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The nature of aroma compounds produced in a cheese model by glutamate dehydrogenase positive *Lactobacillus* INF15D depends on its relative aminotransferase activities towards the different amino acids

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Abstract

Recently, we demonstrated that mesophilic lactobacilli exhibiting glutamate dehydrogenase (GDH) activity were capable of degrading aromatic amino acids (ArAAs) and branched-chain amino acids (BcAAs) in vitro to precursors of aroma compounds and to aroma compounds, when they were combined with *Lactococcus lactis*. Indeed, they produced via their GDH activity the α-ketoglutarate required for the amino acid transamination. In the present study, we compared the ability of two *Lactobacillus* strains, with and without GDH activity, to produce aroma compounds from amino acids in a cheese model. The GDH-positive *Lactobacillus* INF15D produced mainly diacetyl and acetoin from catabolism of aspartate (Asp). However, there were no aroma compounds produced from BcAAs, even when *Lactobacillus* was combined with *L. lactis*. In fact, *Lactobacillus* INF15D exhibited 5–10-fold higher aminotransferase (AT) activity towards Asp than towards BcAAs. We concluded that due to competition of ATs for the α-ketoglutarate produced by GDH, the aroma compounds produced in cheese depends on the relative AT activities towards Asp, BcAAs and ArAAs of the GDH-positive strain.

Keywords: Cheese flavour; Amino acid catabolism; Glutamate dehydrogenase; *Lactobacillus*

1. Introduction

Flavour formation in cheeses such as Cheddar or Gouda is often associated with the presence of non-starter lactic acid bacteria (NSLAB) such as *Lactobacillus plantarum, Lb. casei* and *Lb. paracasei* (Fox, McSweeney, & Lynch, 1998; Rehman et al., 2000). Therefore, strains of NSLAB have been used as flavour-producing adjuncts (Crow, Curry, & Hayes, 2001; Lynch, McSweeney, Fox, Cogan, & Drinan, 1996; Lynch, Muir, Banks, McSweeney, & Fox, 1999; Skeie, Lindberg, & Narvhus, 2001). However, the mechanisms of their contribution to cheese flavour formation have not been elucidated. It is not clear if the flavouring properties of the adjunct are due to *Lactobacillus* itself or if they result from an interaction with the starter lactococci (Muir, Banks, & Hunter, 1996). So far the choice of NSLAB as an adjunct has been empirical. It is based on cheese trials combined with sensory analyses, which are very time-consuming (Crow et al., 2001).

Amino acid catabolism is a major process in the formation of aroma compounds in Cheddar and Gouda-type cheese (Yvon & Rijnen, 2001). Glutamate dehydrogenase (GDH) activity appears to be a key activity in the amino acid catabolism by lactic acid bacteria (LAB) since it produces α-ketoglutarate, which is essential for amino acid transamination. It has previously been shown that the expression of a heterologous GDH gene in *Lactococcus lactis* considerably increased the amino acid conversion to aroma compounds in a cheese model (Rijnen, Courtin, Gripon, & Yvon, 2000). Moreover, LAB strains exhibiting natural GDH activity are capable of catabolising the amino acid precursors of aroma compounds in vitro (Tanous,

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Kieronczyk, Helinck, Chambellon, & Yvon, 2002). Therefore, the GDH activity was proposed as a major criterion for selecting aroma-producing LAB (Tanous et al., 2002).

Recently, it was demonstrated that two lactobacilli strains exhibiting GDH activity degraded phenylalanine (Phe), leucine (Leu) and methionine (Met) in a simple reaction medium containing one of these amino acids in addition to glutamate, but did not excrete α-ketoglutarate produced from glutamate (Kieronczyk, Skeie, Langsrud, & Yvon, 2003). These two strains mainly produced from Phe, Leu and Met α-keto and hydroxy acids, which are not aroma compounds. Interestingly, their combination with *L. lactis* induced a high production of carboxylic acids from the selected amino acids. This indicates that the α-keto and hydroxy acids produced by GDH-positive *Lactobacillus* strains were subsequently converted into aroma compounds by *L. lactis*.

The objective of the present study was to evaluate the ability of two *Lactobacillus* strains (one exhibiting GDH activity and one without GDH activity) to produce aroma compounds from amino acids in a cheese model, when they were used alone or in combination with *L. lactis*.

2. Materials and methods

2.1. Bacterial strains

L. lactis ssp. *cremoris* NCDO763 (*L. lactis* NCDO763) was obtained from the National Collection of Food Bacteria (Shinfield, Reading, England). *Lb. paracasei* 1244 (*Lb*. 1244) was obtained from the CNRZ culture collection (INRA, Jouy-en-Josas, France). *Lb. paracasei* ssp. *paracasei* INF15D (*Lb*. INF15D), isolated from Norvegia cheese was obtained from the culture collection of the Department of Food Science (Agricultural University of Norway, Aas, Norway). *Lactobacillus* INF15D has previously been shown to possess significant NADP-dependent glutamate dehydrogenase (GDH) activity and was selected for this study as a GDH-positive strain (GDH+), *Lactobacillus* 1244 was selected for this study as a GDH-negative strain (GDH−) (Kieronczyk et al., 2003).

2.2. Preparation of cell suspension

L. lactis NCDO763 was grown (2% inoculum) in 10% reconstituted skimmed milk (TINE Norske Meieri, Voll, Norway) buffered with 75 mM sodium β-glycerophosphate. *Lactobacillus* strains were grown (1% inoculum) in MRS broth (Difco Lab., Detroit, MI, USA). Cells of each culture, in late exponential growth phase, were harvested by centrifugation (4100*g*, 15 min, 4°C) and washed twice with 50 mM sodium β-glycerophosphate buffer (pH 7.5). For experiments in the cheese model system, the cells were suspended in the same buffer to an OD_{480} of 100 (cellular concentration of 10^{10} cells mL^{-1}), while for in vitro experiments the cells were suspended to an OD_{480} of 200.

2.3. Incubations in the cheese model system

Amino acid catabolism and formation of volatile compounds were studied under cheese ripening conditions using the cheese model (Ch-easy), produced commercially from young Gouda cheese (pH 5.6, water content 50–52%) (Smit, Braber, Van Spronsen, Van den Berg, & Exterkate, 1995). The trials were performed in sterile tubes containing 10 ± 0.25 g of cheese paste. In incubations made with single strains, *L. lactis* NCDO763 was added to the cheese paste at a cell concentration of $\sim 3.3 \times 10^9$ cfu g^{-1} and each single *Lactobacillus* strain was added at a cell concentration of $\sim 1.3 \times 10^9$ cfu g^{-1}. To study interactions between *L. lactis* NCDO763 and *Lactobacillus* strains, the cheese paste was inoculated with cells of *L. lactis* NCDO763 at a concentration of $\sim 3.3 \times 10^9$ cfu g^{-1} and cells of either *Lb*. INF15D (GDH+) or *Lb*. 1244 (GDH−) at concentrations of $\sim 1.3 \times 10^9$, $\sim 1.3 \times 10^7$ or $\sim 1.3 \times 10^3$ cfu g^{-1} of cheese paste (Table 1). The different quantities of *Lactobacillus* cells were used in order to mimic the population of lactobacilli found in cheese, e.g. adventitious lactobacilli growing from low numbers during cheese ripening (1.3×10^3 cfu g^{-1}) or lactobacilli used as adjuncts added at high cell concentrations during cheese making (1.3×10^9 and 1.3×10^7 cfu g^{-1}). The cheese paste mixture was homogenized and incubated for 6 weeks at 13°C. All Ch-easy model preparations were carried out under aseptic conditions. The analyses were performed at time zero and after 6 weeks of incubation. The Ch-easy experiments were repeated three times by using for each strain the cells harvested from three different cultures.

2.4. Analysis of free amino acids, organic acids and microbial counts of Ch-easy

The cheese paste (10 g) was blended with 30 mL of cold (4°C) sterile H_2O and homogenized with an Ultraturrax at 11 000 rpm twice for 45 s with 60 s cooling on ice between each run. One millilitre of the suspension was used immediately for bacterial counts. The remaining suspension was used for analysis of free amino acids, organic acids and volatile compounds. The cells of *L. lactis* NCDO763 were enumerated on M17 agar (Difco Lab., Detroit, MI, USA) supplemented with lactose after incubation for 24 h at 30°C. *Lactobacillus* strains were enumerated on MRS agar (Difco Lab., Detroit, MI, USA) acidified to pH 5.4, after incubation for 48 h

Table 1
Viable cell counts and free amino acid (FAA) production in Ch-easy inoculated with *L. lactis* ssp. *cremoris* NCDO763 (*L.* NCDO763), *Lb. paracasei* ssp. *paracasei* INF15D (*Lb.* INF15D), *Lb. paracasei* 1244 (*Lb.* 1244) or their combination

Strain(s)	Viable cells at the start of the experiment[a]		Viable cell count after 6 weeks		
	Lactococci[c] (cfu g^{-1})	Lactobacilli[d] (cfu g^{-1})	Lactococci[c] (cfu g^{-1})	Lactobacilli[d] (cfu g^{-1})	FAA production after 6 weeks (mmol kg^{-1})[b]
L. NCDO763	3.3×10^9		2.2×10^6		34.32
Lb. INF15D		1.3×10^9		4.3×10^8	4.00
Lb. 1244		1.3×10^9		6.0×10^7	5.42
L. NCDO763 + *Lb.* INF15D	3.3×10^9	1.3×10^9	ND	3.3×10^8	33.64
	3.3×10^9	1.3×10^7	ND	6.6×10^7	28.30
	3.3×10^9	1.3×10^3	ND	4.0×10^7	9.62
L. NCDO763 + *Lb.* 1244	3.3×10^9	1.3×10^9	ND	3.0×10^7	10.64
	3.3×10^9	1.3×10^7	ND	5.3×10^7	13.30
	3.3×10^9	1.3×10^3	ND	4.6×10^7	15.59

Results are the means from three independent experiments.
ND, not determined.
[a] Estimated by bacteria count in the cell suspension used.
[b] Calculated as the difference between FAA contents after 6 weeks of incubation and FAA content in Ch-easy before cell addition.
[c] Enumerated on M17 agar-added lactose.
[d] Enumerated on MRS agar (pH 5.4).

at 30°C. For Ch-easy prepared with a strain mixture, the cells were enumerated on both MRS and M17 media. The number of cells added to the cheese paste at the start of the experiments was estimated by counting bacteria in the cell suspension at an OD_{480} of 100.

Free amino acid contents were determined using a BIOTRONIK LC 3000 automatic analyser as previously described (Yvon, Berthelot, & Gripon, 1998). Organic acids were determined by HPLC analysis as previously described (Marsili, Ostapenko, Simmons, & Green, 1981; Narvhus, Østeraas, Mutukumira, & Abrahamsen, 1998).

2.5. Analysis of volatile compounds by dynamic headspace extraction and GC-MS

Volatile compounds were analysed as previously described (Berger, Khan, Molimard, Martin, & Spinnler, 1999) using a system composed of a 3100 Purge and Trap Concentrator (Tekmar and Dohrmann, Cincinnati, OH, USA) fitted with a sorbent trap (Tenax, 60–80 mesh, 0.25 g, 30 cm × 0.32 cm, Tekmar Inc., Cincinnati, OH, USA) and a cryofocusing module. The concentrator was coupled to a gas chromatograph (GC 6890+, Agilent Technologies Inc., Palo Alto, CA, USA) connected to a mass spectrometer detector (MS 5973N Agilent Technologies Inc., Palo Alto, CA, USA).

Twenty millilitres of the sample prepared as described above was placed in a 25 mL needle sparger tube and afterwards 2.5 μL of pure octanol was added to prevent foaming. The sample was purged with helium at a flow rate of 60 mL min^{-1} for 30 min at 37°C. The volatile compounds were trapped by adsorption to the Tenax trap maintained at 30°C. Water was then removed by flushing the trap with helium for 5 min (dry-purge). The volatile compounds were desorbed from the trap by heating at 225°C for 2 min and were transferred to the head of the capillary column, where they were condensed by cryofocusing at −150°C. They were then injected onto the non-polar capillary column (HP-5MS, 30.0 m × 0.25 mm, 0.25 μm film thickness), by heating the interface to 225°C for 0.75 min, with a helium pressure of 5 psi. The oven temperature was held at 5°C for 8 min and then increased from 5°C to 20°C at 3°C min^{-1}, followed by a gradient of 10°C min^{-1} up to 200°C and held at 200°C for 10 min. The column was directly connected to the mass-sensitive detector by an interface heated at 280°C. The electron impact energy was set at 70 eV and the data were collected in the range of 20–200 atomic mass units. Components were identified by their retention times and by comparison of their mass spectra with those in the NIST98 library. Specific mass ions (ion 43 for 2-methylpropanal, ion 44 for 3-methylbutanal, ion 57 for 2-methylbutanal) were used in quantification to eliminate potential inference with other peaks and thus to enhance the sensitivity of detection.

2.6. Analysis of volatile compounds by solid-phase microextraction (SPME) and GC-MS

Extraction of volatile compounds from the headspace phase was performed with a Multi-Purpose Sampler MPS2 (Gerstel, GmbH&Co., Mulheim an der Ruhr, Germany). Five millilitres of Ch-easy suspension was acidified to pH 2.0 with 1 mL of 1 N H_2SO_4. The sample

was preheated for 15 min at 60°C and an SPME fibre (Carboxen/PDMS 75 μm, Supelco, Bellfonte, PA, USA) was exposed for 30 min at 60°C in the headspace above the sample, which was agitated (500 rpm for 30 s with intervals of 10 s). After exposure, the SPME fibre was withdrawn from the sample and inserted into the injector of the gas chromatograph heated at 240°C. The compounds were separated onto an HP-INNO-WAX column (30.0 m × 0.25 mm, 0.5 μm film thickness, Agilent Technologies Inc., Palo Alto, CA, USA). Helium flow was maintained at 0.5 mL min^{-1} for 10 min, then it was increased in 2 min to 2.5 mL min^{-1} and kept constant for the rest of the time. The oven temperature was held at 40°C for 3 min, increased to 100°C at the rate of 5°C min^{-1} and then to 240°C at the rate of 10°C min^{-1}. The separated compounds were detected and identified as described above for the purge and trap method. Their concentration was calculated by extraction of specific mass ions: 74 for propionic acid, 43 for butanoic acid, 43 for 2-methylpropanoic acid, 60 for 3-methylbutanoic acid, 88 for acetoin and 86 for diacetyl. The concentrations of carboxylic acid were expressed as peak areas. Quantitative analyses of acetoin and diacetyl were made by external standard calibration. Samples were analysed in duplicate.

2.7. Amino acid catabolism in vitro

Amino acid catabolism by *Lb*. INF15D was studied as previously described (Rijnen, Bonneau, & Yvon, 1999; Yvon, Thirouin, Rijnen, Fromentier, & Gripon, 1997), by incubating the resting cells in different reaction media containing either L-[4,5-^{3}H]Leu or L-[2,3-^{3}H]Aspartic acid. Reaction medium I consisted of 70 mM potassium phosphate buffer (pH 5.5), 0.05 μM of radiolabelled amino acid, 3 mM of unlabelled Leu or Asp, 5 mM of Glu and 0.05 mM of pyridoxal phosphate. The reaction medium II consisted of the components of reaction medium I, except Glu and the unlabelled amino acid, which were replaced by an amino acid mixture similar to the composition of free amino acids (FAAs) in Ch-easy at the start of the experiment. The amino acid concentrations (mmol L^{-1}) were as follows: Asp 0.44, Thr 0.55, Ser 0.54; Asn 1.38; Glu 3.43, Gln 0.46, Pro 0.71, Gly, 0.59, Ala 0.65; Val 1.03; Met 0.40; Ile 0.52; Leu 2.19; Tyr 0.34; Phe 1.05; His 0.21; Trp 0.41; Lys 1.20 and Arg 0.11. Fifty microlitres of the cell suspension was added to the different reaction media and incubated at 37°C for 40 h. Aliquots of the reaction media were taken after 0 and 40 h and the cells were removed by centrifugation (8000*g*, 5 min). Samples containing radiolabelled Leu or Asp were then analysed by ion-exclusion HPLC, with both UV and radioactivity detection as described previously with minor modification (IC Pack ion-exclusion column, kept at 60°C, with 0.1% H_3PO_4 as eluent, at flow 0.8 mL min^{-1} for elution of metabolites of Asp and 1.0 mL min^{-1} for elution of metabolites of Leu) (Yvon et al., 1998). The quantitative analysis was made by calculating the percentage of total radioactivity of the sample associated with each peak of metabolites. The total radioactivity of the sample was determined under similar HPLC conditions, but without column separation. Identification was made by comparison of the retention times with those of appropriate standard compounds as previously described (Rijnen et al., 2000; Yvon et al., 1997). The standard components were obtained from Sigma Chemical Co. (St. Louis, MO, USA.).

2.8. Aminotransferase (AT) activity assay

AT activities towards Ile, Leu, Val, Phe and Asp in cell extracts of experimental strains were determined as previously described (Rijnen et al., 1999). In this test, formation of glutamate was measured with an automatic amino acid analyser after incubation of the cell extract for 15 min at 37°C in the reaction mixture containing 70 mM Tris buffer (pH 8.0), 0.05 mM pyridoxal phosphate, 10 mM α-ketoglutarate (sodium salt) and the amino acid substrate (3 mM). The data reported are the means of at least two replicate assays.

2.9. Statistical analysis

Significant influences from *Lactococcus, Lactobacillus* and the inoculation level of *Lactobacillus* and their interactions on the volatile compounds and FAAs were examined by analysis of variance (ANOVA) and Tukey's studentized range test (Snedecor & Cochran, 1989) using the SAS/Stat 8.01 (proc GLM) package (SAS Institute Inc., Cary, NC, USA). The differences between time zero and 6 weeks of incubations were used for the statistical analysis. The significance of the following treatments were tested A; addition of *L. lactis* NCDO763 at two levels: 0 (no addition) or 1 (addition of 3.3×10^9 cfu g^{-1}), treatment B: addition of *Lactobacillus* at three levels: 0 (no addition), 1 (*Lb*. INF15D) or 2 (*Lb*. 1244), treatment C: inoculation level of *Lactobacillus*; 0 (no addition), 1 (1.3×10^3 cfu g^{-1}), 2 (1.3×10^7 cfu g^{-1}) and 3 (1.3×10^9 cfu g^{-1}). The interaction between treatments A and B and between B and C were also tested.

3. Results

3.1. The viable cell counts and production of free amino acids and organic acids in Ch-easy

The viable cell counts and free amino acid (FAA) production are shown in Table 1. In Ch-easy inoculated with 3.3×10^9 cfu g^{-1} of a single strain of *L. lactis*

NCDO763, the number of cells decreased to 2.2×10^6 cfu g^{-1} after 6 weeks of incubation. The survival of the *Lactococcus* strain in the samples inoculated with a mixture of *Lactococcus* and *Lactobacillus* was not determined since both grew on M17 agar. The number of lactobacilli added to the cheese paste at a high concentration (1.3×10^9 cfu g^{-1}) decreased, by about one log for *Lb*. INF15D and two log for *Lb*. 1244. However, in samples inoculated at a level of 1.3×10^7 cfu g^{-1} of lactobacilli, their final number after 6 weeks remained at about the same level. In contrast, lactobacilli strains inoculated at a concentration of 1.3×10^3 cfu g^{-1} increased to numbers of approximately 4×10^7 cfu g^{-1} after 6 weeks of incubation. In this case, experimental conditions were similar to those found in cheese since after 6 weeks, the lactobacilli level reached the NSLAB level found in Gouda cheese after 4 weeks of ripening and because most of the lactobacilli cells were grown in cheese paste.

The concentration of FAAs in the Ch-easy paste at the beginning of the experiment, before addition of cells, was about 25 mmol kg^{-1}, but increased in all samples after addition of cells and incubation. However, *L. lactis* NCDO763 produced significantly ($p \leqslant 0.001$) more FAAs than lactobacilli, indicating that lactobacilli were weakly peptidolytic. Indeed, lactobacilli strains inoculated alone at a high level did not grow and therefore did not utilize FAAs for growth. The FAA content was generally lower in the Ch-easy samples inoculated with mixtures of strains, than in Ch-easy samples inoculated with *L. lactis* NCDO763 alone. However, the FAA content appeared to vary with the *Lactobacillus* strain (INF15D or 1244) and inoculation level, especially in the case of *Lb*. INF15D. These results suggest that *Lb*. INF15D utilized FAAs for growing when inoculated at a low level. We did not observe clear differences between Ch-easy samples in the relative concentrations of individual amino acids, except arginine concentration, which decreased in all samples inoculated with *L. lactis* NCDO763 and serine concentration, which decreased in all samples inoculated with *Lb*. INF15D (data not shown).

The analyses of organic acids showed that the concentration of lactic (140 mmol kg^{-1}) and citric (0.195 mmol kg^{-1}) acids did not change significantly ($p \leqslant 0.05$) in any of the cheeses during incubation. Moreover, α-ketoglutarate was neither detected at the beginning nor at the end of the experiment.

3.2. Production of aroma compounds in Ch-easy

The carboxylic acids derived from aromatic amino acids and Met were not detected in any cheese (data not shown). The 3-methylbutanoic acid derived from Leu was produced in similar quantities by *L. lactis* NCDO763, both *Lactobacillus* strains and by the mixtures of strains (data not shown). The 2-methylpropanoic acid derived from Val was mainly produced by *L. lactis* NCDO763 and the addition of lactobacilli cells at a high concentration decreased the quantity produced (Fig. 1A).

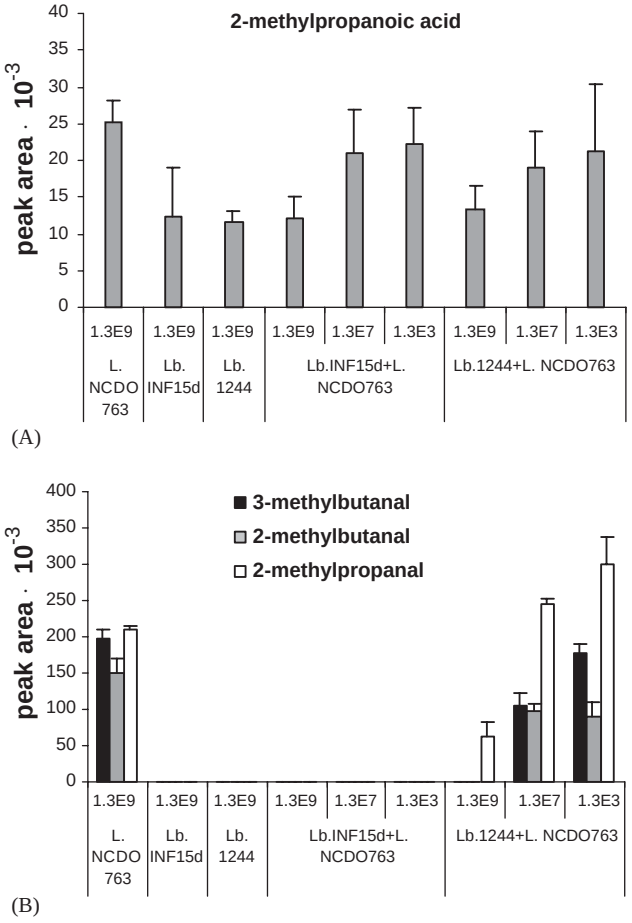

Fig. 1. Production of some metabolites from BcAAs by *L. lactis* ssp. *cremoris* NCDO763 (*L*. NCDO763), the GDH-positive strain *Lb. paracasei* ssp. *paracasei* INF15D, (*Lb*. INF15D), the GDH-negative strain *Lb. paracasei* 1244 (*Lb*. 1244), and their combinations in Ch-easy. Values noted in the *x*-axis indicate the inoculation level with cells of each strain (for mixtures of strains only the inoculation level with *Lactobacillus* is indicated). Data are the means of three replicate trials. The results are expressed as peak areas of specific ions: (A) 2-methylpropanoic acid, (B) aldehydes derived from BcAAs.

The alcohols derived from branched-chain amino acids (BcAAs) were only detected in a few samples. The aldehydes derived from the three BcAAs (Leu, Ile and Val) were only produced by *L. lactis* NCDO763, but their production was significantly ($p \leqslant 0.001$) decreased by the combination of *Lactobacillus* strains with *L. lactis* (Fig. 1B). Indeed, the production of aldehydes was close to zero, when *Lb*. INF15D was added, while it was reduced when *Lb*. 1244 was combined with *L. lactis*, depending on the number of lactobacilli cells added. In fact, the higher the number of lactobacilli cells added to Ch-easy, the lower the amount of aldehydes produced.

These results could be explained by the difference in concentration of viable cells (metabolically active) of *Lactobacillus* and *L. lactis*. Indeed, the concentration of viable cells of *Lactobacillus* was 20–100-fold higher than that of *L. lactis* NCDO763, considering the *Lactococcus* count in Ch-easy with *L. lactis* NCDO763 alone. Therefore, it is possible that the metabolism of lactobacilli dominated. Moreover, the number of viable (or metabolically active) cells of *L. lactis* NCDO763 in the Ch-easy containing mixture of strains was not determined, but it might be reduced by the presence of the *Lactobacillus* strain.

The GDH-positive strain (*Lb*. INF15D), alone or in combination with *L. lactis* NCDO763, produced large amounts of acetoin and diacetyl, which are important cheese aroma compounds (Fig. 2). The concentration of acetoin and diacetyl in the samples inoculated with the mixed culture was clearly related to the number of lactobacilli cells added and was highest for an inoculation of 1.3×10^9 cfu g^{-1}. In contrast, *Lb*. 1244 and *L. lactis* NCDO763 alone and in a mixture produced only small amounts of these compounds.

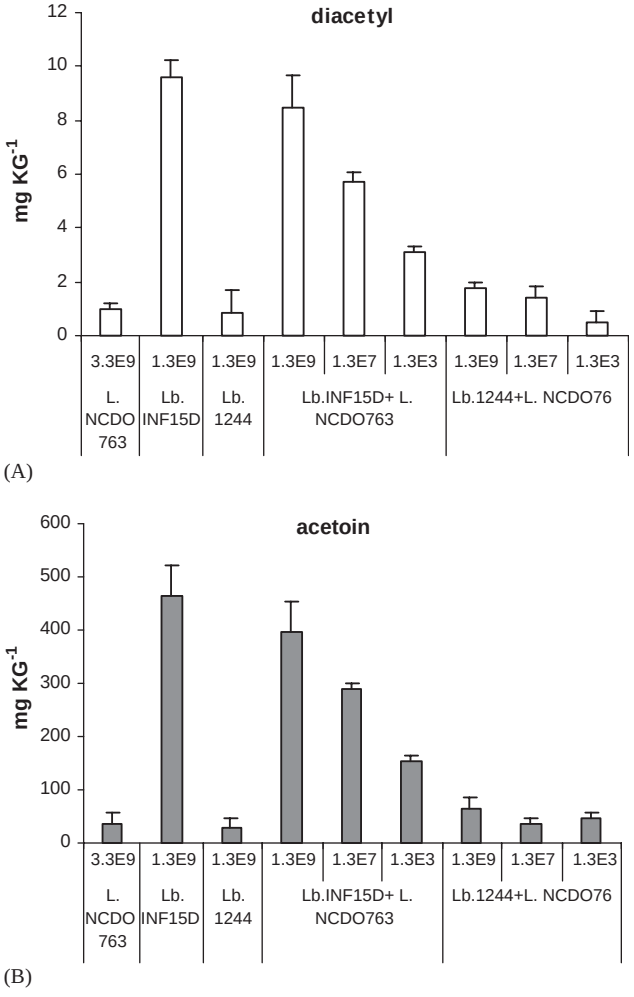

Fig. 2. Production of diacetyl (A) and acetoin (B) by *L. lactis* ssp. *cremoris* NCDO763 (*L*. NCDO763), the GDH-positive strain *Lb. paracasei* ssp. *paracasei* INF15D (*Lb*. INF15D), GDH-negative strain *Lb. paracasei* 1244 (*Lb*. 1244), and their combinations in Ch-easy. Values noted in the *x*-axis indicate the inoculation level with cells of each strain (for mixtures of strains only the inoculation level with *Lactobacillus* is indicated). Data are the means of three replicate trials.

3.3. AT activities of experimental strains

Experimental strains differed in their AT activities towards BcAAs, Phe and aspartate (Asp) (Fig. 3). The two *Lactobacillus* strains exhibited much lower AT activities towards BcAAs than *L. lactis* NCDO763. However, the GDH-positive *Lb*. INF15D exhibited a relatively high AT activity towards Asp and Phe. In fact, in this strain the Asp AT activity was about five-fold higher than its Leu AT activity. In contrast, *Lb*. 1244, exhibited only a low AT activity towards all the amino acids tested.

3.4. Catabolism of Asp and Leu in vitro

Considering the high Asp AT activity of *Lb*. INF15D, we assumed that the α-ketoglutarate produced inside the cells by its GDH activity would primarily be used for transamination of Asp rather than for transamination of BcAAs. This would especially occur in complex media such as the Ch-easy, which contains all common amino acids.

To verify this hypothesis, we compared the catabolism of Leu and Asp by *Lb*. INF15D in two different reaction media. The first one contained a single amino acid (Leu or Asp) in addition to Glu, while the other contained an FAA mixture with a composition similar to that found in the Ch-easy. *Lactobacillus* INF15D degraded Asp

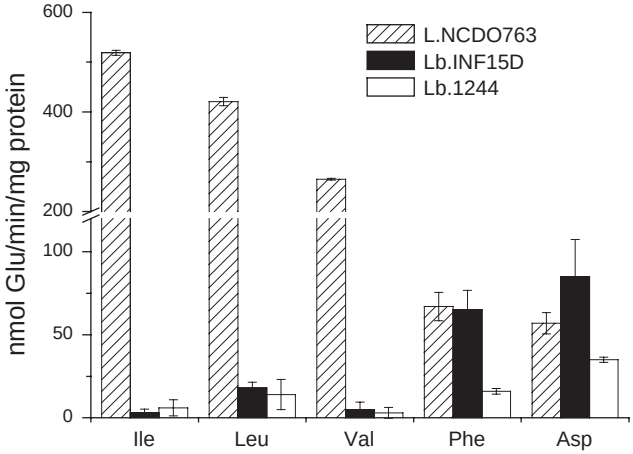

Fig. 3. AT activities of *L. lactis* ssp. *cremoris* NCDO763 (*L*. NCDO763), *Lb. paracasei* ssp. *paracasei* INF15D (*Lb*. INF15D), *Lb. paracasei* 1244 (*Lb*. 1244), measured with Ile, Leu, Val, Phe and Asp as substrate and α-ketoglutarate as co-substrate. Results are the means of at least two replicate tests.

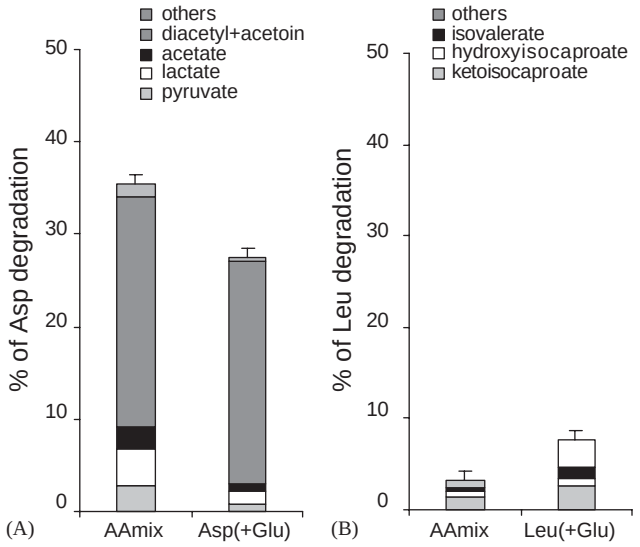

Fig. 4. Degradation of Asp (A) or Leu (B) by *Lb. paracasei* ssp. *paracasei* INF15D in reaction medium containing a single amino acid in addition to Glu (Asp + (Glu) or Leu + (Glu)) and in a medium containing a free amino acid mixture (AAmix). Results are the means of at least two replicate experiments.

extensively in both reaction media, but somewhat more in the medium containing all amino acids than in that containing only Asp and Glu (Fig. 4A). The major metabolites produced were diacetyl and acetoin (corresponding to about 70–90% of the degraded Asp and 25% of the Asp present in the sample). The other metabolites formed from Asp were pyruvate, lactate and acetate. We did not observe significant production of fumarate, malate or succinate.

In comparison, *Lb*. INF15D degraded three-fold less Leu in the reaction mixture containing the FAAs than in that containing only Leu and Glu (Fig. 4B). In fact, in the medium containing the mixture of FAAs, *Lb*. INF15D degraded about 10-fold less Leu than Asp, hence confirming our hypothesis about the competition of AT for α-ketoglutarate.

4. Discussion

Our previous in vitro study performed in simple medium containing only one amino acid (Leu or Phe) demonstrated that the combination of *Lb*. INF15D exhibiting GDH activity with *L. lactis* NCDO763 produced more carboxylic acids from amino acids than each strain separately (Kieronczyk et al., 2003). However, in the present study performed in a cheese model (Ch-easy), these results were not confirmed. Indeed, the production of carboxylic acids derived from BcAAs was not higher in Ch-easy containing both *L. lactis* NCDO763 and the GDH-positive *Lb*. INF15D than in the Ch-easy containing *L. lactis* NCDO763 alone. Moreover, the production of aldehydes derived from BcAAs was less in the samples containing a mixture of these strains than in that containing *Lactococcus* alone. The production of metabolites from BcAAs by *L. lactis* NCDO763 is probably due to its high AT activity towards BcAAs. However, their production is very low compared to the production previously observed in Ch-easy in the presence of α-ketoglutarate (Rijnen et al., 2000). The production of metabolites from BcAAs was not increased by addition of *Lb*. INF15D mainly because *Lb*. INF15D did not carry out transamination of the BcAAs. In fact, α-ketoglutarate produced by its GDH activity inside the cells was essentially used for the transamination of other amino acids such as Asp. Indeed, we demonstrated in vitro that *Lb*. INF15D degraded 10-fold more Asp than Leu in the reaction medium containing the mixture of FAAs due to its high Asp AT activity. Therefore, in Ch-easy, the combination of *Lb*. INF15D and *L. lactis* NCDO763 could not improve the production of carboxylic acids from BcAA since the *Lactobacillus* strain did not produce precursor metabolites (α-keto and hydroxy acids), which could have been transformed to carboxylic acids by *L. lactis*.

The differences between our previous results obtained in vitro and those obtained in the cheese model can be explained by differences in experimental conditions of both studies. As we have outlined, the Ch-easy contains a mixture of FAAs, while the in vitro study was performed in a medium containing only one amino acid. We observed that under the later conditions, Leu was two-fold more transaminated by *Lb*. INF15D than in a medium containing a mixture of FAAs. Moreover, diffusion of precursor metabolites (α-keto and hydroxy acids) produced by *Lactobacillus* strain was probably more difficult in a solid medium such as Ch-easy than in the liquid medium used for in vitro study. Also, survival of the strains in the cheese model incubated for 6 weeks is probably different than their survival in the in vitro experiments which lasted only 40 h. Finally, the enzyme activities involved in the amino acid conversion to carboxylic acids (either from *Lactobacillus* or *Lactococcus* strains) could be lower under Ch-easy conditions than under the in vitro conditions used previously. However, from these results we cannot conclude that no cooperation in amino acid catabolism can occur between any GDH-positive *Lactobacillus* and *L. lactis* in cheese. Further experiments should be done using a GDH-positive strain with high AT activity towards BcAAs and low AT activity towards Asp. This strain should be capable of producing precursor metabolites from BcAAs for further transformation to carboxylic acids by *L. lactis*.

Interestingly, this study revealed that *Lb*. INF15D with GDH activity was an efficient producer of diacetyl and acetoin. These two compounds have been identified as major contributors to the pleasant, creamy and sweet

aroma of Cheddar and Gouda-type cheese (Engels, Dekker, DeJong, Neeter, & Visser, 1997; Milo & Reineccius, 1997). In fact, the quantity of diacetyl produced in Ch-easy by this strain ($10\,mg\,kg^{-1}$) was about 40-fold higher than the amount found in mild-flavoured Cheddar cheese (Milo & Reineccius, 1997). Our results suggest that *Lb*. INF15D produced these compounds from Asp. Usually, formation of diacetyl and acetoin in cheese and other dairy products is linked to citrate metabolism by LAB (McSweeney & Sousa, 2000; Urbach, 1995). However, the Ch-easy paste contained only a very low amount of citrate ($0.2\,mmol\,kg^{-1}$), which was 12-fold lower than the quantity of acetoin produced by *Lb*. INF15D. Moreover, we observed the production of diacetyl and acetoin in vitro by *Lb*. INF15D in the reaction medium containing only Asp (no citrate was present). A recent study of D. Le Bars (results not published) also demonstrated that diacetyl and acetoin were produced by *L. lactis* via the catabolism of Asp. Indeed, Asp is transformed by Asp AT into oxaloacetate, which usually is transformed into diacetyl and acetoin, by the pathway described for citrate metabolism. α-Ketoglutarate produced by GDH activity of *Lb*. INF15D was probably used for the transamination of Asp since this strain exhibits a high Asp AT activity. Therefore, we conclude that the formation of diacetyl and acetoin in Ch-easy most likely derives from the catabolism of Asp by the GDH-positive *Lb*. INF15D.

Recent studies using adjuncts report the beneficial effect of lactobacilli on cheese flavour development (Crow et al., 2001; Lynch et al., 1999; Muir et al., 1996). However, many authors underline the importance of selecting strains, since only certain strains of lactobacilli can improve cheese flavour (Crow et al., 2001). Recently, the GDH activity was identified as a major criterion for the selection of aroma-producing adjuncts (Tanous et al., 2002). Also, it has previously been suggested to use strains with different ratios between AT activities towards ArAAs and BcAAs to diversify aroma formation (Yvon & Rijnen, 2001). Our results indicate that the relative AT activities of GDH-positive strains towards BcAAs, ArAAs and also towards Asp are of major importance for the selection of adjuncts, since they determine which amino acids will be degraded and therefore which aroma compounds will be produced in cheese.

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