensorial evaluation of the cheeses primarily revealed that *L. parabuchneri* FAM 21731 resulted in an unpleasant burning taste in the mouth (Bachmann et al. 2011). The burning taste was mirrored in the high

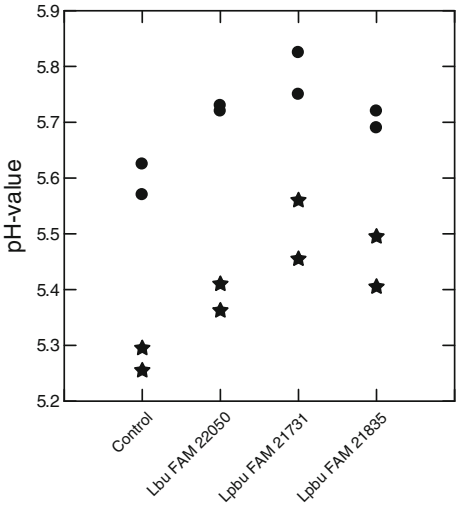

Fig. 5 Determination of the pH value in 90-day aged Tilsit-type model standard cheeses (*black circle*) and acidic cheeses (*black star*). Both groups of cheeses included cheeses without adjuncts (control) and with adjuncts of *Lactobacillus parabuchneri* FAM 21731 (Lpbu FAM 21731), *L. parabuchneri* FAM 21835 (Lpbu FAM 21835) or *L. buchneri* FAM 22050 (Lbu FAM 22050)

scores for the attribute 'bitter' as the closing discussion within the panel revealed. As expected, the acidic cheeses did not meet the quality expectations of the panellists: Compared to the standard cheeses, they were sour, less aromatic, less salty, had more flavour defects and were brittle in texture. Therefore, they were all downgraded (Table 5).

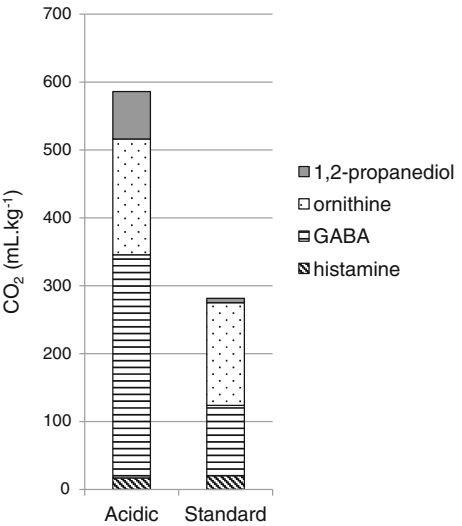

Fig. 6 Estimation of theoretically produced amounts of CO_2 ($mL.kg^{-1}$ under atmospheric pressure at room temperature) in the 90-day aged Tilsit-type model standard and acidic cheeses (N=8 each). The calculation of estimated CO_2 was made on the basis of the following metabolites: 1,2-propanediol, ornithine, γ-aminobutyric acid (GABA) and histamine

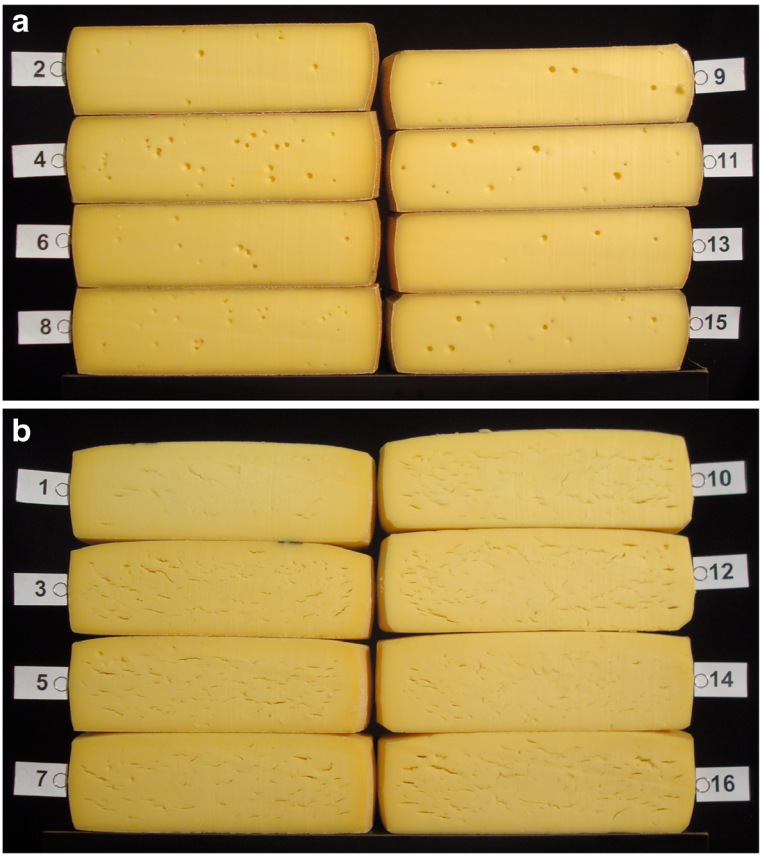

Fig. 7 Sectional view of the 90-day aged Tilsit-type model standard cheeses (**a**) and acidic cheeses (**b**). Both groups of cheeses included cheeses with adjuncts of *Lactobacillus parabuchneri* FAM 21731 (cheese nos. *3*, *4*, *15* and *16*), *L. parabuchneri* FAM 21835 (cheese nos. *5*, *6*, *9* and *10*) or *L. buchneri* FAM 22050 (cheese nos. *7*, *8*, *11* and *12*). The control cheeses (nos. *1*, *2*, *13* and *14*) were made without an adjunct

4 Conclusions

The process of cheese making involves a number of steps, such as heating, acidification and brine salting. These steps cause stress to microorganisms, inhibit their growth and can even lead to their inactivation. The bacterial stress response enables bacteria to survive adverse and fluctuating conditions in their respective habitats. Lactic acid fermentation leads to a drastic drop in the pH value of cheese and induces acidic stress on most microorganisms involved in cheese ripening. Additionally, the fast conversion of available carbohydrates into organic acids results in carbohydrate starvation. The microorganisms present in the starter, adjunct and surface cultures, the growth of NSLAB and the actions of indigenous milk and clotting enzymes all influence the complex biochemical process of cheese ripening. The degradation of arginine by the ADI pathway, decarboxylation of histidine or glutamate into the corresponding biogenic amine and degradation of lactate into 1,2-propandiol by *L. buchneri* and *L. parabuchneri* are important metabolic pathways in the response to acidic stress. The growth of these NSLAB in cheese accelerates the increase in pH and promotes the

production of CO_2 during cheese ripening, thereby affecting the texture, openness and flavour of cheese. Recently, the growth of histamine-producing strains of *L. parabuchneri* has been associated with serious cheese defects, such as crack formation and a burning taste. However, only a few studies have investigated the metabolic activity of these NSLAB in cheese so far. In the present study, the metabolic stress response varied considerably among the three investigated strains. For example, *L. parabuchneri* FAM 21731, a histamine-positive strain, produced only small amounts of 1,2-propanediol under acidic conditions, whereas *L. parabuchneri* FAM 21835, a histamine-negative strain, produced the highest amounts of 1,2-propanediol. This example illustrates that strain-specific properties play an important role in the response to acidic stress. Despite the differences in the individual mechanisms used, the results clearly showed that acidic conditions favoured the degradation of lactate and arginine. An estimation of the amount of CO_2 produced revealed that the ADI metabolism and the decarboxylation of glutamic acid were the most important sources of gas production. Although this study clearly demonstrated the importance of the cheese pH in the metabolic activity of strains of *L. buchneri* and *L. parabuchneri* during cheese ripening, further studies are needed to understand the role of these two species, which are frequently found in silage and therefore in milk, in the overall quality of cheese.

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