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Growth and production of volatiles by *Staphylococcus carnosus* in dry sausages: Influence of inoculation level and ripening time

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Abstract

Three sausage batches inoculated with normal inoculation level of *Pediococcus pentosaceus* (5×10^6 CFU/g) and with low, intermediate, and high inoculation levels of *Staphylococcus carnosus* (10^5, 5×10^6, 5×10^7 CFU/g, respectively) were produced. Cell counts and formation of volatiles were followed throughout a ripening period of three weeks. The staphylococci exhibited the fastest growth in sausages with a low inoculation level, whereas growth was only moderate in sausages with a high initial level. Analysis of volatiles showed that methyl-branched aldehydes and acids, phenylacetaldehyde, 2-methyl-1-butanol, dimethyldisulphide and dimethyltrisulphide were produced in higher amounts in sausages with a high inoculation level of *S. carnosus*, whereas a low inoculation level correlated with high amounts of diacetyl, ethanol and ethyl esters. The levels of most compounds increased over time, but the amount of diacetyl was negatively correlated to ripening time. A negative interaction effect between inoculation level and ripening time was observed for the amounts of methyl-branched aldehydes.

Keywords: Fermented sausage; Volatiles; *Staphylococcus carnosus*; *Pediococcus pentosaceus*

1. Introduction

Staphylococci are widely used as starter cultures for dry sausage manufacture, often in combination with lactic acid bacteria (LAB). While LAB primarily ensure product safety by acid production, the roles of staphylococci are formation and stabilisation of colour, prevention of rancidity, and formation of flavour-intensive volatiles (Jessen, 1995; Lücke, 1998). Staphylococci possess nitrate reductase activity and thereby the ability to reduce nitrate to nitrite, which is important for the formation of nitrosylmyoglobin, the compound responsible for the characteristic red colour of fermented sausages (Skibsted, 1992). Furthermore, as facultative aerobes, staphylococci consume the free oxygen present in sausages after stuffing, thereby creating the reductive conditions that are necessary for colour stability (Lücke, 1986). The prevention of rancidity is also linked to the oxygen consumption and moreover most staphylococci are catalase positive (Kloos & Schleifer, 1986) and thus able to decompose hydrogen peroxide that otherwise could oxidise unsaturated fatty acids. The volatiles so far recognised as being produced by staphylococci are primarily amino acid catabolites, pyruvate metabolites and methylketones from β-oxidation of fatty acids (Montel, Masson, & Talon, 1998; Stahnke, 2002). In particular the methyl-branched aldehydes and acids from degradation of branched-chain amino acids have received attention, as these compounds have been correlated with dry sausage odour (Montel, Reitz, Talon, Berdagué, & Rousset-Akrim, 1996; Stahnke, 1995b; Stahnke, Holck, Jensen, Nilsen, & Zanardi, 2002).

The typical inoculation level of staphylococci is 10^6 to 10^7 CFU/g, a level probably based on trial and error experience. To our knowledge no data have ever been published on the influence of the inoculation level of staphylococci on colour and flavour formation in sausages, though the ability to obtain increased flavour

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and colour by increasing inoculum or reduce costs by decreased inoculum must have an economic significance to manufacturers of both starter cultures and fermented sausages.

The objective of this study was to investigate the influence of inoculation level of *Staphylococcus carnosus* on the formation of volatiles during sausage processing. The study shows that significant differences in volatile profiles are obtained from sausages with a low, intermediate or high inoculation level of *S. carnosus*. The reasons for and implications of these results are discussed in detail.

2. Materials and methods

Sausages with a low, intermediate and high inoculation level of staphylococci were produced (1×10^5, 5×10^6 and 5×10^7 CFU/g, respectively), and the microbiological and volatile profiles studied during the entire ripening period. In order to simplify volatile analysis, spices were omitted from the sausage recipe.

2.1. Sausage processing

Three sausage batches consisting of pork shoulder (32% w/w), beef back rib (32% w/w), pork back fat (31.3% w/w), NaCl with 0.6% w/w $NaNO_2$ (1.7% w/w), NaCl (1.0% w/w), potato starch (1.25% w/w), glucose (0.4% w/w), sodium ascorbate (0.05% w/w) and $MnSO_4$ H_2O (0.1 μg/g Mn) [1] were produced. Freeze-dried starter cultures of *Pediococcus pentosaceus* PC-1 and *S. carnosus* MIII (Chr. Hansen A/S, Denmark) were added to levels of 5×10^6 CFU/g of the *Pediococcus* and 10^5, 5×10^6 and 5×10^7 CFU/g, respectively, of the *Staphylococcus*. The sausages were stuffed into 48 mm cellulose casings (SFK amba, Denmark), fermented and ripened in a climate chamber (Multimat MC1000, Deutsch, Germany) with initial temperature and humidity of 24 °C and 95% RH. Temperature and humidity were gradually reduced to 17 °C and 75% RH during the first 11 days of production and then kept constant for the following 10 days. Fifteen minutes of smoke from beech chips was applied at day 2, 3 and 6. Two sausages from each of the three batches were sampled at day 0, 1, 2, 3, 7, 14 and 21, vacuum-packed and stored at 50 °C until microbiological and volatile analysis.

2.2. Cell count and pH

Cell counts of LAB and staphylococci were determined by 10-fold dilution of 35 g of sample with subsequent pour plating in MRS (de Man, Rogosa, Sharpe, Oxoid, UK) and S-110 (Difco, Germany) agar media, respectively. Plates were incubated for 3 days anaerobically (MRS) and for 2 days aerobically (S-110) at 30 °C.

Sausage pH was measured at day 0, 1, 2, 3, 5, 7, 9, 14, 17, and 21 on one sausage from each batch (Knick Portamess®, Knick Elektronische Meßgeräte GmbH, Germany, with Metrohm 6.0226.100 electrode, Metrohm Ltd., Switzerland).

2.3. Volatile analysis

Sausage (100 g) was cut into cubes (of approx. 1 cm^3), added to 20 g of NaCl, frozen with liquid nitrogen, homogenized with a domestic chopper (Krups Speedy Pro, Krups GmbH, Germany), and transferred to glass bottles (3×30 g). Bottles were sealed with a glass stopper and placed in a 42 °C water bath for equilibration. After 30 min, glass stoppers were replaced by glass purge heads connected with Swagelok® unions/Teflon ferrules to Tenax TA® tubes (200 mg, 60/80 mesh, Chrompack, Holland) and purged with nitrogen (grade $N_2 > 99.999\%$, flow rate 50 ml/min) for 30 min at 42 °C. Prior to sampling, Tenax TA® tubes were conditioned by purging with helium (99.9995%, flow rate 75 ml/min) for 20 min at 340 °C.

Tenax TA® tubes were desorbed by thermal desorption (ATD50, Perkin–Elmer Ltd., UK) in a two-step manner (first desorption: 250 °C for 3 min onto Tenax TA® cold trap (20 mg, −30 °C), second desorption: 250 °C for 60 s, line temp: 225 °C) and automatically injected into a GC (Hewlett–Packard 5890 series II, Agilent Technologies, USA). Separation was performed on a 30 m × 0.25 mm id. DB 1701 (1 μm film) fused silica capillary column (J & W Sci., USA), detection was by an MS detector (Hewlett–Packard 5972, ionisation energy 70 eV, 3.4 scans/s, source 160 °C, scan range 33–250 AMU). GC oven programme was 35 °C, 1 min, 4 °C/min until 175 °C, 10 °C/min from 175 to 260 °C, 260 ° C for 5 min. Identification was based on MS spectra compared to the NBS75k-database. Volatile responses were measured as single-ion responses in arbitrary units (eV). Data analysis was based on Log_{10}-transformed volatile responses.

3. Results and discussion

Three sausage batches were produced with low, intermediate and high inoculation levels of *S. carnosus*, respectively, by otherwise identical recipe and processing conditions. After three weeks of ripening all sausages irrespective of *Staphylococcus* inoculation level were characterised by a final pH of 5.29 ± 0.04 and an average weight-loss of 31.7 ± 2.2%.

[1] Manganese was added as substitute for the amount typically supplied by spices.

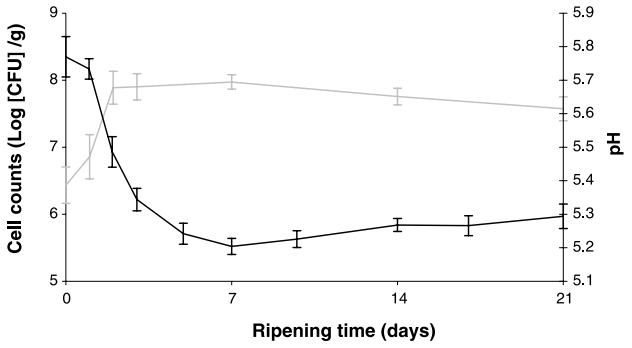

Fig. 1. (□) LAB cell counts and (■) pH of sausages to which were added three different levels of *S. carnosus*. Both measures are based on average values of three sausages.

The course of LAB cell counts and pH is shown in Fig. 1. The LAB increased rapidly in number until day 2 after which they remained at a relatively constant level of approximately 10^8 CFU/g. Sausages with low, intermediate and high inoculation levels of staphylococci were randomly used in the determinations of both pH and LAB. The standard deviations shown in Fig. 1 are not larger than typical batch to batch variations (Demeyer et al., 2000; Gimeno, Astiasarán, & Bello, 2001; Montel, 1999; Sanz, Flores, Toldra, & Feria, 1997), which indicate that staphylococcal inoculation level had no major influence on growth and acidifying activity of LAB.

In Fig. 2 the cell counts of staphylococci in sausages with low, intermediate and high inoculum are shown. In all three sausage batches the levels of staphylococci peaked at day two, after which they slowly decreased. For sausages with low inoculum the cell numbers rapidly increased during the first two days of fermentation whereas there was almost no initial increase in cell numbers for sausages with a high inoculum.

Sausages were analysed for 22 volatile compounds most of which were selected because of their relation to microbial flavour generating metabolism in fermented sausages (Montel et al., 1998; Stahnke, 2002).

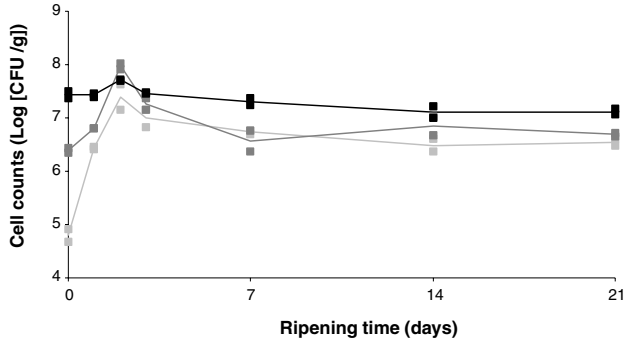

Fig. 2. Cell counts of staphylococci in sausages with three different inoculation levels of *S. carnosus*: (□) 1×10^5, (▩) 5×10^6, and (■) 5×10^7 CFU/g. Lines are drawn between average counts.

Volatile responses are given in Log_{10} values of single ion counts (eV). The average coefficient of variance (CV) between replicates was 2. Results from analysis of variance are shown in Table 1. The levels of most components increased significantly over time, but diacetyl and methyl-branched aldehyde levels were negatively correlated to time, showing that the amount of these compounds was reduced during the last part of fermentation. A likely fate of diacetyl is reduction to acetoin and possibly further to 2,3-butanediol in a NAD^+ generating process, whereas the methyl-branched aldehydes could be either reduced or oxidised into the corresponding alcohols or acids that all show a positive correlation to ripening time (Table 1) (Beck, Hansen, & Lauritsen, 2002; Yvon & Rijnen, 2001). Decrease in methyl-branched aldehyde levels was also seen in sausages inoculated with *S. xylosus* after three days of ripening (Tjener, Stahnke, Andersen, & Martinussen, 2003a). In contrast, (Olesen, Meyer, & Stahnke, 2004) reported increased levels of methyl-branched aldehydes throughout five weeks of ripening of sausages inoculated with either *S. xylosus* or *S. carnosus*.

The straight chain aldehydes showed no significant correlation to time, i.e., lipid oxidation was not

Table 1
Influence of ripening time and inoculation level on the amount of volatiles – analysis of variance of volatile responses versus design [a,b]

Volatile	Ripening time	Inoculation level	Time *Inoculation level
Hexanal			
Decanal			
2-Methyl propanal	−−−	+++	−−−
2-Methyl butanal	−−−	+++	−−−
3-Methyl butanal	−−−	+++	−−−
Phenylacetaldehyde	+++	+++	−−−
Acetic acid	+++		
2-Methyl propanoic acid	+++	+	
2-Methyl butanoic acid	+++	+	
3-Methyl butanoic acid	+++	++	
Ethanol	++	−−−	
2-Methyl-1-butanol	++	+++	
3-Methyl-1-butanol	++		
2-Phenylethanol	+++		
Ethyl acetate	+++	−−−	
Ethyl butanoate	+++	−−−	
Ethyl-2-methyl butanoate	+++	−−−	−−
Ethyl-3-methyl butanoate	+++	−−−	−−
2-Butanone	+++		
Diacetyl	−−	−−−	
2-Pentanone	+++		
Methional	+++	−	
Dimethyldisulphide		+++	
Dimethyltrisulphide		+++	+

[a] Significance levels: +++, ++ and + represent *p*-values below 0.001, 0.01 and 0.05, respectively.

[b] The symbols + and − indicate positive or negative impact on volatile level, respectively.

pronounced within the time span of this study. Sulphides also showed no correlation to ripening time.

High inoculation levels gave a significant increase in the levels of methyl-branched aldehydes and acids, 2-methyl-1-butanol, phenylacetaldehyde and sulphides, whereas diacetyl, methional, ethanol and the ethyl esters were negatively influenced. Furthermore, a strong negative interaction effect between time and inoculation level was seen for the methyl-branched aldehydes. All compounds that correlated positively to high inoculum were products of amino acid catabolism (Stahnke, 2002; Yvon & Rijnen, 2001), and it is very plausible that high inoculum provides higher amino acid catabolic capacity.

Both ethanol and diacetyl formation are most likely linked to pyruvate metabolism. Three factors that vary with inoculation level of staphylococci and that possibly could alter pyruvate metabolism are (1) the specific growth rate of the staphylococci (Fig. 2), (2) carbohydrate availability and (3) the redox potential in the initial phase of fermentation. Factors 2 and 3 can influence the pyruvate metabolism of LAB (Liu, 2003) and presumably also of staphylococci. Furthermore, the higher redox potential (less oxygen consumption) expected in sausages with a low inoculation level of staphylococci may result in increased growth and activity of indigenous bacteria or yeasts that could produce ethanol.

Ethyl ester formation is linked to the level of ethanol (Stahnke, 1994; Stahnke, 1995a; Tjener, Stahnke, Andersen, & Martinussen, 2003b) and thus also negatively influenced by a high inoculation level of staphylococci.

It is interesting to note, that increased inoculum size did not result in higher levels of 2-pentanone, as methylketone formation during meat fermentation often has been ascribed to the activity of staphylococci (Engelvin, Feron, Perrin, Mollé, & Talon, 2000; Montel et al., 1996; Stahnke, 1999a; Søndergaard & Stahnke, 2002). In a resting cell study, (Fadda, Lebert, Leroy-Sétrin, & Talon, 2002) showed that *S. carnosus* produced less of the methylketones when redox potential was low, i.e., low aeration rate and low level of nitrate. As mentioned above, a high inoculation level may result in a lower redox potential and thus lower methylketone formation. Also, the formation of the precursors for methylketones, the β-keto- and hydroxy acids, may be influenced by the redox potential. In addition to the lower redox potential, a high inoculation level probably also results in more of the methylketone forming enzymes. The oppositely directed effects of inoculum size of staphylococci, oxygen consumption and enzymatic activity, thus may nullify each other.

The negative interaction effect of ripening time and inoculation level on the level of methyl-branched aldehydes is illustrated in Fig. 3 with the levels of 3-methyl butanal as example (2-methyl propanal, 2-methyl butanal and phenylacetaldehyde showed the same course as 3-methyl butanal). The level of 3-methyl butanal increased most rapidly in sausages with a low inoculation of staphylococci, whereas almost no increase was seen in sausages with a high inoculum size. From day 2 and 3 the level of 3-methyl butanal started to decrease in sausages with high and intermediate inoculation levels, respectively, whereas no decrease was seen in sausages with the lowest inoculum. Note the striking similarity between the levels of 3-methyl butanal and cell numbers (Fig. 2) suggesting a high correlation between the level of staphylococci and the amount of methyl-branched aldehydes.

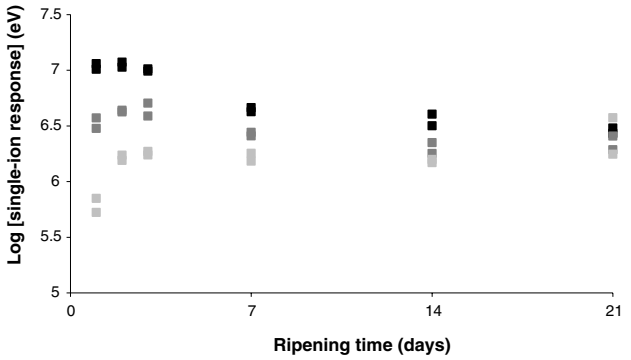

Fig. 3. Level of 3-methyl butanal in relation to *S. carnosus* inoculation level and ripening time. (□) 1×10^5, (▩) 5×10^6, and (■) 5×10^7 CFU/g. Please note the Log_{10} scale of the Y-axis.

However, the relation between growth and volatile formation by staphylococci is not well established. Stahnke (1999b) observed an inverse correlation between the levels of volatile amino acid degradation products and *S. carnosus* cell counts in model minces and suggested that growing cells use amino acids to build cell mass and therefore produce less of the volatile amino acid degradation products than non-growing cells. In opposition to this, Petersen, Beck, and Lauritsen (2003) found, by on-line measurement in a liquid culture broth, that *S. xylosus* produces methyl-branched aldehydes in highest amounts during exponential, aerobic growth, thus indicating a positive correlation between growth and volatile formation.

In the present study, it looks as if cell number and volatile production are proportional (Figs. 2 and 3), which supports the findings of Petersen et al. (2003) more than those of Stahnke (1999b). Nevertheless, care should be taken when using cell counts as a measure of growth (i.e., number of cell divisions), and more appropriate experimental setups should be used to study this important relation.

At the end of ripening, the levels of 3-methyl butanal (plus 2-methyl propanal, 2-methyl butanal and phenylacetaldehyde) were comparable for sausages with low, intermediate and high inoculum levels, and the practical implication of increasing inoculum size is therefore limited to fast-ripened sausages with respect to the methyl-branched aldehydes.

However, several other volatiles with high sensory impact were significantly influenced by staphylococcal inoculation level throughout the ripening phase. Formation of methyl-branched acids (sweaty, cheesy odour notes) and sulphides (sulphury, cabbage-like odour notes) were favoured by high inoculation level whereas diacetyl (buttery odour note) and ethyl esters (fruity odour notes) were favoured by low inoculation level. [2] Presuming the above-mentioned volatiles are present at levels above their sensory threshold levels, it is thus possible to change sausage aroma profiles by changing the inoculation level of this *Staphylococcus* starter culture.

A change of staphylococcal inoculation level will, in combination with the potential effect on flavour, possibly also influence oxygen consumption and nitrate reduction, which again is important for colour formation and lipid oxidation. In this study, no conclusive data on nitrate reductase activity and oxygen consumption were obtained, but indications were given: at day one, sausages with the highest inoculum size were more red than sausages with intermediate and low inoculation level. From day 2–21 all sausages had the same red colour (visual observations, data not shown). The faster colour formation in sausages with a high inoculation level of staphylococci could indicate a higher nitrate reductase activity, faster oxygen consumption, or both. Thus, although aroma profiles can be changed by increasing or decreasing the inoculation level of staphylococci, one should carefully consider the other effects of such a change, for instance the influence on colour formation.

Also, it should be kept in mind that sausages in the current study were produced without spices. It is not possible to rule out effects of spices on growth and activity of LAB and staphylococci, and consequently, further work in the presence of spices may be needed before applying current results to industrial sausage production.

4. Conclusion

The inoculation level of *S. carnosus* significantly affected the volatile profiles of fermented sausages. Low inoculation level resulted in high contents of diacetyl, ethanol and ethyl esters whereas high inoculation resulted in high levels of methyl-branched aldehydes and acids, 2-methyl-butanol, phenylacetaldehyde and sulphides.

The levels of most compounds increased over time, but diacetyl and, with exception of sausages with a low inoculation level, also methyl-branched aldehydes were negatively correlated to ripening time.

[2] Odour notes of volatiles were found in (Rychlik, Schieberle, & Grosch, 1998).

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