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Amino acid conversions by coagulase-negative staphylococci in a rich medium: Assessment of inter- and intraspecies heterogeneity

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ABSTRACT

The ability of coagulase-negative staphylococci (CNS) to convert amino acids into volatile compounds and biogenic amines was investigated after 24 h and 48 h of incubation in a rich medium (brain heart infusion). Volatile compounds were measured with static-headspace gas chromatography and mass spectrometry (SH-GC–MS); biogenic amine measurements were carried out with a newly developed method based on ultra-performance liquid chromatography coupled to tandem mass spectrometry (UPLC–MS/MS). In total, 56 CNS strains from five different species were used, namely *Staphylococcus carnosus*, *Staphylococcus epidermidis*, *Staphylococcus equorum*, *Staphylococcus saprophyticus*, and *Staphylococcus xylosus*. With respect to the production of volatile compounds, the leucine-derived 3-methyl butanol was produced over time by most CNS strains, up to 52 μM for *S. xylosus* W1-1 after 48 h of incubation. The average production by strains of *S. xylosus* was significantly higher than for strains of *S. carnosus*, whereas strains of *S. epidermidis* turned out to be poor producers. Yet, differences between species were blurred to a large degree because of the high strain variability. A few strains also produced 3-methyl butanal on top of the amount that was already present in the medium background, although most CNS led to a decrease of this compound. Concerning biogenic amines, the average total concentrations per species remained below 100 μM after 48 h of incubation. The most abundant variant was 2-phenylethylamine (PEA), especially within *S. carnosus* (average of 65 μM after 48 h of incubation). Yet, some individual strains were able to produce higher concentrations, as found for the PEA production of 295 μM by *S. epidermidis* ATCC 12228 after 48 h of incubation. The insights obtained during this study indicate heterogeneity and are of importance in view of both starter culture development and the evaluation of a spontaneously established CNS microbiota in artisan-type meat fermentations.

1. Introduction

Catalase-positive cocci, in particular the coagulase-negative staphylococci (CNS), are important bacteria for the development of colour and aroma in fermented meats, through their nitrate reductase activity and amino acid and fatty acid metabolism, respectively (Leroy et al., 2006; Montel et al., 1998). Although industrial application is usually limited to the use of *Staphylococcus carnosus* and *Staphylococcus xylosus* as meat starter cultures, a number of other species have been isolated from artisan-type, spontaneously fermented meats too, including *Staphylococcus epidermidis*, *Staphylococcus equorum*, and *Staphylococcus saprophyticus* (Cocolin et al., 2011; Ravyts et al., 2012; Talon et al., 2007).

During meat fermentation, peptides and amino acids are generated due to the action of endogenous meat proteases and microbial enzymes. Subsequently, the CNS convert amino acids into different compounds, encompassing several volatile compounds (Leroy et al., 2006) and biogenic amines (Talon and Leroy, 2011). In general, bacterial compounds arising from the metabolism of branched-chain amino acids are known to be key-flavour compounds (Ardo, 2006; Smit et al., 2009). The leucine-derived compounds 3-methyl butanoic acid, 3-methyl butanal, and 3-methyl butanol, for instance, are typical markers for the metabolic activity of CNS in fermented sausages (Berdague et al., 1993; Stahnke, 1995a) and correlate with sausage aroma (Stahnke, 1995b). Although several authors have looked into the conversion of amino acids into aroma compounds by CNS (Beck et al., 2004; Masson et al., 1999; Møller et al., 1998; Olesen and Stahnke, 2003; Tjener et al., 2004), a description of the heterogeneity on species level remains sketchy (Janssens et al., 2013; Larrouture et al., 2000; Ravyts et al., 2009, 2010; Søndergaard and Stahnke, 2002).

In contrast to aroma formation, conversion of amino acids into biogenic amines by CNS in meat has not been receiving a lot of attention (Bermúdez et al., 2012; Landeta et al., 2007; Masson et al., 1996), especially as compared to lactic acid bacteria and *Enterobacteriaceae* (Arena and Manca de Nadra, 2001; Bover-Cid et al., 2000, 2001; Maijala and Eerola, 1993). Decarboxylation of amino acids into biogenic amines in foods is undesirable, as these compounds exert physiological effects that may lead to health concerns (Shalaby, 1996; Silla Santos, 1996).

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In fermented meats, tyramine and histamine are sometimes considered as problematic (Latorre-Moratalla et al., 2012; Suzzi and Gardini, 2003).

The aim of the present study was to evaluate the ability of different CNS species to generate volatile compounds and biogenic amines from amino acids and to explore the inter- and intra-species heterogeneity of these two features.

2. Materials and methods

2.1. Microorganisms

In total, 56 CNS strains belonging to five species (*S. carnosus*, *S. epidermidis*, *S. equorum*, *S. saprophyticus*, and *S. xylosus*) were investigated as to their production of volatile compounds and biogenic amines from amino acids. All strains were present in the collection of the research group Industrial Microbiology and Food Biotechnology (Vrije Universiteit Brussel, Brussels, Belgium) and originated from different ecosystems, including fermented meats, meat starter cultures, milk, and bovine teat apices (Table S1). The authenticity of all strains was confirmed at the species level through *rpoB* gene sequencing. All strains were stored at −80 °C in glycerol-containing (25%, v/v) brain heart infusion (BHI) medium (Oxoid, Basingstoke, Hampshire, UK).

2.2. Culturing of coagulase-negative staphylococci

For all culturing experiments, BHI medium was used as a rich growth medium. The typical formulation of BHI medium consists of brain infusion solids (12.5 g/l), beef heart infusion solids (5.0 g/l), proteose peptone (10.0 g/l), glucose (2.0 g/l), sodium chloride (5.0 g/l), and di-sodium phosphate (2.5 g/l). Prior to experimental use, all strains were propagated twice in BHI medium and incubated at 30 °C overnight. Precultures of all investigated strains were then inoculated (1%, v/v) into 30 ml of BHI medium and incubated at 30 °C for 48 h. Samples to determine medium pH and cell growth were taken after 24 h and 48 h. In addition, cell-free culture supernatants were obtained via centrifugation (8041 × *g* at 4 °C for 20 min) and preserved at −20 °C till further analysis of the concentrations of volatile compounds and biogenic amines. Non-inoculated BHI medium was used as a control to determine the initial background concentrations of any native volatile compounds and biogenic amines that were not due to culturing with CNS. All experiments were done in triplicate.

2.3. Analyses

The medium pH was recorded using a DY-P10 pH meter (Sartorius AG, Göttingen, Germany) equipped with a pH probe (VWR International, Darmstadt, Germany). Cell growth was monitored as colony forming units (cfu) per ml by plating on mannitol salt phenol-red agar (MSA; VWR International), which was subsequently incubated at 30 °C for at least 24 h.

Relevant amino acids were determined through high-performance liquid chromatography–electrospray ionization mass spectrometry (HPLC–ESI/MS) using a Waters 2695 liquid chromatograph coupled to a Quattro Micro™ mass spectrometer (Waters Corp., Milford, MA, USA). Samples were prepared by adding 200 μl of cell-free culture supernatant or medium to 300 μl of internal standard (2-aminobutyric acid, 0.02 g/l) and 500 μl of acetonitrile. These solutions were then filtered (0.2-μm filters; Minisart high-flow, Sartorius AG), transferred into microvials (VWR International), and injected into the column. The column (ChirobioticT, Sigma Aldrich, Steinheim, Germany) was kept at 30 °C during analysis. The mobile phase, at a flow rate of 1.0 ml/min, was composed of 4 mM formic acid and 4 mM ammonium formate in deionized water at pH 4.0 with 5% (v/v) acetonitrile (eluent A) and of 4 mM formic acid in pure (100%) acetonitrile (eluent B). The following gradient was used for the determination of 2-aminobutyric acid, arginine, histidine, lysine, and ornithine [composition (v/v) at different time points with a linear profile imposed between different time points]: 0.0 min, 40% A and 60% B; 10.0 min, 100% A and 0% B; 20.0 min, 100% A and 0% B; 25.0 min, 40% A and 60% B; and 30.0 min, 40% A and 60% B. For the determination of isoleucine, leucine, tryptophan, tyrosine, and valine this gradient equalled [composition (v/v) at different time points with a linear profile imposed between different time points]: 0.0 min, 20% A and 80% B; 10.0 min, 20% A and 80% B; 15.0 min, 35% A and 65% B; 20.0 min, 100% A and 0% B; 23.0 min, 100% A and 0% B; 25.0 min, 20% A and 80% B; and 30.0 min, 20% A and 80% B. Settings of the mass spectrometer were as follows: capillary voltage, 0.4 kV; cone voltage, 10–22 V; source temperature, 120 °C; desolvation temperature, 350 °C; cone gas flow, 50 l/h; desolvation gas flow, 750 l/h; and collision energy, 10–29 eV. The following mother and daughter ions were monitored: 2-aminobutyric acid: 104.1 > 57.8; arginine: 175.2 > 69.9; histidine: 156.2 > 110; isoleucine and leucine: 132.2 > 85.9; lysine: 147.2 > 83.9; ornithine: 133.1 > 69.8; tryptophan: 205.2 > 187.9; tyrosine: 182.1 > 164.9; and valine: 118.1 > 71.9. The amino acid concentrations were calculated using a calibration curve obtained after adding known concentrations of the respective compounds to ultrapure water, and correcting based on the internal standard. All values were expressed in μM.

The concentrations of the volatile compounds were determined by static-headspace gas chromatography coupled to mass spectrometry (SH-GC–MS) (Ravyts et al., 2009). Samples were prepared in headspace vials containing 5 ml of the culture medium or of the different cell-free culture supernatants, 1 g of sodium chloride to enhance volatility (Kolb and Ettre, 2006), and a known amount of 4-methyl-2-pentanol (Fluka, Buchs, Switzerland) as internal standard. Analyses were performed with an Agilent 6890 gas chromatograph (Agilent Technologies; Santa Clara, CA, USA), equipped with a DB-WAXetr capillary column (30 m × 0.25 mm × 0.5 μm; Agilent Technologies) and a MPS2 Gerstel autosampler (Gerstel GmbH & Co. KG, Mülheim-an-der-Ruhr, Germany), and coupled to an Agilent 5973N mass spectrometer (Agilent Technologies). Helium was used as carrier gas with a flow rate of 1 ml/min. Samples were equilibrated by agitation at 80 °C for 30 min prior to injection. The injection port was set in split mode with a ratio of 30:1 and the needle temperature was set at 90 °C. The injection volume was set at 1 ml, at a rate of 500 μl/s. The oven temperature programme consisted of an initial step at 40 °C for 5 min, followed by a linear increase from 40 to 120 °C at 20 °C/min and a linear increase from 120 to 225 °C at 10 °C/min. Finally, the temperature remained constant at 225 °C. The temperature of the transfer tube was held at 280 °C. Detection was done by an MS detector (ionization energy 70 eV, 4.1 scans/s, source 230 °C, scan range 29–200 m/z). Identification of the peaks was done by comparison with standard compounds that were injected separately and with library data (NIST98, http://www.nist.gov; Stein et al., 1999). The peak areas of the examined compounds were considered only in case they exceeded three times the baseline signal (Aguinaga et al., 2008). The concentrations of volatile compounds were calculated using a calibration curve obtained after adding known concentrations of the respective compounds to ultrapure water, and correcting based on the internal standard. All values were expressed in μM.

Biogenic amine concentrations were measured with ultra-performance liquid chromatography coupled to tandem mass spectrometry (UPLC–MS/MS), using Waters' Acquity model with a TQD mass spectrometer (Waters Corp.). Ionisation for mass spectrometry was established by positive electron ionisation (ESI+). An Acquity UPLC HSS T3 column (Waters Corp.) was used for separation and was kept at 40 °C. The mobile phase, at a flow rate of 0.4 ml/min, was composed of 10 mM ammonium acetate with 0.2% (v/v) of formic acid and 5% (v/v) of acetonitrile in ultrapure water (eluent A) and 10 mM ammonium acetate with 0.2% (v/v) formic acid and 5% (v/v) ultrapure water in acetonitrile (eluent B). The following gradient was applied [composition (% in v/v) at different time points with a linear profile imposed in between]: 0.0 min, 100% A; 1 min, 100% A; 1.6 min, 90% A and 10% B; 3.4 min, 90% A and 10% B; and 3.5 min, 40% A and 60% B; 4.5 min, 40%