International Journal of Food Microbiology 212 (2015) 60–66

Contents lists available at ScienceDirect

International Journal of Food Microbiology

journal homepage: www.elsevier.com/locate/ijfoodmicro

Process-driven bacterial community dynamics are key to cured meat colour formation by coagulase-negative staphylococci via nitrate reductase or nitric oxide synthase activities

María Sánchez Mainar, Frédéric Leroy *

Research Group of Industrial Microbiology and Food Biotechnology (IMDO), Faculty of Sciences and Bioengineering Sciences, Vrije Universiteit Brussel, Pleinlaan 2, B-1050 Brussels, Belgium

ARTICLE INFO

Available online 12 March 2015

Keywords:
Coagulase-negative staphylococci
Meat fermentation
Fermented sausage
Arginine
Nitrate reductase
Nitric oxide synthase

ABSTRACT

The cured colour of European raw fermented meats is usually achieved by nitrate-into-nitrite reduction by coagulase-negative staphylococci (CNS), subsequently generating nitric oxide to form the relatively stable nitrosomyoglobin pigment. The present study aimed at comparing this classical curing procedure, based on nitrate reductase activity, with a potential alternative colour formation mechanism, based on nitric oxide synthase (NOS) activity, under different acidification profiles. To this end, meat models with and without added nitrate were fermented with cultures of an acidifying strain (*Lactobacillus sakei* CTC 494) and either a nitrate-reducing *Staphylococcus carnosus* strain or a rare NOS-positive CNS strain (*Staphylococcus haemolyticus* G110), or by relying on the background microbiota. Satisfactory colour was obtained in the models prepared with added nitrate and *S. carnosus*. In the presence of nitrate but absence of added CNS, however, cured colour was only obtained when *L. sakei* CTC 494 was also omitted. This was ascribed to the pH dependency of the emerging CNS background microbiota, selecting for nitrate-reducing *Staphylococcus equorum* strains at mild acidification conditions but for *Staphylococcus saprophyticus* strains with poor colour formation capability when the pH decrease was more rapid. This reliance of colour formation on the composition of the background microbiota was further explored by a side experiment, demonstrating the heterogeneity in nitrate reduction of a set of 88 CNS strains from different species. Finally, in all batches prepared with *S. haemolyticus* G110, colour generation failed as the strain was systematically outcompeted by the background microbiota, even when imposing milder acidification profiles. Thus, when aiming at colour formation through CNS metabolism, technological processing can severely interfere with the composition and functionality of the meat-associated CNS communities, for both nitrate reductase and NOS activities. Several major bottlenecks, among which the rareness of phenotypic NOS activity in meat-compatible CNS, need to be considered, which is seriously questioning the relevance of this pathway in fermented meats.

1. Introduction

Fermented meats are foods with a long-standing tradition due their stability and convenience, their nutritional value, and their attractive sensory properties (Leroy et al., 2013). They owe the development of their typical characteristics largely to the enzymatic activities of the microorganisms that take part in the fermentation course. As a major example, their distinctive colour is usually based on the nitrate reductase activity of catalase-positive cocci, encompassing coagulase-negative staphylococci (CNS) and, sometimes, *Kocuria* species (Ravyts et al., 2012). At the beginning of the production process of most European-style fermented meats, nitrate is added as a curing agent and subsequently reduced by bacterial nitrate reductase to nitrite (Sebranek, 2009). Nitrite generates nitric oxide (NO) that is needed to form the characteristic nitrosomyoglobin pigment, based on either bacterial nitrite reductase activities or chemical conversion under acidic conditions (Gøtterup et al., 2008; Honikel, 2007). Besides colour formation, this process also yields antimicrobial action towards food-borne pathogens that may prevail in the meat batter (Tompkin, 2005).

Nitrate reductase activity is dependent on the overall environmental conditions and is hence affected by the process technology applied during meat fermentation. Although nitrate reduction can be achieved at 15–20 °C, the process seems to be more effective at temperatures above 30 °C (Casaburi et al., 2005), at no too acidic pH values (Talon et al., 2004), and when using sodium rather than potassium chloride salt (Ibañez et al., 1996). In addition, the efficiency of the myoglobin nitrosation process in dry fermented sausages depends on many meat-related parameters, such as the water content, pigment concentration, and redox potential of the animal muscle (Chasco et al., 1996). Based on the above, it is expected that optimization of process conditions for improved colour formation will be of particular importance

* Corresponding author. Tel.: +32 2 6293612; fax: +32 2 6292720.
E-mail address: fleroy@vub.ac.be (F. Leroy).

http://dx.doi.org/10.1016/j.ijfoodmicro.2015.03.009
0168-1605/

when the nitrate levels applied are low or when the CNS community structure is suboptimal. The latter could for instance be the case during those spontaneous fermentation processes that are dominated by CNS with low nitrate reductase activity levels, as this is a variable threat within this group of bacteria (Götz et al., 2006).

Meat curing is nowadays often under scrutiny, as it has been epidemiologically related to colorectal cancer incidence (Chan et al., 2011), resulting in the dietary advice to "avoid processed meat" (World Cancer Research Fund, 2007). The presence of haem iron and the formation of *N*-nitroso compounds derived from curing salts have been suggested as potential carcinogenic drivers (Demeyer et al., 2008). Therefore, the search for alternatives for nitrate and nitrite is ongoing (Pegg and Shahidi, 2008). One of them could be the technological exploitation of the nitric oxide synthase (NOS) pathway, which is present in certain bacteria (Crane et al., 2010). NOS activity converts the amino acid *L*-arginine into *L*-citrulline in the presence of oxygen and NADPH, generating NO that could then form nitrosylmyoglobin. Some authors have suggested that lactic acid bacteria can display NOS activity in fermented meats (Arihara et al., 1993; Gündoğdu et al., 2006; Møller et al., 2003; Morita et al., 1997; Zhang et al., 2007). This remains nevertheless unconvincing due to potential methodological flaws (Xu and Verstraete, 2001) and because none of the currently available genome sequences of *Lactobacillus* spp. harbour a gene encoding a NOS enzyme homologue (Acland et al., 2014). With respect to staphylococci, activity of NOS has been shown in *Staphylococcus aureus* (Choi et al., 1997) and has also been suggested for CNS in meat (Li et al., 2013). Yet, phenotypic NOS activity in CNS seems to be a very rare feature despite a widespread genetic potential; out of a collection of 86 CNS strains cultured in a meat simulation medium, only one strain (*S. haemolyticus* G110) displayed NOS activity (Sánchez Mainar et al., 2014).

The present study aimed at exploring if *S. haemolyticus* G110 can act as a colour-generating model strain in fermented meat models based on its NOS activity, in view of functional starter culture development for meat fermentation (Leroy et al., 2006). This was contrasted with nitrate reductase activity from either a selected strain of *Staphylococcus carnosus* or from CNS strains originating from the background microbiota, for different degrees of acidification. Moreover, heterogeneity in nitrate reductase activity was assessed for a set of CNS from different species, to further illustrate the potential impact of the composition of the background microbiota on colour formation.

2. Materials and methods

2.1. Fermentation experiments in meat models

2.1.1. Preparation and setup of the meat models

Meat models were prepared with fresh pork mince containing (m/m): 3.0% of sodium chloride (VWR International, Darmstadt, Germany), 0.3% of arginine (Sigma-Aldrich, St. Louis, MO, USA), 0.05% of sodium ascorbate (Sigma-Aldrich), and 0.02% of $MnSO_4.4H_2O$ (VWR International). A first series was prepared with an additional 0.5% (m/m) of glucose (VWR International) and without added sodium nitrate, encompassing four batches (A, B, C, and D); a second series was prepared with an additional 0.5% (m/m) of glucose and 200 ppm of added sodium nitrate (VWR International), also encompassing four batches (An, Bn, Cn, and Dn). Each batch consisted of twelve petri dishes, containing about 40 g of mince each, for each sampling moment (after 0 h, 24 h, 48 h, and 72 h) in triplicate. A third series of experiments consisted of three batches (E1, E2, and E3), with the same composition as mentioned above, but with three different levels of added glucose (0.1, 0.2, or 0.3%, m/m) and without added sodium nitrate. Each of these three batches consisted of six petri dishes, with about 40 g of mince each, for each sampling moment (after 0 h and 70 h) in triplicate. Since NOS is an oxygen-dependent pathway, an air-containing headspace was allowed above the meat. This also contributed to the reinforcement of oxidative colour degeneration in the control samples without nitrate, as compared to the cured variants.

Two CNS strains were tested for colour generation in the meat models, namely the starter culture *S. carnosus* Combistart 1505 (Chr. Hansen, Hørsholm, Denmark) for its nitrate reductase activity, and *S. haemolyticus* G110, a rare CNS strain, originating from bovine teat apex skin, and displaying phenotypic NOS activity as previously shown (Sánchez Mainar et al., 2014). In addition, *Lactobacillus sakei* CTC 494 was used as a starter culture for the acidification of some of the batches (Rimaux et al., 2011). All three strains were present in the laboratory collection of the Research Group of Industrial Microbiology and Food Biotechnology (Vrije Universiteit Brussel, Brussels, Belgium). They were stored in cryovials at −80 °C, containing brain heart infusion (BHI) medium (Oxoid, Basingstoke, UK) for both CNS strains or de Man-Rogosa-Sharpe (MRS) medium (Oxoid) for *L. sakei* CTC 494, supplemented with 25% (*v*/*v*) glycerol as a cryoprotectant. The inocula for the meat model experiments were prepared through two subcultures of the applied strains from the cryovials into 10 ml of their respective growth media, followed by incubation at 30 °C for 12 h. Next, 1 ml of the first subculture was added to 100 ml of either BHI or MRS, followed by incubation at 30 °C for 12 h. Cell pellets were obtained by centrifugation of these cultures at 7200 ×*g* for 20 min at 4 °C and were resuspended in sterile peptone water (0.85% of NaCl and 0.1% of peptone, *m*/*v*). Aliquots of these resuspensions were homogenously distributed in the corresponding meat batches, as to obtain inoculation levels between 10^5 and 10^6 cfu/g of meat.

Batches A and An were inoculated with both *L. sakei* CTC 494 and *S. carnosus* Combistart 1505; batches B and Bn were inoculated with both *L. sakei* CTC 494 and *S. haemolyticus* G110; batches C and Cn were inoculated with *L. sakei* CTC 494 solely; and batches D and Dn were not inoculated but subjected to spontaneous fermentation. Batches E1, E2, and E3 were inoculated with *S. haemolyticus* G110 solely. All fermentations were carried out at a temperature of 21 °C.

2.1.2. Measurement of technological parameters

For each meat model sample, the pH, cured colour, and bacterial counts were measured. To evaluate cured colour formation, the Cie L*, a*, and b* colour space values were measured with a Chroma meter CR-400 (Minolta, Osaka, Japan) by reflectance on the surface of the meat models. Three different points were targeted and the resulting value from each point was the mean of five measurements. Determination of pH was performed on three different points of the meat model samples with a DY-P10 pH meter (Sartorius AG, Göttingen, Germany) equipped with an insertion pH probe (VWR International). For bacterial enumeration, a 10-g meat sample from the central portion of each petri dish was aseptically added to 90 g of sterile peptone water and homogenized in a stomacher (Stomacher 400, Seward, Worthington, UK) for 2 min at 230 rpm (±5%). The appropriate decimal dilutions, made in sterile saline (0.85% of NaCl, *m*/*v*), were spread on agar media. Mannitol salt phenol red agar (MSA; VWR International) and MRS agar were used for the respective enumeration of CNS and lactic acid bacteria, expressed in colony forming units (cfu) per g. Incubation of the MSA and MRS agar plates was performed at 30 °C for 48–72 h.

2.1.3. Bacterial community analysis through $(GTG)_5$-PCR fingerprinting of genomic DNA

To evaluate the microbial diversity of the CNS communities in the meat models, 10% of the colonies on MSA from each sampling point were randomly selected. The number of isolates per sampling point varied between 5 and 23. In total, 288 bacterial isolates were obtained for the first two experimental series (batches A/An, B/Bn, C/Cn, and D/Dn); for the third series (batches E1, E2, and E3), an additional 106 isolates were obtained. The isolates were grown overnight in BHI medium at 30 °C, upon which 1.5 ml of the cultures was transferred to cryovials, containing 25% (*v*/*v*) of glycerol for storage at −80 °C until further use. The rest of the culture was subjected to genomic DNA extraction with the Nucleospin 96 tissue kit (Macherey Nagel GmbH, Düren, Germany), according to the manufacturer's instructions. Identification of the isolates