



## Natural diversity of aminotransferases and dehydrogenase activity in a large collection of *Lactococcus lactis* strains

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### ABSTRACT

The natural diversity of *Lactococcus lactis* was studied with respect to the flavour-generating enzymes leucine (Leu-AT) and aromatic (AraT) aminotransferase, and hydroxyisocaproic acid dehydrogenase (HicDH). The screening of 175 *L. lactis* strains required the development of enzyme assays to enable the use of high throughput screening facilities. Natural diversity was expressed as the difference between 10% of the strains with the highest and lowest enzyme activity. The natural diversity found for Leu-AT, AraT and HicDH activity was 13-, 49- and 30-fold, respectively. Leu-AT activity showed a weak correlation with the AraT and HicDH activity, whereas there was no correlation between AraT and HicDH activity. In the group of strains with the highest Leu-AT activity, 5- and 6-fold differences of AraT and HicDH activities were found, respectively. These results demonstrated that it is possible to select strains with specific combinations of important ripening enzymes that could enhance cheese flavour formation.

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### 1. Introduction

Soluble proteins released from casein by the action of chymosin and plasmin are degraded by lactic acid bacteria (LAB) and play a major role in the flavour development of matured cheese. With a complex set of proteolytic and peptidolytic enzymes, LAB are able to degrade the released proteins in several steps from oligopeptides via di- and tripeptides to, finally, amino acids (Baankreis, 1992; Kunji, Mierau, Hagting, Poolman, & Konings, 1996). Amino acids are converted by aminotransferases to  $\alpha$ -ketoacids that can be degraded by enzymatic or non-enzymatic reactions to flavour-generating compounds (Alting, Engels, van Schalkwijk, & Exterkate, 1995; Engels et al., 2000; Rijnen et al., 2003; Smit et al., 2004; Yvon, Thirouin, Rijnen, Fromentier, & Gripon, 1997).

The typical mature flavour of Gouda and Cheddar cheeses depends on the degradation of branched chain amino acids (BcAA) and methionine (Engels et al., 2000; Weimer, Seefeldt, & Dias, 1999). The  $\alpha$ -ketoacids derived from aromatic amino acids (ArAA) are converted to floral generating flavours (Rijnen et al., 1999) and are generally considered as off-flavours in these types of cheeses (Broadbent et al., 2004). As well as the formation of flavour-generating compounds,  $\alpha$ -ketoacids can also be converted to compounds that do not contribute to flavour (Yvon & Rijnen, 2001).

The enzymatic conversion of  $\alpha$ -ketoacids originating from BcAA and methionine towards hydroxy acids is especially considered to be unwanted. An important enzyme in this respect is hydroxyisocaproic acid dehydrogenase (HicDH), which converts  $\alpha$ -ketoacids from BcAA, methionine and also ArAA into hydroxy acids (Hummel, Schutte, & Kula, 1985). Broadbent et al. (2004) suggested the use of strains with high HicDH activity to prevent the formation of floral off-flavours in Cheddar. Unfortunately, in cheese experiments, it was demonstrated that the overall flavour development was less appreciated, probably due to the reduction of  $\alpha$ -ketoacids originating from BcAA and methionine. It was demonstrated by Rijnen et al. (2003) that BcAA, methionine and ArAA degradation can also be influenced in cheese by *Lactococcus* strains with different relative activities of branched chain (BcaT) and aromatic aminotransferase (AraT). This suggests that the aroma profile and aroma intensity of cheese can be manipulated by selecting *Lactococcus* strains with specific combinations of BcaT, AraT and HicDH activities. Therefore, such work emphasizes the relevance of studying the biodiversity and correlation of these enzymes in *L. lactis* strains.

The natural diversity of proteolytic enzyme activities in *Lactococcus* strains has been studied mainly in a limited number of strains at the level of proteinases and peptidases. Coolbear, Pillidge, and Crow (1994) found that the total proteinase activity in *L. lactis* subsp. *cremoris* and subsp. *lactis* varies with a factor of 8- and 15-fold, respectively. In the same set of *Lactococcus* strains, the diversity of six peptidases was also determined. The variation in levels of

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peptidase activity was between 2- and 22-fold (Crow, Holland, Pritchard, & Coolbear, 1994). The BcaT and AraT activity was studied by Fernández de Palencia, de la Plaza, Amárta, Requena, and Peláez (2006) for *L. lactis* strains isolated from traditional Spanish produced cheeses. These authors found a BcaT and AraT variation of 1.5- and 14-fold, respectively.

The aim of this study was to determine the natural diversity of *L. lactis* strains to transaminate BcAA and ArAA, and HicDH activity. Enzyme assays were developed or adapted to enable the use of high throughput facilities. The natural enzyme diversity was measured in a large collection of strains of dairy origin. Furthermore, the possibility of selecting strains with high aminotransferase activity towards BcAA, low activity towards ArAA and low HicDH activity was determined.

## 2. Materials and methods

### 2.1. Chemicals

Pyridoxal-5'-phosphate, L-leucine, L-tryptophan and  $\alpha$ -ketoglutarate were obtained from Sigma-Aldrich (Zwijndrecht, The Netherlands); other chemicals were obtained from Merck (Darmstadt, Germany).

### 2.2. Strains and growth conditions

A collection of 175 *L. lactis* strains was obtained from NIZO food research (Ede, The Netherlands). *L. lactis* subsp. *cremoris* B643 (NCDO 763) was used as a reference strain and incubated on all microtitre plates to determine the relative specific enzyme activities. Strains were stored in M17 + 1% lactose and with 30% glycerol on 96-wells microtitre plates at  $-80^{\circ}\text{C}$ . In order to determine the incubation time for high throughput screening, the growth rate and outgrowth during 24 h of incubation were measured for 14 strains grown in quadruplicate on 200  $\mu\text{L}$  M17 + 1% lactose in a microtitre plate reader Spectramax M2 (Molecular Devices, Wokingham, England). Growth rate ( $\mu$ ) was calculated with the growth equation:

$$\ln(\text{OD}_{600\text{ nm}})_{t=t} = \ln(\text{OD}_{600\text{ nm}})_{t=0} + \mu t$$

and expressed as  $\text{h}^{-1}$ . Enzyme assays were validated with the wild type (WT) strain *L. lactis* subsp. *cremoris* TIL46 (NCDO 763 cured of its 2-kb plasmid) and modified strains (Chambellon & Yvon, 2003). The genes disrupted from the WT strain were *araT* (TIL313), *bcaT* (TIL357) and the double disruption of *araT* and *bcaT* (TIL358). The overexpressed genes in the WT strain were *bcaT* (WT with pTIL252 plasmid) and *araT* (WT with pTIL200 plasmid). All strains were kindly provided by INRA (Jouy-en-Josas, France). The WT and modified strains were grown in M17 + 1% glucose with erythromycin ( $5\text{ mg L}^{-1}$ ) and chloramphenicol ( $10\text{ mg L}^{-1}$ ) when necessary. Overexpression was induced by adding nisin ( $2.5\text{ }\mu\text{g L}^{-1}$ ). Specific enzyme activities were expressed as  $\mu\text{mol min}^{-1}\text{ mg}^{-1}$  protein by measuring the protein content of the cell extract (BCA Protein Assay, Pierce Biotechnology, Rockford, IL, USA).

The linearity between the cell extract of B643 and enzyme activities was determined in duplicate. Cells were grown in M17 + 1% lactose for 24 h at  $30^{\circ}\text{C}$ , washed, concentrated 10 times, re-suspended in 50 mM sodium phosphate buffer (pH 7.2) and stored at  $-40^{\circ}\text{C}$ . Cell extracts were prepared by beating cells (Fastprep FP120, ThermoSavant, Ramsey, MN, USA) with 1–1.2 g, 0.1 mm Zirconia beads (Biospec Products, Bartlesville, OK, USA) five times for 30 s with intensity 4. After beating, cells were stored for 2 min on melting ice. Cell extracts were obtained by centrifugation (Eppendorf 5417R, Hamburg, Germany) at  $10,000 \times g$  for 5 min.

### 2.3. Enzyme assays

#### 2.3.1. Branched chain aminotransferase assay

The aminotransferase (AT) activity was determined by the maximum reduction rate of NADH measured by the absorbance at 340 nm for 15 min at  $30^{\circ}\text{C}$ . The specific AT activity was expressed as  $\mu\text{mol min}^{-1}\text{ OD}_{600\text{ nm}}^{-1}$ . The assay reaction mixture contained 25 mM potassium phosphate at pH 7.5, 0.05 mM pyridoxal-5'-phosphate, 50 mM ammonium sulphate, 0.5 mM NADH, 10 mM  $\alpha$ -ketoglutarate, 100  $\mu\text{L}$  cell extract and 20 mM amino acid as substrate in a final volume of 250  $\mu\text{L}$ . The AT activity for L-leucine was determined in duplicate with and without the addition of 2.5 units of leucine dehydrogenase (LeuDH) from *Bacillus cereus* (Calbiochem, VWR International, Roden, The Netherlands) to the assay reaction mixture. The AT activity towards L-leucine, L-isoleucine and L-valine was determined in duplicate without the addition of LeuDH.

#### 2.3.2. Aromatic aminotransferase assay

The AraT activity was determined by following the conversion of L-tryptophan to 4-hydroxyphenylpyruvate and indole-3-pyruvate. The assay mixture contained 25 mM sodium tetraborate buffer at pH 8.5, 0.05 mM pyridoxal-5'-phosphate, 20 mM L-tryptophan, 10 mM  $\alpha$ -ketoglutarate, 0.5 mM sodium-EDTA and 100  $\mu\text{L}$  cell extract in a final volume of 250  $\mu\text{L}$ . The AraT activity was determined by measuring the maximum indole-3-pyruvate production at 327 nm for 30 min at  $37^{\circ}\text{C}$ , and expressed as  $\text{OD}_{327\text{ nm}}\text{ min}^{-1}\text{ OD}_{600\text{ nm}}^{-1}$ .

#### 2.3.3. Hydroxyisocaproic acid dehydrogenase assay

The HicDH activity was measured by determining the maximum NADH reduction rate at 340 nm for 15 min at  $30^{\circ}\text{C}$  used to convert  $\alpha$ -ketoacids into hydroxy acids. The assay reaction mixture contained 25 mM potassium phosphate at pH 7.5, 0.5 mM NADH, 10 mM  $\alpha$ -ketoisocaproic acid and 10  $\mu\text{L}$  cell extract in a final volume of 250  $\mu\text{L}$ . The HicDH activity was expressed as  $\mu\text{mol min}^{-1}\text{ OD}_{600\text{ nm}}^{-1}$ .

### 2.4. High throughput screening of the *L. lactis* collection

Strains were incubated in quadruplicate in 1.5 mL M17 + 1% lactose in deepwell plates and grown at  $30^{\circ}\text{C}$  for 24 h. Cells were centrifuged (Beckman Coulter Avanti JS-5.3, Fullerton, CA, USA) for 10 min at  $2500 \times g$ , washed three times and re-suspended in 1.2 mL 50 mM sodium phosphate buffer (pH 7.2). The optical density ( $\text{OD}_{600\text{ nm}}$ ) was determined and cells were stored at  $-20^{\circ}\text{C}$ . Cell extracts were prepared by incubation with lysozyme ( $20\text{ g L}^{-1}$ ), mutanolysin ( $100\text{ U mL}^{-1}$ ) and tetracyclin ( $2.5\text{ mg L}^{-1}$ ) for 1 h at  $30^{\circ}\text{C}$ . The handling of cultures was performed with Genesis workstation 150/8 (Tecan Benelux, Giessen, The Netherlands) and spectrophotometrical measurements with Spectramax Plus384 (Molecular Devices).

### 2.5. Data analysis

The possibilities of choosing strains with different combinations of enzyme activities, and the effect of outgrowth ( $\text{OD}_{600\text{ nm}}$ ) on enzyme activities were determined. The average values of two or more Leu-AT, AraT, HicDH activities and  $\text{OD}_{600\text{ nm}}$  measurements were analysed using linear regression. A linear model was made for all combinations of enzyme activities and/or  $\text{OD}_{600\text{ nm}}$  with the least squares method. The Fisher distribution ( $P < 0.05$ ) was used to determine if the combination was significantly correlated, and the level of correlation was expressed by the correlation coefficient ( $r^2$ ).

### 3. Results

#### 3.1. Assay development for high throughput screening facilities

Assays were developed to determine the enzyme activities of multiple *L. lactis* strains with high throughput screening (HTS) facilities. The capacity of strains to convert branched chain amino acids (BcAA) into  $\alpha$ -ketoacids was based on the branched chain aminotransferase (BcaT) activity assay described by Cooper, Conway, and Hutson (2002). In this assay, leucine was transaminated in the presence of  $\alpha$ -ketoglutarate into  $\alpha$ -ketoisocaproic acid. The BcaT activity was indirectly measured by the NADH reduction of the leucine dehydrogenase (LeuDh) catalyzed conversion of  $\alpha$ -ketoisocaproic acid into leucine. Assuming that the natural occurring dehydrogenase activity of *L. lactis* was high enough, the BcaT assay was simplified for HTS purposes by omitting the use of LeuDh. The simplified assay was validated with 20 randomly chosen strains from the *L. lactis* collection. It was found that transamination of leucine with and without LeuDh was comparable (Fig. 1). Furthermore, it was shown that the reduction of  $\alpha$ -ketoisocaproic acid by HicDH of all strains was more than 3-fold higher compared with the production of  $\alpha$ -ketoisocaproic acid from leucine by aminotransferase (AT) activity. This demonstrated that transamination of leucine into  $\alpha$ -ketoisocaproic acid was the rate-limiting reaction and that natural occurring dehydrogenase activity of *L. lactis* strains was enough to remove LeuDh from the assay reaction mixture.

Modified strains were used to study the AT activity for BcAA. The *bcaT*-overexpressing strain showed a significantly higher activity for all BcAA compared with the wild type (WT) strain. In the *bcaT*-disrupted strain the conversion of isoleucine and valine was strongly reduced, whereas the effect on leucine conversion was limited. A negligible conversion of leucine was only found when both *bcaT* and *araT* genes were disrupted. The *araT*-overexpressing strain converted leucine around 10 times faster, whereas the conversion of isoleucine and valine was comparable with the WT strain, showing that both BcaT and AraT were able to transaminate leucine (Fig. 2). These results correspond with observations made by Yvon, Chambellon, Bolotin, and Roudot-Algaron (2000). Therefore, to determine the total capacity of *L. lactis* strains to convert BcAA, leucine was used as a substrate for the high throughput AT activity assay, catalysed by Leu-AT.

The AraT activity was determined by measuring the conversion of tryptophan into idole-3-pyruvate. Validation experiments with

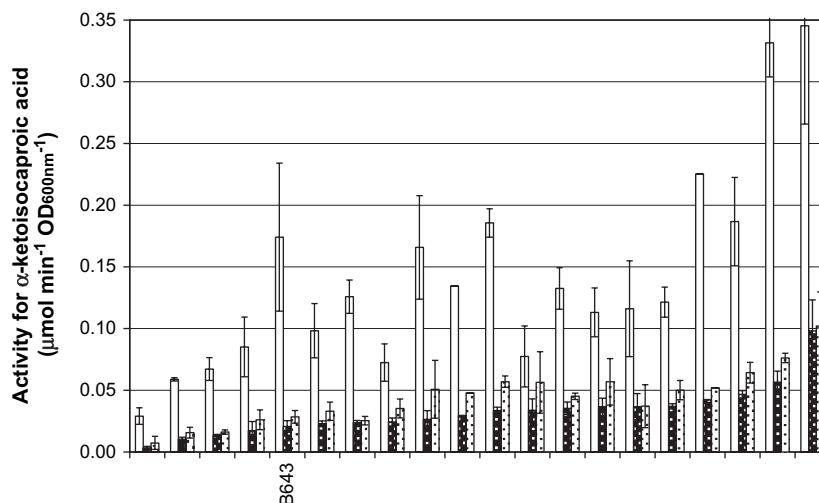
WT and *araT*-overexpressing strains showed that the AraT activity increased from 0.38 (S.D. = 0.01) to 4.45 (S.D. = 0.04) OD<sub>327 nm</sub> min<sup>-1</sup> mg<sup>-1</sup> protein, respectively. Overexpression of the *bcaT* gene had a small effect on the AraT activity (0.67, S.D. = 0.02). The AraT activity of both the *araT*- and *araT/bcaT*-disrupted strains was below 0.05 OD<sub>327 nm</sub> min<sup>-1</sup> mg<sup>-1</sup> protein. This indicated that BcaT activity had a negligible effect on the AraT measurement, which corresponded with the results performed with comparable AraT activity measurements (Chambellon & Yvon, 2003; Rijnen, Bonneau, & Yvon, 1999).

The hydroxyketoacid dehydrogenase activity was studied for  $\alpha$ -ketoacids originating from BcAA.  $\alpha$ -Ketoisocaproic acid, the  $\alpha$ -ketoacid originating from leucine, gave the highest dehydrogenase activity, 0.9  $\mu$ mol min<sup>-1</sup> mg<sup>-1</sup> protein, for the WT strain. The influence of AraT and BcaT activity on hydroxyisocaproic acid dehydrogenase (HicDH) activity was studied with the modified strains. The WT strain and the *araT*- and *bcaT*-overexpressing and disrupted strains had HicDH activities ranging from 0.7 to 1  $\mu$ mol min<sup>-1</sup> mg<sup>-1</sup> protein. This showed that both BcaT and AraT activity levels had no disturbing effect on HicDH activity measurement.

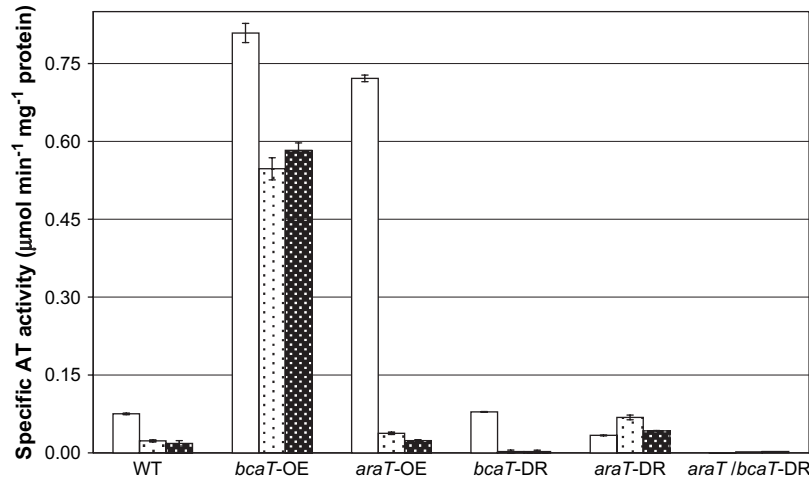
The Leu-AT, AraT and HicDH assays were quantitatively validated by determining the enzyme activity in relation to the amount of cell extract. It was found that the amount of cell extract was linear with respect to the enzyme activities (Fig. 3). This enabled screening of strains with a large difference in enzyme activities.

#### 3.2. High throughput screening of *L. lactis* strains

The growth of 14 randomly selected strains from the *L. lactis* collection was determined. The growth rates varied between 0.250 h<sup>-1</sup> (S.D. = 0.004) and 0.528 h<sup>-1</sup> (S.D. = 0.004). All cells reached the stationary growth phase after 6–16 h of incubation. In addition, after 24 h of incubation, the OD<sub>600 nm</sub> was at least 90% of the maximum obtained OD<sub>600 nm</sub>. The collection of 175 *L. lactis* strains was incubated for 24 h to ensure that cells were in the stationary phase. Twelve strains did not grow or the enzyme activity measurement failed. The outgrowth of 163 of the 175 *L. lactis* strains showed a normal distribution between OD<sub>600 nm</sub> 0.5 and 3 (Fig. 4). There was no significant correlation ( $P < 0.05$ ) found between the outgrowth of individual strains and the specific Leu-AT, AraT or HicDH activity. The average standard deviation of the specific Leu-AT, AraT and HicDH activities found for the collection of *L. lactis* strains was 31, 20 and 22% of the mean, respectively.



**Fig. 1.** The specific reduction of  $\alpha$ -ketoisocaproic acid by hydroxyisocaproic acid dehydrogenase (HicDH) ( $\square$ ) was determined for 19 *Lactococcus lactis* strains and the reference strain B643. The specific production of  $\alpha$ -ketoisocaproic acid from leucine was measured by the Leu-aminotransferase assay with (▨) or without (■) the addition of leucine dehydrogenase to the assay mixture.



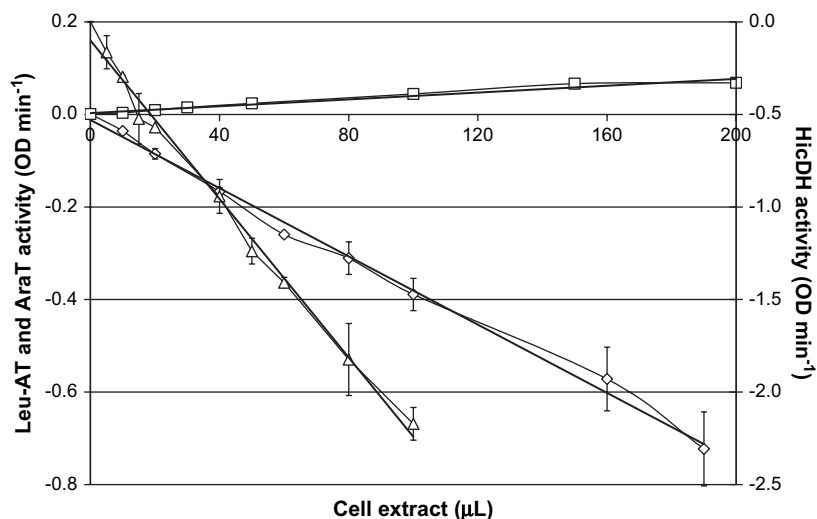
**Fig. 2.** The specific aminotransferase (AT) activity towards leucine (□), isoleucine (▨) and valine (■) determined using the Leu-AT assay. The effects of overexpression (OE) and disruption (DR) of *bcaT* and/or *araT* genes on enzyme activities were studied in relation to the wild type (WT) strain.

The natural diversity of the *L. lactis* collection was expressed by the difference between 10% ( $n = 16$ ) of the strains with the highest and lowest enzyme activity. The Leu-AT and AraT activities showed a Gaussian distribution (Fig. 5) with a natural diversity of 13- and 49-fold, respectively. A weak ( $r^2 = 0.32$ ), but significant ( $P < 0.05$ ) correlation was found between the Leu-AT and AraT activity. A similar distribution, with a natural diversity of 30-fold, was found for the HicDH activity (data not shown). The HicDH activity did not significantly ( $P < 0.05$ ) correlate with AraT, whereas a small ( $r^2 = 0.12$ ) but significant ( $P < 0.05$ ) correlation was found with Leu-AT activity.

Strains with relatively high Leu-AT, low AraT and HicDH activities have the potency to enhance the intensity of cheese flavour. The variation in the level of AraT and HicDH activity was determined for the group ( $n = 16$ ) of strains with the highest Leu-AT activity. In this respect, the most favourable subgroup of strains had a relative HicDH and AraT activity of around 1 (Fig. 6). Strains with a relative AraT activity below 1 were mainly found in combination with a higher HicDH activity. The overall difference between strains with the highest and lowest AraT and HicDH activity was 5- and 6-fold, respectively.

#### 4. Discussion

Degradation of casein has a significant influence on the flavour development in cheese. The conversion of branched chain amino acids (BcAA) to flavour compounds catalyzed by enzymes originating from lactic acid bacteria is especially of major importance for matured Gouda and Cheddar cheeses (Banks et al., 2001; Engels et al., 2000; Yvon et al., 1997). The first rate-limiting step for the production of these aromatic compounds is the availability of  $\alpha$ -ketoglutarate for the transamination of amino acids (Yvon, Berthelot, & Gripon, 1998). In this respect, all the available  $\alpha$ -ketoglutarate is preferably used for the transamination of BcAA. Thus, low AraT and HicDH activities are required to minimize the loss of  $\alpha$ -ketoglutarate by the production of unwanted floral flavours or non-aromatic hydroxy acids, respectively. Previous studies with relatively small collections of *L. lactis* showed that the variation of proteolytic activities was limited (Coolbear et al., 1994; Crow et al., 1994; Fernández de Palencia et al., 2006). Therefore, in this study the natural diversity of enzymes involved in the first rate-limiting step of flavour formation was studied in a large collection of *L. lactis* strains.



**Fig. 3.** The quantitative validations of the Leu-aminotransferase (Leu-AT) (◇), aromatic aminotransferase (AraT) (□) and hydroxyisocaproic acid dehydrogenase (HicDH) (△) assays performed by measuring the enzyme activities of the reference strain B643 grown on M17 + 1% lactose for 24 h at 30 °C, in relation to the amount of cell extract used in the assay.

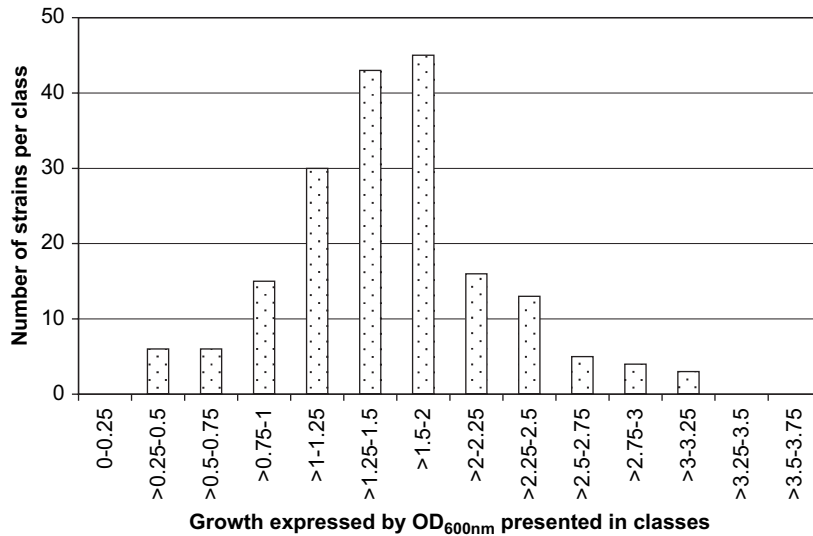


Fig. 4. Growth of the *Lactococcus lactis* strains in microtitre plates as expressed by the optical density (OD) at 600 nm. Strains were classified in classes with an OD<sub>600 nm</sub> of 0.25.

In recent years, screening and selection facilities have developed enormously, enabling the handling of large collections of bacteria (Fletcher, 2006). The screening of lactic acid bacteria for dairy applications is ideally performed on milk-incubated cells. Unfortunately, milk is difficult to handle due to clotting, which interferes with spectrophotometrical measurements. To enable HTS, the *L. lactis* strains were grown on laboratory media which will, to a certain extent, influence the enzyme activities. Proteolytic activity of *L. lactis* is influenced by the growth phase (Meijer, Marugg, & Hugenholtz, 1996). Williams, Noble, Tammam, Lloyd, and Banks (2002) showed that aminotransferase activity of *Lactobacillus* sp. increased between the end-exponential and stationary phase. Here, the enzyme activities were measured after 24 h to ensure that all cells were in the stationary phase. The outgrowth of individual strains and the enzyme activities did not significantly correlate, which further demonstrated that the natural diversity found in this study was not the result of the growth phase. Nevertheless, strains selected with specific combinations of enzyme activities by high throughput screening should be further studied in milk incubations.

Leucine was the only branched chain amino acid that was converted by BcaT and AraT. Therefore, to obtain a good estimate of the total capacity of *L. lactis* strains to convert BCAA, leucine was selected as substrate. In this way, the Leu-AT activity enables the selection of strains with the highest total capacity to produce important intermediates for typical mature cheese flavours. Consequently, this approach could also explain the relative large natural diversity of the Leu-AT activity (13-fold) compared with the small (1.5-fold) BcaT diversity found by Fernández de Palencia et al. (2006) for *L. lactis*, where isoleucine was used as substrate. On the other hand, the AraT activity of *L. lactis* found by Fernández de Palencia et al. (2006) was low (14-fold) compared with the results (49-fold) presented here, suggesting that the relative high number of strains used in this study was responsible for the larger natural diversity.

Important for the selection of strains is variety of combinations between Leu-AT, AraT and HicDH activity. The results showed a weak correlation between the Leu-AT and AraT activity which can partly be explained by the affinity of AraT for leucine. In addition, both enzymes are part of the CodY regulon, which could also have

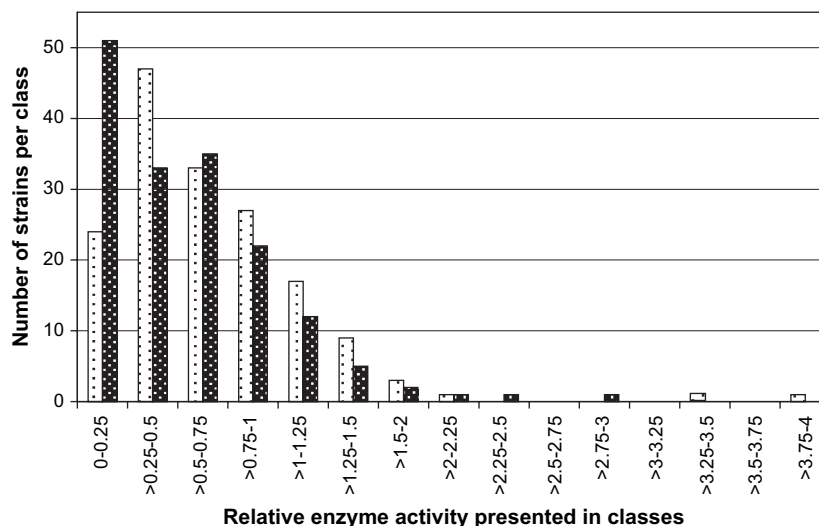


Fig. 5. The relative Leu-aminotransferase (stippled) and aromatic aminotransferase (solid) activity of *Lactococcus lactis* strains. The strains were classified in classes with an OD<sub>600nm</sub> of 0.25 and the relative enzyme activity of the reference strain B643 was 1.

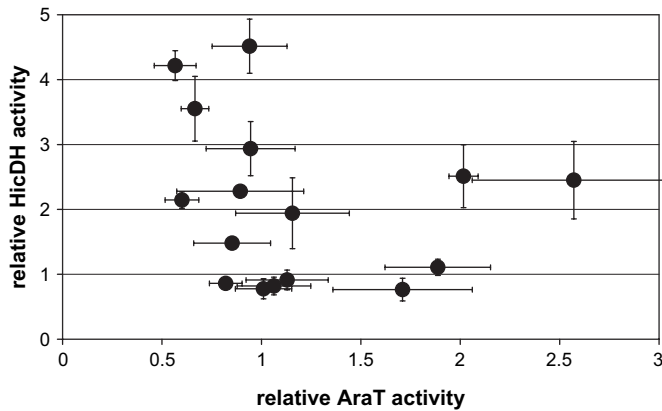


Fig. 6. The relative aromatic aminotransferase (AraT) and hydroxyisocaproic acid dehydrogenase (HicDH) activity of 10% ( $n = 16$ ) of the *Lactococcus lactis* strains in the collection with the highest Leu-aminotransferase activity.

an effect on the correlation (Chambellon & Yvon, 2003). Nevertheless, it was shown that 10% of the strains with the highest Leu-AT activity still had a considerable 5- to 6-fold difference in AraT and HicDH activity.

The Leu-AT, AraT and HicDH activities showed an unbalanced distribution around the mean value, suggesting that *L. lactis* needed a minimal level of activity for growth. This is in agreement with the double BcaT and AraT inactivated *L. lactis* strain that is unable to grow in milk supplemented with amino acids (Chambellon & Yvon, 2003). The role of HicDH in the metabolism is not yet clear. In addition to the conversion of  $\alpha$ -ketoisocaproic acid, HicDH also regenerates NAD<sup>+</sup> by the conversion of pyruvate into D-lactate (Viana, Yebra, Galán, Monedero, & Pérez-Martínez, 2005). Broadbent et al. (2004) suggested on the basis of cheese ripening studies, and thus under lactose depletion, that HicDH might play an important role in maintaining the NADH/NAD<sup>+</sup> balance. However, here the strains were grown in the presence of lactose and at low lactate levels. Therefore, it is expected that lactate dehydrogenase activity was enough to maintain the NADH/NAD<sup>+</sup> balance. This suggests that the HicDH activity also serves other purposes, which is further supported by the observation that HicDH has a higher affinity towards  $\alpha$ -ketoacids than to pyruvate (Bernard et al., 1994). The strains screened in this work appeared to have a minimal HicDH activity. It can be speculated that HicDH plays a role in the removal of excessive intercellular amino acids by converting  $\alpha$ -ketoacids into secretable end-products.

## 5. Conclusions

This work showed a 13-, 49- and 30-fold difference for the Leu-AT, AraT and HicDH activity in *L. lactis* strains. It was shown for the first time that it is possible to select strains with a high Leu-AT activity and relatively low AraT and HicDH activities, which have the potency to enhance cheese flavour.

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