Enzymatic activities of non-starter lactic acid bacteria isolated from a traditional Portuguese cheese

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Abstract

Four strains of lactic acid bacteria, previously isolated from traditional Serra da Estrela cheese and duly identified as *Lactobacillus paracasei* ssp. *paracasei*, *Leuconostoc mesenteroides* ssp. *dextranicum*, *Lactococcus lactis* ssp. *lactis* and *Enterococcus faecium*, were tested for their aminotransferase, oxidase and dehydrogenase activities towards five amino acids in free form: two branched-chain (leucine and valine), one sulfur-containing (methionine) and two aromatic (phenylalanine and tryptophan) amino acids. For this purpose, both resting cells (RC) and crude cell-free extracts (CFE) were considered; their lyase and demethiolase activities (towards methionine and α-keto-γ-methiolbutyric acid (KMBA), respectively) were also evaluated. Aminotransferase activity (AT) of RC was ca. 10-fold higher towards Met than towards Leu or Val. No AT activity was found in CFE, although these extracts displayed high dehydrogenase activity (DA) towards Phe, especially *L. lactis* ssp. *lactis* and *E. faecium*. In the case of *L. paracasei* ssp. *paracasei* and *L. mesenteroides* ssp. *dextranicum*, they showed high DA activity, which was ca. 10-fold higher towards Met than towards Leu or Val. *E. faecium* displayed high oxidase activity towards Met, and produced nine-fold more methanethiol from Met and five-fold more methanethiol from KMBA than the other three isolates under study.

1. Introduction

A wide variety of lactic acid bacteria (LAB) are currently employed in the manufacture of several fermented dairy products. In addition to producing lactic acid (as happens with starter LAB), the enzyme machineries of those microrganisms often play a direct role in determining flavor and texture of the final products (as happens with non-starter LAB). Due to a growing demand of foods that are organoleptically appealing and microbiologically safe, the development of new, or the improvement of existing products has been under scrutiny. One of the most important fermented dairy products worldwide is cheese, and one of the (still) poorly studied one is traditional cheese from small ruminants' milks.

Portuguese traditional cheeses have been manufactured for ages from raw milk, following artisanal practices which barely ensure that the microbiological safety standards imposed by the most demanding markets are met. In attempts to improve their organoleptic and microbiological quality, a thorough understanding of the pathways involved in formation of flavor compounds is in order; the final goal is to guarantee a balance between the various microrganisms present and between their biochemical activities during cheese ripening.

Cell lysis leads to release of intracellular enzymes into the cheese matrix, often causing natural enzymatic pathways to become unbalanced or even incomplete, and thus resulting in end products that are quantitatively and qualitatively different from those produced by intact bacteria [1]. In particular, the enzymatic conversion of free amino acids (AA) is important towards synthesis of compounds that possess a relevant role in flavor formation throughout cheese ripening [2]. However, the addition of those AA as supplementary substrates does not usually produce a significant effect on flavor development [1], hence suggesting that there should be a rate-limiting factor in that conversion. Yvon et al. [3] have recently demonstrated that the catabolism of AA via transamination requires an α-ketoacid acceptor; this reaction is responsible for the generation of flavor compounds and precursors thereof. Later, Tammam et al. [4] showed that lactobacilli possess the capacity to utilize a range of

Abbreviations: LAB, lactic acid bacteria; RC, resting cells; CFE, cell-free extract; AT, aminotransferase; DH, dehydrogenase; OX, oxidase; AA, amino acids; Leu, leucine; Val, valine; Met, methionine; Trp, tryptophan; Phe, phenylalanine; KMBA, α-keto-γ-methiolbutyric acid; SSA, sulfosalicylic acid

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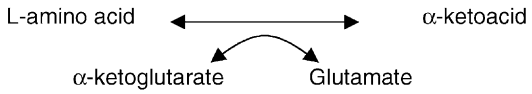
AA, hence confirming that transamination is a non-specific process. Lactobacilli have been used as adjunct starters to enhance proteolysis and improve flavor formation [5], especially in reduced-fat cheese varieties [6]. The metabolic potential of non-starter lactic acid bacteria lies on their dominance in the viable microrganism pool in mature cheeses [7], coupled with their metabolic versatility [8].

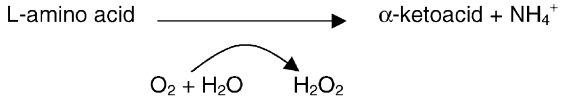
Therefore, it is essential that in vitro studies regarding AA catabolism be conducted using different microrganisms—both with whole resting cells (RC) and cell-free extracts (CFE) thereof, since catabolic activities seem to be highly strain-dependent [9].

Although AA do not contribute directly to the typical cheese flavors associated with the volatile fraction of cheese [10], they are precursors of such volatile aroma compounds as aldehydes, acids, alcohols, esters and thiols. Among all AA, those which are aromatic, branched-chain and sulfur-containing impart floral, cheesy and sulfurous flavors, respectively [11], which are easily detected by consumers. Several typical cheese flavors have been attributed to the presence of sulfur compounds of varying complexity; these compounds arise from the enzymatic conversion of specific free AA brought about by the adventitious microflora, as emphasized in the chemical scheme below.

Aminotransferase-mediated process:

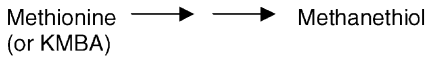


Oxidase-mediated process:



Dehydrogenase-mediated process:

Demethiolase-mediated process:



In particular, the methionine catabolic pathway in microrganisms is known to involve demethiolation of either methionine or α-keto-γ-methiolbutyric acid (KMBA).

In this research effort, whole RC and CFE of four LAB isolated from Serra da Estrela, the most famous Portuguese traditional cheese [12]—manufactured from raw milk and clotted with a plant protease, were used, and their aminotransferase (AT), oxidase (OX) and dehydrogenase (DA) activities towards target AA were duly monitored; for this purpose, two branched-chain AA (leucine and valine), two aromatic AA (phenylalanine and tryptophan) and one sulfur-containing AA (methionine) were selected. Methanethiol production from methionine and KMBA, also via RC and CFE, was also monitored.

2. Materials and methods

2.1. Microbial isolates

Four strains were tested in this study, which had been previously isolated from traditional Serra da Estrela cheese as described in detail elsewhere [13], and duly identified in chemotaxonomical terms via their protein profile (SDS–PAGE). The strains were stored as a stock culture in 30%(v/v) aqueous glycerol at −80 °C.

2.2. Preparation of resting cells

Colonies from the aforementioned isolates were grown in MRS agar; they were afterwards transferred to MRS broth using a 2% (v/v) inoculum, and allowed to grow for 18 h. The medium was then centrifuged in a Sorvall Centrifuge RC5 (Wilmington DL, USA) for 20 min at 9000 × *g*, and the cell-containing pellet was washed three times with 0.01 M potassium phosphate buffer (pH 7.0). The pellet was reconstituted in phosphate buffer (pH 8.0) to an optical density of 20 at 480 nm.

2.3. Preparation of cell-free extracts

Cells were grown in MRS broth for 18 h, in 1-l flasks using a 2% (v/v) inoculum. The broth was then centrifuged for 20 min at 10,000 × *g*, and the cell-containing pellet was washed twice with 0.01 M potassium phosphate buffer (pH 7.0). The pellet was then ground with alumina type 305 (Sigma, St. Louis, MO, USA) using a mortar and pestle, in the proportion pellet:alumina of 1:5 (w/w). The extracts were suspended in 50 ml of buffer (i.e. 5% of the initial volume), and then centrifuged for 50 min at 9000 × *g* to remove alumina and cell debris. The supernatant, which will hereafter be designated as cell-free extract, was kept frozen at −30 °C until use. The amount of protein in the CFE was determined by the BioRad protein assay kit (Munich, Germany).

2.4. Assay for aminotransferase activity

The RC (250 μl) or the CFE (1 $mg_{protein}$ ml^{-1}) was incubated at 37 °C in 0.2 M Tris–HCl buffer (pH 8.0) containing 1 mM pyridoxal-5′-phosphate, 200 mM α-ketoglutarate and 10 mM of the AA tested. The RC-containing samples were centrifuged at 8000 × *g* for 5 min, whereas in the CFE-containing counterparts the reaction was quenched by addition of 3% sulfosalicylic acid (SSA); samples of the latter were then centrifuged at 10,000 × *g* for 5 min, and glutamic acid present in the supernatant was assayed for

via the Boehringer assay kit (Mannheim, Germany). Controls consisted of the same reaction mixture but with 250 μl of sterile distilled water instead of RC. The corresponding ketoacids were analyzed by HPLC using an IC-Pak column (7.8 mm × 300 mm, Waters, Milford, MA, USA) for those containing tryptophan and phenylalanine adducts, and an RP18 column for those containing leucine, valine and methionine adducts; detection was by absorbance at 214 nm, using aqueous 0.1% (w/v) H_3PO_4 as eluant.

2.5. Assay for dehydrogenase activity

The RC (250 μl) or the CFE (1 $mg_{protein}$ ml^{-1}) was incubated at 25 °C in 100 mM glycine/NaOH buffer (pH 10.5) containing 1 mM NAD and 3 mM of the AA tested [14]. Controls consisted of the same reaction mixture as above but with the addition of 250 μl of sterile distilled water instead of RC. In the CFE-containing samples, the reaction was quenched by addition of 3% (w/v) SSA. Samples were centrifuged at 10,000 × *g* for 5 min, and NH_4^+ in the supernatant was assayed for using the Boehringer assay kit. The corresponding α-ketoacids were analyzed as described above.

2.6. Assay for oxidase activity

The RC (250 μl) or the CFE (1 $mg_{protein}$ ml^{-1}) was incubated at 37 °C in 80 mM of 0.2 M Tris–HCl buffer (pH 8.0) containing 60 units of catalase and 3 mM of the AA tested. Controls consisted of the same reaction mixture as above but with addition of 250 μl of sterile distilled water instead of RC. Samples were withdrawn at selected time intervals; in the CFE-containing samples, the reaction was quenched by addition of 3% (w/v) SSA. Samples were centrifuged at 10,000 × *g* for 5 min. The corresponding α-ketoacid and NH_4^+ were determined in the supernatant as described above.

2.7. Assay for demethiolase activity

The RC (10 μl) or the CFE (1 $mg_{protein}$ ml^{-1}) was incubated at 30 °C in 0.1 M potassium phosphate buffer (pH 8.0) containing 20 mM pyridoxal-5′-phosphate, 0.2 mM 5,5′-dithiobis-2-nitrobenzoic acid and 25 mM methionine (or KMBA) in a 3-ml glass cuvette. Controls consisted of the same reaction mixture as above but with addition of 10 μl of sterile distilled water instead of RC. The methanethiol formed was assayed for by measuring absorbance at 412 nm, which was recorded continuously for 1 h [15].

3. Results and discussion

The AT, DH and OX activities of RC of the four isolates under study towards Leu, Val and Met, respectively, assayed for via determination of the corresponding α-ketoacid are depicted in Figs. 1–3. The amount of glutamic acid formed (which reflects the AT activity) is tabulated in Table 1, whereas the DH and OX activities determined via measurement of the amounts of ammonia formed are depicted in Table 2 (CFE) upon Leu, Val, Met, Phe and Trp, and in Table 3 (RC) upon Leu, Val and Met.

The AT activity of RC towards Met was ca. 10-fold that towards Leu and Val. *Lactococcus lactis* ssp. *lactis* displayed a very weak AT activity towards Leu and Val when compared with the other three isolates; this point was further confirmed by the data pertaining to Glu (Table 1).

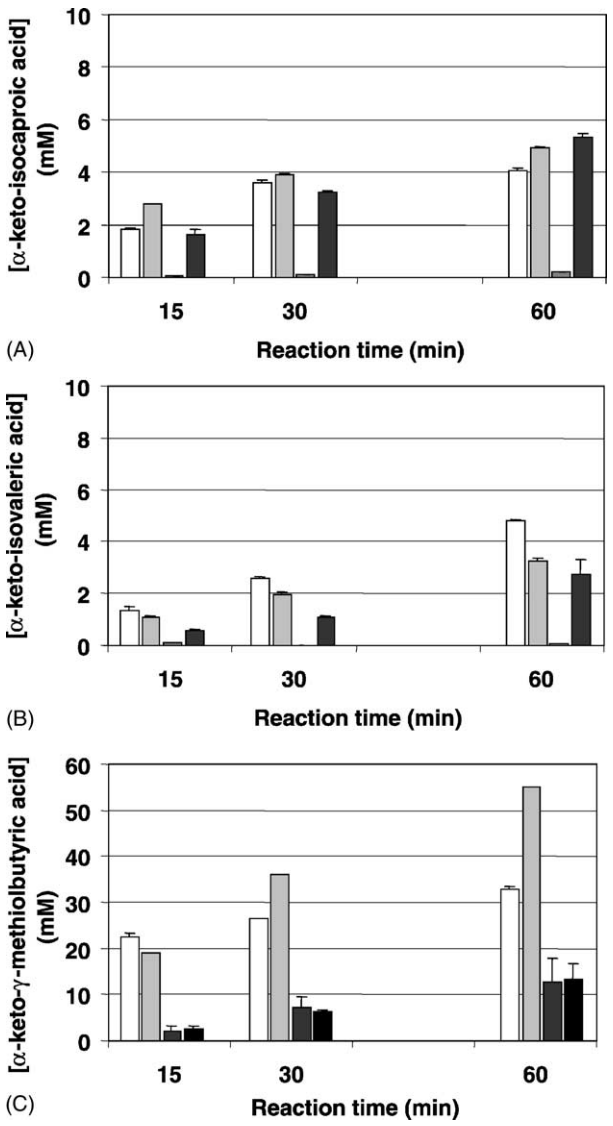


Fig. 1. Evolution with incubation time of the concentration of α-ketoacid formed via aminotransferase activity towards Leu (A), Val (B) and Met (C) by resting cells of *L. paracasei* ssp. *paracasei* (□), *L. mesenteroides* ssp. *dextranicum* (▨), *L. lactis* ssp. *lactis* (■) and *E. faecium* (■). Each datum represents a mean, and the error bar represents its standard deviation based on two replicates.

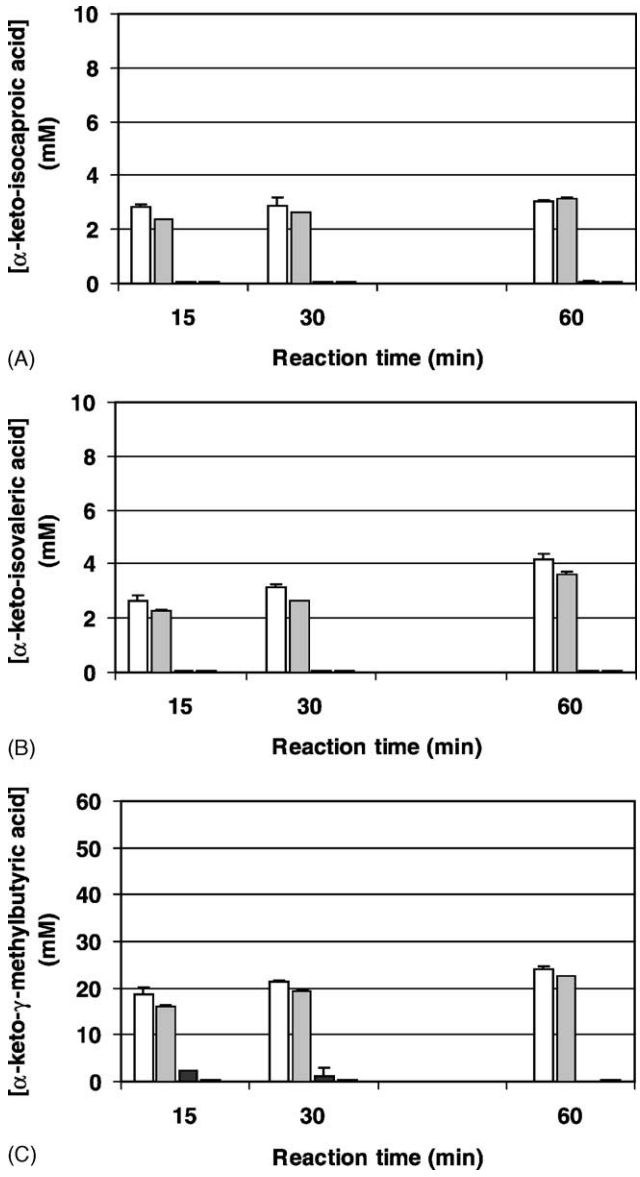


Fig. 2. Evolution with incubation time of the concentration of α-ketoacid formed via dehydrogenase activity towards Leu (A), Val (B) and Met (C) by resting cells of *L. paracasei* ssp. *paracasei* (□), *L. mesenteroides* ssp. *dextranicum* (▨), *L. lactis* ssp. *lactis* (■) and *E. faecium* (■). Each datum represents a mean, and the error bar represents its standard deviation based on two replicates.

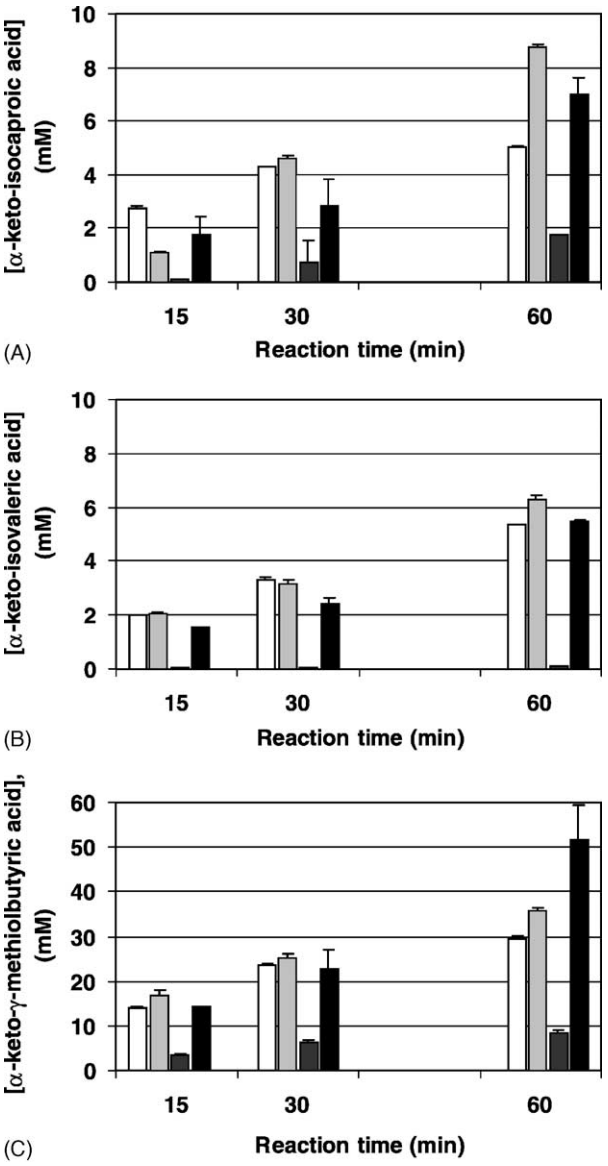


Fig. 3. Evolution with incubation time of the concentration of α-ketoacid formed via oxidase activity towards Leu (A), Val (B) and Met (C) by resting cells of *L. paracasei* ssp. *paracasei* (□), *L. mesenteroides* ssp. *dextranicum* (▨), *L. lactis* ssp. *lactis* (■) and *E. faecium* (■). Each datum represents a mean, and the error bar represents its standard deviation based on two replicates.

Enterococcus faecium was very active towards Leu (Fig. 1, Table 1), but *L. paracasei* ssp. *paracasei* was most active towards Val (based on α-ketoacid production). The AT activity was always much higher for RC than for CFE; however, comparison between the two is difficult owing to absence of a common comparison basis. The AT activity for Met was statistically different ($P < 0.05$) from that upon Leu and Val. The RC of *L. lactis* ssp. *lactis* and of *E. faecium* displayed almost no DH activity towards Leu, Val and Met (Fig. 2); this realization was further confirmed by the ammonia data (Table 2). On the other hand, *L. paracasei* ssp. *paracasei* and *L. mesenteroides* ssp. *dextranicum* exhibited high DH activity, ca. 6-fold higher towards Met than Val and Leu — which is not, however, confirmed by the data pertaining to NH_4^+ in Table 2, probably because the assay method used is linear only within a limited range.

The AT, DH and OX activities of CFE of all isolates upon five AA are depicted in Table 4. The AT activity (determined via measurement of the amount of α-ketoacid formed per unit time and per milligram of protein) of CFE was virtually none (or very weak) towards all AA tested; this again was confirmed by the Glu data (Table 1). However, these CFE displayed highest DH activity towards Phe; *L. lactis* ssp. *lactis* and *E. faecium* yielded activities ca.

Table 1
(Specific) rate of production of glutamic acid from aminotransferase activity of RC ($mM\,min^{-1}$) and of CFE ($mM\,min^{-1}\,mg_{protein}{}^{-1}$)

Isolate	Source type	Target AA				
		Leu	Val	Met	Trp	Phe
L. paracasei ssp. *paracasei*	RC	0.0065	0.0024	0.059	0.0006	0.0012
	CFE	0.003	0.004	ND	0.002	ND
L. mesenteroides ssp. *dextranicum*	RC	0.0122	0.0017	0.0085	0.0057	0.0077
	CFE	0.002	0.001	ND	ND	ND
L. lactis ssp. *lactis*	RC	0.0027	0.0007	0.0133	0.010	0.014
	CFE	ND	ND	ND	0.001	ND
E. faecium	RC	0.0422	0.0032	0.0084	0.011	0.0086
	CFE	ND	0.002	ND	0.001	ND

ND, not detected.

Table 2
(Specific) rate of production of ammonia from dehydrogenase (DH) and oxidase (OX) activities of RC ($mM\,min^{-1}$) and of CFE ($mM\,min^{-1}\,mg_{protein}{}^{-1}$)

Isolate	Activity type	Source type	Target AA				
			Leu	Val	Met	Trp	Phe
L. paracasei ssp. *paracasei*	DH	RC	0.026	0.031	0.054	ND	ND
		CFE	0.017	0.015	0.013	0.012	0.008
	OX	RC	0.035	0.041	0.026	ND	ND
		CFE	0.034	0.016	0.008	0.017	0.004
L. mesenteroides ssp. *dextranicum*	DH	RC	0.052	0.054	0.039	ND	ND
		CFE	0.013	0.013	0.007	0.015	0.021
	OX	RC	0.059	0.033	0.026	ND	ND
		CFE	0.039	0.015	0.014	0.019	0.013
L. lactis ssp. *lactis*	DH	RC	0.011	0.009	0.015	ND	ND
		CFE	0.015	0.014	0.010	0.002	0.026
	OX	RC	0.015	0.015	0.019	ND	ND
		CFE	0.074	0.013	0.056	0.021	0.000
E. faecium	DH	RC	0.020	0.009	0.019	ND	ND
		CFE	0.017	0.014	0.002	0.012	0.014
	OX	RC	0.020	0.033	0.024	ND	ND
		CFE	0.036	0.017	0.039	0.019	0.014

ND, not determined.

Table 3
Rate of production of ketoacid ($\mu M\,min^{-1}$) via aminotransferase (AT), dehydrogenase (DH) and oxidase (OX) activities of RC

Isolate	Activity type	Target AA		
		Met	Leu	Val
L. paracasei ssp. *paracasei*	AT	1497 ± 64	122.1 ± 4.4	89.0 ± 9.3
	DH	1248 ± 96	187.8 ± 8.9	174.9 ± 12.4
	OX	930 ± 32	184.7 ± 4.4	132.7 ± 0.6
L. mesenteroides ssp. *dextranicum*	AT	1270.7 ± 0.0	187.8 ± 0.0	73.3 ± 3.1
	DH	1067 ± 32	156.5 ± 0.0	150.9 ± 3.1
	OX	1112 ± 96	72.0 ± 4.4	138.2 ± 1.3
L. lactis ssp. *lactis*	AT	132 ± 83	Neg	6.1 ± 0.0
	DH	22.3 ± 1.7	Neg	4.0 ± 0.0
	OX	236.0 ± 6.4	6.2 ± 1.0	2.7 ± 0.0
E. faecium	AT	166 ± 54	109.5 ± 13.3	39.0 ± 1.1
	DH	22.1 ± 0.032	Neg	3.8 ± 0.0
	OX	953.0 ± 0.0	119.0 ± 44.3	103.3 ± 1.3

Each datum represents a mean ± standard deviation based on two replicates. Neg, negligible; rate estimates are based on two datapoints within initial period of linearity.

Table 4
Specific rate of production of ketoacid ($\mu M\,min^{-1}\,mg_{protein}{}^{-1}$), via aminotransferase (AT), dehydrogenase (DH) and oxidase (OX) activities of CFE

Isolate	Activity type	Target AA				
		Met	Leu	Val	Trp	Phe
L. paracasei ssp. *paracasei*	AT	4.0 ± 0.0	2.4 ± 0.1	Neg	Neg	Neg
	DH	6.1 ± 0.0	6.5 ± 0.4	0.2 ± 0.0	4.4 ± 0.2	8.9 ± 0.0
	OX	0.8 ± 0.0	0.2 ± 0.1	2.1 ± 0.0	4.9 ± 0.3	16.9 ± 0.4
L. mesenteroides ssp. *dextranicum*	AT	Neg	0.9 ± 0.0	Neg	Neg	Neg
	DH	10.9 ± 0.2	10.8 ± 0.7	4.6 ± 0.0	9.9 ± 2.0	12.2 ± 0.3
	OX	1.8 ± 0.1	0.3 ± 0.0	3.8 ± 0.9	3.3 ± 0.3	19.6 ± 1.9
L. lactis ssp. *lactis*	AT	Neg	Neg	Neg	Neg	Neg
	DH	2.3 ± 0.3	4.9 ± 0.4	Neg	1.6 ± 0.0	34.0 ± 0.4
	OX	0.3 ± 0.0	Neg	Neg	4.3 ± 0.4	16.1 ± 0.0
E. faecium	AT	Neg	Neg	Neg	Neg	Neg
	DH	1.8 ± 0.3	3.4 ± 0.5	Neg	1.8 ± 0.1	36.8 ± 1.2
	OX	0.3 ± 0.0	Neg	Neg	5.6 ± 1.4	15.2 ± 1.9

Each datum represents a mean ± standard deviation based on two replicates. Neg, negligible; rate estimates are based on two datapoints within initial period of linearity.

3-fold those exhibited by *L. paracasei* ssp. *paracasei* and *L. mesenteroides* ssp. *dextranicum*. The catabolism of Phe, effected by microrganisms that belong to the cheese native microflora, to generate phenethyl alcohol has been studied by Lee and Richard [16]. Gummalla and Broadbent [17] assayed CFE of *Lactobacillus helveticus* and *Lactobacillus casei* for Trp catabolism, and concluded that both degraded Trp to indole-3-lactic acid via sequential transamination and dehydrogenation reactions. *Leuconostoc* spp. are, on the other hand, slow acid producers; cheeses manufactured with these strains demand long maturation times, but develop in turn interesting flavors (owing to their heterofermentative metabolism, and concomitant diacetyl production). Aminotransferases catalyze transamination reactions, which have been demonstrated [18] to be the first step in the degradation of Leu, Met and aromatic amino acids by lactococci. Therefore, the higher activity observed towards Met may positively contribute to the generation of sulfur-containing flavor compounds.

In terms of OX activity, *L. lactis* ssp. *lactis* exhibited a poor performance; conversely, *E. faecium* showed a strong activity, especially towards Met (between 8- and 10-fold). The CFE of *L. mesenteroides* ssp. *dextranicum* possessed relevant DH activity towards Leu and Met; however, only a small amount of ammonia (ca. $0.013\,mM\,min^{-1}\,mg_{protein}{}^{-1}$) was thus formed (Table 2).

Methanethiol formation from Met and KMBA is shown in Figs. 4 and 5, from RC and CFE, respectively. Whole cells were not active towards Met; Ferchichi et al. [19] followed methanethiol production during growth of *Brevibacterium linens*, and reported demethiolation of Met only during the exponential growth phase. The transport mechanism of Met into the cell may be more complex than that of KMBA, or alternatively Met suffers rapid chemical breakdown into volatile components. On the other hand, RC were rather active towards KMBA; *L. lactis* ssp. *lactis* and *E. faecium* produced twice as much methanethiol as the other two isolates.

The CFE of *E. faecium* produced nine-fold more methanethiol from Met, and five-fold more from KMBA than the CFE of the other three isolates. These results contrast with those reported by Dias and Weimer [20] for *B.*

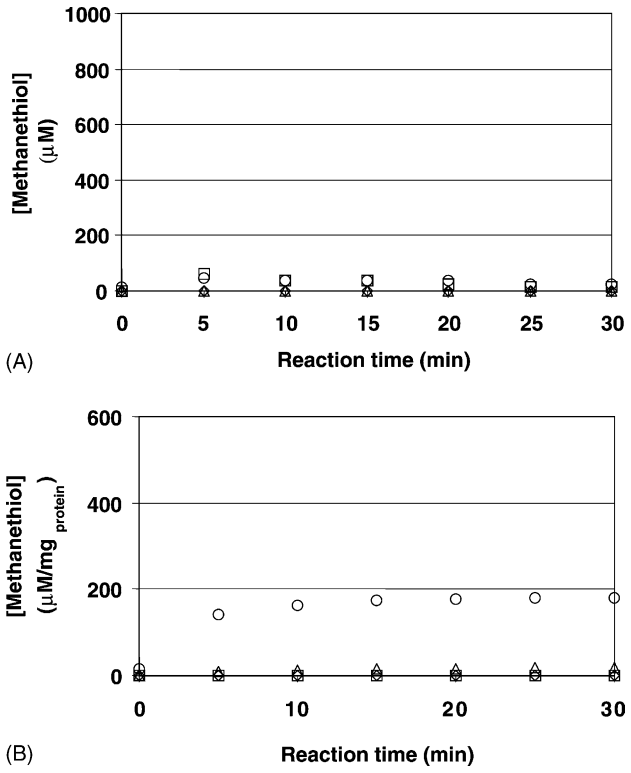


Fig. 4. Evolution with incubation time of the concentration of methanethiol formed from Met by resting cells (A) and cell-free extracts (B) of *L. paracasei* ssp. *paracasei* (◇), *L. mesenteroides* ssp. *dextranicum* (□), *L. lactis* ssp. *lactis* (△) and *E. faecium* (○).

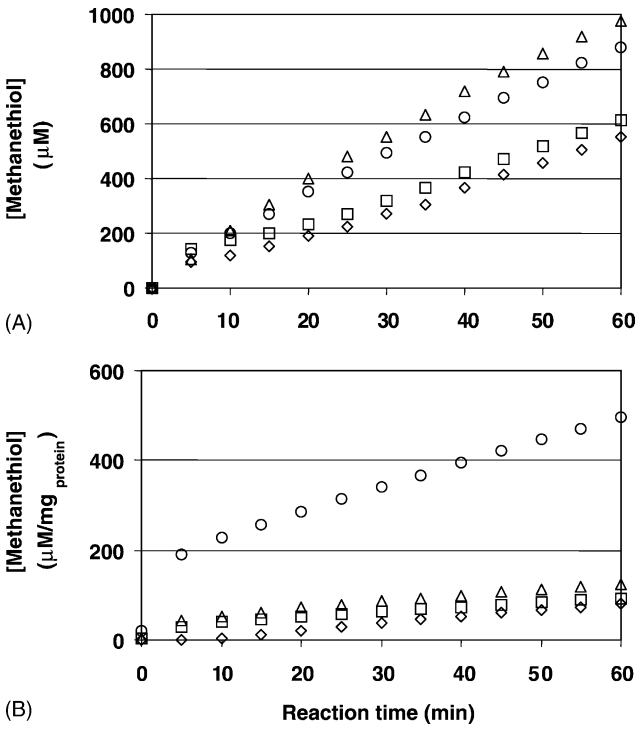


Fig. 5. Evolution with incubation time of the concentration of methanethiol formed from KMBA by resting cells (A) and cell-free extracts (B) of *L. paracasei* ssp. *paracasei* (◇), *L. mesenteroides* ssp. *dextranicum* (□), *L. lactis* ssp. *lactis* (△) and *E. faecium* (○).

linens BL2, hence suggesting that specific conditions may be required in order to activate the transport mechanism across the cell wall. The enzyme activities of RC are dependent on the AA used as substrate and on the strain employed [9], as expected (i.e. enzyme-mediated transport mechanisms for a particular AA are strain-specific); activities using CFE are less specific because no transport mechanism is involved, and are thus strain-dependent but not AA-dependent.

Methanethiol production from KMBA and from Met was also monitored for increased amounts of RC (20, 50, 100, 200 and 500 μl) and CFE (10, 20 and 50 $mg_{protein}$) (data not shown). Methanethiol concentration increased with increased cell concentration of both substrates, except for *L. lactis* ssp. *lactis* towards KMBA. This strain exhibited a weaker production of methanethiol with higher cell density, probably due to a decrease in oxygen availability [19]. Interestingly, all four strains considered in our study are auxotrophic for Leu, Val and either both (or at least one of) the aromatic AA (Phe and Trp), but none for Met. The specific enzyme activities for methanethiol production towards Met and KMBA for CFE, and enzyme activities for RC are shown in Table 5. The highest activities were observed in CFE of *E. faecium* and RC of *L. mesenteroides* ssp. *dextranicum* towards Met, and in RC of *E. faecium* and *L. lactis* ssp. *lactis* towards KMBA.

Table 5
(Specific) Activity of CFE ($\mu M\,min^{-1}\,mg_{protein}{}^{-1}$) and activity of RC ($\mu M\,min^{-1}$) towards Met and KMBA

Isolate	Source type	Target substrate	
		Met	KMBA
L. paracasei ssp. *paracasei*	RC	Neg	8.74
	CFE	Neg	1.44
L. mesenteroides ssp. *dextranicum*	RC	11.77	9.27
	CFE	Neg	1.10
L. lactis ssp. *lactis*	RC	Neg	16.21
	CFE	1.33	1.47
E. faecium	RC	7.06	14.20
	CFE	25.24	5.47

Neg, negligible; rate estimates are based on two datapoints within initial period of linearity.

The synthesis of methanethiol from Met is generally believed to involve the one-step degradation of the latter by the enzyme methionine γ-lyase [21]; this is a pyridoxal 5′-phosphate-dependent enzyme, which is able to catalyze the α-,γ-elimination of Met to form α-ketobutyrate, ammonia and methanethiol. Methanethiol can also be generated from Met, in a two-step degradation pathway initiated by an aminotransferase; this enzyme requires the presence of an amino acceptor (e.g. α-ketoglutarate), hence yielding KMBA which is then transformed to methanethiol via an (yet) unknown mechanism [22]. These authors further concluded that methanethiol formation from Met occurs via an AT pathway which converts Met to KMBA, followed either by enzymatic conversion or chemical decomposition of KMBA to methanethiol. In a subsequent work [23], and unlike what happens with KMBA demethiolating and Met AT specific activities, the specific demethiolating activity upon Met was reported to remain constant regardless of culture age, while the other two increased. These results are also confirmed by our data: Met AT activity increased with time, or equivalently, with culture age (Fig. 1), as well as KMBA demethiolating activity (Fig. 5A), whereas Met demethiolating activity (Fig. 4A) remained constant. Such realisation suggests that this is probably a constitutive enzyme system, whereas the former two are not [23]. Gao et al. [22] suggested that both RC and CFE are capable of methanethiol formation, yet via distinct pathways: in whole cells, KMBA is enzymatically converted to methanethiol, while in CFE the release of AT could lead to accumulation of KMBA from Met, which would then be decomposed to methanethiol directly, or enzymatically by RC.

Although cell autolysis plays an important role in flavor development because of the release of enzymes into the medium—where they are responsible for the range of reactions that ultimately lead to volatile formation, it is the balance of lysed and intact cells that apparently is responsible for the desired cheese-ripening events.

4. Conclusions

Both RC and CFE are able to catabolize free AA, but postulation of a clearcut relationship between them has not proven feasible. Methionine, one of the most important AA in the development of flavor compounds in cheese, is also the one most rapidly and extensively catabolized by our strains; hence, evidence was provided that said strains are potentially implicated in the generation of relevant flavor notes. Due to the relevance of Met breakdown and the concomitant formation of sulfurous volatile compounds, these isolates (especially *E. faecium*, strain ESB 338) are good candidates for inclusion in a formulated starter for cheese production.

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