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ORIGINAL ARTICLE

Selection of *Staphylococcus carnosus* strains based on in vitro analysis of technologically relevant physiological activities

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Abstract The focus of this study was the taxonomic identification of a collection of 40 *Staphylococcus carnosus* strains to the species, subspecies and strain level. These strains were screened for physiological activities suitable for their use as starter cultures, in particular as starter cultures for the processing of raw ham. Consequently, the strains were tested for their ability to grow in Brain Heart Infusion broth at different NaCl and $NaNO_2$ concentrations. We found that most of the *S. carnosus* strains tested were able to grow at NaCl concentrations of up to 150 g l^{-1} and at $NaNO_2$ concentrations of up to 400 mg l^{-1}. Essential metabolic activities, such as nitrate reduction, proteolysis and lipolysis, were analysed in vitro. Three *S. carnosus* subsp. *carnosus* strains produced > 0.6 mol NO_2 per 1.0×10^7 colony forming units. Twenty-six *S. carnosus* strains showed proteolytic activity on calcium caseinate agar, and one *S. carnosus* subsp. *utilis* strain showed activity on agar supplemented with sarcoplasmic proteins. Lipolytic activity was found in 34 strains. Of these 40 *S. carnosus* strains four were found to fulfil the multiple metabolic requirements necessary for their use in industrial meat fermentation processes.

Keywords *Staphylococcus carnosus* · Subspecies differentiation · Metabolic activity · Raw ham · Meat starter culture

Introduction

A characteristic microbiota develops during the ripening process of spontaneously fermented meat products, with *Staphylococcus* spp. as one of the dominant genera (Rantsiou and Cocolin 2006). Since spontaneous fermentation cannot be controlled well, defined starter cultures are commonly used in food processing applications to guarantee constant product quality attributes. In addition to a reduced fermentation time (Stahnke et al. 2002), starter cultures ensure standardized organoleptic properties and reduce the growth of pathogenic and spoilage bacteria (Hammes 1990), thereby enhancing shelf life and safety. Strains of the coagulase-negative species *Staphylococcus xylosus* and *S. carnosus* are frequently used as starter cultures in the production of fermented sausage (Corbiere Morot-Bizot et al. 2007). Several studies have investigated the application of starter cultures, mostly lactic acid bacteria, coagulase negative staphylococci, yeasts or a combination of these, in the industrial processing of raw ham (Sánchez-Molinero and Arnau 2008; Scanell et al. 2004; Toledano et al. 2011). Nevertheless, raw ham is still produced without the deliberate application of starter cultures. Raw ham is a meat product that is manufactured by subjecting the raw material to various processing steps, such as curing, drying, smoking (optional) and ripening. Contrary to fermented sausage production, processing times last up to several months (Arnau et al. 2007). Additionally, high doses of curing salt, which consists of approximately 99 % sodium chloride and 0.4–0.9 % sodium nitrite, are applied to control the growth of pathogenic and

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spoilage bacteria (Leistner 2000) and to obtain the typically red colour (Hammes 2012). Very few microorganisms are physiologically active at sodium chloride concentrations of 10–15 %, amongst these are strains of the species *S. carnosus* (Casaburi et al. 2005). The typically red colour of raw ham is one of the most important quality criteria (Mancini and Hunt 2005), and defective (in terms of colouration) lots, even those completely meeting all other quality criteria, are considered unacceptable by the consumer. As nitrate-reducing microorganisms, such as *S. carnosus*, contribute to colour development (Gøtterup et al. 2007) and colour stability, they have the potential to be an effective means of increasing the product quality.

The aim of this study was to select *S. carnosus* strains as candidates for starter cultures in raw ham fermentation based on the results of an in vitro analysis. To this end, we taxonomically classified and characterized 40 distinct *S. carnosus* strains for their ability to grow at NaCl and $NaNO_2$ concentrations relevant for raw ham production, as well as for their proteolytic, lipolytic and nitrate reductase activities.

Material and methods

Bacterial strains and culture conditions

The 40 *Staphylococcus carnosus* strains (Table 1) analysed in this study include the *S. carnosus* subsp. *carnosus* type strain LTH 7089 and the completely sequenced strain LTH 7024 (Rosenstein et al. 2009), as well as 38 isolates. All strains were grown in Brain Heart Infusion (BHI) broth or on BHI agar (both Merck KGaA, Darmstadt, Germany) at 37 °C for 24 h under aerobic conditions. For the determination of the viable count, the overnight culture in BHI broth was adjusted to an optical density at 600 nm (OD_{600}) of 0.1 and serially diluted in 0.9 % (w/v) NaCl solution. A 100-µl aliquot of each dilution step was spread-plated on BHI agar in duplicate. Colony forming units (CFU) were counted after a 48 h incubation at 37 °C from plates containing between 30 and 300 colonies. The weighted arithmetic mean was calculated from these data.

Preparation of bacterial DNA

Total bacterial DNA was isolated as described by Wilson (1997) with minor modifications. Instead of using bacterial pellets directly, we stored the pellets at −20 °C for at least 12 h before re-suspending them in 567 µl lysozyme solution (3 mg ml^{-1}; Serva Electrophoresis GmbH, Heidelberg, Germany). An additional RNase digestion was performed with 50 µl ribonuclease A solution (3 mg ml^{-1}; Serva Electrophoresis GmbH), with a 1 h incubation at 37 °C. The DNA was precipitated with 600 µl isopropanol (Carl Roth GmbH+Co KG, Karlsruhe, Germany) at −20 °C overnight. A DNA pellet was obtained by centrifugation at 18,800*g* for 5 min at 4 °C and washed with 1 ml 70 % (v/v) ethanol. The pellet was re-suspended in 50 µl TE-buffer (10 mmol l^{-1} Tris, 1 mmol l^{-1} EDTA, pH 8.0). The DNA concentration was determined with a NanoDrop 2000 UV–VIS spectrophotometer (Thermo Fisher Scientific Inc., Waltham, MA) and adjusted to a concentration of 50 ng $µl^{-1}$ with TE-buffer. DNA solutions were stored at 4 °C.

Identification of bacterial strains by 16S rRNA gene sequencing and sequencing of the superoxide dismutase gene *sodA*

Amplification of the 16S rRNA gene was performed with primers 616V and 630R as described in Table 2. Each 16S rRNA gene amplicon (5 µl aliquot) was treated with 0.5 µl exonuclease I (20 U $µl^{-1}$) and 1 µl fast alkaline phosphatase (1 U $µl^{-1}$; both Thermo Fisher Scientific Inc.). This mixture was incubated at 37 °C for 15 min, followed by heat inactivation of the enzymes at 85 °C for 15 min. Sequencing of the 16S rRNA gene was performed with a CEQ™ 8000 analysis system (Beckman Coulter Inc., Brea, CA), as described earlier (Sterr et al. 2009) with primers 616V and 630R (Table 2), 610V, 699V, 97K (all Sterr et al. 2009) and S890R (5′-GCA CTC AAG TTT TCC AGT TTC C-3′; this study; all by Eurofins Genomics, Ebersberg, Germany). The raw sequences were processed as described by Sterr et al. (2009). The 1391-bp partial sequences of the 16S rRNA genes of 38 *S. carnosus* isolates are available in the ENA database (http://www.ebi.ac.uk/ena/data/view/LN610133-LN610170).

Table 1 *Staphylococcus carnosus* strains analysed in this study

Origin	Strain no.
Fermented fish	LTH 3729, LTH 3730, LTH 3838, LTH 4407, LTH 4408, LTH 4409, LTH 4410, LTH 4411
Fermented meat	LTH 6175, LTH 6176, LTH 6180, LTH 6181, LTH 6182, LTH 6183, LTH 6184, LTH 7012, LTH 7013[a], LTH 7034, LTH 7035, LTH 7036, LTH 7037, LTH 7089 (= DSM 20501^T)
Unknown	LTH 3697, LTH 3739, LTH 3740, LTH 3741, LTH 3742, LTH 3743, LTH 3744
Starter culture	LTH 1574, LTH 7024 (= TM300), LTH 7027, LTH 7038, LTH 7039, LTH 7040, LTH 7041, LTH 7042, LTH 7044, LTH 7053, LTH 7057

[a] Müller et al. 2015

Table 2 Conditions and primer sequences for 16S rRNA gene amplification, superoxide dismutase gene amplification and random amplification polymorphism DNA-PCR

Primer	Sequence (5′→3′)	Reference	Additional $MgCl_2$ [25 mM]	Primer (concentration)	PCR conditions[a]	Number of cycles
616V 630R	AGA GTT TGA TYM TGG CTC AAG GAG GTG ATC CAR CC	Lehner et al. 2004 Sterr et al. 2009	None	0.5 μl each (10 pmol μl^{-1})	95 °C, 0.5 min; 58 °C, 1 min; 72 °C, 2 min	30
sodA-fwd sodA-rev	CCA TAC GAG TTT GAT GCA TTG AGT AAG CGT GTT CCC AAA CAT	This study	None	0.5 μl each (10 pmol μl^{-1})	95 °C, 1 min; 54 °C, 1 min; 68 °C, 2 min	30
RAPD1	AGC AGG GTC G	Cocconcelli et al. 1995	2 μl	0.5 μl (100 pmol μl^{-1})	94 °C, 1 min; 34 °C, 1 min; 68 °C, 2 min	35
RAPD2	AGC AGC GTC G	Cocconcelli et al. 1995	2 μl	0.5 μl (100 pmol μl^{-1})	94 °C, 1 min; 34 °C, 1 min; 68 °C, 2 min	30
RAPD3	GTA GAC CCG T	Wiese et al. 2004	None	2 μl (12.5 pmol μl^{-1})	94 °C, 1 min; 36 °C, 1.5 min; 68 °C, 2 min	40
RAPD7	AGC AGC GTG G	Cocconcelli et al. 1995	2 μl	0.5 μl (100 pmol μl^{-1})	94 °C, 1 min; 36 °C, 1 min; 68 °C, 2 min	37
RAPD8	ACG CGC CCT	Johansson et al. 1995	None	2 μl (12.5 pmol μl^{-1})	94 °C, 1 min; 36 °C, 1.5 min; 68 °C, 2 min	40
XD9[b]	GAA GTC GTC G	Moschetti et al. 1998	3 μl	1 μl (100 pmol μl^{-1})	94 °C, 1 min; 36 °C, 1 min; 68 °C, 2 min	35
M13	GAG GGT GGC GGT TCT	Huey and Hall 1989	2 μl	0.5 μl (100 pmol μl^{-1})	94 °C, 1 min; 45 °C, 1 min; 68 °C, 2 min	35

[a] 16S rRNA gene PCR: initial denaturation: 4 min at 95 °C; final elongation: 5 min at 72 °C; superoxide dismutase gene (*sodA*): initial denaturation: 5 min at 95 °C; final elongation: 5 min at 68 °C; random amplified polymorphic DNA (RAPD): initial denaturation: 5 min at 94 °C; final elongation: 5 min at 68 °C. Each 25 μl reaction consisted of 2.5 μl 10×ThermoPol™ reaction buffer, 0.5 μl dNTPs (10 mM each), 0.125 μl Taq Polymerase (5 U μl^{-1}; all New England BioLabs GmbH, Frankfurt, Germany) and 1 μl DNA (50 ng μl^{-1})

[b] 2 μl DNA (50 ng μl^{-1}) was used as the template

The identification of the isolates at the subspecies level was performed by amplification of a 482 bp fragment of the manganese-dependent superoxide dismutase gene *sodA* under the conditions described in Table 2. Sequencing of the *sodA* fragment with the primers sodA-fwd and sodA-rev and the processing of the sequences were the same as those described for the 16S rRNA gene. The *sodA* sequence of strain LTH 3728 (= DSM 11676^T), the type strain of *S. carnosus* subsp. *utilis*, was also determined and included as a reference sequence for the subspecies *utilis*. The 384 bp partial sequences of *sodA* are available in the ENA database at http://www.ebi.ac.uk/ena/data/view/LN868466-LN868503.

Typing of *S. carnosus* strains by random amplified polymorphic DNA-PCR

Random amplified polymorphic DNA (RAPD)-PCR was performed in a T1 thermocycler (Biometra GmbH, Göttingen, Germany) as described in Table 2. The PCR products were separated by electrophoresis on 2 % (w/v) agarose gels (LE agarose; Biozym Scientific GmbH, Hessisch Oldendorf, Germany) at 80 V for 105 min. Gels were stained with 1 μg ml^{-1} ethidium bromide (Carl Roth GmbH+Co KG) for 20 min, visualized under UV-light at 302 nm and photographed (E.A.S.Y Win32 Analyse Software; Herolab GmbH, Wiesloch, Germany). The digitalized images were processed and normalized, and band patterns were defined with the GelCompar II software package version 6.6 (Applied Maths NV, Sint-Martens-Latem, Belgium). Dice was chosen as the similarity coefficient with an optimization setting of 2 % and a band matching tolerance of 1 %. Cluster analysis was performed with the unweighted pair group method with arithmetic mean (UPGMA).

Net growth at different NaCl and $NaNO_2$ conditions

Growth in the presence of different NaCl and $NaNO_2$ concentrations was tested by adding 150 μl of an overnight culture of each strain, adjusted to an OD_{600} of 0.1, to 150 μl of BHI broth supplemented with different amounts of NaCl and $NaNO_2$ to achieve final concentrations of 5–150 g l^{-1} NaCl or 0–400 mg l^{-1} $NaNO_2$ (Merck KGaA). BHI broth without bacteria was used as a negative control, and BHI broth inoculated with *S. carnosus* strain LTH 7089 served as a positive control. The experiments were incubated in microtiter plates at 37 °C. The OD_{600} was measured immediately after inoculation, and after 2, 4, 6, 24 and 48 h of incubation with a microplate reader (Infinite® 200 Pro; Tecan Trading AG, Männedorf, Switzerland).

The net growth was obtained by calculating the difference in the arithmetic mean values of the OD_{600} of three replicates of the 0 h and 48 h incubations, respectively.

Lipolytic activity

Lipolytic activity was analysed on agar containing tributyrin modified from Anderson (1939). It contained 2.5 g l^{-1} peptone from meat (pancreatic; Merck KGaA), 2.5 g l^{-1} Bacto™ peptone, 3 g l^{-1} yeast extract (both Becton Dickinson and Co., Franklin Lakes, NY), 10 ml l^{-1} glyceryl tributyrate (Sigma-Aldrich) and 12 g l^{-1} Bacto™ agar (Becton, Dickinson and Co.). *Bacillus cereus* ATCC 14579^T was used as a positive control. Bacterial strains were cultured overnight in BHI broth as described above. A sterile filter disc (diameter 5 mm) was deposited on the agar plate and inoculated with 10 µl of an overnight culture with an OD_{600} of 0.1. Agar plates were incubated for 72 h at 37 °C. Clear zones (synonym: halos), which are formed by lipolytic strains, were measured from the edge of the colony in millimetres as described by Martín et al. (2006). The arithmetic mean was calculated from three different measurements for each strain.

Proteolytic activity

The proteolysis of calcium caseinate was determined on calcium caseinate agar (Oxoid, Thermo Scientific, Waltham, MA) with *B. cereus* ATCC 14579^T as a positive control.

The proteolysis of pork sarcoplasmic proteins was studied on modified nutrient agar. Sarcoplasmic proteins from pork escalope were extracted according to Molina and Toldrá (1992) and their concentration was determined according to Bradford (1976). Sarcoplasmic protein extract (20 % v/v; 5 mg ml^{-1}) was added to the nutrient agar (5 g l^{-1} peptone from meat (pancreatic; Merck KGaA), 2.5 g l^{-1} meat extract and 15 g l^{-1} Bacto™ agar (Becton, Dickinson and Co.)). The OD_{600} of the overnight cultures was adjusted to 0.1, and a 10 µl aliquot was pipetted onto a sterile filter disc (diameter 5 mm) that had been deposited on the agar plate. All plates were incubated at 37 °C for 96 h and then stained as described by Mauriello et al. (2002). The extent of the halos around the colonies was measured from the edge of the colony in millimetres. The data of three replicates per strain were combined as the arithmetic mean.

Nitrate reductase activity

Nitrate reductase activity was determined according to Casaburi et al. (2005) with modifications. An overnight culture of each strain was centrifuged for 10 min at 2500*g* and 4 °C. The cell pellet was re-suspended in nitrate broth consisting of 8.6 g l^{-1} peptone from meat (pancreatic; Merck KGaA), 6.4 g l^{-1} NaCl and 1.5 g l^{-1} KNO_3 (Sigma-Aldrich). One hundered microlitre of this suspension were transferred to 10 ml fresh nitrate broth and incubated at 37 °C for 24 h. Then, 100 µl of this culture, 2 ml sterile ultrapure water (Millipore type I; Merck Millipore, Merck KGaA) and 500 µl Grieß-Ilosvay's reagent for nitrite (Merck KGaA) were mixed for 1 min. After incubation at room temperature for 15 min, the development of a red colour was measured at 540 nm as OD_{540}.

A standard curve was constructed to facilitate a correlation between the nitrate reductase activity, expressed as the amount of nitrite produced, and the viable counts. For this purpose 100 µl nitrate broth spiked with 0.01, 0.05, 0.1, 0.25, 0.5, 1, 5, 10, 50 or 100 mmol l^{-1} nitrite were treated identically to the samples. The measured values were used to calculate a standard curve. The production of nitrite was then expressed as mol NO_2 per 1.0×10^7 CFU.

Statistical analysis

Statistical analyses were performed with the R software version 3.1.1 (http://www.R-project.org/). All *S. carnosus* strains were divided into groups depending on their origin. The Kruskal–Wallis test was performed to determine differences in the mean values of these groups in terms of their physiological characteristics and enzyme activities. Pairwise comparison of the mean values of significant different groups was done with the Wilcoxon rank sum test, where the Bonferroni method was used for *p*-value adjustment.

Significant differences in the mean values of physiological characteristics and enzyme activity between the two subspecies *S. carnosus* subsp. *carnosus* and *S. carnosus* subsp. *utilis* were determined with the Welch two-sample *t* test.

Results and discussion

Taxonomic identification and typing of *S. carnosus* strains

Taxonomic identity is a prerequisite for the application of bacteria in food fermentation processes. The isolates were therefore identified by sequencing a 1391 bp fragment of the 16S rRNA gene. Since members of the Simulans–Carnosus cluster group (Lamers et al. 2012), especially *S. carnosus* and *S. condimenti*, are highly identical at the 16S rRNA gene level, strains were further analysed by sequencing *sodA*, as suggested by Poyart et al. (2001). Using the 384 bp fragment of *sodA* obtained in our analysis, we identified the strains at subspecies level [Electronic Supplementary Material (ESM) 2)]. One strain, LTH 3730, was clearly identified as *S. carnosus*, but could not be assigned to one of the two subspecies *S. carnosus* subsp. *carnosus* or *S. carnosus* subsp. *utilis*.

The 38 *S. carnosus* isolates were investigated at the strain level with RAPD-PCR using seven different primers. Of these 38 *S. carnosus* isolates, we were able to identify 36 as distinct strains; only isolates LTH 3697 and LTH 6184 showed the same band patterns with all of the primers tested (Fig. 1).

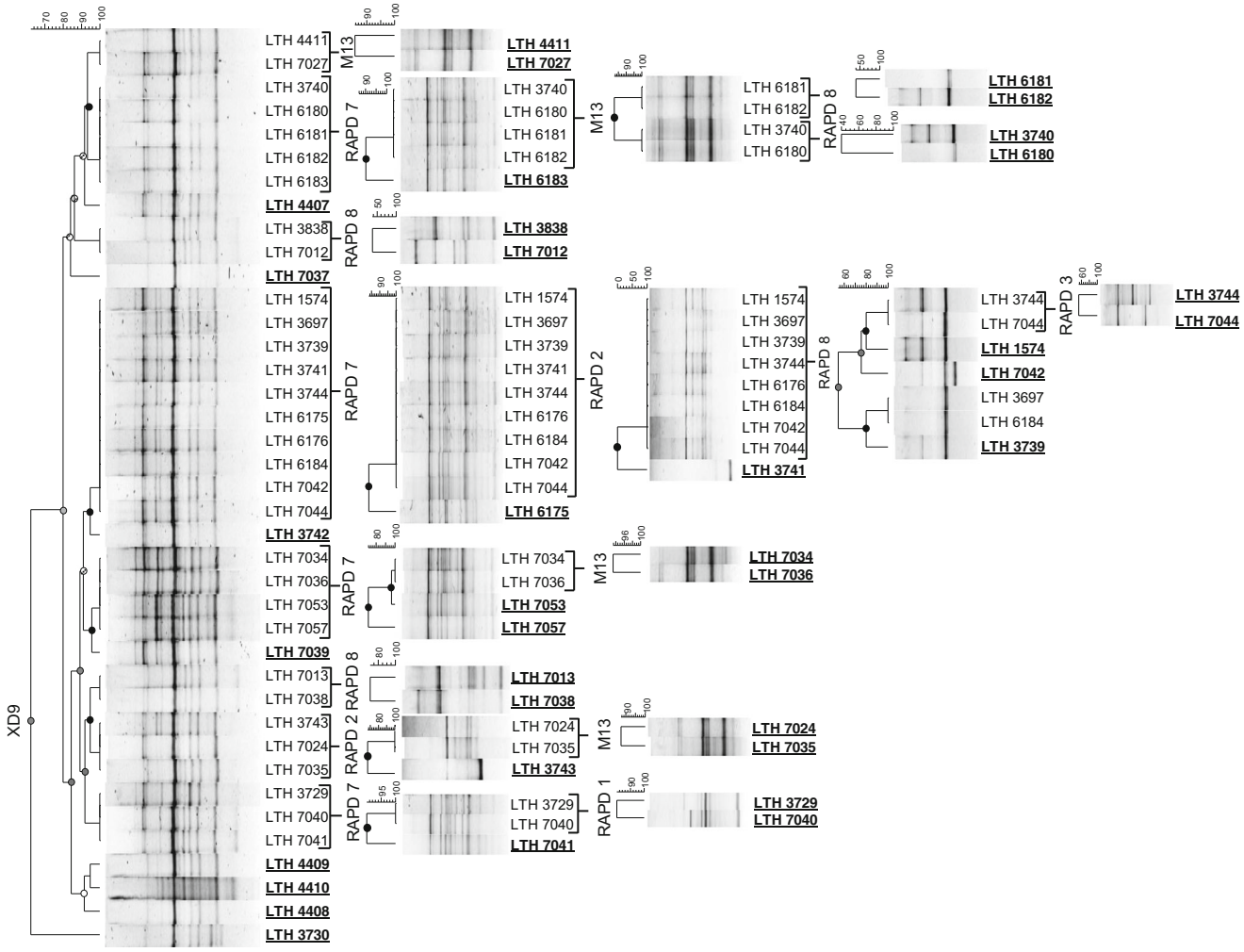

Fig. 1 Dendrogram obtained by random amplified polymorphic DNA (RAPD)-PCR using primer XD9. The unweighted pair group method with arithmetic mean (UPGMA) was used for cluster analysis, and Dice was used as the similarity coefficient. Clusters were defined as band patterns with 100 % similarity and analysed separately with primers RAPD7, RAPD1, RAPD2, M13, RAPD8 and RAPD3

Growth of *S. carnosus* strains at different physiological conditions

In this study, the growth of the different *S. carnosus* strains was tested at NaCl concentrations of up to 15 % in BHI broth. Of these, seven strains, all isolated from fish, showed excellent net growth (ESM 1), indicating that they were well adapted to salty environments. Most of the strains grew well but to a different extent in BHI broth in the presence of 5–150 g l^{-1} NaCl, as indicated by differences in OD_{600} values (Table 3). The only exception was strain LTH 7012, which did not grow at 150 g l^{-1} NaCl (Table 3; ESM 1). The selection of coagulase-negative staphylococci as starter cultures often includes testing growth at high NaCl concentrations, as NaCl is one of the main ingredients added to sausage or ham. Growth in medium containing up to 15 % NaCl has been described for *Staphylococcus equorum* and *S. saprophyticus* (Cachaldora et al. 2013); for *S. carnosus*, growth at 20 % NaCl has been described (Casaburi et al. 2005).

A similar result was obtained when the strains were grown at different $NaNO_2$ concentrations. Almost all *S. carnosus* strains, with the exception of LTH 3744, were able to grow well at $NaNO_2$ concentrations of up to 400 mg l^{-1}, with only

Table 3 Net growth of 40 *S. carnosus* strains in Brain Heart Infusion broth supplemented with NaCl at concentrations ranging from 5 to 150 g l^{-1}

OD_{600}[a]	NaCl (g l^{-1})						
	5	25	50	75	100	125	150[b]
0.1–0.39	2.5	0.0	0.0	0.0	20.0	57.5	65.0
0.4– 0.79	40.0	87.5	97.5	100	80.0	42.5	32.5
≥ 0.8	57.5	12.5	2.5	0.0	0.0	0.0	0.0

Data in table are presented as the percentage

[a] Net growth to OD_{600} values of 0.1–0.39, 0.4–0.79 and≥0.8 were assessed as weak, good and excellent, respectively

[b] Strain LTH 7012 did not grow at a NaCl concentration of 150 g l^{-1}

Table 4 Net growth of 40 *S. carnosus* strains in Brain Heart Infusion broth supplemented with $NaNO_2$ at concentrations ranging from 0 to 400 mg l^{-1}

OD_{600}[a]	$NaNO_2$ (mg l^{-1})								
	0	50[b]	100	150	200	250	300	350[b]	400
0.1–0.39	2.5	17.5	2.5	2.5	2.5	2.5	2.5	0.0	2.5
0.4–0.79	77.5	65.0	77.5	87.5	87.5	87.5	87.5	87.5	87.5
≥ 0.8	20.0	15.0	20.0	10.0	10.0	10.0	10.0	10.0	10.0

Data in table are presented as the percentage

[a] Net growth to OD_{600} values of 0.1–0.39, 0.4–0.79 and≥0.8 were assessed as weak, good and excellent, respectively

[b] Strains LTH 7012 and LTH 3744 did not grow at $NaNO_2$ concentrations of 50 mg l^{-1} and 350 mg l^{-1}, respectively

variations in their final cell densities (Table 4). LTH 3744 grew only weakly over the whole concentration range or did not grow at all (Table 4; ESM 1). Again, *S. carnosus* strains LTH 4408, LTH 4409, LTH 4410 and LTH 4411, which were isolated from fermented fish, grew excellent at high $NaNO_2$ concentrations ranging from 150 to 400 mg l^{-1} (ESM 1). Raw ham is usually fermented with curing salt which contains 0.4–0.9 % nitrite, at a dose of 40 g curing salt per 1 kg meat. Taking this into account, a ham starter culture should be able to survive nitrite concentrations of 0.04 % (w/v), which all of the tested strains, except LTH 3744, were able to do.

Determination of selected metabolic traits

Lipolytic, proteolytic and nitrate reductase activities were investigated. Twenty-six strains showed proteolytic activity on calcium caseinate agar, but not on agar supplemented with sarcoplasmic proteins (ESM 2). Mauriello et al. (2002) suggested the use of muscle protein extract, either sarcoplasmic or myofibrillar, rather than casein for detecting proteolytic activity of bacteria from meat products. Only strain LTH 3838, a fermented fish isolate, digested sarcoplasmic proteins (ESM 2). Although proteolysis in fermented meat products

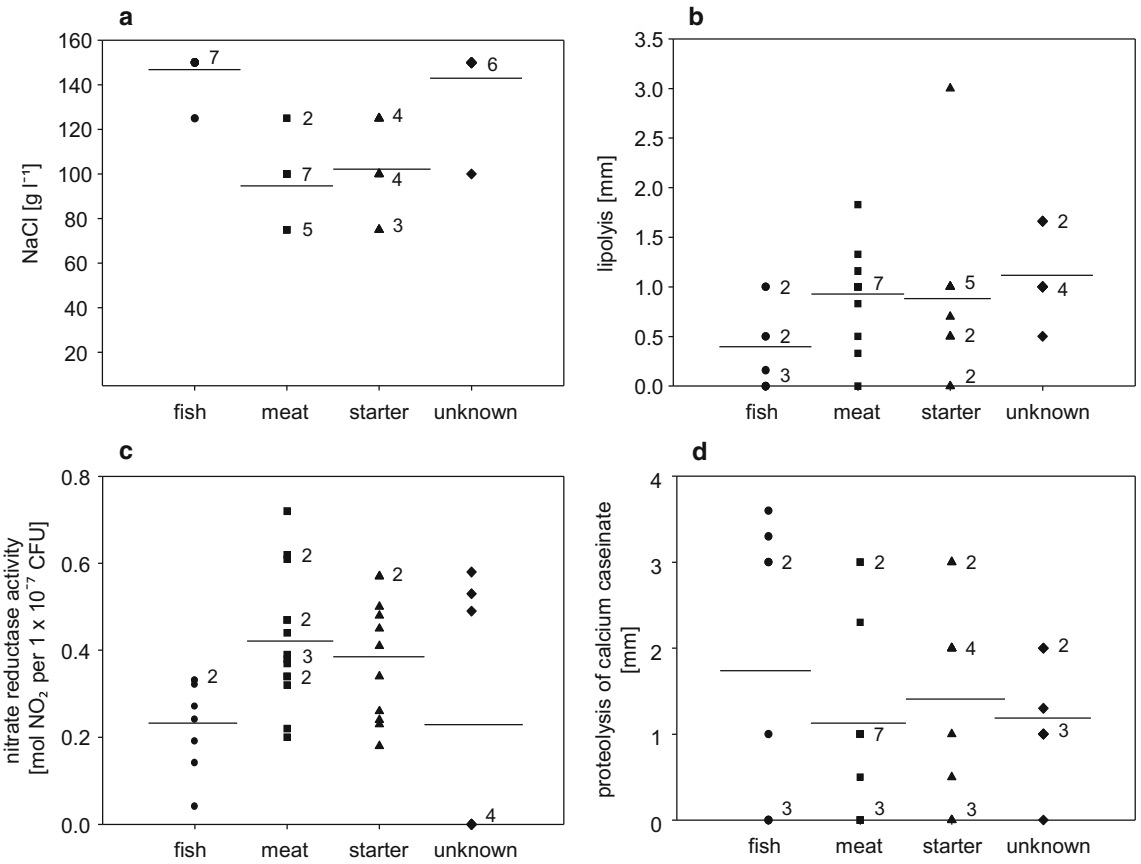

Fig. 2 Comparison of 40 *Staphylococcus carnosus* strains of different origin in terms of net growth at different NaCl concentrations (**a**), lipolytic activity (**b**), nitrate reductase activity (**c**) and proteolytic activity on calcium caseinate agar (**d**). Strains are divided into groups depending on their origin: fish (8 strains), meat (14 strains), starter culture (11 strains) and unknown (7 strains). *Horizontal lines* Mean values of each group, *numbers next to data points* number of strains per data point if several strains showed the same result. **a** Plot of the highest NaCl concentration (g l^{-1}) at which good growth (OD_{600}≥ 0.4) was still measured. **b**, **d** Plots of lipolytic (**b**) and proteolytic activity of calcium caseinate (**d**), displayed as diameter of the halo (in mm). **c** Plot of nitrate reductase activity [mol NO_2 per 1.0×10^7 colony forming units (CFU)]. The graphs were drawn with SigmaPlot version 10.0 (Systat Software, San Jose, CA)

is mainly caused by endogenous muscle proteases, Scanell et al. (2004) showed that the addition of proteolytic starter cultures had an influence on the amino acid profile of dry-cured ham and may also influence the overall sensory attributes. Taking this into consideration, LTH 3838 could be used for further investigations of volatile compound formation in raw ham.

Lipolysis not only is an important factor in proteolytic processes, but it also plays an important role in flavour development. Of the 40 strains tested, 33 showed moderate lipolytic activity on tributyrin agar. Only six strains were considered to have no lipolytic activity. One of the starter cultures, strain LTH 1574, was remarkable in terms of its good lipolytic activity (Fig. 2b; ESM 2).

The results of in vitro screening for lipolytic and proteolytic activity can indicate the behaviour in vivo. Due to the more complex substrate composition of the meat itself and the presence of meat indigenous enzymes and an autochthonous microbiota, the confirmation of the sensory effect can only be obtained after the application in a specific food (Casaburi et al. 2008). Nevertheless, Lorenzo et al. (2015) showed that the use of different starter cultures led to significantly different effects in terms of volatile compounds and overall sensory analysis of foal sausage. This study showed that using microorganisms with different characteristics on the same raw material can have a major impact on the final product. Thus, the identification of starter cultures with different abilities is the basis for developing different flavours and products.

The formation of the typical red colour plays a major role in the overall organoleptic profile of meat products. Nitrate reductase is an enzyme which contributes to colour development and stabilization and, consequently, is one of the more important criteria in the evaluation of bacterial strains as potential starter cultures. The majority of strains tested in our analysis exhibited a nitrate reductase activity that led to the formation of >0.4 mol NO_2 per 1.0×10^7 CFU. Three strains, LTH 6175, LTH 7035 and LTH 7036, stood out with a nitrite formation of >0.6 mol NO_2 per 1.0×10^7 CFU (ESM 2). These findings suggest that nitrate reductase activity is a strain-specific property, similar to findings for other coagulase-

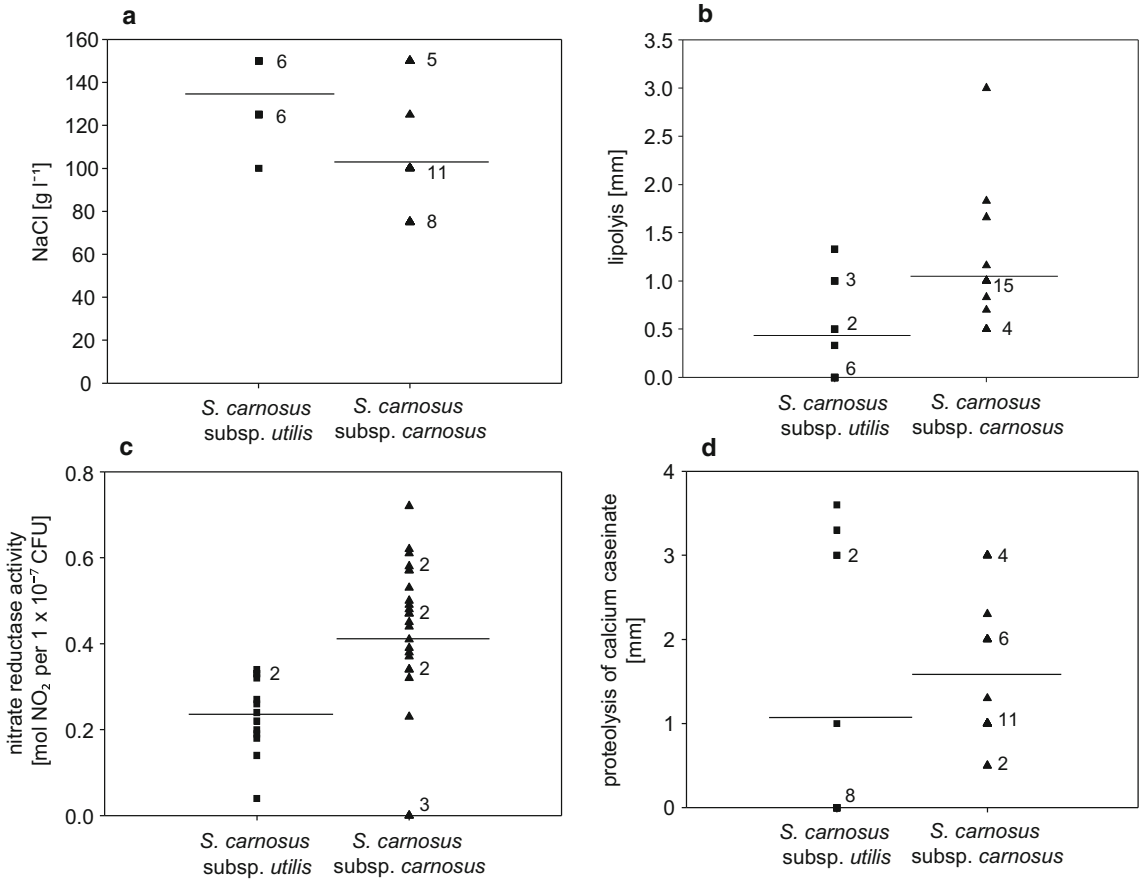

Fig. 3 Comparison of strains of the subspecies *S. carnosus* subsp. *carnosus* (25 strains) and *S. carnosus* subsp. *utilis* (13 strains) in terms of net growth at different NaCl concentrations (**a**), lipolytic activity (**b**), nitrate reductase activity (**c**) and proteolytic activity on calcium caseinate agar (**d**). *Horizontal lines* Mean values of each group, *numbers next to data points* number of strains per data point if several strains showed the same result. **a** Plot of the highest NaCl concentration (g l^{-1}) at which good growth ($OD_{600}\geq0.4$) was still measured. **b**, **d** Plots of lipolytic (**b**) and proteolytic activity of calcium caseinate (**d**), displayed as diameter of the halo (in mm). **c** Plot of nitrate reductase activity (mol NO_2 per 1.0×10^7 CFU). The graphs were drawn with SigmaPlot version 10.0 (Systat Software)

negative staphylococci such as *S. equorum*, *S. warneri*, *S. vitulinus* and *S. xylosus* by Casaburi et al. (2005), Jeong et al. (2014), Landeta et al. (2013) and Marty et al. (2012). A recent study using a meat model demonstrated that the combination of added nitrate and a nitrate-reducing *S. carnosus* strain resulted in the formation of the typical red colour (Sánchez Mainar and Leroy 2015). As our aim was to identify starter cultures which can be applied to reduce colour defects in ham, strains LTH 6175, LTH 7035 and LTH 7036 are promising candidates for similar experiments.

Comparison of *S. carnosus* strains in terms of the different origins and subspecies affiliation

All *S. carnosus* strains were divided into groups according to their origin (Fig. 2) or their respective subspecies (Fig. 3) and then compared to each other in terms of growth to an OD_{600} of ≥0.4 in BHI broth supplemented with different NaCl concentrations, the intensity of lipolysis, nitrate reduction and proteolysis of calcium caseinate (Figs. 2, 3). Only the growth at high NaCl concentrations could be related to the origin of the strains in a given group (Fig. 2a). As expected, the proteolytic, lipolytic and nitrate reductase activities of the *S. carnosus* strains were strain specific and varied strongly within the groups (Fig. 2). Due to this high variation, no significant differences between the mean values were observed ($p>0.05$). Although the variation of lipolytic, proteolytic and nitrate reductase activity varied within the subspecies as well, the mean values of lipolysis and nitrate reductase differed significantly between the subspecies ($p<0.05$). Nevertheless, it has to be noted that different numbers of strains were tested for *S. carnosus* subsp. *carnosus* (25 strains) and *S. carnosus* subsp. *utilis* (13 strains).

These findings confirm our hypothesis that some strains of the species *S. carnosus* might be more suitable for raw ham fermentation than others and that these strains are not necessarily identical to those already being used as commercial starter cultures.

Conclusion

Based on the results of our in vitro screening procedure we identified *S. carnosus* strains with properties that are prerequisites for their use in industrial-level raw ham fermentation processes. The taxonomic characterization in combination with the analysis of metabolic activities led to the identification of specialized strains, such as LTH 3838 which is able to cleave sarcoplasmic proteins and may have an impact on aroma production of raw ham. Strains LTH 6175, LTH 7035 and LTH 7036, which showed a high nitrate reductase activity, are candidates for in vivo experiments on colour stabilization. Combinations of these strains might yield an even more tailored fermented product. Thus, in future studies we will be investigating these strains for the presence of pathogenicity factors and antibiotic resistance to fulfil the requirements of food safety prior to testing their properties in fermented ham.

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Conflict of interest The authors declare that there are no conflicts of interest.

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