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The influence of precultivation parameters on the catabolism of branched-chain amino acids by *Staphylococcus xylosus* and *Staphylococcus carnosus*

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Abstract

The influence of precultivation parameters on the ability of *Staphylococcus xylosus* and *Staphylococcus carnosus* to convert branched-chain amino acids—leucine, isoleucine and valine—into volatile flavour compounds was investigated using resting cells in a defined reaction medium. The studied precultivation parameters were: growth phase, temperature, NaCl concentration and the concentration of leucine, isoleucine and valine (only for *S. xylosus*). Flavour compounds were sampled by automatic static headspace collection and separated/quantified using gas chromatography/flame ionization detection (GC/FID).

Main catabolic products from degradation of leucine, isoleucine and valine were the flavour intensive branched-chain acids: 2- and 3-methylbutanoic and 2-methylpropanoic acids. The precultivation parameters altered the production of the branched-chain acids significantly, but to various degrees for *S. xylosus* and *S. carnosus*.

Production of branched-chain acids by *S. carnosus* was only influenced slightly by the growth phase and not by changing the NaCl concentration between 4.0% and 10.0% (w/w). Lowering the temperature from 28°C to 18°C significantly decreased *S. carnosus'* generation of branched-chain acids. In contrast, *S. xylosus* was significantly influenced by all precultivation parameters, in particular by the growth phase. Cells taken from growing cultures had a much higher production of branched-chain acids compared to cells taken from stationary cultures. Addition of leucine and valine to the precultivation medium enhanced the production of branched-chain acids whereas addition of isoleucine had the opposite effect.

Keywords: Fermented sausage; Precultivation; Starter culture; *Staphylococcus xylosus*; *Staphylococcus carnosus*; flavour; Branched-chain amino acid

1. Introduction

Along with lactic acid bacteria, *Staphylococcus* species such as *Staphylococcus xylosus* and *Staphylococcus carnosus* are commonly used as starter cultures in fermented meat products. The advantage of staphylococci is their nitrite/nitrate reductase activity, catalase activity and oxygen consumption, which improve colour stability and reduce rancidity (Geisen et al., 1992). Finally staphylococci contribute to the formation of typical fermented sausage flavour (Montel et al., 1996).

Several researchers have identified flavour intensive aldehydes, acids and esters in fermented sausages derived from the degradation of amino acids (Montel et al., 1996; Schmidt and Berger, 1998; Stahnke, 1995b). Also, a correlation between sausage aroma and several of those compounds has been reported (Stahnke, 1995c). It has been shown that both *S. xylosus* and *S. carnosus* degrade branched-chain amino acids (BCAAs) into methyl branched-chain alcohols, aldehydes, acids and their corresponding esters but the exact degradation pathway is not firmly established (Larrouture et al., 2000; Møller et al., 1998; Vergnais et al., 1998).

The initial step in the degradation of BCAAs into flavour compounds is a transamination step catalysed by a BCAA aminotransferase. As shown recently for *S. carnosus* by Madsen et al. (2002) the BCAAs are deaminated into the corresponding α-keto acids, followed by decarboxylation into methyl-branched aldehydes. The aldehydes can either oxidized into the corresponding acids or reduced into the corresponding alcohols (Beck et al., 2002; Vergnais et al., 1998).

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Staphylococcus starter cultures added to fermented sausages may not grow or only grow to a limited extent, in particular in rapidly ripened sausages and in the core of the sausages (Lücke, 1998; Montel et al., 1993; Stahnke, 1995a). We can, therefore, assume that it is essential that the culture possesses an optimal aroma-forming capacity at the time of inoculation in the sausage mince. Precultivation conditions of the starter culture is one factor that could influence the catabolic activity of the culture in the fermented products. Presently, little information exists about the relationship between the state of the *Staphylococcus* cultures and their ability to generate methyl-branched flavour compounds.

The objective of the present study was to examine the effect of various precultivation parameters on the ability of *S. xylosus* and *S. carnosus* to catabolize leucine, isoleucine and valine into volatile components. At first, growth phase was studied by harvesting cells of *S. xylosus* and *S. carnosus* from either growing or stationary cell cultures. Secondly, the influence of temperature, NaCl concentration and varied amounts of leucine, isoleucine and valine were investigated for stationary cell cultures.

2. Materials and methods

2.1. Experimental design and analysis

Experiments were conducted by growing cells in different precultivation media under different conditions, harvesting the cells and measuring their activity in reaction medium (details in following sections). All experimental designs were fully randomized and data analysed according to a fixed effect model. The influence of growth phase, NaCl concentration and temperature was analysed according to a simple single factor design, whereas the influence of leucine, isoleucine and valine was analysed according to a full 2^3 factorial design. Analysis of variance was used to verify any effects of the factorials, and Duncan's multiple range test was used to detect differences between means (Montgomery, 1997).

2.2. Bacterial strains and chemicals

Two commercial starter cultures were investigated: *S. carnosus* S1 (Wisby, Niebüll, Germany) and *S. xylosus* DD-34 (Chr. Hansen, Hørsholm, Denmark). Chemicals were supplied from Applichem (Darmstadt, Germany), Merck KGaaA (Darmstadt, Germany), Sigma-Aldrich Corp. (St. Louis, MO, USA), J.T Baker (Deventer, Holland), BDH (Poole, England) or TCI (Tokyo, Japan).

2.3. Preparation and precultivation of cell cultures

The commercial cultures were isolated on MSA agar (Mannitol Salt Agar, Difco 0306-17-2), propagated in modified basic medium as described by Neubauer and Götz (1996) and stored at −80°C in cryo-tubes in basic medium added 20% glycerol. For each experiment cell material was scraped from the cryo-tubes and streaked onto MSA agar. Cells were incubated for 3 days at 30°C, and material from one colony were transferred to growth (MH) medium (described in Table 1) for further propagation. Growth took place at 27°C in Erlenmeyer flasks with MH-medium (pH 6.4, shaking at 110 RPM). The flasks were closed with water repellent cotton. After 24 h the optical density of the culture was measured at 600 nm (ultrospec 4050, LKB biochrom, UK), and cell material transferred to new MH-medium, this time incubated according to the experimental set-up (see following sections). The inoculation level was 1×10^5 cfu/ml for all experiments, ensuring that cells would grow at least 10 generations in the second cultivation step before harvesting.

2.4. Growth phase study

S. xylosus and *S. carnosus* were grown at 27°C in MH-medium, pH 6.4 and 3.5% (w/w) NaCl under shaking (110 RPM). Stationary cells were harvested after 48 h. Growing cells were harvested when the cells still had a high growth rate at a cell concentration of 1×10^8 cfu/ml. Each treatment was made in triplicate.

2.5. NaCl and temperature study

S. xylosus and *S. carnosus* were grown in MH-medium, pH 6.2, under shaking (110 RPM) and harvested in the stationary growth phase (48 h). Three different growth conditions were applied: 4.0% (w/w) NaCl at 28°C, 4.0% (w/w) NaCl at 18°C and 10.0% (w/w) NaCl at 28°C. Each treatment was made in duplicate.

2.6. BCAA study

S. xylosus was grown at 27°C in MH-medium, pH 6.2, 3.5% (w/w) NaCl under shaking (110 RPM) and harvested in the stationary growth phase (48 h). Yeast extract concentration was reduced to 100 mg/l in the MH-medium to minimize background amount of leucine, isoleucine and valine to 0.03–0.04 mM (Table 1). The high and low levels of leucine, isoleucine and valine in the MH-growth medium were adjusted to 2.5 and 0.1 mM, respectively. Each treatment was made in duplicate. *S. carnosus* was not investigated since it did not grow well at the low yeast extract concentration necessary for this experiment.

Table 1
Growth and reaction media used in the experiments

	MH medium[a] (mg/l)	Reaction medium (mg/l)
Buffers/salts/sugar		
MES-buffer	20,000	10,000
$Na_2HPO_4 \cdot 2H_2O$	10,000	10,000
KH_2PO_4	3000	3000
NaCl[b]	35,000	35,000
$MgSO_4$ $7H_2O$	500	500
D(+)-Glucose	2000	—
Yeast extract[c]	1000	—
Amino acids		
L-Alanine	100.0	—
L-Arginine	100.0	—
L-Aspartic acid	150.0	—
L-Cysteine	50.0	—
L-Glutamic acid	150.0	—
L-Glycine	100.0	—
L-Histidine	100.0	—
L-Isoleucine[c]	150.0	250
L-Leucine[c]	150.0	250
L-Lysine H_2O	112.0	—
L-Methionine	100.0	—
L-Phenylalanine	100.0	—
L-Proline	150.0	—
L-Serine	100.0	—
L-Threonine	150.0	—
L-Tryptophane	100.0	—
L-Tyrosine	100.0	—
L-Valine[c]	150.0	250
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-Panthotenic 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 B12	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
$(NH_4)_2Fe(SO_4)_2 \cdot 6H_2O$	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
$(NH_4)_6 \cdot Mo_7O_{24} \cdot 4H_2O$	0.11	0.11

The pH was adjusted with HCl/NaOH and subsequently sterile filtered through a cellulose acetate 0.2 μm filter (Frisenette, Denmark) in all media.

[a] Modified from Hussain et al. (1991).

[b] Special: NaCl and temperature study.

[c] Special: BCAA study.

2.7. Reaction procedure

The cells from the precultivation medium were centrifuged (7000*g*, 4°C, 10 min), washed and suspended in reaction medium (Table 1) without BCAAs (ice-cold). The suspension of resting cells was added to sterile 11 ml glass tubes with Teflon faced rubber lined screw caps (Wheaton, USA) containing room tempered reaction medium at pH 5.8 (pH 6.2 in growth phase study) to a final volume of 8.0 ml and a cell concentration of 2×10^8 cfu/ml. Tubes were incubated for 24 h at 25°C under agitation. The bacteria did not grow in the reaction medium. The tubes were centrifuged (5500*g*, 10 min. at 4°C), and supernatant transferred to 22 ml headspace vials, capped (PTFE coated butyl rubber septa) and stored at −80°C until analysis.

2.8. Analysis of flavour compounds

Analysis of the supernatant was conducted for branched-chain alcohols/aldehydes/esters and acids in two separate static headspace analyses. The acids had to undergo an ethyl esterification since they are not sufficiently volatile for direct analysis. In all the analyses 2.0 ml supernatant was mixed with 2.0 g of NaCl. For the acid analysis the mixture was also added 0.50 g of $NaHSO_4$, $1H_2O$ and 120 μl of ethanol (99.8%). Reaction medium without added cell culture was used as blank.

Samples were analysed by a static headspace (HS) sampler (HS 40XL, Perkin-Elmer Ltd., UK) connected to a gas chromatograph (AutoSystem XL, Perkin-Elmer Ltd, UK). Samples were kept below 15°C until analysis. The headspace components were transferred splitless into a polar deactivated guard column (RE-10067, Restek, USA) and separated on a (5%-phenyl)-methyl-polysiloxane column (DB-5, 1 μm, 60 m, 0.25 mm, J&W Sci., USA). In order to lower detection limit compounds were cryofocused (accessory HS 40XL) directly onto the column (flow 35 cm/s). Components were detected by flame ionization detection (300°C) and quantified/identified by using external standards. The HS/GC parameters depended upon the compounds analysed for.

2.9. Alcohol/aldehyde/ester analysis

Thermostating time: 25.0 min (shaking), thermostating/needle/transfer line temperature: 65/75/85°C, carrier gas pressure: 44 psi, pressurization time: 3.0 min, injection time: 1.20 min, withdrawal time: 0.50 min, precryofocusing time: 1.90 min, post-cryofocusing time: 0.50 min, injection temperature: 100°C. GC-programme: 35°C for 1 min, 15°C/min to 50°C, 5°C/min to 120°C, 20°C/min to 220°C, 6.0 min.

2.10. Acid analysis

Thermostating/esterification time: 360.0 min (shaking), thermostating/needle/transfer line temperature: 95/100/105°C, carrier gas pressure: 44 psi, pressurization time: 3.0 min, injection time: 0.15 min, withdrawal time: 0.5 min, precryofocusing time: 0.90 min, post-cryofocusing

time: 0.50 min, injection temperature: 110°C. GC-programme: 35°C for 1 min, 20°C/min to 85°C, 2°C/min to 110°C, 20°C/min to 220°C, 4.5 min.

3. Results

In all experiments the main volatile catabolites generated by *S. xylosus* and *S. carnosus* from the degradation of isoleucine, leucine and valine were 2- and 3-methylbutanoic acids, and 2-methylpropanoic acid, respectively, with 2-methylpropanoic acid as the least favoured end product (Figs. 1–3). Despite a detection limit around 1 μg/kg, there was no sign of the respective branched-chain aldehydes, esters or alcohols except for a small amount of 3-methyl-1-butanol produced by *S. xylosus*. The concentration of 3-methyl-1-butanol increased when the concentration of 3-methylbutanoic acid increased, and vice versa (Figs. 1–3). Only a small amount of the BCAAs in the reaction medium was catabolized into branched-chain acids during the reaction. The highest value of a branched-chain acid measured at any time (2-methylbutanoic acid) corresponded to 6.6% (mol/mol) of the added isoleucine pool.

3.1. The influence of growth phase

The growth phase of *S. xylosus* had a marked influence on its ability to generate branched-chain acids. The difference between the growing and stationary cultures resulted in a total difference in the acid concentration by more than a factor 7 (Fig. 1). This effect could be seen for all three acids and in particularly for 2-methylbutanoic acid.

For *S. carnosus* there was only a small, if any, difference in the total concentration of acids in relation to growth phase (Fig. 1 and Table 2). However, for the individual acids the concentration of 3-methylbutanoic and 2-methylpropanoic acids was higher in the stationary cultures than in the growing cultures, whereas the amount of 2-methylbutanoic acid was lower. For both staphylococci there was a shift in the relative distribution between the acids, from 2-methylbutanoic acid being the predominant acid produced by the growth phase culture, to 3-methylbutanoic acid being the predominant acid produced by the stationary phase culture (Fig. 1 and Table 2). The changes in the concentration of 2-methylpropanoic acid seemed largely to follow the changes in the concentration of 3-methylbutanoic acid.

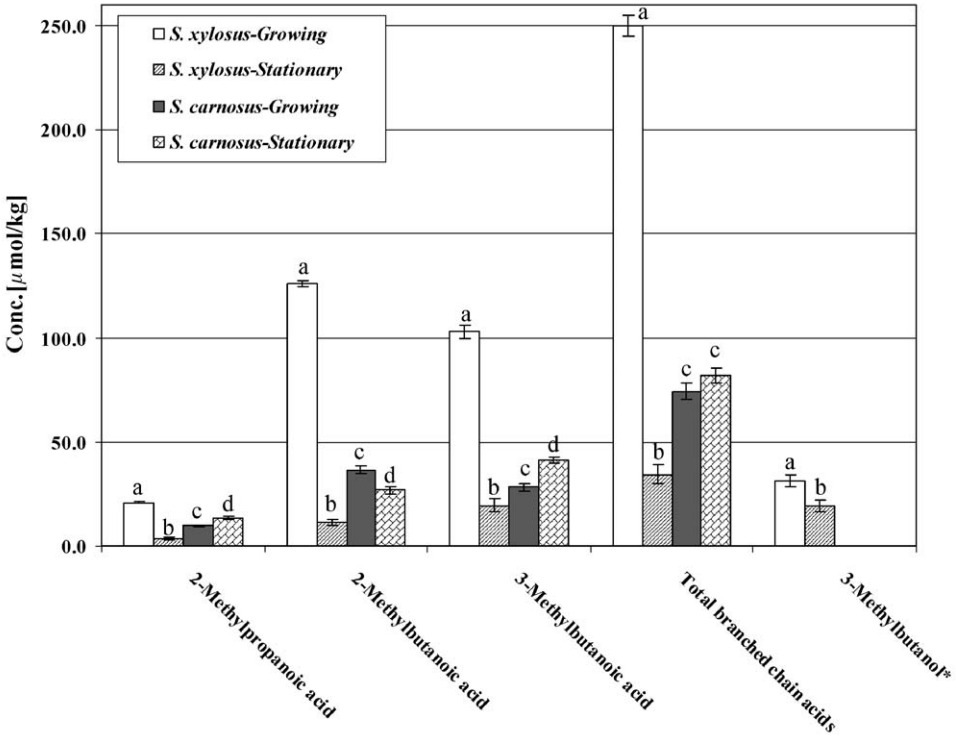

Fig. 1. Catabolites produced by *S. xylosus*-DD-34 and *S. carnosus*-S1 from branched-chain amino acids (pooled SD). Cells were harvested from either growing or stationary cells cultures. *Concentration of 3-methylbutanol is multiplied by a factor of 100 to bring it into scale. **Letters indicate whether the acid concentrations are significantly ($P<0.01$) different according to Duncan's multiple range test.

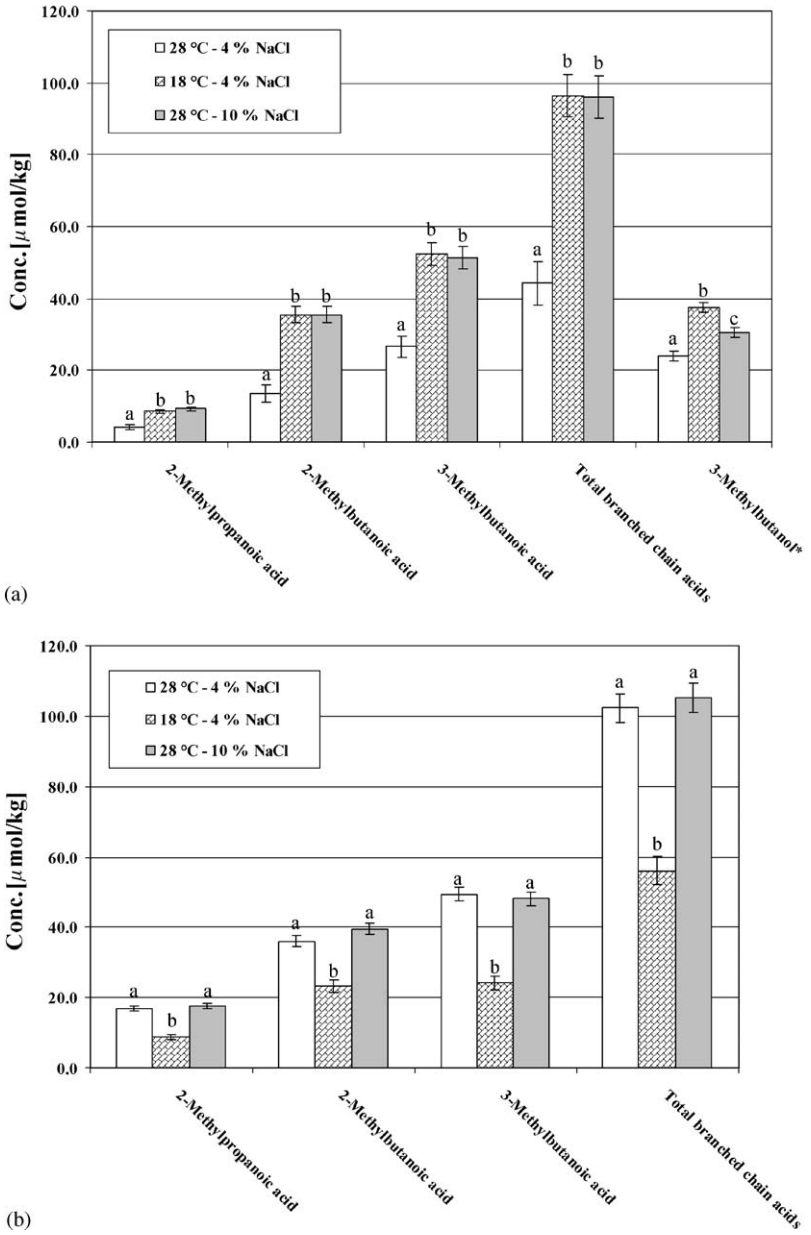

Fig. 2. (a) Catabolites produced by *S. xylosus*-DD-34 from branched-chain amino acids (pooled SD). Cells were harvested from stationary cells cultures cultivated at different temperatures and NaCl concentrations. *Concentration of 3-methylbutanol is multiplied by a factor of 100 to bring it into scale. **Letters indicate whether the acid concentrations are significantly ($P<0.05$) different according to Duncan's multiple range test. (b) Catabolites produced by *S. carnosus*-S1 from branched-chain amino acids (pooled SD). Cells were harvested from stationary cells cultures cultivated at different temperatures and NaCl concentrations. **Letters indicate whether the acid concentrations are significantly ($P<0.05$) different according to Duncan's multiple range test.

3.2. The influence of NaCl and temperature

The ability of *S. xylosus* to degrade the branched-chain amino acids was strongly affected by changing the NaCl concentration or the temperature during precultivation (Fig. 2a and Table 3). Increasing the concentration of NaCl or decreasing the temperature, both resulted in an increased generation of all acids and 3-methyl-1-butanol. It is important to note, however, that by increasing the NaCl concentration or lowering the temperature the growth rate of *S. xylosus* was reduced (McMeekin et al., 1987; Søndergaard and Stahnke, 2002; Sørensen and Jakobsen, 1996). This meant that the culture grown at 28°C and 4% NaCl reached the stationary phase faster and grew to a higher final cell concentration. Differences between the samples could therefore to some extent be influenced by differences in the precultivation growth phase (see previous section). However, all cultures had reached the stationary phase within 24 h of incubation during the precultivation and the relative distribution of acids resembled that of a stationary culture (Table 2).

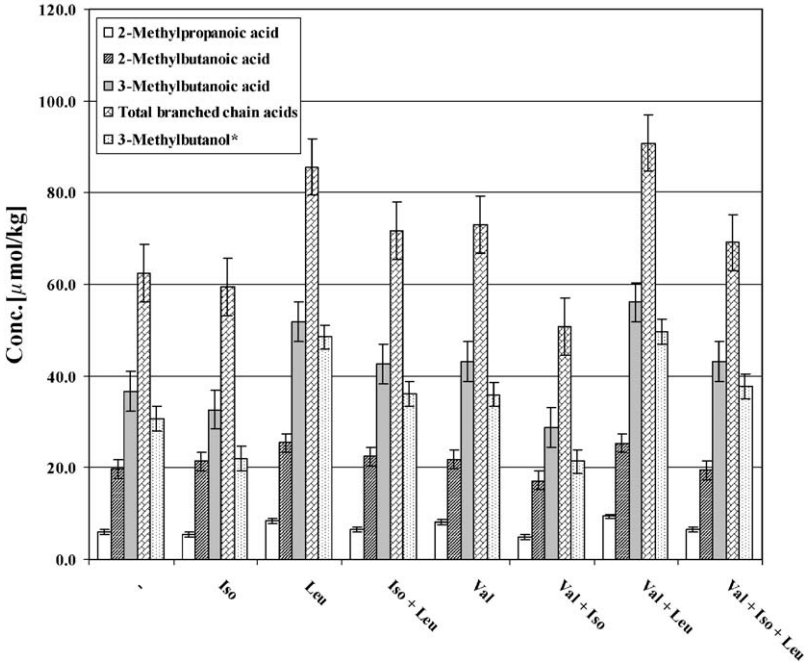

Fig. 3. Catabolites produced by *S. xylosus*-DD-34 from branched-chain amino acids (pooled SD). Cells were harvested from stationary cells cultures cultivated with varying leucine, isoleucine and valine concentrations. *Concentration of 3-methylbutanol is multiplied by a factor of 100 to bring it into scale.

Table 2
The relative distribution among the branched-chain acids formed by *S. xylosus* and *S. carnosus* respectively that were harvested in either the growing or stationary growth phase

	S. xylosus		*S. carnosus*	
	Growing (%)	Stationary (%)	Growing (%)	Stationary (%)
2-Methylpropanoic acid	8	10	13	16
2-Methylbutanoic acid	50	33	49	33
3-Methylbutanoic acid	41	57	38	51
Total branched-chain acids	100	100	100	100

The generation of acids by *S. carnosus* was unaffected by changes in the concentration of NaCl (Fig. 2b and Table 3). Lowering the temperature resulted in a reduced concentration of acids. This was particularly pronounced for 3-methylbutanoic acid (Table 3), which may be due to the influence of growth phase variation. It cannot, however, explain the change in the total acid concentration.

3.3. The influence of adding leucine, isoleucine and valine to the precultivation medium

All cultures grew well during the precultivation step and reached the same final cell concentration (2.9×10^8 cfu/ml $\pm 0.1 \times 10^8$ cfu/ml). Increasing the leucine concentration led to an increase of the total amount of acids and 3-methyl-1-butanol (Fig. 3 and Table 4). The same effect could be seen for valine, though to a lesser extent. High levels of leucine and valine together resulted in the highest acid concentration. In contrast, isoleucine at a high level decreased the generation of the acids. The lowering effect of isoleucine appeared to be small if isoleucine was added alone, but it clearly inhibited the positive effect of leucine and valine on the acid production. In fact, the negative interaction between valine and isoleucine generated the lowest concentration of acids in the experiment. It should be noted that the main factor effects in the factorial analysis of isoleucine and valine are obscured by the interaction between the two amino acids. The relative distribution of the three acids was fairly stable in all experimental combinations, but there was a slight tendency for valine, isoleucine and leucine to induce an increase in the concentration of their corresponding branched-chain acids in the reaction medium.

4. Discussion

In the last decade increasing focus has been directed toward clarifying the role of staphylococci in the flavour development of fermented sausages. However, to fully evaluate the potential of staphylococci it is necessary to understand how staphylococci are influenced by changes in the sausage environment and how changes in the precultivation step affect their flavour-forming capacity

Table 3
The relative distribution among the branched-chain acids formed by *S. xylosus* and *S. carnosus* respectively grown at different temperature and NaCl concentrations

	S. xylosus			*S. carnosus*		
	4% NaCl 28°C (%)	4% NaCl 18°C (%)	10% NaCl 28°C (%)	4% NaCl 28°C (%)	4% NaCl 18°C (%)	10% NaCl 28°C (%)
2-Methylpropanoic acid	9	9	10	17	16	17
2-Methylbutanoic acid	31	37	37	35	41	37
3-Methylbutanoic acid	60	54	53	48	43	46
Total branched-chain acids	100	100	100	100	100	100

Table 4
ANOVA analysis showing the effect of adding valine, leucine or isoleucine to the precultivation medium on the concentration of branched-chain acids formed by *S. xylosus* in reaction medium

Factor	2-Methylpropanoic acid		2-Methylbutanoic acid		3-Methylbutanoic acid		Total branched-chain acids		3-Methyl-1-butanol	
	Effect[a]	Significances[b]	Effect	Significances	Effect	Significances	Effect	Significances	Effect	Significances
Val	0.68	0.022	—	0.206	—	0.407		0.702		0.168
Leu	1.64	<0.001	3.11	0.010	13.09	<0.001	17.84	<0.001	0.155	<0.001
Iso	−2.13	<0.001	−2.96	0.013	−10.11	<0.001	−15.21	<0.001	−0.119	<0.001
Val∗Iso	−0.93	0.004	−2.25	0.044	—	0.119	−6.72	0.046	—	0.322
Leu∗Iso	—	0.429	—	0.168	—	0.660	—	0.399	—	0.854
Val∗Iso∗Leu	—	0.095	—	0.406	—	0.448	—	0.342	—	0.241

All Val∗Leu interactions was negligible ($P > 0.5$) and therefore pooled with the error estimate.
[a] Regression coefficients of main factors and their interactions in μmol/kg (only significant regression values displayed, $P \leqslant 0.05$).
[b] Analysis of variance, significance levels given as *P*-values.

after inoculation in the sausage. Comparing the results of the present experiments with other resting cells studies and research conducted on sausages, it is noteworthy that the production of branched-chain acids in reaction medium is always highly favoured compared to the generation of the flavour important branched-chain aldehydes which have been frequently detected in sausages (Beck et al., 2002; Berdagué et al., 1993; Johansson et al., 1994; Larrouture et al., 2000; Masson et al., 1999; Olesen and Stahnke, 2000; Stahnke, 1995b; Vergnais et al., 1998). In fact, Beck et al. (2002) showed by kinetic studies of resting cells that the α-ketoacids generated by transamination of the branched-chain amino acids in *S. xylosus* DD-34 were decarboxylated into the corresponding branched-chain aldehydes, but quickly oxidized into the corresponding acids ($V_{max} > 5$ μmol/min/10^{12} cfu). So far there is no established explanation for the differences in aldehyde/acid ratio between sausages and resting cells experiments, but one explanation could be the disparity in redox balance between sausage and reaction medium (microaerophilic versus aerobic). Another explanation could be that 3-methylbutanal diffuses into the lipid phase of the sausages making molecules inaccessible for further oxidation by the micro-organisms. However, the amount of branched-chain acids formed in resting cells experiments should give us a general view of different staphylococci's capability to degrade branched-chain amino acids into their corresponding methyl-branched flavour compounds as a whole.

The shift in the relative distribution between 2- and 3-methylbutanoic acids from a growing to a stationary culture could be related to changes in metabolic transport or catabolic products being drawn away into other pathways. Many bacteria regulate the fluidity of their membranes by changing the ratio of *iso* and *anteiso* branched-chain fatty acids, and the fluidity of the membrane affects the metabolite transport across the cellular membrane (Kaneda, 1991; McElhaney, 1985). In staphylococci a large portion of the cell membrane is composed of branched-chain fatty acids (O'Donnel et al., 1985). Branched-chain acids, and in particular branched-chain α-keto acids generated by the degradation of BCAAs, are used in the anabolism of long branched-chain fatty acids for the cell membrane (Beck pers. comm.; Kaneda, 1991). The cell membrane composition is known to be influenced by growth conditions such as growth phase, temperature and excessive supply of leucine, isoleucine and valine (Kaneda, 1991). Thus during growth, 2- and 3-methylbutanoic acids and 2-methylpropanoic acid or their α-keto precursors can be utilized in cellular membrane synthesis, which explains why there is a likely relationship between growth parameters, growth

phase and the production of volatile branched-chain acids.

The present study showed that temperature, NaCl and growth phase in the precultivation step affected the generation of branched-chain acids by *S. carnosus* and *S. xylosus* to a different extent (Figs. 1, 2a and b). This might be due to the direct effect of growth conditions on the metabolic systems at the time of harvesting, but also to differences in the way *S. carnosus* and *S. xylosus* adapt their membranes to environmental changes. Further investigation is needed, however, to determine how the anabolism of branched-chain fatty acids affect BCAA degradation and vice versa.

The addition of leucine, isoleucine and valine (Fig. 3) to the precultivation medium affected the ability of *S. xylosus* to generate branched-chain acids. Bearing in mind what was previously discussed, this should come as no surprise. However, even though the actual effect and interaction between those amino acids are not readily explainable, increasing the leucine concentration in the precultivation medium seemed to be the best way to promote the generation of branched-chain acids in the reaction medium.

Only two other investigations have studied the influence of precultivation parameters on degradation products from BCAAs. Møller et al. (1998) showed that growing *S. xylosus* with agitation in contrast to no agitation significantly increased the production of 3-methylbutanol in minimal medium and meat extracts. No significant effect was observed for 3-methylbutanoic acid, though. Masson et al. (1999) showed that the production of 3-methylbutanoic acid by *S. carnosus* was increased by a factor of 2 when using a complex medium instead of a defined medium.

In sausages produced with staphylococci, the generation of 3-methylbutanal and 3-methylbutanoic acid is favoured compared to the generation of 2-methylbutanal and 2-methylbutanoic acid (Ansorena et al., 2000; Berdagué et al., 1993; Johansson et al., 1994; Olesen and Stahnke, 2000; Viallon et al., 1996). This was consistent with the metabolic profile of the stationary cell culture (Tables 2 and 3), indicating that the majority of branched chain flavour compounds in sausages are generated after the growth of the staphylococci has ceased. This is not surprising since in most sausages staphylococci do not grow after the initial fermentation stage, if they grow at all (Lücke, 1998; Montel et al., 1993; Stahnke, 1995a). Research carried out after the growth phase experiment was therefore conducted using stationary cell cultures.

The ratio between 2- and 3-methylbutanoic acids is of some importance. The odour thresholds of 2-methylbutanoic and 2-methylpropanoic acid are much higher than for 3-methylbutanoic acid (Brennand et al., 1989) and the odour threshold is higher for 2-methylbutanal than for 3-methylbutanal (Berlitz and Grosch, 1999). Therefore, it seems reasonable that the degradation of leucine is of more importance for the flavour formation of fermented sausages than the degradation of isoleucine and valine.

5. Conclusion

It is clear from the experiments that the potential of the *Staphylococcus* starter cultures for degrading leucine, isoleucine and valine into flavour compounds can be manipulated during the precultivation of the starter cultures. In particular changing the precultivation temperature seems promising since the temperature interval allowing growth of staphylococci is much wider than the interval examined in this study (Bergey's, 1994). This could be of relevance for manufactures of starter cultures. Since the number of staphylococci decreases shortly after inoculation in many modern sausage productions, it could be of importance that the staphylococci starter cultures have a high initial activity toward the degradation of BCAAs. It should be kept in mind, though, that those changes might not endure for long in the sausage environment since the staphylococci may adapt their BCAA degradation potential to the sausage environment. To date, research in *Staphylococcus* starter cultures has not addressed this issue.

Only one strain of *S. xylosus* and *S. carnosus* has been investigated in this study, and therefore caution must be taken to extent the present results to other *S. xylosus* and *S. carnosus* strains. In particular for *S. carnosus* due to the large variation within this species (Probst et al., 1998).

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References

Ansorena, D., Astiasarán, I., Bello, J., 2000. Changes in volatile compounds during ripening of chorizo de Pamplona elaborated with *Lactobacillus plantarum* and *Staphylococcus carnosus*. Food Sci. Technol. Int. 6, 439–447.

Beck, H.C., Personal Communication, Biotechnological Institute, DK-6000, Kolding, Denmark.

Beck, H.C., Hansen, A.M., Lauritsen, F.R., 2002. Metabolite production and kinetics of branched-chain aldehyde oxidation in *Staphylococcus xylosus*. Enzyme Microbial Technol. 31, 94–101.

Berdagué, J.L., Monteil, P., Montel, M.C., Talon, R., 1993. Effects of starter cultures on the formation of flavour compounds in dry sausage. Meat Sci. 35, 275–287.

Bergey's Manual of Determinative Bacteriology, 1994, 9th Edition. Williams and Wilkins, Baltimore, USA, p. 545.

Berlitz, H.D., Grosch, W., 1999. Food Chemistry 2nd Edition. Springer, Berlin, Germany, p. 333.

Brennand, C.P., Ha, J.K., Lindsay, R.C., 1989. Aroma properties and thresholds of some branched-chain and other minor volatile fatty acids occurring in milkfat and meat lipids. J. Sensory Studies 4, 105–120.

Geisen, R., Lücke, F.K., Kröckel, L., 1992. Starter and protective cultures for meat and meat products. Fleischwirtschaft 72, 894–898.

Hussain, M., Hastings, J.G.M., White, P.J., 1991. A chemically defined medium for slime production by coagulase-negative staphylococci. J. Med. Microbiol. 34, 143–147.

Johansson, G., Berdagué, J.L., Larsson, M., Tran, N., Borch, E., 1994. Lipolysis, proteolysis and formation of volatile components during ripening of a fermented sausage with *Pediococcus pentosaceus* and *Staphylococcus xylosus* as starter cultures. Meat Sci. 38, 203–218.

Kaneda, T., 1991. Iso- and anteiso-fatty acids in bacteria: biosynthesis, function, and taxonomic significance. Microbiol. Rev. 55, 288–302.

Larrouture, C., Ardaillon, V., Pépin, M., Montel, M.C., 2000. Ability of meat starter cultures to catabolize leucine and evaluation of the degradation products by using an HPLC method. Food Microbiol. 17, 563–570 doi:10.1006/fmic.2000.0353.

Lücke, F.K., 1998. Fermented sausages. In: Wood, B.J.B. (Ed.), Microbiology of Fermented Foods. Blackie Academic & Professional, London, UK, pp. 441–483.

Madsen, S.M., Beck, H.C., Ravn, P., Vrang, A., Hansen, A.M., Israelsen, H., 2002. Cloning and inactivation of a branched-chain-amino-acid aminotransferase gene from *Staphylococcus carnosus* and characterization of the enzyme. Appl. Environ. Microbiol. 68, 4007–4014.

Masson, F., Hinrichsen, L., Talon, R., Montel, M.C., 1999. Factors influencing leucine catabolism by a strain of *Staphylococcus carnosus*. Int. J. Food Microbiol. 49, 173–178.

McElhaney, R.N., 1985. The effect of membrane lipids on permeability and transport in prokaryotes. In: Benga, G. (Ed.), Structure and Properties of Cell Membranes, Vol. 2. CRC Press, Boca Raton, FL, pp. 20–51.

McMeekin, T.A., Chandler, R.E., Doe, P.E., Garland, C.D., Olley, J., Putros, S., Ratkowsky, D.A., 1987. Model for combined effect of temperature and salt concentration/water activity on the growth rate of *Staphylococcus xylosus*. J. Appl. Bacteriol. 62, 543–550.

Montel, M.C., Talon, R., Berdagué, J.L., Cantonnet, M., 1993. Effects of starter cultures on the biochemical characteristics of French dry sausages. Meat Sci. 35, 229–240.

Montel, M.C., Reitz, J., Talon, R., Berdagué, J.L., Rousset-Akrim, S., 1996. Biochemical activities of *Micrococcaceae* and their effects on the aromatic profiles and odours of a dry sausage model. Food Microbiol. 13, 489–499.

Montgomery, D.C., 1997. Design and Analysis of Experiments, 4th Edition. Wiley, New York, USA.

Møller, J.K.S., Hinrichsen, L.L., Andersen, H.J., 1998. Formation of amino acid (L-leucine, L-phenylalanine) derived volatile flavour compounds by *Moraxella phenylpyruvica* and *Staphylococcus xylosus* in cured meat model systems. Int. J. Food Microbiol. 42, 101–117.

Neubauer, H., Götz, F., 1996. Physiology and interaction of nitrate and nitrite reduction in *Staphylococcus carnosus*. J. Bacteriol. 178, 2005–2009.

O'Donnel, A.G., Nahie, M.R., Goodfellow, M., Minnikin, D.E., Hajek, V., 1985. Numerical analysis of fatty acid profiles in the identification of staphylococci. J. Gen. Microbiol. 131, 2023–2033.

Olesen, P.T., Stahnke, L.H., 2000. The influence of *Debaryomyces hansenii* and *Candidautilis* on the aroma formation in garlic spiced fermented sausages and model minces. Meat Sci. 56, 357–368.

Probst, A.J., Hertel, C., Richter, L., Wassill, L., Ludwig, W., Hammes, W.P., 1998. *Staphylococcus condimenti* sp. nov., from soy sauce mash and *Staphylococcus carnosus* (Schleifer and Fischer 1982) subsp. utilis subsp. nov. Int. J. Syst. Bacteriol. 48, 651–658.

Schmidt, S., Berger, R.G., 1998. Aroma compounds in fermented sausages of different origins. Lebensm. Wiss. Technol. 31, 559–567.

Søndergaard, A.K., Stahnke, L.H., 2002. Growth and aroma production by *Staphylococcus xylosus*, *S. carnosus* and *S. equorum*—a comparative study in model systems. Int. J. Food Microbiol. 75, 99–109.

Sørensen, B.B., Jakobsen, M., 1996. The combined effects of environmental conditions related to meat fermentation on growth and lipase production by the starter culture *Staphylococcus xylosus*. Food Microbiol. 13, 265–274.

Stahnke, L.H., 1995a. Dried sausages fermented with *Staphylococcus xylosus* at different temperatures and with different ingredient levels. Part I. Chemical and bacteriological data. Meat Sci. 41, 179–191.

Stahnke, L.H., 1995b. Dried sausages fermented with *Staphylococcus xylosus* at different temperatures and with different ingredient levels. part II. Volatile components. Meat Sci. 41, 193–209.

Stahnke, L.H., 1995c. Dried sausages fermented with *Staphylococcus xylosus* at different temperatures and with different ingredient levels. Part III. sensory evaluation. Meat Sci. 41, 211–223.

Vergnais, L., Masson, F., Montel, M.C., Berdagué, J.L., Talon, R., 1998. Evaluation of solid-phase microextraction for analysis of volatile metabolites produced by staphylococci. J. Agric. Food Chem. 46, 228–234.

Viallon, C., Berdagué, J.L., Montel, M.C., Talon, R., Martin, J.F., Kondjoyan, N., Denoyer, C., 1996. The effect of stage of ripening and packaging on volatile content and flavour of dry sausage. Food Res. Int. 29, 667–674.