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Expression of amino acid converting enzymes and production of volatile compounds by *Lactococcus lactis* IFPL953

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ABSTRACT

Lactococcus lactis contains a large number of key enzymes responsible for the formation of volatile compounds characteristic of cheese flavour. In the present work we have investigated the expression of genes codifying amino acid converting enzymes (AACE) and the formation of volatile compounds by *L. lactis* IFPL953 and its mutant lacking the hydroxy acid dehydrogenase PanE (*L. lactis* IFPL953Δ*panE*). The growth in absence of isoleucine was the main induction factor in the expression of *araT*, *bcaT*, *panE*, and *kivD*. The expression of the *gdh* gene of *L. lactis* IFPL953Δ*panE* increased remarkably during the stationary growth phase, particularly under isoleucine and valine starvation conditions. *L. lactis* IFPL953Δ*panE* showed an enhanced formation of 2- and 3-methylbutanal and their corresponding alcohols and an increase in the formation of ketones. These findings contribute to a better knowledge of *L. lactis* AACE regulation and its potential application in cheese flavour formation.

1. Introduction

Lactococcus lactis is the species most used in industrial starters for cheese manufacture. Casein is metabolised by microbial proteinases and peptidases during cheese manufacture being finally converted into amino acids. These are further catabolised by microbial amino acid converting enzymes (AACE) and transformed into volatile components characteristic of the flavour and aroma of semi-ripened cheeses (Smid & Kleerebezem, 2014; Van de Bunt, Bron, Sijtsma, De Vos, & Hugenholtz, 2014). The lactococcal proteinase and peptidase systems had been extensively investigated over the second half of the last century (Kunji, Mierau, Hagting, Poolman, & Konings, 1996). Since then, the research has focused primarily on the microbial enzymes able to convert amino acids into specific flavour molecules of significant importance for the organoleptic quality of cheeses (Liu et al., 2014; Smit, Smit, & Engels, 2005; Ziadi et al., 2010).

Two major pathways of amino acid catabolism are well recognised in *L. lactis*. The first series of reactions are initiated by the activity of an aminotransferase that transfers the amino group from an amino acid to an α-keto acid (usually α-ketoglutaric acid) and results in the production of a new α-keto acid and an amino acid (usually glutamic acid). The aromatic aminotransferase (AraT) and branched chain aminotransferase (BcaT) catalyse the transamination of aromatic and branched-chain amino acids, respectively (Yvon & Rijnen, 2001). The enzyme glutamate dehydrogenase (GDH) catalyses the reversible conversion of glutamate into α-ketoglutarate (Gómez de Cadiñanos, Peláez, Martínez-Cuesta, García-Cayuela, & Requena, 2018; Tanous, Chambellon, Sepulchre, & Yvon, 2005). The α-keto acids formed by amino acid transamination play a central role in the biosynthetic and catabolic routes and are limiting factors for aroma formation (Yvon & Rijnen, 2001). Further catabolism of these α-keto acids is catalysed by an α-ketoacid decarboxylase (KivD) responsible for their conversion into aldehydes (De la Plaza, Fernández de Palencia, Peláez, & Requena, 2004; Smit et al., 2005). Hydroxy acid dehydrogenases (HA-DHs) negatively affect aroma production by competing with decarboxylases for α-keto acids to convert them into the non-aromatic α-hydroxy acids (Broadbent et al., 2004; Chambellon et al., 2009; Gómez de Cadiñanos et al., 2013).

The second major series of reactions by which, particularly, sulphur amino acids are catabolised by *L. lactis* is initiated by the activity of C–S lyases (cystathionine β- or γ-lyase and YtjE), which catalyse the simultaneous deamination and demethylthiolation of

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methionine to methanethiol (Bustos et al., 2011; Hanniffy, Peláez, Martínez-Bartolomé, Requena, & Martínez-Cuesta, 2009; Martínez-Cuesta, Peláez, & Requena, 2013).

The nitrogen metabolism of *L. lactis* is influenced by the level of intracellular branched-chain amino acids (BCAAs), isoleucine, leucine and valine, through the pleiotropic transcription regulator CodY that controls several genes implicated in proteolysis (proteinase, oligopeptide transporters, peptidases) and amino acid pool regulation (Guédon, Serror, Ehrlich, Renault, & Delorme, 2001; Kok et al., 2017; Petranovic et al., 2004). It has also been demonstrated that dipeptides containing BCAAs are the sources of free intracellular BCAAs, which in turn provides the intracellular signal recognised by CodY. This pleiotropic regulator represses gene expression in Gram-positive bacteria during logarithmic phase in nitrogen enriched media (Sonenshein, 2005). Among CodY-dependent genes, 45% encode enzymes involved in amino acid biosynthesis pathways, while most of the other genes are involved in functions related to nitrogen supply. This global control leads to growth inhibition in several amino acid-limited media containing an excess of isoleucine (Guédon, Sperandio, Pons, Ehrlich, & Renault, 2005).

In previous studies, we showed that when *L. lactis* IFPL730, a wild strain isolated from raw goats' milk cheese, was grown in chemically defined medium (CDM) under isoleucine starvation conditions, it increased the KivD activity 4-fold compared with cells grown in CDM with casitone as nitrogen source (De la Plaza, Peláez, & Requena, 2009). Later, we studied the effect of BCAA content on the growth of *L. lactis* IFPL730 and we observed that the expression of some functional genes related to amino acid catabolism (*kivD*, *bcaT* and *ytjE*) increased markedly when growing under both isoleucine and valine starvation conditions (García-Cayuela, Gómez de Cadiñanos, Peláez, & Requena, 2012).

Aminotransferase activities are widespread among *L. lactis*, whereas GDH and KivD are key aroma enzymes that show low prevalence and great strain dependence (De la Plaza et al., 2006; Gómez de Cadiñanos et al., 2018). *L. lactis* IFPL953 is a wild strain isolated from raw milk cheese characterised by a wide enzymatic background for aroma production, including AraT and BcaT activities, the GDH activity to produce α-ketoglutarate required for transamination reactions, and the KivD and C–S lyase (YtjE) activities. In addition, we have inactivated its HA-DH activity (encoded by the *panE* gene) to generate *L. lactis* IFPL953Δ*pan*E to optimise the degradation of α-keto acids into volatile compounds (Gómez de Cadiñanos et al., 2013). The objective of the present work has been to investigate the effect of BCAA content in the growth medium on the expression of genes codifying AACE and the formation of volatile compounds by *L. lactis* IFPL953 and the mutant *L. lactis* IFPL953Δ*panE*.

2. Materials and methods

2.1. Lactococcus lactis *strains and growth conditions*

The strains were maintained at −80 °C and routinely grown in 10 mL M17 broth (Pronadisa, Madrid, Spain) supplemented with 0.5% glucose (M17G) at 30 °C. For the analysis of gene expression and volatile formation, the strains were grown in the buffered chemically defined medium (CDM) described by García-Cayuela et al. (2012), where the concentration of amino acids could be modified. The changes in media were CDM-Ile, CDM without isoleucine; CDM-Val, containing 33 mg L^{-1} valine (10-fold reduction of basal content) and CDM-Leu, containing 4.7 mg L^{-1} leucine (100-fold reduction of basal content).

The growth of *L. lactis* IFPL953 and IFPL953Δ*panE* in CDM at 30 °C was monitored for 30 h at 480 nm (OD_{480}) in triplicate 300 μL-wells of sterile 96-well microplates (Sarstedt, La Roca del Vallès, Spain) in an automated microplate reader (Varioskan Flash, Thermo Fisher Scientific, Waltham, MA, USA). The maximum growth rate (μ_{max}), maximum OD_{480} (OD_{max}) and lag parameter (lag) of strains were calculated by fitting the curves to a sigmoid model using the Microsoft Excel add-in DMfit v.3.5 (available at http://www.ifr.ac.uk/safety/DMfit/default.html). Cultures used for gene expression and volatile compound analyses were performed in 300 mL. Samples were taken at different growth phases to determine viable counts on M17G-agar plates, incubated aerobically at 30 °C for 48 h. The pH of the CDM was kept at 7 using 0.19 M 3-(N-morpholino)propanesulfonic acid (MOPS; Sigma–Aldrich, St Louis, MO, USA) as described previously (García-Cayuela et al., 2012).

2.2. RNA extraction, reverse transcription (RT) and quantification of gene expression by real-time RT-PCR

Total RNA from *L. lactis* IFPL953 and IFPL953Δ*panE* cultures in all CDMs was extracted at early- and late-exponential growth phase and during stationary phase. RNA extraction was performed using RNeasy kit (Qiagen, Hilden, Germany) including an additional lysis step as described by García-Cayuela et al. (2012). Before reverse transcription, purified RNA was treated with DNase Treatment and Removal Reagents (Ambion, Austin, TX, USA) to eliminate DNA from the samples and cDNA was synthesised using a ThermoScript RT-PCR system (Invitrogen, Paisley, UK).

The quantification of gene expression by real-time RT-PCR was carried out on an iQ™5 Multicolor Real-Time PCR detection system Cycler (Bio-Rad, Hercules, CA, USA) with IQ™ SYBR® Green Supermix (Bio-Rad) in 96-well plates. The primers were previously designed to target the *araT* and *bcaT* genes encoding aromatic and branched-chain amino acid specific aminotranferases, respectively, the *kivD* gene encoding an α-keto acid decarboxylase, the *ytjE* gene encoding a C–S lyase, the *panE* gene encoding a D-2-hidroxyacid dehydrogenase and the *gdh* gene encoding the *L. lactis* glutamate dehydrogenase (García-Cayuela et al., 2012; Gómez de Cadiñanos et al., 2018). Primer annealing was set at 52 °C (30 s) and fluorescence was recorded during each extension step (72 °C, 60 s). Negative controls with RNA treated with DNAse and a non-template control were included. Results were recorded as threshold cycles (CT) and analysed using the method described by Pfaffl (2001). The results obtained under BCAA starvation conditions were compared with the control condition (cells growing in CDM). The results were normalised using the *L. lactis tuf* gene encoding for elongation factor TU (García-Cayuela et al., 2012).

2.3. Analysis of volatile compounds

Volatile compounds produced by *L. lactis* IFPL953 and IFPL953Δ*panE* cultures in all CDMs after 30 h of incubation were determined by solid phase microextraction (SPME) and gas chromatography–mass spectrometry (GC–MS) as described previously by García-Cayuela et al. (2012). Volatiles were sampled with a divinylbenzene/carboxen/polydimethylsiloxane (DVB/CAR/PDMS) fibre (Supelco, Dorset, UK) and injected onto a HP-INNOWAX 136 (60 m × 0.25 mm, 0.50 μm film thickness) (Agilent, Cheshire, UK) column at a helium flow rate of 54 mL min^{-1}. Volatile compounds were determined by GC using an Agilent 7890 GC system with a CTC CombiPAL autosampler coupled to a single quadrupole mass spectrometer (Agilent 5975). The mass selective detector was scanned from 29 to 500 *m/z* at a speed of 1.1 scans s^{-1}. Volatile compounds were identified by comparing mass spectra with that obtained using the data base NIST98 from Chemstation software (Agilent Technologies Ltd., UK). Results were expressed as percentages of the abundance of 4-methyl-2-pentanol

used as internal control peak. Samples were analysed in duplicate from three independent experiments.

2.4. Statistical analysis

Results are expressed as mean ± standard deviation. Differences were determined by one-way ANOVA followed by Tukey's post hoc test using SPSS software package (SPSS Inc., Chicago, IL, USA).

3. Results

3.1. Effect of inactivation of panE on the growth of L. lactis IFPL953 under BCAA starvation conditions

The growth of *L. lactis* IFPL953 and its mutant IFPL953Δ*panE* in CDM and with minimal BCAA content supporting growth is shown in Fig. 1. *L. lactis* IFPL953 did grow in the absence of isoleucine or with minimum content of valine or leucine. The lack of PanE activity did not change this capability, although all the growth parameters (OD_{max}, μ_{max} and lag) were diminished in *L. lactis* IFPL953Δ*panE* for all the assayed conditions. The growth of the strains in the absence of isoleucine or with minimum content of valine delayed the lag time up to three extra hours when compared with the strains growing in CDM containing all amino acids. Growth with minimum content of leucine also extended the lag phase, but to a lesser extent (Fig. 1). The highest value of OD_{max} for both strains was obtained using CDM (2.6 and 2.2 for IFPL953 and IFPL953Δ*panE*, respectively). Deficiency of valine or leucine in the CDM reduced the maximal growth of both strains, being the lowest value obtained under leucine starvation (OD_{max} 1.9 and 1.8 for IFPL953 and IFPL953Δ*panE*, respectively). Accordingly, the highest μ_{max} rates for both strains were attained when growing in CDM (μ_{max} 1.00 for IFPL953 and 0.86 for IFPL953Δ*panE*).

3.2. Effect of BCAA content and panE inactivation in the relative expression of L. lactis genes involved in amino acid catabolism

The effect of *L. lactis* IFPL953 and IFPL953Δ*panE* growth under BCAA starvation conditions in the expression of genes encoding the enzymes AraT, BcaT, GDH, KivD, YtjE and PanE is shown in Fig. 2. The *tuf* gene codifying the elongation factor TU was used as a reference gene (Guédon et al., 2005; Sperandio, Polard, Ehrlich, Renault, & Guédon, 2005). Gene expression was analysed over the bacterial growth at different stages: early exponential phase (Fig. 2A), late exponential phase (Fig. 2B) and stationary phase (Fig. 2C). The results of gene expression are relative to those when the strains were grown in CDM.

In general, expression of the genes *araT*, *bcaT*, *gdh*, *kivD* and *ytjE* increased during growth of both strains in CDM-Ile, CDM-Leu and CDM-Val, indicating overexpression of these genes under BCAA starvation conditions. In all cases, gene expression was higher during late exponential and stationary phases (Fig. 2B and C) than at the early exponential phase (Fig. 2A). Expression of the *panE* gene in *L. lactis* IFPL953 was highest at the stationary phase and, as expected, there was not *panE* expression in *L. lactis* IFPL953Δ*panE*.

The growth in absence of isoleucine was the main induction factor in gene regulation, particularly *araT*, *bcaT* and *panE*, and similar with valine starvation in the expression of *kivD* and *ytjE*. Leucine minimal content during growth induced higher overexpression values of *ytjE* and *gdh* during the exponential growth phase (Fig. 2). The inactivation of the *panE* gene, which limits the formation of α-hydroxy acids (Gómez de Cadiñanos et al., 2013), was related with higher expression of the *kivD* and *ytjE* genes and decreased expression of *araT* and *bcaT* (Fig. 2). On the other hand, the relative expression of the *gdh* gene of *L. lactis* IFPL953Δ*panE* was lower than that of IFPL953 during the exponential growth phase, but increased remarkably during the stationary growth phase, particularly under isoleucine and valine starvation conditions (21.87 ± 1.78 and 32.05 ± 14.19, respectively).

3.3. Formation of volatile compounds by L. lactis IFPL953 and IFPL953ΔpanE

Table 1 shows the most relevant volatile organic compounds (VOCs) identified using SPME-GC-MS generated by *L. lactis* IFPL953 and IFPL953Δ*panE* after 30 h of growth (stationary phase) in CDM with different BCAA content (CDM, CDM-Ile, CDM-Val and CDM-Leu). Overall, *L. lactis* IFPL953Δ*panE* gave rise to a higher number of VOCs than the wild strain IFPL953. The α-ketoisocaproate acid (KIC), resulting from the transamination of leucine and the preferred substrate of the HA-DH, was accumulated by the mutant strain in all cultures, except in CDM-Leu (minimum leucine content). Other acids such as propanoic and succinic were also detected with higher relative abundances in the CDM cultures with the mutant strain (Table 1).

Among the aldehydes, 2-methylbutanal, which derives from isoleucine, was only identified in the CDM and CDM-Leu cultures, and in both cases the relative abundances were significantly higher after the growth of *L. lactis* IFPL953Δ*panE* than those obtained with IFPL953. On the other hand, 3-methylbutanal, which comes from leucine, was produced abundantly by the two *L. lactis* strains, except in CDM-Leu, where it was not detected. Another aldehyde detected was benzaldehyde, which derives from the aromatic amino acid phenylalanine, and it was also produced in higher abundance by *L. lactis* IFPL953Δ*panE* in all growth conditions,

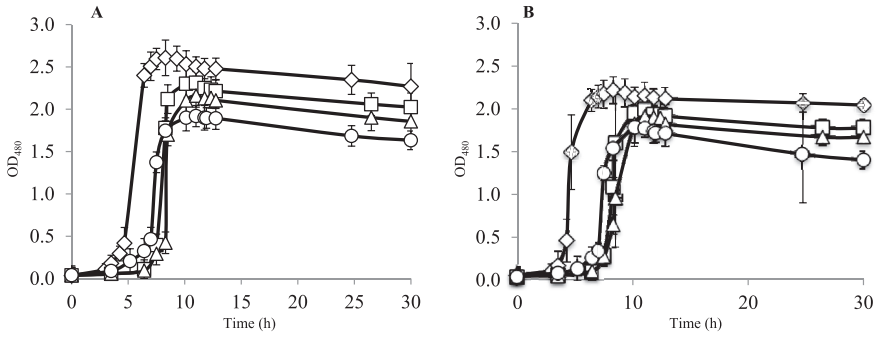

Fig. 1. Growth curves for *L. lactis* IFPL953 (A) and IFPL953Δ*panE* (B) incubated in CDM with all amino acids (◇), CDM without isoleucine (□) and CDM with minimal supporting growth content of valine (△) and leucine (○). Values are the mean of three independent experiments.

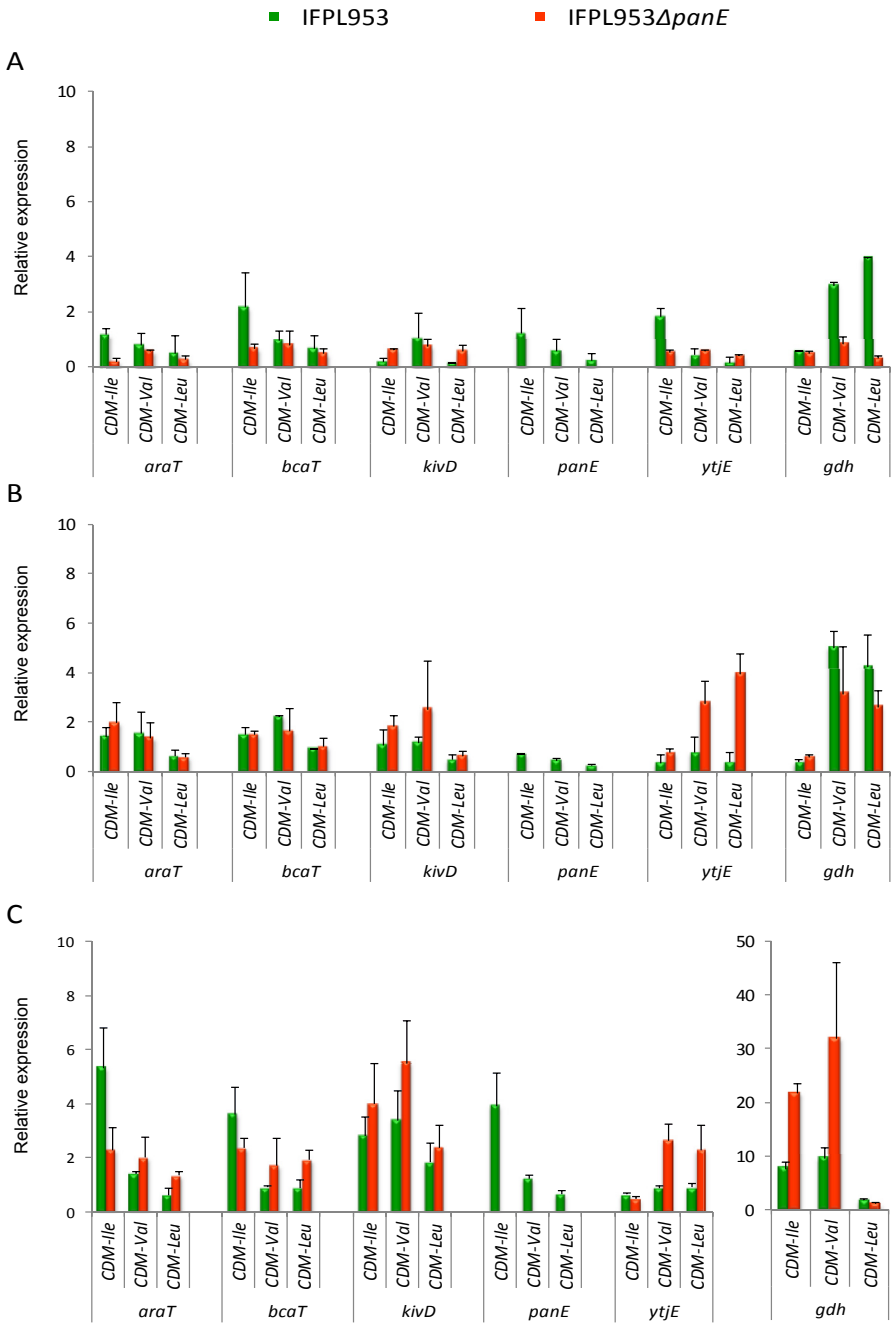

Fig. 2. Comparison of the relative expression levels of *araT, bcaT, kivD, panE, ytjE* and *gdh* of *L. lactis* IFPL953 (■) and IFPL953Δ*panE* (■) in cells growing in CDM without isoleucine (CDM-Ile) and CDM with minimal supporting growth content of valine (CDM-Val) and leucine (CDM-Leu). The analysis was carried out at early exponential phase (A), late exponential phase (B) and stationary phase (C). Gene expression was quantified using real-time RT-PCR and the growth condition in CDM with all amino acids was defined as the calibrator. Values are the mean of three experiments performed in triplicate for each gene.

except in CDM-Ile (Table 1). The formation of 2-methylbutanol and 3-methylbutanol, generated after the reduction of 2-methylbutanal and 3-methylbutanal, respectively, was higher by *L. lactis* IFPL953Δ*panE* than the wild strain IFPL953, which only produced 2-methylbutanol in CDM-Leu. Other alcohols detected were 2-ethyl-1-hexanol, 2-hexanol and 1-nonanol, mainly after the growth of the strains in basal CDM (Table 1).

In relation to the ketones detected after the growth of *L. lactis* IFPL953 and IFPL953Δ*panE*, it is noteworthy that it was the group of major VOCs produced by the two strains (Table 1). In general, the mutant strain produced more ketones than the wild type. Some compounds, such as 2-heptanone and 2-nonanone, were only detected after the growth of the mutant strain. Another ketone that was only detected after the growth of *L. lactis* IFPL953Δ*panE* was 2,3-pentanedione, which is derived from α-aceto-α-hydroxybutyrate (an intermediate of isoleucine biosynthesis). This VOC has been detected in cheeses manufactured with *L. lactis* (Garde, Ávila, Fernández-García, Medina, & Núñez, 2007).

Table 1
Volatile compounds detected by SPME-GC-MS in *L. lactis* IFPL953 and IFPL953Δ*panE* grown in CDM with all amino acids, CDM without isoleucine (CDM-Ile) and CDM with minimal supporting growth content of valine (CDM-Val) and leucine (CDM-Leu).

Compound	CDM		CDM-Ile		CDM-Val		CDM-Leu	
	IFPL953	IFPL953Δ*panE*	IFPL953	IFPL953Δ*panE*	IFPL953	IFPL953Δ*panE*	IFPL953	IFPL953Δ*panE*
Acids								
Pyruvic	5.81 ± 1.30	12.49 ± 2.15*	0.48 ± 0.18	1.33 ± 0.39*	–	1.89 ± 0.26*	–	–
Propanoic	2.45 ± 1.01	8.78 ± 0.96*	1.24 ± 0.26	5.54 ± 1.42*	–	4.21 ± 2.01*	27.80 ± 3.60*	20.48 ± 2.40
α-Ketoisocaproic	–	38.08 ± 7.12*	–	45.63 ± 5.38*	–	42.62 ± 6.31*	–	–
Succinic	–	13.15 ± 1.73*	–	12.59 ± 2.78*	–	35.52 ± 2.87*	–	10.46 ± 1.65*
Aldehydes								
Benzaldehyde	10.38 ± 3.11	21.18 ± 3.41*	15.56 ± 5.23*	1.46 ± 0.65	30.62 ± 7.19	52.59 ± 6.45*	27.51 ± 5.18	31.32 ± 5.73
2-Methylbutanal	5.28 ± 1.05	10.10 ± 2.15*	–	–	–	–	8.67 ± 1.94	20.10 ± 4.14*
3-Methylbutanal	23.43 ± 1.74	30.13 ± 5.02	46.38 ± 6.34	54.12 ± 8.62	29.74 ± 1.41	60.76 ± 8.08*	–	–
Alcohols								
2-Methylbutanol	–	10.52 ± 1.74*	–	–	–	3.22 ± 0.31*	6.73 ± 1.67	17.74 ± 2.51*
3-Methylbutanol	10.49 ± 2.15	17.94 ± 1.17*	15.24 ± 1.32	32.58 ± 5.60*	18.35 ± 2.07	29.10 ± 3.81*	–	–
2-Hexanol	5.24 ± 1.58	50.07 ± 5.38*	–	–	–	–	–	–
2-Ethyl-1-hexanol	2.27 ± 0.77	12.24 ± 1.73*	–	–	–	–	–	–
1-Nonanol	10.11 ± 3.15	9.78 ± 0.87	–	–	–	13.17 ± 1.86*	–	12.92 ± 1.30*
Ketones								
Acetone	13.29 ± 2.52	17.86 ± 2.63	22.91 ± 1.61	31.61 ± 3.10*	14.40 ± 1.43	21.53 ± 2.63*	12.13 ± 1.84	16.08 ± 2.27*
2-Butanone	30.18 ± 0.52	45.52 ± 2.87*	–	10.32 ± 1.60*	2.61 ± 0.38	14.54 ± 1.78*	2.71 ± 0.19	10.24 ± 1.09*
Diacetyl	2.60 ± 0.46	3.00 ± 0.57	–	–	2.78 ± 0.44	4.04 ± 0.43*	2.89 ± 0.71	6.39 ± 1.90*
2-Pentanone	–	21.34 ± 1.89*	–	21.66 ± 1.78*	–	19.19 ± 1.98*	–	17.79 ± 1.35*
2.3-Pentanedione	–	20.28 ± 1.87*	–	–	–	–	–	12.31 ± 1.43*
4-Methyl-2-pentanone	18.99 ± 1.40	26.49 ± 2.61	10.56 ± 2.62	12.15 ± 4.60	18.43 ± 2.36	28.49 ± 3.70*	11.43 ± 1.33	20.64 ± 2.41
2-Methyl-5-hexanone	21.98 ± 4.60	26.54 ± 1.78	16.51 ± 2.72	17.78 ± 1.50	15.19 ± 1.03	14.20 ± 1.58	–	–
2-Heptanone	–	12.16 ± 1.72*	–	17.45 ± 2.47*	–	–	–	–
4-Methyl-2-heptanone	8.44 ± 5.13	10.89 ± 2.95	15.20 ± 3.28	18.38 ± 2.60	10.17 ± 2.24	15.94 ± 2.25	16.15 ± 3.21	13.74 ± 1.94
4.6-Dimethylheptanone	24.21 ± 7.00	28.35 ± 3.10	–	35.73 ± 1.90*	25.12 ± 5.71	28.55 ± 5.82	20.09 ± 6.13	20.16 ± 2.14
2-Nonanone	10.35 ± 1.14	15.54 ± 3.05	–	22.21 ± 3.14*	–	–	–	–

[a] Data (mean ± std) represent relative abundance (%) against 4-methyl-2-pentanol (internal control) from duplicate determinations in three experiments; -, not detected; an asterisk indicates significant difference ($P < 0.05$) between *L. lactis* IFPL953 and IFPL953Δ*panE*.

4. Discussion

The BCAAs represent important nutrients in the bacterial adaptation to amino acid starvation (Kaiser & Heinrichs, 2018). Overall, the lack of the α-hydroxyacid dehydrogenase (HA-DH or PanE) activity did not change the *L. lactis* IFPL953 growth profile under BCAA starvation conditions. Similar growth behaviour was previously observed when both strains were grown in milk (Gómez de Cadiñanos et al., 2013). Inactivation of some genes encoding enzymes involved in amino acid catabolism such as the *gdh* gene of *Lactobacillus plantarum* negatively affects bacterial growth (Siragusa et al., 2011).

The fact that we observed growth of *L. lactis* IFPL953 and IFPL953Δ*panE* in absence of isoleucine supports the hypothesis that the species is not auxotrophic for this amino acid (Dressaire et al., 2011). Godon et al. (1993) postulated that *L. lactis* strains isolated from dairy products are auxotrophic for all BCAAs. On the other hand, strains isolated from raw-milk cheeses or non-dairy environments are prototrophic for isoleucine (García-Cayuela et al., 2012; Machii et al., 2013), and auxotrophic for valine and leucine despite encoding the BCAA biosynthetic operon. Regarding this, it has been demonstrated that the apparent auxotrophy of *Staphylococcus aureus* for leucine and valine is due to the fact that the biosynthesis of both amino acids is inhibited by isoleucine via CodY repression of the BCAA biosynthetic genes, whereas inactivation of CodY results in growth of *S. aureus* in media lacking either isoleucine, leucine or valine (Kaiser et al., 2018). Likewise, the expression of the complete operon encoding BCAA biosynthesis pathway in *L. lactis* IL1403 is strongly increased during isoleucine starvation (Dressaire et al., 2011). Therefore, isoleucine plays a dominant role in controlling the expression of genes involved in BCAA synthesis and transport and then the growth under BCAA starvation conditions.

The results observed in our study regarding BCAA regulation are in accordance with previous results observed for the genus *Lactococcus* (Chambellon & Yvon, 2003; Guédon et al., 2005). These expression patterns suggest that most genes encoding AACE may be subject to regulation by the global regulator of nitrogen metabolism CodY. This global regulator represses amino acid biosynthesis and its activity would stop when bacteria grow in a BCAA deficient media (Guédon et al., 2001). We previously demonstrated that expression of the *kivD*, *bcaT* and *ytjE* genes from *L. lactis* IPL730 was also markedly increased under isoleucine and valine starvation conditions (García-Cayuela et al., 2012). A putative CodY-box sequence in the promoter region of the *kivD* gene from *L. lactis* IFPL730 that was regulated at the transcription level has been previously identified (De la Plaza et al., 2009). Our results indicated that *gdh* was the most affected gene by BCAA starvation in both strains with a clear induction of the gene expression. Induction of genes involved in glutamate catabolism was also observed by Dressaire et al. (2011) during growth of *L. lactis* IL1403 under isoleucine starvation. The overexpression of the *gdh* gene found during the stationary phase of *L. lactis* IFPL953Δ*panE* growing under isoleucine and valine deficiencies can be explained by the absence of HA-DH activity in the mutant strain. HA-DH is an enzyme playing a physiological role in NAD^+ regeneration needed for BCAA catabolism (Chambellon et al., 2009). The absence of this HA-DH activity in IFPL953Δ*panE* could induce the expression of GDH activity to keep playing the dehydrogenase activity and maintenance of the balance NAD/NADH. In this sense, Siragusa et al. (2011) observed that mutation of the *gdh* gene in *L. plantarum* leads to an increase of the NAD-dependent dehydrogenase activity.

The increased expression of the *bcaT*, *gdh* and *kivD* genes during the stationary phase of the two strains growing in CDM-Ile (Fig. 2) was manifested in the abundances obtained for 3-methylbutanal and 3-methylbutanol (Table 1). This increase in

volatile formation was even more evident with *L. lactis* IFPL953Δ*panE*, which due to the lack of HA-DH activity produced high amounts of KIC, a direct product of the transamination of leucine and precursor of 3-methylbutanal and 3-methylbutanol. 3-Methylbutanal has been identified as a potent aroma compound in Camembert, Cheddar, Emmental and Gruyere cheeses (Rychlik & Bosset, 2001) and 3-methylbutanol has been positively related to fresh Mozzarella aroma (Moio, Dekimpe, Etievant, & Addeo, 1993) and caramel-like aroma in some European cheese varieties (Lawlor, Delahunty, Wilkinson, & Sheehan, 2001). The higher production of benzaldehyde by *L. lactis* IFPL953Δ*panE* in CDM-Val (Table 1) could be related to the increased expression of *gdh* and *kivD* (Fig. 2), which together with the inactivation of *panE* would favour the accumulation of phenylpyruvate and its subsequent degradation to benzaldehyde. The use in Cheddar cheese manufacture of *Lactobacillus casei* that overexpressed HA-DH activity reduced the formation of benzaldehyde during ripening (Broadbent et al., 2004).

L. lactis IFPL953Δ*panE* produced more ketones than the wild strain, which can be related to the accumulation of branched-chain α-keto acids that are the preferred substrate of the *L. lactis* HA-DH (Chambellon et al., 2009). The accumulation of α-keto acids from the catabolism of BCAAs in *Bacillus subtilis* enhances the production of branched chain fatty acids and their subsequent degradation to ketones by oxidative decarboxylation (Oku & Kaneda, 1988). Ganesan and Weimer (2004) also demonstrated the participation of *L. lactis* BcaT in the synthesis of branched chain fatty acids from α-keto acids. This connection between metabolic pathways would explain the greater increase in the formation of certain ketones, such as 2-pentanone, 2-heptanone and its 4-methyl derivatives, in the incubations with the mutant strain IFPL953Δ*panE* than with the wild strain (Table 1). The increase of ketones would be favoured by the greater availability of α-keto acids caused by the combined effect of the inactivation of HA-DH activity and the increase of *gdh* expression. In this sense, Broadbent et al. (2004) observed that overexpression of HA-DH in *L. casei* significantly decreased the formation of 2-heptanone, while the content of this ketone was restored by the addition of α-ketoglutarate.

5. Conclusions

From the results of this work we can conclude that the absence or very limited amounts of the BCCA Leu, Ile and Val in the growth medium of *L. lactis* IFPL953 and its mutant IFPL953 Δ*panE* deficient in HA-DH activity, triggers an increase of the expression of genes involved in amino acid catabolism. This indicates, therefore, that these genes may be subject to regulation by the global regulator of nitrogen metabolism CodY. The highest gene expression is observed with the *gdh* gene under isoleucine and valine starvation conditions. The absence of HA-DH activity enhances the formation of metabolites from BCAAs, such as 2- and 3-methylbutanal and their corresponding alcohols. Furthermore, the greater availability of α-keto acids in the medium caused by the inactivation of HA-DH and the increase of *gdh* expression in IFPL953Δ*panE* leads to an increase in the formation of ketones. These findings enhance the knowledge of AACE regulation of *L. lactis* and its potential contribution to cheese flavour under cheesemaking conditions.

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