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THE POTENTIAL ROLE OF ARGININE IN COMPETITIVENESS AND FUNCTIONALITY OF COAGULASE-NEGATIVE STAPHYLOCOCCI DURING MEAT FERMENTATION

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The capacity for arginine metabolism during meat fermentation offers a potential bonus in fitness to coagulase-negative staphylococci (CNS) in the absence of carbohydrates, as is particularly so for the arginine deiminase (ADI) pathway. An alternative is provided by the nitric oxide synthase (NOS) enzyme, which converts arginine into citrulline and nitrogen oxide (NO). Since NO causes colour-stabilizing nitrosylation of myoglobine, this pathway could potentially be used for the generation of nitrosomyoglobine in clean label products without added nitrate and/or nitrite. A genotypic and physiological screening of 88 CNS strains indicated that active ADI machinery is often found in this CNS group, although in a strain-specific manner. In contrast, phenotypic NOS-like activity was only present in one of the strains (*Staphylococcus haemolyticus* G110), although the genetic potential for NOS was widespread among CNS strains. Attempts to express NOS activity in some of the latter strains were unsuccessful, suggesting that the genetic potential for NOS is not commonly expressed by CNS. Both ADI and NOS kinetics were investigated in a meat simulation medium, indicating the need for sufficient oxygen for NOS activity. The use of NOS-positive CNS cultures as a curing alternative in fermented meats is thus not clear-cut.

Key Words – meat fermentation, coagulase-negative staphylococci, arginine deiminase, nitric oxide synthase, colour

I. INTRODUCTION

During the production of traditionally fermented sausages, spontaneous fermentation takes place that is dominated by communities of lactic acid bacteria (LAB) and catalase-positive cocci, mostly coagulase-negative staphylococci (CNS) (1). During this process, LAB cause a desirable acidification of the sausage batter, whereas CNS produce a stable cured colour via nitrate reductase activity, generate aroma, and protect against oxidation. In such spontaneous processes, *Lactobacillus sakei* is the most dominant LAB species and thus frequently used as a starter culture for meat fermentation. A wider species variety exists amongst the meat-associated CNS communities but, in general, *Staphylococcus xylosus*, *S. equorum*, and *S. saprophyticus* are predominant. Yet, several other CNS may also occur (1).

Because meat is generally poor in carbohydrates and added sugars are rapidly depleted, differences in the use of alternative energy sources may lead to improved competitiveness for certain meat bacteria (2). The arginine deiminase (ADI) pathway, which is present in certain CNS, is an interesting option, as it generates ATP and protects against acid stress through ammonia production. Whereas ornithine is the end-metabolite, the intermediate citrulline may be partly excreted too, as is the case for *Lb. sakei* (3).

An alternative but poorly explored arginine-converting pathway could potentially be based on the action of nitric oxide synthase (NOS) (4). As a result of NOS activity, arginine is converted into citrulline and nitric oxide (NO). From a technological point of view, this could be of interest for colour formation in fermented meat products prepared without nitrate and nitrite (4). Indeed, the cured colour of fermented meat products is obtained via the formation of nitrosomyoglobin, resulting from the interaction between muscle-based myoglobin and NO that generally originates from the addition of nitrate and/or nitrite as curing agents under low pH conditions. Thus far, most of the studies looking into colour generation via NOS in fermented meat

products have been rather preliminary and definite proof is lacking (4, 5).

Therefore, the present study investigated the use of arginine via ADI and NOS activities by CNS and explored the heterogeneity on species and strain level, both from a phenotypical and genotypical perspective. It also explored the kinetic patterns of arginine metabolism as a function of the prevailing glucose and oxygen levels.

II. MATERIALS AND METHODS

A genotypic screening for *nos* (NOS-encoding) and *arcA* (ADI-encoding) genes was performed in a set of 88 strains of CNS. Four primer sets for the *nos* gene and one primer set for the *arcA* gene were designed, based on Genbank entries for staphylococci, and the associated PCR assay conditions were optimised.

Second, a phenotypic screening of the ability of these CNS strains to use the precursor arginine was carried out. Each CNS strain was cultivated at 30 °C in 100 ml of meat simulation medium (MSM), containing 3 g/l of added arginine (17.2 mM), under microaerobic or aerobic (shaker incubator) conditions, with sampling after 72 h.

Third, a further screening under possibly NOS-stimulating conditions [including growth on solid agar, the use of different temperatures (12, 20, and 40 ºC) and initial pH values (pH 6.5, 7.0, and 7.5), and the presence of added glucose, hemin, methanol, myoglobin, different minerals (Fe^{++}, Mg^{++}, Ca^{++}), and tetrahydrobiopterin] was performed on a subset of 12 *nos*-positive *S. carnosus* strains.

Fourth, kinetic analyses of possible NOS activities in CNS (*S. haemolyticus* G110, *S. pasteuri* αS3-13, and *S. carnosus* 833), as a function of glucose and oxygen levels, were done in laboratory fermentors. Fermentations were carried out at 30 °C in 10 l of MSM containing 3 g/l of added arginine and 5 g/l of added glucose, and with an initial pH of 5.8 under micro-aerobic or aerobic conditions. Bacterial growth was followed by the determination of colony forming units (cfu) per ml on mannitol salt agar (MSA).

In all experiments, concentrations of arginine, citrulline, and ornithine were measured with liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS). Glucose consumption was measured with high-performance liquid chromatography (HPLC) with refractive index (RI) detection.

III. RESULTS AND DISCUSSION

Among the 88 strains of CNS tested, the *nos* gene was present in 74 % of the strains. The genetic potential for ADI activity was also frequently found, *i.e.*, amongst 44 % of the CNS strains tested. The phenotypic screening experiments confirmed that arginine metabolism was indeed common, resulting in mixtures of citrulline and mostly ornithine, even though considerable variability was found on species and even strain level. Although microaerobic conditions seemed to stimulate ADI activity, this pathway generally also took place under aerobic conditions. The production of citrulline without ornithine formation, indicative of potential NOS activity, was not found under the conditions tested for any of the CNS strains, except for *S. haemolyticus* G110. The latter strain showed modest citrulline production (9 ± 4 mM) without ornithine under aerobic conditions.

The absence of phenotypic NOS activity amongst the other strains was remarkable, considering the fact that the *nos* gene was quite often present. Further attempts to express the *nos* potential, by applying growth on solid agar or by modifying the medium composition or the prevailing temperature and pH, were unsuccessful. This indicates that the genetic potential for NOS activity is not readily expressed under a range of conditions.

Kinetic experiments in MSM confirmed that NOS-like activity, indicated by citrulline production without ornithine, was obtained with *S. haemolyticus* G110 (Fig. 1). This pattern was found under aerobic conditions, but no manifest consumption of arginine was seen under micro-aerobic conditions, further confirming the NOS-like behaviour of the strains since this pathway requires oxygen.

With *S. pasteuri* αS3-13 and *S. carnosus* 833, combinations of citrulline and ornithine were obtained under both aerobic and micro-aerobic

conditions, indicating ADI activity. The example of *S. carnosus* 833 is given in Fig. 2, but results for *S. pasteuri* aS3-13 were similar. ADI activity only started after glucose depletion, indicating carbon catabolite repression. This suggests a role for ADI activity in the CNS metabolism of alternative energy substrates under glucose-depleted conditions, commonly encountered in meat fermentation.

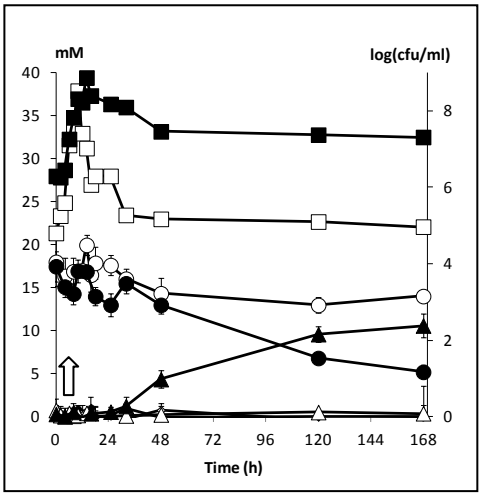

Fig. 1. Conversion of arginine (mM; circles) into citrulline (mM; triangles) by *S. haemolyticus* G110 [cell counts in log (cfu/ml); squares] under aerobic (full symbols) and micro-aerobic conditions (open symbols) in MSM at 30 °C. The arrow indicates the moment of glucose depletion for both cases.

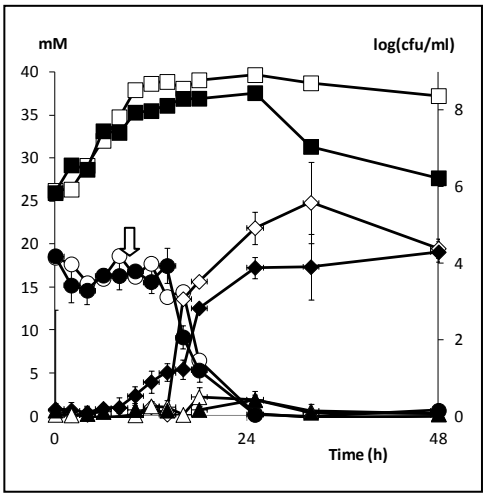

Fig. 2. Conversion of arginine (mM; circles) into citrulline (mM; triangles) and ornithine (mM; diamonds) by *S. carnosus* 833 [cell counts in log (cfu/ml); squares] under aerobic (full symbols) and micro-aerobic conditions (open symbols) in MSM at 30 °C. The arrow indicates the moment of glucose depletion for both cases.

Although NOS activity leading to NO generation could be of interest for colour formation in fermented meats with clean labels, its successful implementation in fermented meat products has thus far been insufficiently proven (5). It remains unclear to which degree NOS activity can be modulated by environmental conditions and process factors. The need for oxygen represents a major potential hurdle, besides the fact that expression of NOS activity is absent in the large majority of the CNS strains possessing the required gene under a wide range of tested conditions.

IV. CONCLUSION

Arginine consumption by CNS generally relied on the ADI pathway, although considerable variability existed on species and strain level. The ADI pathway only became active in the absence of glucose, which has physiological implications with respect to ecosystem adaptation. The genetic potential for NOS activity was present in many CNS strains too, but this was not reflected in the phenotypic behaviour, except for one strain, indicating that the bottleneck was likely to be on the gene expression level. The use of NOS-positive CNS cultures for nitrate and nitrite cutback in fermented meats is therefore not straightforward and its industrial application could be problematic. From an ecological perspective, further research is needed to assess to which degree differences in arginine metabolism play a role in the establishment of specific CNS communities during meat fermentation.

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REFERENCES

1. Ravyts, F., De Vuyst, L. & Leroy, F. (2012). Bacterial diversity and functionalities in food

fermentations. Engineering in Life Sciences 12: 356-367.
2. Janssens, M., Van der Mijnsbrugge, A., Sánchez Mainar M., Balzarini T., De Vuyst L. & Leroy, F. (2014). The use of nucleosides and arginine as alternative energy sources by coagulase-negative staphylococci in view of meat fermentation. Food Microbiology 39: 53-60.
3. Rimaux, T., Vrancken, G., Pothakos, V., Maes, D., De Vuyst, L. & Leroy, F. (2011). The kinetics of the arginine deiminase pathway in the meat starter culture *Lactobacillus sakei* CTC 494 are pH-dependent. Food Microbiology 28: 597-604.
4. Morita, H., Yoshikawa, H., Sakata, R., Nagata, Y. & Tanaka, H. (1997). Synthesis of nitric oxide from the two equivalent guanidino nitrogens of L-arginine by *Lactobacillus fermentum*. Journal of Bacteriology 179: 7812–7815.
5. Sudhamsu, J., & Crane, B. (2009). Bacterial nitric oxide synthases: what are they good for? Trends in Microbiology 17: 212-218.