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Effect of ascorbate, nitrate and nitrite on the amount of flavour compounds produced from leucine by *Staphylococcus xylosus* and *Staphylococcus carnosus*

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Abstract

Resting cells of *Staphylococcus xylosus* and *S. carnosus* were incubated with ascorbate, nitrate and nitrite in defined reaction medium and their degradation of ^{3}H-labelled leucine into methyl-branched catabolites were studied using HPLC/radiometric detection. The experiments were carried out with and without addition of α-ketoglutarate. The main catabolic product of leucine degradation was 3-methylbutanoic acid but also small amounts of α-hydroxy isocaproic acid were produced. Nitrite addition lowered the concentration of 3-methylbutanoic acid for both *Staphylococcus* species and this effect was strongly amplified by ascorbate for *S. xylosus* but not for *S. carnosus*. For both species ascorbate alone had little if any effect. Also nitrate lowered the concentration of 3-methylbutanoic acid for *S. xylosus*. The concentration of α-hydroxy isocaproic acid was, however, increased by addition of nitrite and nitrate for *S. xylosus*. Addition of α-ketoglutarate generally increased the concentration of 3-methylbutanoic acid for both *S. xylosus* and *S. carnosus*

Keywords: Fermented sausage; Starter culture; *Staphylococcus xylosus*; *Staphylococcus carnosus*; Flavour; Leucine; Nitrite; Nitrate; Ascorbate

1. Introduction

In the manufacturing of fermented sausages nitrate and/or nitrite are commonly added to the sausage mince. Ascorbate is a potent antioxidant usually added along with nitrite to stabilise the red colour (Alley, Cours, & Demeyer, 1992; Lücke, 1998). Nitrate and nitrite are primarily added to ensure a satisfactory colour development but also, in the case of nitrite, to protect the sausage against oxidation and thereby rancidity. In addition nitrite and nitrate also affect the cured sausage flavour (Wirth, 1991). In a simplified explanation nitrite oxidises myoglobin or oxymyoglobin into metmyoglobin (MMb) and is reduced to nitric oxide (NO), which reacts with MMb, creating the red nitrosyl myoglobin (Pegg & Shahidi, 1997). Nitrate does not directly take part in the colour developing reactions and must be reduced to the active agent nitrite. Therefore nitrate reducing *Staphylococcus* or *Kocuria* (formerly *Micrococcus*) species are required if nitrate is used instead of nitrite (Lücke, 1998). It has been shown that *Staphylococcus carnosus* can also use nitrate and nitrite as terminal electron acceptors. Nitrate reductase activity is strongly enhanced by anaerobic conditions but activity is still present under aerobic conditions (Neubauer & Götz, 1996).

Increased focus has been directed toward understanding the role of staphylococci in regard to amino acid degradation and flavour generation. The degradation of the branched-chain amino acids (BCAA) leucine, isoleucine and valine into flavour intensive methyl-branched aldehydes, acids and the less flavorous alcohols by staphylococci has been shown by Larrouture, Ardaillon, Pépin, and Montel (2000) and Vergnais,

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Masson, Montel, Berdagué, and Talon (1998), and has been linked to development of fermented sausage flavour (Montel, Reitz, Talon, Berdagué, & Rousset-Akrim, 1996; Stahnke, 1995b).

Presently, little information is available about how ascorbate and nitrite directly affect staphylococci's generation of these flavour compounds. But studies have been conducted on the effect of nitrate with resting cells in aqueous reaction media. The degradation of leucine into 3-methylbutanoic acid by *S. carnosus* was clearly inhibited by the presence of nitrate as shown by Larrouture et al. (2000). Masson, Hinrichsen, Talon, and Montel (1999) demonstrated the same influence of nitrate for *S. carnosus* but also detected an increase in the concentration of 3-methylbutanal. In contrast Møller, Hinrichsen, and Andersen (1998) showed for *S. xylosus*, that presence of nitrate decreased the concentration of 3-methylbutanal and 3-methylbutanol and slightly increased the concentration of 3-methylbutanoic acid in meat extract.

The aim of this study was to examine the direct effect of ascorbate, nitrate and nitrite on the ability of *S. xylosus* and *S. carnosus* to catabolise the branched-chain amino acid leucine into important flavour intensive compounds.

2. Materials and methods

2.1. Experimental design

All experimental designs were fully randomised and data analysed according to a fixed effect model. Analysis of ascorbate, nitrate and nitrite was conducted according to a full $3\times2\times2$ factorial design with each level replicated twice, for both *S. xylosus* and *S. carnosus*. The factorial experiment was done using a defined reaction medium with leucine as the only amino acid. The experimental design was carried out with and without α-ketoglutarate, which is believed to affect the initial transaminase step in the degradation of leucine by staphylococci (Madsen et al., 2002). Since nitrate is occasionally used in rather large concentrations, the effect of increasing nitrate concentration was also studied for *S. xylosus* in a single factor experiment with five levels, analysed in triplicate. Analysis of variance was used to verify any effects of the factorials and Duncan's multiple range test was used to detect differences between concentration means (Montgomery, 1997).

2.2. Bacterial strains and chemicals

The strains used for the experiment were two commercial starter cultures *S. carnosus* S1 (Wisby, Germany) and *S. xylosus* DD-34 (Chr. Hansen, Denmark). Chemicals were supplied either from Applichem (Darmstadt, Germany), Merck KGaaA (Darmstadt, Germany), Sigma–Aldrich Corp. (St. Louis, MO, USA) or TCI (Tokyo, Japan). L-(4-5 H^3) leucine was supplied by Amersham Pharmacia Biotech (Little Chalnoth, UK).

2.3. Preparation of cell cultures

Staphylococcus cells were streaked from a cryotube onto MSA plates (Mannitol Salt Agar, Difco 0306-17-2) and incubated for three days at 30 °C. Cells from a single colony were propagated twice in a modified medium (Table 1) of Hussain, Hastings, and White (1991) by incubation at pH 7.2, 30 °C with shaking (170 rpm). After 24 h, bacteria were centrifuged (7000*g*, 10 min at 4 °C), the pellets washed three times and the bacteria suspended in reaction medium (Table 1) without leucine. The optical density (600 nm) of the resting cell suspension was adjusted to 100 and this suspension was kept ice cold until addition into the reaction medium where the reaction procedure took place (see below).

2.4. Reaction procedure

L-ascorbic acid (0–250–500 mg/l), $NaNO_2$ (0–150 mg/l), $NaNO_3$ (0–150 mg/l) and α-ketoglutaric acid (5 mM) were added to 2 ml Eppendorf tubes according to the experimental design for the factorial experiment and $NaNO_3$ (0–150–300–600–1200 mg/l) for the single factor experiment (numbers in brackets is final concentrations). The resting cell suspension was added to the tubes containing the above components along with reaction medium to a final volume of 1.5 ml and an OD of 10. The final concentrations of the non-factorial components are listed in Table 1.

Finally L-(4-5 H^3) leucine was added to the tubes to achieve a concentration of 5 μCi/ml. The tubes were wrapped in parafilm and incubated for 22 h at 30 °C, pH 5.8 under shaking conditions (1200 rpm). To stop the reaction, 100 μl 6.4 M H_2SO_4 was added to the reaction mixture (0.4 M) and the mixture was centrifuged at 10 000*g* for 10 min. The supernatant was then removed and stored at −20 °C until final analysis.

2.5. Analysis of leucine catabolites

From each sample 100 μl of supernatant was injected into a Kontron (Saint-Quentin en Yvelines, France) HPLC system consisting of an autosampler model 360 and HPLC Pump model 325. The mobile phase consisted of an isocratic elution of filtered (0.45 μm filter) and degassed H_2SO_4 (0.0075 N) with a flow rate of 0.7 ml/min. The guard column was a microguard H^+ cartridge and the column was an Aminex HPX 87H ion exchange column both from Biorad Labs (Richmond, CA, USA). Column temperature was kept at 55 °C. The radioactive metabolites were detected by a Flo-one/Beta A-515TR radio-

Table 1
Growth and reaction media used in the experiments

	Growth medium (mg/l)	Reaction medium (mg/l)
Buffers/salts/sugar		
MES-buffer	–	10 000
$Na_2HPO_4 \cdot 2H_2O$	10 000	10 000
KH_2PO_4	3000	3000
NaCl	35 000	35 000
$MgSO_4 \cdot 7H_2O$	500	500
D-Glucose	2000	–
Yeast extract	1000	–
Amino acids		
L-Alanine	100.0	–
L-Arginine · HCl	121.0	–
L-Aspartic acid	150.0	–
L-Cysteine · HCl	65.0	–
D/L-Glutamic acid	150.0	–
L-Glycine	100.0	–
L-Histidine	100.0	–
D/L-Isoleucine	150.0	–
L-Leucine	150.0	250
L-Lysine · HCl	124.9	–
L-Methionine	100.0	–
L-Phenylalanine	100.0	–
L-Proline	150.0	–
L-Serine	100.0	–
L-Threonine	150.0	–
L-Tryptophan	100.0	–
L-Tyrosine	100.0	–
L-Valine	150.0	–
Growth factors		
Adenine sulfate	20.0	20.0
Guanine hydrochloride	20.0	20.0
Biotin	0.10	0.10
Nicotinic acid	2.00	2.00
D-Pantothenic acid (Ca-salt)	2.00	2.00
Pyridoxal hydrochloride	4.90	4.90
Pyridoxamine dihydrochloride	4.00	4.00
Riboflavin	2.00	2.00
Thiamine hydrochloride	2.00	2.00
Vitamin B_{12}	0.10	0.10
Folic acid	0.10	0.10
Trace metals		
$CaCl_2 \cdot 2H_2O$	6.70	6.70
$MnSO_4 \cdot 1H_2O$	5.60	5.60
$(NH4)_2Fe(SO_4)_2 \cdot 6H_2O$	6.00	6.00
$CoCl_2 \cdot 6H_2O$	0.41	0.41
$CuSO_4 \cdot 5H_2O$	0.04	0.04
$ZnSO_4 \cdot 7H_2O$	0.87	0.87

HPLC detector using Ultima-Flo^{tm} AP scintillation liquid (Packard instruments Co., Meridan Connecticut, USA). Two injections were made for each sample and the average used as one measurement. Results are expressed in arbitrary units, counts per minute (cpm).

3. Results and discussion

Both *Staphylococcus* species produced 3-methylbutanoic acid as the major catabolite from degradation of leucine. α-Hydroxy isocaproic acid was produced in small amounts. There were no signs of α-ketoisocaproic acid, 3-methylbutanol or 3-methylbutanal which are other known catabolites from the degradation of leucine (Beck, Hansen, & Lauritsen, 2002). From a flavour aspect the generation of 3-methylbutanoic acid is of primary importance, since this catabolite along with its precursor 3-methylbutanal has been correlated with the characteristic aroma of fermented sausages (Berdagué, Monteil, Montel, & Talon, 1993; Montel et al., 1996; Stahnke, 1995b). Generation of α-hydroxy isocaproic acid from degradation of leucine by *S. xylosus*, *S. carnosus* and *S. warneri* has previously been reported by Larrouture et al. (2000). α-Hydroxy isocaproic is generated by reduction of α-ketoisocaproic acid formed through transamination of leucine. The α-hydroxy isocaproic acid is not a known flavour compound or precursor of such, but it is nevertheless of some interest in regard to aroma because its generation draws α-ketoisocaproic acid away from degradation into aroma compounds (Yvon & Rijnen, 2001). As expected, addition of α-ketoglutarate generally increased the 3-methylbutanoic acid concentration, though the effect was considerably more pronounced for *S. xylosus* than for *S. carnosus* (Fig. 1(a) and (b)).

3.1. The effect of ascorbate on 3-methylbutanoic acid formation

For *S. xylosus*, ascorbate addition alone had no effect on the concentration of 3-methylbutanoic acid, even though there was a strong significant main factor effect of ascorbate (Fig. 1(a) and Table 2). This was due to the strong negative influence by the interaction between ascorbate and nitrite/nitrate. In combination with α-ketoglutarate there was a small increase in the concentration of 3-methylbutanoic acid by ascorbate (Fig. 1(a)). For *S. carnosus*, ascorbate had no effect at all on the production of 3-methylbutanoic acid (Fig. 1(b) and Table 2). These results are in agreement with experiments using sausage minces with *S. xylosus* or *S. carnosus* culture, where addition of ascorbate had no effect in regard to generation of branched-chain amino acid degradation products (Stahnke, 1999).

3.2. The effect of nitrate on 3-methylbutanoic acid formation

Nitrate had a limited but significant effect on *S. xylosus* by reducing the concentration of 3-methylbutanoic acid irrespective of the presence of α-ketoglutarate (Table 2 and Fig. 1(a)). This observation is in accordance with the results of Larrouture et al. (2000), and this effect of nitrate was also reported for *S. carnosus* by Larrouture et al. (2000) and Masson et al. (1999).

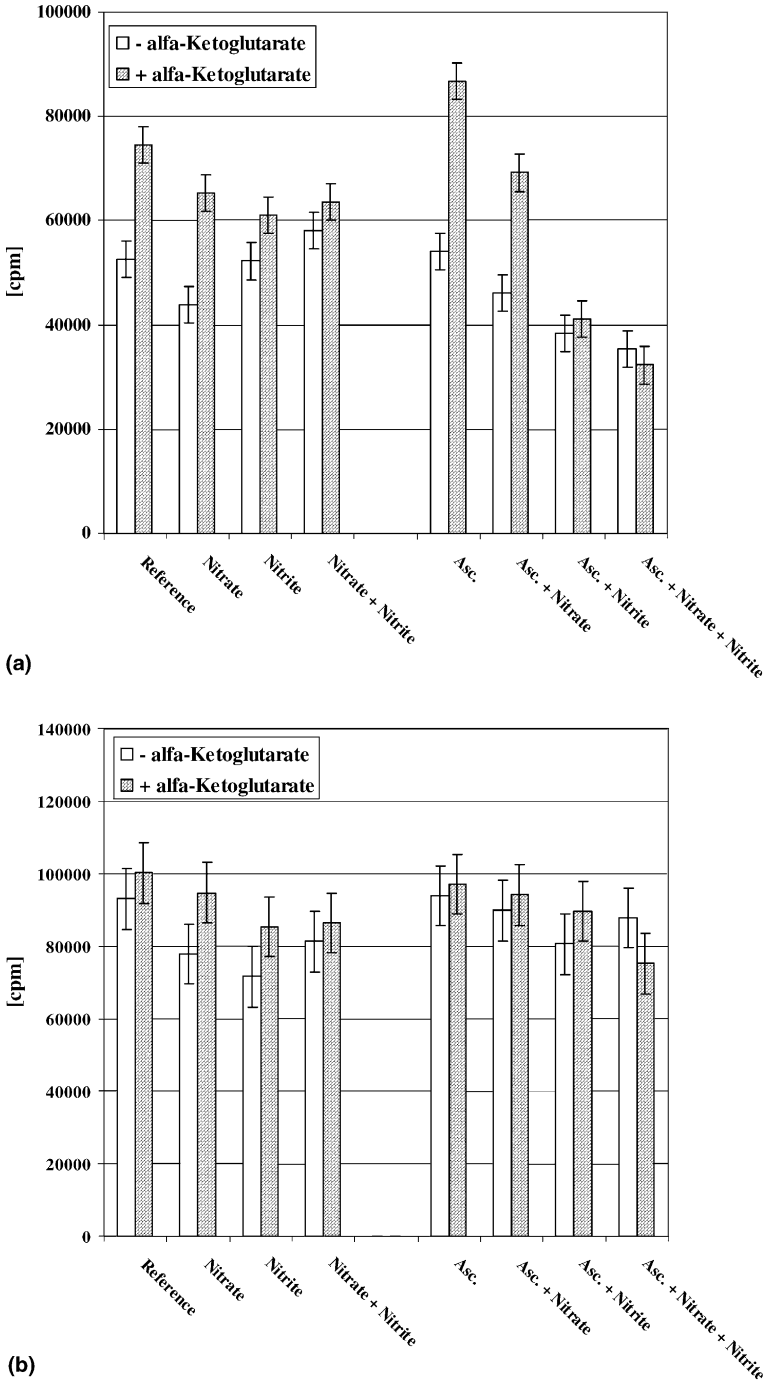

Fig. 1. (a) Concentration of 3-methylbutanoic acid produced by *S. xylosus* from degradation of leucine under the effect of 500 mg/l ascorbate (Asc.), 150 mg/l nitrate ($NaNO_3$) and 150 mg/l nitrite ($NaNO_2$). Error bars are based on pooled standard deviation. (b) Concentration of 3-methylbutanoic acid produced by *S. carnosus* from degradation of leucine under the effect of 500 mg/l ascorbate (Asc.), 150 mg/l nitrate ($NaNO_3$) and 150 mg/l nitrite ($NaNO_2$). Error bars are based on pooled standard deviation.

In the present study however, there was no statistically significant effect of nitrate on *S. carnosus* (Table 2) even though the addition of nitrate appeared to lower the 3-methylbutanoic acid concentration somewhat, especially in the absence of α-ketoglutarate (Fig. 1(b)). However, the strain of *S. carnosus* used in the present study, was different from the strain used in the two previously mentioned studies. It should be noted that *S. carnosus* is able to utilise nitrate as terminal electron acceptor instead of oxygen (Neubauer & Götz, 1996). This ability might enhance their metabolic activity in the oxygen limited sausage environment.

Nitrate is often added to sausages in much larger quantity than used in these factorial experiments (Lücke,

Table 2
ANOVA-analysis of the effect of ascorbate, nitrite and nitrate upon the amount of 3-methylbutanoic acid produced by *S. xylosus* and *S. carnosus* in reaction medium

	Regression coefficients[a] and significance levels[b]			
	S. xylosus		*S. carnosus*	
	–	+α-Ketoglutaric acid	–	+α-Ketoglutaric acid
Factor				
Ascorbate	−4098***	−4400**	–	–
NO_2^-	−3724***	−25 237***	−10 173**	−15 231**
NO_3^-	−4404***	−9661***	–	–
Asc. × NO_2^-	−5312***	−8800***	–	–
Asc. × NO_3^-	–	–	–	–
NO_2^- × NO_3^-	4188*	4203*	10 396**	–
Asc. × NO_2^- × NO_3^-	−1583*	–	–	–

[a] Only significant regression coefficients displayed, $P < 0.05$.
[b] Significance levels: Not significant (–), $P < 0.05$(*), $P < 0.01$(**), $P < 0.001$(***).

1998). Therefore another experiment was carried out for *S. xylosus* to examine the effect of high nitrate levels. The concentration of 3-methylbutanoic acid decreased significantly with addition of nitrate (150 mg/l), as it was shown in the factorial experiment (Fig. 1(b) and Table 2), but higher nitrate concentration up to 12 000 mg/l had no additional effect (Fig. 2).

It could be suspected that it was nitrite and not nitrate that exerted the negative effect upon 3-methylbutanoic acid production. Indeed Talon, Walter, Chartier, Barrière, and Montel (1999) showed that nitrate reductase activity and subsequent production of nitrite was prevalent in several strains of *S. carnosus* and *S. xylosus* in resting cell cultures. However from Fig. 1(a) it can be seen that nitrite in the absence of ascorbate and α-ketoglutarate had no apparent effect on the production of 3-methylbutanoic acid for *S. xylosus*. Therefore it seems more plausible that nitrate might act as a direct or indirect regulator of the branched-chain amino acid catabolism. At concentrations above 150 mg $NaNO_3$/l the regulatory mechanisms might be saturated.

3.3. The effect of nitrite and interaction effects on 3-methylbutanoic acid formation

For *S. carnosus* nitrite had a significant effect by reducing the concentration of 3-methylbutanoic acid. This contrasted the effects observed for *S. xylosus*, where addition of nitrite only reduced the 3-methylbutanoic acid concentration when added together with α-ketoglutarate or ascorbate. When nitrite and nitrate were added together (without ascorbate and α-ketoglutarate) they increased the concentration of 3-methylbutanoic acid compared to the addition of these compounds individually (Fig. 1(a)). Though the direct effect of ascorbate or nitrite alone was small in this study their interaction was very pronounced for *S. xylosus*. Ascorbate markedly intensified the negative effect of nitrite on the generation of 3-methylbutanoic acid for *S. xylosus* as shown in Fig. 1(a). The combination of ascorbate, nitrite and nitrate produced the lowest 3-methylbutanoic acid concentration, though this effect only penetrated as statistically significant in the absence of α-ketoglutarate

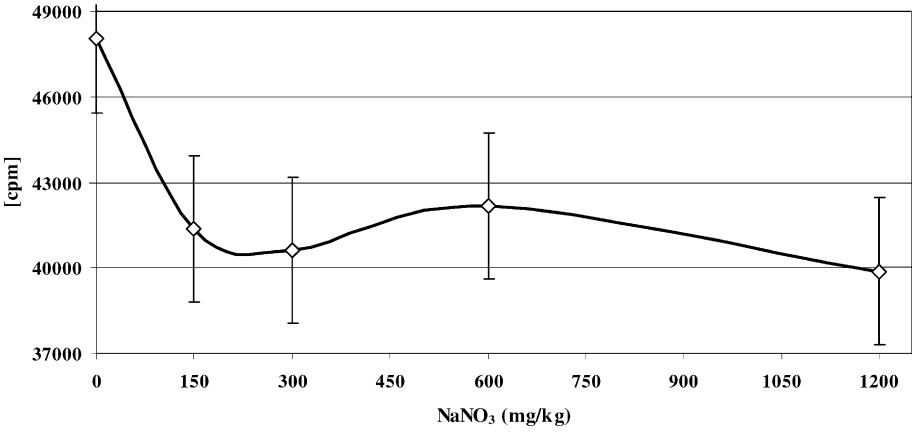

Fig. 2. Concentration of 3-methylbutanoic acid (pooled standard deviation) produced by *S. xylosus* from degradation of leucine under the effect of increasing nitrate concentration.

(Table 2). Ascorbate and nitrite also nullified the effect of α-ketoglutarate (Fig. 1(a)). High levels of nitrite (300 mg/kg) have previously been shown to reduce the concentration of 3-methylbutanal, 3-methylbutanoic acid and 2-methylpropanic acid in sausages inoculated with *S. xylosus* starter culture (Stahnke, 1995a). Also in reaction media inoculated with *S. carnosus* cells, addition of nitrite (0.01%) clearly decreased the production of 3-methylbutanoic acid at pH 5.4 where nitrite is partly protonised, but at pH 8.6 nitrite actually increased the concentration of 3-methylbutanoic acid (Montel et al., 2000). Nitrite is a well-known antimicrobial agent and its antimicrobial effect is related to its ability to diffuse through the cell wall in its protonated form (Skovgaard, 1992). This property could explain the negative effect of nitrite seen for *S. carnosus*. But this mechanism cannot explain the nitrite/ascorbate interaction observed in the present work. However, nitrite can be reduced by ascorbate into nitric oxide (Fox, Fiddler, & Wasserman, 1981). The nitrite/ascorbate interaction therefore could be linked to generation of nitric oxide, a known metabolic regulator (Zhumabaeva, Baider, Volodina, & Kuropteva, 2001). Nitric oxide has also been proposed to damage cells by reacting with porphyrin-containing compounds such as catalase, peroxidase or cytochromes in a similar way it reacts to myoglobin (Jay, 1992). If nitric oxide was truly the detrimental agent, it might be suspected that in fermented sausages there would be no such effect since nitric oxide quickly reacts with myoglobin. In contrast to *S. xylosus*, the only significant interaction for *S. carnosus* was between nitrite and nitrate (Fig. 1(b) and Table 2). It seemed that nitrate reduced or nullified the effect of nitrite. This interaction however disappeared with the addition of α-ketoglutarate where the effect of nitrite was retained despite addition of nitrate. The interaction between nitrite and nitrate is not readily explained. It is known that *S. carnosus* reduction of nitrite into ammonia is inhibited by addition of nitrate but the nitrite reductase activity is likely shutdown in the aerobically grown cell cultures (Neubauer & Götz, 1996) and it is obscure whether ammonia should affect the degradation of leucine in any way.

3.4. Generation of α-hydroxy isocaproic acid

Though the concentration of α-hydroxy isocaproic acid was small it was clearly influenced by all factors for *S. xylosus* (Tables 3 and 4). The most striking result was the very strong effect of nitrite (and to a lesser degree nitrate and ascorbate) that clearly increased the α-hydroxy isocaproic acid concentration. Nitrate enhanced the effect of nitrite and so did ascorbate when mixed with both nitrite and nitrate (Table 3). When ascorbate was added with either nitrite or nitrate it actually reduced the

Table 3
ANOVA-analysis of the effect of ascorbate, nitrite and nitrate upon the amount of α-hydroxy isocaproic acid produced by *S. xylosus* and *S. carnosus* in reaction medium

	Regression coefficients[a] and significance levels[b]			
	S. xylosus		*S. carnosus*	
	–	+α-Ketoglutaric acid	–	+α-Ketoglutaric acid
Factor				
Ascorbate	28***	–	−49*	–
NO_2^-	787***	583***	93**	–
NO_3^-	179***	374***	–	–
Asc. × NO_2^-	103***	–	−35**	–
Asc. × NO_3^-	83***	–	−76*	–
NO_2^- × NO_3^-	166***	268***	–	–
Asc. × NO_2^- × NO_3^-	130***	–	–	–

[a] Only significant regression coefficients displayed, $P < 0.05$.
[b] Significance levels: Not significant (–), $P < 0.05$(*), $P < 0.01$(**), $P < 0.001$(***).

Table 4
The production of α-hydroxy isocaproic acid [cpm] by *S. xylosus* and *S. carnosus* in reaction medium ±α-ketoglutaric acid

		0 mg/l $NaNO_2$		150 mg/l $NaNO_2$	
		0 mg/l $NaNO_3$	150 mg/l $NaNO_3$	0 mg/l $NaNO_3$	150 mg/l $NaNO_3$
S. xylosus	0 mg/l Asc.	43/27	150/119	717/376	637/875
	250 mg/l Asc.	46/96	48/239	715/483	1311/1123
	500 mg/l Asc.	71/33	1/116	592/243	1111/1030
S. carnosus	0 mg/l Asc.	770/1014	1089/914	1075/932	1110/923
	250 mg/l Asc.	791/1020	807/915	1013/795	963/959
	500 mg/l Asc.	1004/1050	896/1031	904/892	847/877

concentration of α-hydroxy isocaproic acid (Table 4), showing that positive effect of ascorbate in Table 3 was almost entirely derived from the three-factor interaction. However, any effect of ascorbate disappeared with addition of α-ketoglutarate. α-Ketoglutarate did not seem to have any other effects upon the generation of α-hydroxy isocaproic acid. The effects of nitrite and nitrate are in direct contrast to the results for the generation of 3-methylbutanoic acid, indicating an inverse relationship between the concentration of 3-methylbutanoic acid and α-hydroxy isocaproic acid. The concentration of α-hydroxy isocaproic acid was however so small that it cannot directly explain changes in the concentration of 3-methylbutanoic acid unless α-hydroxy isocaproic is an intermediate compound which is further metabolised into undetected metabolites.

The concentration level of α-hydroxy isocaproic acid was generally higher for *S. carnosus* than for *S. xylosus* and concentration levels were much less affected by ascorbate, nitrate and nitrite. The concentration was slightly raised by nitrite and reduced by ascorbate in combination with nitrite or nitrate. With addition of α-ketoglutarate all these effects disappeared.

4. Conclusion

Catabolism of leucine was reduced by addition of nitrate for *S. xylosus* and nitrite for *S. carnosus*. Ascorbate alone had only little effect but in combination with nitrite it had a strong influence, lowering the concentration of 3-methylbutanoic acid for *S. xylosus* but not for *S. carnosus* showing a considerable difference in response between the two examined *Staphylococcus* species. Additional research would be desirable to better examine whether any of these effects can be found in sausages, in particular the effect of ascorbate. An inverse relationship existed between the concentration of 3-methylbutanoic acid and α-hydroxy isocaproic acid, in particular for *S. xylosus*.

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