Food Research International 85 (2016) 113–120

Contents lists available at ScienceDirect

Food Research International

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

Nitrate reductase activity of *Staphylococcus carnosus* affecting the color formation in cured raw ham

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ARTICLE INFO

Article history:
Received 29 January 2016
Received in revised form 19 April 2016
Accepted 21 April 2016
Available online 22 April 2016

Keywords:
Meat starter cultures
Staphylococcus carnosus
Raw ham curing
Specific enzyme activities
Meat fermentation
Nitrosylmyoglobin

ABSTRACT

The influence of the nitrate reductase activity of two *Staphylococcus carnosus* strains used as starter cultures on the formation of nitrate, nitrite and color pigments in cured raw ham was investigated. In this context, microbiological, chemical and multivariate image analyses were carried out on cured raw hams, which were injected with different brines containing either nitrite or nitrate, with or without the *S. carnosus* starter cultures. During processing and storage, the viable counts of staphylococci remained constant at 6.5 log cfu/g in the hams inoculated with starter cultures, while the background microbiota of the hams processed without the starter cultures developed after 14 days. Those cured hams inoculated with *S. carnosus* LTH 7036 (high nitrate reductase activity) showed the highest decrease in nitrate and high nitrite concentrations in the end product, but were still in the range of the legal European level. The hams cured with nitrate and without starter culture or with the other strain, *S. carnosus* LTH 3838 (low nitrate reductase activity) showed higher residual nitrate levels and a lower nitrite content in the end product. The multivariate image analysis identified spatial and temporal differences in the meat pigment profiles of the differently cured hams. The cured hams inoculated with *S. carnosus* LTH 3838 showed an uncured core due to a delay in pigment formation. Therefore, the selection of starter cultures based on their nitrate reductase activity is a key point in the formation of curing compounds and color pigments in cured raw ham manufacture.

1. Introduction

Meat curing with is an old and traditional technique to preserve meat from pathogens, as the addition of nitrate and/or nitrite prevents growth of anaerobic bacteria, such as *Clostridium botulinum* (Pegg, 2004; Shahidi & Samaranayaka, 2004). On the other hand, the usage of nitrite and nitrate is strictly regulated by governments due to the toxicity of nitrite in higher concentrations and the formation of N-nitrosamines, which are formed by reactions of nitrite with amino acids and amines (Hammes, 2012; Shahidi & Samaranayaka, 2004).

Additionally, quality attributes of meat products, such as typical taste and convincing appearance, are the main criteria for manufacturers and consumers and can be controlled by using starter cultures (Hammes, 2012; Honikel, 2008). In particular, *Staphylococcus carnosus* has been used since the 1950s as starter culture, especially in the processing of raw fermented sausages, due to its ability to reduce nitrates, contribute to the characteristic fermented flavor, and convert hydrogen peroxide to oxygen and water (Pegg, 2004; Shahidi & Samaranayaka, 2004). The membrane-bound enzyme nitrate reductase of *S. carnosus* reduces nitrate to nitrite under anaerobic conditions to generate energy. Nitrite can be reduced further to ammonium by the enzyme nitrite reductase to prevent cells of nitrite intoxication and reoxidize NADH to NAD^+ (Nicotinamide adenine dinucleotide) (Neubauer & Götz, 1996; Schlag, Nerz, Birkenstock, Altenberend, & Götz, 2007). Furthermore, the reducing agents in the meat matrix, like NADH, reduce nitrite to nitric oxide (NO) and nitrosylmyoglobin (MbFe(II)NO), the typical bright-red to pink meat pigment, is formed in a complex process that is not yet fully understood (Mancini & Hunt, 2005; Møller & Skibsted, 2002; Skibsted, 2011). Additionally, during the oxidation of nitrosylmyoglobin, the brown metmyoglobin (MbFe(III)) and nitrate (NO_3^-) are produced and are available for further reactions (Mancini & Hunt, 2005; Møller & Skibsted, 2002; Skibsted, 2011). Color formation of fermented meat products can be measured by several different methods: for example, color measurements using CIE L*a*b*, or spectral analysis of meat pigments (Cornforth & Jayasingh, 2004). Another non-invasive low-cost tool is image analysis, which has previously been used to investigate meat color pigments (Jackman & Sun, 2013; Jackman, Sun, & Allen, 2011; Prats-Montalbán, de Juan, & Ferrer, 2011). Demos et al. demonstrated that there is a relationship between the red color intensity measured by a* value and the oxidation state of meat pigments (Demos, Gerrard, Gao, Tan, & Mandigo, 1996).

A new approach for raw fermented sausages was described by Fongaro et al., who used multivariate image analysis (MIA) to

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http://dx.doi.org/10.1016/j.foodres.2016.04.021
0963-9969/

distinguish between three oxidation states of meat pigments (Fongaro, Alamprese, & Casiraghi, 2015). MIA is utilized for images of heterogeneous material with more than one measurement per pixel, for example red, green and blue (RGB) channels in an image. The most important information is described in a decreasing order to explore the image and classify regions of interest (Geladi & Grahn, 2006; Prats-Montalbán et al., 2011). Principal component analysis (PCA) is the most-used technique in MIA to reduce many variables to a few important principal components (PC) that describe most of the variability in the image (Geladi & Grahn, 2006). The PCs can easily be transferred into pseudocolor images or score plots for analysis (Geladi & Grahn, 2006; Prats-Montalbán et al., 2011). To the best of our knowledge, there exists no study in literature that discusses the spatial and temporal profile of meat pigments during the manufacture of cured raw hams with the addition of *S. carnosus* strains.

The aim of this study was to investigate the influence of the nitrate reductase activity of selected *Staphylococcus carnosus* strains on the spatial and temporal color formation in cured raw hams. As nitrate reduction is a strain-specific property (Müller et al., 2015), an assessment of the effectiveness in vitro is needed to find suitable strains for the inoculation of cured raw hams. Therefore, *S. carnosus* strain LTH 7036, with a high nitrate reductase activity, and LTH 3838, with a low nitrate reductase activity (Müller et al., 2015), were used as starter cultures in raw ham to investigate nitrate and nitrite content over time. Additionally, the oxidation levels of the meat pigments (via MIA) were determined to get precise information on the spatial and temporal color formation process in cured raw hams.

2. Materials and methods

2.1. Selection and preparation of starter cultures

The selection of the two starter cultures was based on the study of Müller et al., who screened 40 *S. carnosus* strains on their physiological properties (Müller et al., 2015). *S. carnosus* LTH 3838, a *S. carnosus* subsp. *utilis* isolate of fermented fish was selected because of the low nitrate reductase activity (0.04 mol NO_2 per 1×10^7 colony forming units (cfu)). In addition, the strain *S. carnosus* LTH 3838 was the only strain which digested sarcoplasmatic proteins (Müller et al., 2015). In contrast, the second strain, *S. carnosus* subsp. *carnosus* LTH 7036 (origin: fermented sausages) was selected because of its high nitrate reductase activity (0.72 mol NO_2 per 1×10^7 cfu).

The bacterial strains were routinely grown in 100 mL standard 1 nutrient broth (Merck, Darmstadt, Germany) in 300 mL Erlenmeyer flasks at 37 °C for 24 h with shaking at 180 rpm (Innova 42, Eppendorf, Hamburg, Germany). Cells were concentrated by centrifugation of the overnight culture (2500 × g, 10 min, 4 °C; HermLe Z 32 HK, Wehingen, Germany) and resuspended in 1 mL 0.9% (w/v) sodium chloride solution and combined (NaCl solution; Carl Roth, Karlsruhe, Germany). The combined cell suspensions were washed twice and finally resuspended in 1 mL NaCl solution. The concentration of the resuspended cells was adjusted to approx. 1×10^{11} cfu/mL by measuring the optical density at 600 nm (OD_{600}; UV–Vis photometer 8453, Hewlett Packard/Agilent Technologies, Santa Clara, CA, USA; $OD_{600} = 0.1$ refers to 8.6×10^6 cfu/mL for *S. carnosus* LTH 7036 or to 1.7×10^7 cfu/mL for *S. carnosus* LTH 3838 (Müller et al., 2015)).

2.2. Cured raw ham production

Fresh pork loin (*Musculus longissimus dorsi*; pH: 5.46 ± 0.05, Table 1) was obtained from a local central market (MEGA, Stuttgart, Germany) and cured raw ham production was carried out in the pilot plant at the University of Hohenheim (Germany) (Supplementary material 2). For all four batches, the brine (30 L) was prepared with either 10% (w/w) nitrite or nitrate as the curing agent, and one of the two *S. carnosus* strains ($1 \times 10^7 - 10^8$ cfu/mL brine), or no strain. Nitrate curing salt was produced by using 13.18 g KNO_3 (Gewürzmüller, Korntal-Münchingen, Germany) per kg NaCl to get the same molar concentration as sodium nitrite curing salt (Südsalz, Heilbronn, Germany; 0.9% $NaNO_2$). For the first batch (nitrate control), brine was produced with nitrate curing salt without staphylococci. The second batch (nitrite control) contained commercial nitrite curing salt and no starter culture. The brine with *S. carnosus* LTH 3838 (batch 3) was produced by using nitrate curing salt and 1 mL of cell suspension with approx. 1×10^{11} cfu/mL. Batch 4 contained *S. carnosus* LTH 7036 instead of *S. carnosus* LTH 3838. All hams were produced in duplicate by injection using a multi-needle injector 105 MC2 R (Günther, Dieburg, Germany) with the following setting: 105 needles (2 mm diameter, 2 × 0.8 mm hole size), 0.7 bar injection pressure, triple injection in two-way mode. A mean injection weight of 7–8% was intended. Neither spices nor carbohydrates were added during the production of the cured hams to avoid an increase in lactic acid bacteria and observe the effects of the staphylococci. After injection, a dry-curing step was applied to reach a total curing salt content of 40 g per kg fresh meat, and the hams were cured for 7 days at 5 °C. Drying and mild smoking (at 24 °C) took place in the climatic chamber Air Master UK-1800 BE (Reich, Urbach, Germany) for an additional 7 days at temperatures of 10 °C to 15 °C with a relative humidity gradient from 85% to 75% relative humidity (Supplementary material 3). Afterwards, the hams were stored at 15 °C and 75% relative humidity till they reached their final weight loss of approximately 25% (25.9 ± 0.4%). The final vacuum-packed hams were stored at 15 °C until day 34. The weight loss of each batch was examined during the process: sampling took place on day 0 (raw material, cured raw hams after injection and brine), day 1, day 3, day 7 and day 14, as well as during storage on day 21 and day 34. All analyses were done in duplicate.

Table 1
Summary of the production and end product parameters (mean ± standard deviation; total: mean value over all batches) of all cured raw ham batches cured either with (1) nitrate or (2) nitrite without starter cultures as control batches or with nitrate and (3) *S. carnosus* LTH 3838 (low nitrate reductase activity) or (4) *S. carnosus* LTH 7036 (high nitrate reductase activity): pH of raw meat (n = 12), total viable count in brine (log cfu/mL, n = 4), staphylococcal count in brine (log cfu/mL, n = 4) and injection weight (%, n = 4). End product parameters of the cured raw hams were investigated at day 34: weight loss (%, n = 2), pH (n = 6), moisture content (%, n = 6) and water activity (a_w value; n = 6). Different letters indicate significant differences between batches and # indicates significant differences of pH over time (confidence level $\alpha = 0.05$).

Batch	Raw product (day 0)				End product (day 34)			
	pH raw meat ()	Total viable count in brine (log cfu/mL)	Staphylococcal count in brine (log cfu/mL)	Injection weight (%)	pH raw ham ()	Weight loss* (%)	Moisture content (%)	a_w value ()
1 Nitrate control	5.46 ± 0.02^{a}	<2.00	<2.00	7.07 ± 0.49^{a}	5.67 ± 0.01$^{a\#}$	24.7 ± 0.4^{*a}	65.6 ± 0.6^{a}	0.930 ± 0.005^{a}
2 Nitrite control	5.48 ± 0.04^{a}	<2.00	<2.00	8.68 ± 1.51^{a}	5.71 ± 0.02$^{b\#}$	24.6 ± 0.7^{*a}	63.9 ± 0.6^{b}	0.929 ± 0.002^{a}
3 Nitrate + *S. carnosus* LTH 3838	5.51 ± 0.04^{b}	7.7 ± 0.1^{a}	7.0 ± 0.1^{a}	7.79 ± 1.96^{a}	5.69 ± 0.01$^{ab\#}$	25.5 ± 1.6^{*a}	61.8 ± 1.4^{c}	0.915 ± 0.005^{b}
4 Nitrate + *S. carnosus* LTH 7036	5.42 ± 0.02^{c}	7.4 ± 0.2^{a}	6.9 ± 0.1^{a}	7.71 ± 0.79^{a}	5.69 ± 0.02$^{ab\#}$	25.9 ± 0.4^{*a}	61.7 ± 0.5^{c}	0.919 ± 0.004^{b}
Total	5.46 ± 0.05	–	–	7.81 ± 1.32	5.69 ± 0.02	25.9 ± 0.4	63.2 ± 1.8	0.923 ± 0.008

* mean ± mean deviation (n = 2).