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Arginine metabolism in sugar deprived *Lactococcus lactis* enhances survival and cellular activity, while supporting flavour production

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ABSTRACT

Flavour development in cheese is affected by the integrity of *Lactococcus lactis* cells. Disintegrated cells enhance for instance the enzymatic degradation of casein to free amino acids, while integer cells are needed to produce specific flavour compounds from amino acids. The impact of the cellular activity of these integer cells on flavour production remains to be elucidated. In this study we investigated whether lactose-deprived *L. lactis* cells that use arginine as an alternative energy source can extend cellular activity and produce more specific flavours. In cheese experiments we demonstrated that arginine metabolising cells survived about 3 times longer than non-arginine metabolising cells, which suggests prolonged cellular activity. Cellular activity and flavour production of *L. lactis* was further studied *in vitro* to enable controlled arginine supplementation. Comparable with the results found in cheese, the survival rates of *in vitro* incubated cells improved when arginine was metabolised. Furthermore, elongated cellular activity was reflected in 3–4-fold increased activity of flavour generating enzymes. The observed prolonged cellular activity resulted in about 2-fold higher concentrations of typical Gouda cheese flavours. These findings provide new leads for composing starter cultures that will produce specific flavour compounds.

1. Introduction

Lactococcus lactis starter cultures are mainly used to acidify cheese milk, but play also an important role in flavour production. It has been suggested that both disintegrated and integer cells contribute to flavour production in cheese. Disintegrated cells enhance peptidolytic reactions that increase free amino acid concentration (Crow et al., 1995; Kunji et al., 1996, de Ruyter et al., 1997). On the other hand integer cells are necessary to produce specific flavours that depend on a series of enzymatic steps in which a number of co-factors are involved (Klein et al., 2001; Thage et al., 2004). However, integer *L. lactis* cells are likely to loose cellular activity during cheese ripening, with a concomitant decrease in enzymatic activity and availability of co-factors. The role of cellular activity in these flavour-producing pathways remains to be elucidated. In this respect, arginine may play a crucial role, since this amino acid is the only known energy generating substrate for sugar-deprived *L. lactis*. It is conceivable that arginine metabolism enables the maintenance of cellular activity, thus supporting flavour-producing pathways during cheese ripening. However, there is currently a lack of information on the role of arginine metabolism in flavour production in cheese.

Starter cultures that are used for the production of Gouda cheese contain a large variety of undefined *L. lactis* and *Leuconostoc* strains. Undefined mixed strain Gouda starter cultures contain – based on cell numbers – about 90–95% *L. lactis* and 5–10% *Leuconostoc* strains. The *L. lactis* population can be subdivided using classical phenotypic characterisation in arginine metabolising *L. lactis* subsp. *lactis* strains and non-arginine metabolising *L. lactis* subsp. *cremoris* strains. Gouda cheese starters generally contain between 10 and 50% of *L. lactis* subsp. *lactis* strains. The arginine metabolism in these strains can be activated at acidic pH values (Budin-Verneuil et al., 2004) and/or through sugar depletion (Redon et al., 2005), and this enables the generation of precursors for the biosynthesis of nucleotides or the production of energy in the form of ATP. In both cases arginine is first converted to citrulline and then to ornithine and carbamoyl-phosphate, which can be used for pyrimidine synthesis. Alternatively, energy is generated when carbamoyl-phosphate is deaminated to ammonia and CO_2, whereby ADP is converted to ATP (Vaughan and Thomas, 1982). Arginine metabolism generates a maximum of 1 mol ATP per mol arginine, which is

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relatively low compared to glycolysis (4 mol ATP per mol lactose). Nevertheless, the beneficial effect of arginine metabolism on cell viability was demonstrated *in vitro* by Stuart et al. (1999). Sugar-deprived *L. lactis* ML3 cells incubated in a chemical defined medium (pH = 7.0) and supplemented with 2% arginine were able to survive for a longer period, while on the contrary, ML3 cells incubated without sugar and arginine were unable to survive (Ganesan et al., 2007). These results suggest that in cheese, at a much lower pH of about 5.2, arginine metabolising *L. lactis* strains could display prolonged cellular activity and conceivably increase the production of flavours that require complex pathways.

Production of typical Gouda cheese flavours by *L. lactis* cells involves several enzymatic conversions from amino acids to aldehydes and alcohols in which co-factors are involved. Amino acids, for instance branched chain amino acids, are transaminated to α-ketoacids that are further decarboxylated and/or dehydrogenated to volatiles such as 3-methylbutanal and 3-methylbutanol (Rijnen et al., 2003). According to Yvon et al. (1998), the first rate-limiting step in such conversions is the availability of α-ketoglutarate to transaminate amino acids to α-ketoacids (Yvon et al., 1998; Rijnen et al., 1999). α-ketoglutarate is not available in cheese and therefore needs to be produced by *L. lactis*. Some *L. lactis* strains are able to produce α-ketoglutarate from glutamate with glutamate dehydrogenase (Tanous et al., 2002). Another way to generate α-ketoglutarate is through the conversion of citrate into oxaloacetate, which is subsequently transaminated to aspartate, whereby glutamate is converted to α-ketoglutarate (Tanous et al., 2005). In both examples we expect that α-ketoglutarate is only produced by integer and cellular active cells. This expectation is furthermore supported by the observation that disintegrated cells contributed only to flavour production when α-ketoglutarate was added to the medium (Bourdat-Deschamps et al., 2004).

In our study cheese experiments were conducted to determine whether arginine metabolisation can have a significant impact on the cellular activity of *L. lactis* during ripening. Thereafter, the effect of arginine metabolism on cellular activity and the flavour production of lactose-deprived *L. lactis* was studied.

2. Materials and methods

2.1. Cheese experiments

2.1.1. Cheese manufacturing and analysis

Gouda cheeses were manufactured from 1.500 l of pasteurised cheese milk (10 s at 74 °C) by adopting the standard procedure (Walstra et al., 2006). Cheese milk was inoculated with 0.6% (w/w) fresh undefined mixed strain starter culture CO_2 that contains *L. lactis* and *Leuconostoc* strains. (CSK Food Enrichment, Leeuwarden, The Netherlands). Cheeses weighing 12 kg were produced and matured at 13 °C. Chemical analyses that were performed after 2 weeks of ripening showed that the cheese characteristics were according to expected values (moisture was 41.1 ± 0.3% (w/w), fat in dry matter was 50.9 ± 0.3% (w/w), salt in dry matter was 3.35 ± 0.08% (w/w), pH was 5.23 ± 0.03 and citrate concentration was 132 ± 31 mg/kg). Cheese trials were conducted in triplicate.

2.1.2. Determination of cell survival in cheese

The survival of arginine and non-arginine metabolising cells was determined during cheese ripening. Cheese extract samples were therefore obtained by dissolving 5 g of cheese in 45 ml 2% (w/w) sodium citrate with stomaching for 5 min. Discriminatory agar plates were used to determine cell counts, differentiating arginine and non-arginine metabolising cells. The medium contained a limited amount of lactose, which was sufficient to form yellow colonies, representing the non-arginine metabolising cell count. After lactose was depleted, some of the colonies produced ammonia, whereby the pH indicator turned the colony white, representing the arginine metabolising cell count. A discriminatory medium was prepared by first suspending 15 g of agar in 500 ml of distilled water. A second solution was prepared by suspending 6 g of carboxy methyl cellulose C700 (CP Kelco B.V. Nijmegen, The Netherlands) in 500 ml of distillate water. Both solutions were clarified in a water bath at 100 °C and then mixed together. Thereafter, medium components including 5 g of trypton (BD B.V., Breda, The Netherlands), 5 g of yeast extract (BD B.V., Breda, The Netherlands), 4 g of L-arginine hydrochloride, 1 g of K_2HPO_4 and 3 g of $CaCO_3$ were added to the mixture. The mixture was heated with medium components for 10 min at 100 °C to obtain a homogenous medium. After heating a pH of 6.8 (±0.1) was reached. The total amount of medium was divided into portions of 100 ml and sterilised at 121 °C for 15 min. After sterilisation the medium was cooled to 55 °C and then 5 ml 11% sterile fat free milk (Nilac, NIZO food research, Ede, The Netherlands) and 2 ml of 0.1% sterile broomcresolpurper was added to each portion of 100 ml. The petri dishes were filled with medium at 55 °C and subsequently cooled to 5 °C in order to obtain a homogenous distribution of $CaCO_3$. Petri dishes were dried for 18–24 h at 37 °C. Cell counts were determined after incubating the discriminatory agar plates at 32 °C for 3 days under anaerobic conditions. Cells counts were expressed as colony forming units (CFU) per g of cheese.

2.2. In vitro incubation of L. lactis

2.2.1. Strain and set-up in vitro incubation

L. lactis subsp. *lactis* ML3 (NCDO 763) was incubated *in vitro* in order to study the effect of controlled arginine supplementation on cellular activity and flavour production. Inoculum was prepared by incubating *L. lactis* ML3 cells in M17 (Sigma–Aldrich) with 1% (w/w) lactose for 18 h at 30 °C. Incubation experiments were performed in 1-l fermentors (Applikon, Schiedam, The Netherlands) filled with 800 ml M17 medium that contained 0.50% (w/w) lactose. Fresh inoculum was used to inoculate (1%) the incubation media. Incubations were performed at 30 °C for 11 days and constantly stirred at 100 rpm without atmospheric control. During the whole fermentation period the pH in both fermentors was automatically controlled at 5.3 with 1 M HCl. Potential contamination was verified after 7 and 11 days by plating on Difco Nutrient (DN) agar (BD B.V., Breda, The Netherlands), on which lactic acid bacteria are unable to form colonies. DN agar plates were incubated at 30 °C for 4 days under normal atmospheric conditions. No contamination was detected. Incubation experiments were performed in duplicate.

2.2.2. Sample taking and arginine supplementation

Each day, at the same time, samples (32 ml) were taken from the fermentors. Part (25 ml) of each sample was centrifuged (10,000 rpm, 5 min) at 5 °C. Supernatant was directly stored at −40 °C and used for flavour analysis. Biomass pellets were washed twice with phosphate buffer (50 mM, pH 5.3), stored at −40 °C and used for enzyme activity and protein concentration measurements. The remaining part of the sample (7 ml) was used to determine the amount of biomass (cell counts, optical density and microscopic counting). Directly after sampling the incubation medium was supplemented with 32 ml of sterilised H_2O (control) or 32 ml of filter sterilised (0.22 μm) arginine (Sigma–Aldrich) stock solution (500 mM, pH 5.3).

2.2.3. Survival and integrity of the in vitro incubated cells

The survival of arginine and non-arginine metabolising cell counts, which was determined with discriminatory agar plates, was expressed as CFU per ml incubation medium. Cell integrity was