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Technological activities of *Staphylococcus carnosus* and *Staphylococcus simulans* strains isolated from fermented sausages

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Abstract

The aim of this study was to determine the technological properties of 2 strains of *Staphylococcus simulans* (Ssm12, Ssm21) and 4 strains of *S. carnosus* (SC28, SC31, SC54 and SC55) for the selection of a potential starter cultures to employ in the processing of dry fermented sausages. The strains were studied to evaluate nitrate reductase, proteolytic, lipolytic, decarboxylase and antioxidant activities as well as growth ability at different temperatures, pH and NaCl concentrations. Nitrate reductase activity was determined at 15, 20 and 30 °C. By spectrophotometric method all the strains were able to reduce nitrate to nitrite at the different temperatures but these results were not confirmed by the agar plate method. Antioxidant and lipolytic activities were evaluated by spectrophotometric assay. All the strains showed antioxidative enzymes superoxide dismutase (SOD) and catalase whereas all appeared unable to hydrolyse pork fat. Proteolytic activity was determined by agar plate method, spectrophotometric assay (OPA) and sodium dodecyl sulphate gel-electrophoresis (SDS–PAGE) and all strains appeared to be able to hydrolyse sarcoplasmic proteins but not myofibrillar proteins. Finally, all the strains grew at 15 and 20 °C, in presence of 10%, 15% and 20% of NaCl and at pH 5.0 and 5.5 and were unable to produce histamine, cadaverine and putrescine. The results showed that all strains studied possess useful technological activities that would make them eligible as a good starter cultures for fermented sausages.

Keywords: *Staphylococcus carnosus*; *Staphylococcus simulans*; Technological properties; Dry fermented sausages

1. Introduction

Coagulase negative staphylococci (CNS) are commonly found as natural flora of fermented meat products, in relatively high number also in dry fermented sausages produced without addition of starter cultures (Miralles, Flores, & Perez-Martinez, 1996).

CNS play a major role in the development of sensory properties of fermented sausages, by reduction of nitrates to nitrite and then to nitrous oxide, by preventing rancidity through peroxide decomposition, and by producing flavour and aroma compounds through proteolysis and lipolysis (Hammes & Hertel, 1998; Sondergaard & Stahnke, 2002).

Staphylococcus xylosus is the dominating CNS species in many Italian sausages (Cocolin, Manzano, Aggio, Cantoni, & Comi, 2001; Rossi, Tofalo, Torriani, & Suzzi, 2001) and in the Spanish sausage Chorizo (García-Varona, Santos, Jaime, & Rovira, 2000). *S. saprophyticus* and *S. carnosus* the dominating species in traditional Greek sausages (Papamanoli, Kotzekidou, Tzanetakis, & Litopoulou-Tzanetaki, 2002; Samelis, Metaxopoulos, Vlassi, & Pappa, 1998) and in Naples type salami (Coppola, Mauriello, Aponte, Moschetti, & Villani, 2000). Many other CNS species (*S. haemolyticus*, *S. warneri*, *S. equorum*, *S. cohnii*, *S. epidermidis*,

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S. hominis, *S. capitis*, *S. intermedius*) have been reported to occur in one or more sausage types.

Staphylococcus species are commonly used as starters cultures for fermented sausages. Actually Italian law permits the use of *S. xylosus*, *S. carnosus* and *S. simulans* as starter for fermented sausage production (Repubblica Italiana, 1995).

Among starters, only few data are available in the literature on technological properties of *S. simulans* and *S. carnosus* and little attention has been paid to their contribution in sausages fermentation (Sondergaard & Stahnke, 2002; Stahnke, Holck, Jensen, Nilsen, & Zanardi, 2001). Moreover, as some CNS can possess decarboxylase activities and consequently deteriorate the hygienic quality and safety of the meat product, the absence of biogenic amines formation represent an essential selection criterion for starter cultures (Straub, Kicherer, Schilcher, & Hammes, 1995).

The aim of this work was to study some of the technological and safety properties of 2 *S. simulans* and 4 *S. carnosus* strains in order to select the most suitable as starters for fermented dry sausage production.

2. Materials and methods

2.1. Bacterial strains and culture conditions

Four strains of *S. carnosus* and two strains of *S. simulans* (Table 1) isolated from traditional fermented sausages from Vallo Diano (Campania region–Italy) were used in this study. The strains were isolated and identified as reported previously (Mauriello, Casaburi, Blaiotta, & Villani, 2004) Briefly, Micrococcaceae present in dry fermented sausages were enumerated on Mannitol Salt Agar (MSA, Oxoid) after 48 h at 30 °C. Colonies from countable plates were initially tested for morphology, Gram-stain and catalase production. Gram-positive and catalase-positive cocci were purified by streaking on MSA and maintained on P-agar (Phillips & Nash, 1985) slants stored at 4 °C. They were subjected to the oxidation/fermentation test in OF medium and to the anaerobic growth in semisolid thioglycollate medium (Evans & Kloos, 1972). Sensitivity to furazolidone, bacitracin and lysostaphin was determined as described by Kloos and Bannerman (1995). Production of pigment was observed on P-agar. Staphylococci were assayed for coagulase activity using the tube test with coagulase plasma (Becton, Dickinson & Company, NJ, USA) and for novobiocin sensitivity (Kloos, Tornabene, & Schleifer, 1974). Other biochemical properties were studied using API Staph identification strips and API LAB Plus software according to the manufacturer's instructions (API, Biomérieux System).

Species-specific PCR assays were used to confirm the species identifications as reported in previous study (Blaiotta, Casaburi, & Villani, 2005). Briefly, DNA extraction was carried out from a single colony by using an Insta-Gene Matrix (Bio-Rad Laboratories, Hercules, CA) and 5 μl (about 25 ng) were used for PCR amplifications.

Primers couples simF–simR (ATCCTTTCACCTAACTCTGAAGAG and GTAATTGGGTTGTCTTGGTTTGCT) and carF–carR (CTTCTAACACCTAATTCTGAAGA and GATTGGATTGTCTTGGTTAGGA) were used to amplify specific-*sod* A gene fragments of *S. simulans* and *S. carnosus*, respectively. Moreover, specificity of the amplified fragments were further verified by restriction endonucleases analysis of the PCR products by *Eco*RI and *Dra*I restriction enzymes, respectively. Working cultures were grown overnight at 30 °C in Tryptone Soya Broth (TSB Oxoid Ltd, London, England) and were maintained on P-agar (Phillips & Nash, 1985) slants stored at 4 °C.

2.2. Detection of nitrate reductase activity

2.2.1. Agar plate method

Nitrate reductase activity was determined as described by Miralles et al. (1996) on YTA (1.0% tryptone, 0.5% yeast extract, 1.5% agar, pH 7.0) supplemented with 1 g l^{-1} KNO_3. The cell pellet of an overnight culture was resuspended in 10 ml of 50 mM phosphate buffer pH 7.0 and 30 μl loaded into wells (6 mm diameter) bored in YT agar plates. After incubation at 30 °C for 7 h and at 20 and 15 °C for 24 and 72 h, respectively, the plates were flooded with 1 ml of a 1:1 solution of NIT1 (0.8 g of sulphanilic acid in 100 ml of 5 N acetic acid) and NIT 2 (0.6 g of *N*-*N*-dimethyl-1-Naphthylamine in 100 ml of 5 N acetic acid) for the detection of nitrite. The appearance of red haloes surrounding the wells indicate the presence of nitrate reductase activity.

2.2.2. Spectrophotometric assay

Ten millilitres of YT-broth (YTA without agar) supplemented with 250 ppm of KNO_3 were inoculated with 100 μl of an overnight culture of each strain. After incubation at 30 and 20 °C for 24 h and at 15 °C for 72 h a fraction of overnight cultures was used for the determination of dry weight while another fraction was used to detect nitrate reductase activity by spectrophotometric assay according to Gerhardt, Murry, Willis, and Krieg (1994). One hundred microlitres of each overnight culture were added to 250 μl of Griess I (0.5 g of sulphanilic acid in 150 ml of 5 N acetic acid), 250 μl of Griess II (0.5 g of Naphthylamine in 50 ml of distilled water and 100 ml of 5 N acetic acid) and 2 ml of distilled water and incubated at room temperature for 15 min after shaking in a vortex for 1 min. Nitrite production from nitrate was determined by reading optical density (OD) at 540 nm. Relative activity was calculated as the rate: $OD_{540\ nm}$/mg dry weight.

Table 1
Technological activities of *Staphylococcus carnosus* and *S. simulans* strains[a]

Activity	*Staphylococcus carnosus*				*Staphylococcus simulans*	
	SC28	SC31	SC54	SC55	Ssm12	Ssm21
Nitrate-reductase						
Agar-plate method[b]						
15 °C	0^{a1}	7^{b1}	0^{a1}	0^{a1}	0^{a1}	0^{a1}
20 °C	0^{a1}	20^{b2}	0^{a1}	0^{a1}	22^{c2}	18^{d2}
30 °C	8^{a2}	15^{b3}	7^{a2}	7^{a2}	23^{c2}	23^{c3}
Spectrophotometric assay[c]						
15 °C	5.78^{b1}	9.14^{d1}	4.59^{a1}	3.74^{a1}	7.05^{c1}	6.10^{b1}
20 °C	5.04^{a1}	15.3^{d2}	7.02^{b2}	5.36^{a1}	11.35^{c2}	10.5^{c2}
30 °C	10.38^{a2}	10.3^{a1}	14.45^{b3}	10.77^{a2}	19.55^{d3}	18.06^{c3}
Catalase[d]						
YT-broth	20.77^{ab1}	24.80^{bc1}	19.57^{ab1}	16.13^{a1}	27.06^{c1}	27.76^{c1}
YT-broth + 0.25% KNO_3	15.9^{a2}	21.38^{b1}	21.93^{b1}	21.35^{b1}	24.46^{b1}	24.24^{b2}
Superoxide dismutase[e] (SOD)	57.95^{c}	36.01^{b}	64.25^{d}	51.02^{e}	32.90^{b}	23.85^{a}
Lipolytic						
Titration method[f]	6.4^{a}	6.4^{a}	6.4^{a}	6.4^{a}	11.2^{b}	19.2^{c}
Spectrophotometric assay[g]	0.0035^{a}	0.0015^{a}	0.0043^{b}	0.003^{a}	0.249^{c}	0.229^{c}
Proteolytic						
Sarcoplasmic/myofibrillar proteins[h]	7^{a}/0	6^{a}/0	6^{a}/0	7^{b}/0	7^{b}/0	8^{c}/0
Amino-acid and small peptides[i]	0.0831^{e}	0.0458^{c}	0.1153^{f}	0.0720^{d}	0.0027^{a}	0.0049^{b}

Values in row with common letters are not significantly different ($P > 0.05$); values in column with different numbers are significantly different ($P < 0.05$).

[a] The strains were isolated from traditional fermented sausages of Vallo Diano (Campania Region – Italy).

[b] Nitrate reductase activity was checked by the measure (mm) of red haloes surrounding the wells (without the diameter of well). The values are the mean of three independent assays.

[c] Nitrate reductase: mM nitrite × mg^{-1}dry weight.

[d] μmoles of degraded H_2O_2/min/ml of cells with optical density ($OD_{600\ nm}$) = 1.0.

[e] % of optical density at 560 nm.

[f] % of palmitic acid.

[g] mg/ml of palmitic acid.

[h] Proteolytic activity was checked by the measure of clear zone surrounding the wells (mm). The values are the mean of three independent assays.

[i] Free amino acids and small peptides where determined by *o*-phtaldialdehyde (OPA) spectrophotometric assay by reading optical density at 340 nm. Sarcoplasmic extract control showed an $OD_{340\ nm}$ of 0.5374.

2.3. Catalase assay

Catalase activity was measured on resting cells according to Aebi's method (Aebi, 1974) in parallel, after growth in YT broth and in YT broth supplemented with 250 ppm of KNO_3 for 24 h at 30 °C. Five millilitres of each culture (resting cells with a $OD_{600\ nm}$ = 1.0) were centrifuged at 13,000*g* for 5 min and the resulting pellet mixed with 1.5 ml of 60 mM H_2O_2 in 20 mM phosphate buffer pH 7.0. The activity was measured spectrophotometrically at 240 nm after 3 min of incubation at room temperature. Results were expressed in arbitrary units: micromoles of degraded H_2O_2/min/ml/ of cells with OD_{600} = 1.0.

2.4. Superoxide dismutase activity

Superoxide dismutase (SOD) activity was determined by spectrophotometric method. The cell pellet of each strain was harvested by centrifugation of 2 ml of culture at 13,000*g* for 5 min and washed once in 50 mM K_2HPO_4, pH 7.8. For SOD extraction the cell pellet (approximately 100 mg wet weight) was suspended in 1 ml of 50 mM K_2HPO_4 pH 7.8 and disrupted with glass beads (0.10–0.11 mm) on a vortex mixer for 5 min. After lysis, the suspension was centrifuged at 13000*g* for 5 min and the supernatant used for determination of SOD activity as described below. Ten microlitres of cell extract were added to 1.0 ml of 150 μM nitroblue tetrazolium (NBT), 10 mM methionine, 1.2 μM riboflavin, 50 mM K_2HPO_4, pH 7.8 and incubated at room temperature in a light chamber with a 60 W bulb lamp for 8 min. Under this condition, riboflavin is excited by a photon and is able to oxide the methionine. This donation of an electron results in the production of a superoxide molecule (O_2^-) that is able to reduce NBT resulting in a colour change which was measured spectrophotometrically at 560 nm against the appropriate solution. The presence of SOD leads to a reduction in the colour change. Results were expressed as percentage of optical density (OD) at 560 nm according to the following equation:

$$\text{SOD activity} = (1 - s/c)100,$$

where s is the OD of sample and c the OD of the control, consisting of a solution without adding cell extract and incubated under the same conditions.

2.5. Lipolytic activity

2.5.1. Titration method

One ml of an overnight culture of each strain was inoculated into 10 ml of YTF broth containing 1% tryptone, 0.5% yeast extract, 3% NaCl, pH 7.0 supplemented with 4% (w/v) pork fat. After incubation at 30 °C for 7 days the lipolytic activity was measured by titration methods. The lipids were extracted by adding 10 ml of petroleum ether (Merck & Co. Inc., NJ, USA) and shaking for 1 min. The free fatty acids (FFAs) of the upper phase (lipid extract) were titrated with NaOH 0.1 N in ethanol using 1% phenolphthalein ethanol solution as indicator, according to Mauriello et al. (2004) and percentage of palmitic acid, instead of oleic acid, was used to determine lipolytic activity by the following equation:

$$\text{Lipolytic activity} = (a \times N \times 25.6)/g,$$

where a is ml of NaOH used for titration, N is the normality of NaOH, 25.6 is the percent of equivalent weight of palmitic acid and g the quantity of pork fat the in sample.

2.5.2. Spectrophotometric assay

The strains cultivated in YTF broth as described above were used to determine free fatty acids according to Leuschner, Kenneally, and Arendt (1997). Free fatty acids were extracted from a sample volume of 0.5 ml by an organic solvent mixture composed of chloroform: η-heptane: methanol (49:49:2; v:v:v). "Copper salts" were formed by addition of a copper reagent, composed by 10 ml $Cu(NO_3)_2 \cdot 3H_2O$ 1 M, 5 ml of triethanolamine and 85 ml of NaCl 270 g/l. Colour development was achieved by the formation of a complex between copper salts with 0.1% sodium diethyl dithiocarbamate in η-butanol. A standard curve was prepared using palmitic acid. The free fatty acids were determined spectrophotometrically at 440 nm and were expressed as mg/ml of palmitic acid.

2.6. Detection of proteolytic activity

2.6.1. Agar plate method

Sarcoplasmic and myofibrillar proteins, extracted according to the method described by Mauriello, Casaburi, and Villani (2002) were added at concentration of 0.5 mg ml^{-1} to agar medium containing 0.5% tryptone, 0.25% yeast extract, 0.1% glucose, 1.5% agar, pH 6.9. Sarcoplasmic proteins were filter-sterilized and added to the medium after sterilization, while myofibrillar proteins were added to the agar medium that was sterilized at 121 °C for 15 min. The overnight culture (10 ml) of each strain was centrifuged at 13,000g for 5 min and resulting pellet washed once with 20 mM phosphate buffer pH 7.0 and resuspended in a equal volume of the same buffer. Forty microlitres of cell suspension were pipetted into wells bored in agar plates. After incubation at 30 °C for 48 h, the agar layers were removed from Petri dishes and stained for 5 min in 0.05% (w/v) Brillant Blue R (Sigma) in methanol:acetic acid:water (50:40:10) and destained in methanol:acetic acid:water (25:5:70). A clear zone surrounding the inoculated wells indicated proteolytic activity and its diameter was measured in mm.

2.6.2. Spectrophotometric and electrophoretic methods

Overnight cultures of the different strains were centrifuged, the pellets resuspended in 20 mM phosphate buffer pH 7.0 and inoculated in 10 ml of sarcoplasmic extract supplemented with 1% of glucose, to yield an initial number of bacteria of approximately 2×10^6 CFU ml^{-1}. Uninoculated sarcoplasmic extract was used as control and incubated under the same conditions at 30 °C in a shaken bath. Samples (1 ml) were taken at 0 and 192 h to determine pH and proteolytic activity. The latter was evaluated measuring the release of soluble amino acids and small peptides by *o*-phtaldialdehyde (OPA) spectrophotometric assay (Church, Swaisgood, Porter, & Catignani, 1983). Sarcoplasmic proteins degradation was assayed by SDS–PAGE as described by Laemmli (1970) on 12% polyacrylamide gels. One hundred microlitres of each sample was mixed with an equal volume of a sample buffer containing 2% of SDS, 5% of β-mercaptoethanol, 10% of glycerol, 0.05% (w/v) of bromophenol blue in 0.5 mM Tris–HCl pH 6.8 and heated for 5 min at 90 °C. Twelve microlitres of each sarcoplasmic extract inoculated with cell suspensions of the strains, 12 μl of uninoculated sarcoplasmic extract and 10 μl of Precision Plus Protein Standards (Bio-Rad) were applied onto gel into different wells. The electrophoresis was carried out at 200 V until the bromophenol blue marker reached the bottom of the gel. Gels were stained in 0.1% (w/v) Coomassie blue R-250 in methanol:acetic acid:water (40:10:50) and destained until background was obtained.

2.7. Decarboxylase activity

Overnight cultures of each strain cultured in TSB were centrifuged at 5000g for 15 min, the cells were washed with saline solution (NaCl 0.85%) and resuspended in the same solution at a density of 10^7–10^8 cells per ml. An aliquot of suspension (0.2 ml) was inoculated into 2 ml of Maijala's medium (Maijala, 1993) but without agar, supplemented with histidine (0.5%), lysine (0.5%) and tyrosine (0.5%). Maijala's mediums supplemented with cadaverine dihydrochloride, histamine dihydrochloride and tyramine (Sigma) at two different

concentrations (10 and 100 ppm) were used as positive controls. After incubation at 30 °C for 5 days the medium was centrifuged at 8000*g* for 5 min, 1 ml of supernatant was extracted with 1 ml of 0.1 N HCl, centrifuged at 8000*g* for 5 min and 1 ml of the acid extract was freeze dried.

2.7.1. Derivatization

The method described by Galgano et al. (2001) was used. The acid extract was mixed with 150 μl of saturated $NaHCO_3$ solution and the pH was adjusted to 11.5 with about 300 μl 1.0 N NaOH. Two millilitres of dansyl chloride (Merck) solution (10 mg/ml dansyl chloride/acetone,) were added to the alkaline amine extract from staphylococcal growth medium. The mixture was then transferred to an incubator and kept at 40 °C under agitation (195 strokes) for 60 min. The residual dansyl chloride was removed by adding 200 μl of 300 g/l ammonia solution (Fluka). After 30 min at 20 °C and protected from light, each sample was brought up to 5 ml with acetonitrile and filtered through 0.22 μm PTFE filter (Millipore).

2.7.2. HPLC analysis

The method reported by Galgano et al. (2001) was used with some modifications as following described. Samples were analysed for the presence of biogenic amines (BA) by high-performance liquid chromatography (HPLC, system Shimadzu). The chromatographic system consisted of two pumps LC-10 AD VP, a DIODE array detector, a system controller SCL-10A VP and a personal computer running the chromatographic software Data System Version 4:3. The sample (10 μl) was injected onto a Luna 5u C18 (2) column, (150 × 4.60 mm) (Phenomenex, USA). The peaks were detected at 254 nm and the elution programme consisted of the gradient system with a flow-rate of 0.8 ml/min.

The mobile phase was constituted by eluent A: water and eluent B: acetonitrile. The gradient program was implemented as follows: time = 0.01 min, 70% B, time = 1 min, 70% B, time = 11 min, 100% B, time = 19 min, 100% B, time = 20 min, 70% B, time = 25 min, 100% B.

Standard solutions of cadaverine dihydrochloride, histamine dihydrochloride and tyramine (Sigma) were prepared at three different concentrations (10, 100 and 1000 ppm) and were derivatized as described for the samples.

2.8. Effect of temperature, NaCl and pH on microbial growth

The strains were tested for growth ability at different temperatures, pH and NaCl concentrations. Growth was evaluated at 15 and 20 °C in yeast tryptone agar (YTA, tryptone 1%, yeast extract 0.5%, NaCl 0.4%, agar 1.5%, pH 7.0). The effect of NaCl was determined in YTA supplemented with 10%, 15% and 20% NaCl, respectively. The effect of pH on growth in YTA adjusted to pH values of 5.0 and 5.5 by addition of HCl (0.1 M) was evaluated at 15 and 20 °C. Ten microlitres of an overnight culture of each strain were inoculated into the different media described above and the growth response, whether positive or negative, was registered every 12 h for a week.

2.9. Statistical analysis

Statistical analyses were performed using the SPSS 11.5 software for Windows (SPSS Chicago, IL, USA). The analysis of variance (ANOVA test, Duncan test) was applied to determine difference in means. All the values are the mean values obtained from three independent assays for each trait.

3. Results

3.1. Growth characteristics, nitrate reduction and antioxidant (catalase and SOD) activities

All strains grew at temperatures usually used for meat fermentation (15 and 20 °C) in the presence of 10% 15% and 20% NaCl, as well as pH 5.0 and 5.5 (data not shown).

The reduction of nitrate to nitrite by the *Staphylococcus* strains detected by spectrophotometric and agar plate method was found to be temperature dependent. Significant differences ($P < 0.05$) were found between the activity determined at 20 and 30 °C for all the strains assayed, except for the strain *S. simulans* Ssm 12 that showed very similar activity at 20 and 30 °C ($P > 0.05$) when assayed by agar plate method (Table 1). The results obtained by spectrophotometric method showed that all the strains were able to reduce nitrate at 15, 20 and 30 °C but the major quantity of nitrite were released at 30 °C ($P < 0.05$), except for the strain of *S. carnosus* SC31 that showed the major activity at 20 °C ($P < 0.05$).

All the strains of *Staphylococcus* studied showed antioxidative enzymes catalase and superoxide dismutase (SOD) (Table 1). The higher values of catalase activity measured after growth in YT broth were showed by *S. simulans* strains and *S. carnosus* SC31. For all the strains the addition of nitrate in growth media not enhanced the synthesis of the enzyme. The SOD activity seem to be mainly strain-dependent with the higher value showed by the strain SC54 of *S. carnosus*.

3.2. Lipolytic, proteolytic and decarboxylase activities

The results of lipolytic activity are reported in Table 1. All the strains analyzed by both spectrophotometric

and titration methods showed low values of palmitic acid. The proteolytic activity was assayed against sarcoplasmic and myofibrillar proteins. The results obtained by agar plate methods showed that all the strains were able to hydrolyse only sarcoplasmic proteins. On the basis of these results, proteolytic activity by OPA and SDS–PAGE methods was evaluated only in sterile sarcoplasmic extract. The pH of sarcoplasmic extracts inoculated with all strains decrease significantly ($P < 0.05$) from 6.27 to 4.1–4.4 after 192 h of incubation at 30 °C, indicating an active cell metabolism (data not shown). All the strains showed a great consumption of amino acids from the soluble meat extracts (Table 1). Electrophoretic profiles of sarcoplasmic proteins hydrolysed by the strains of *Staphylococcus* are reported in Fig. 1. The bands were identified by their molecular weights, in comparison with the standard proteins marker. Proteolytic activity of endogenus origin was not detected in control samples. After 192 h of incubation all the strains hydrolysed sarcoplasmic proteins showing their action by the disappearance of bands of about 150, 64.4, 31.4, 27 kDa and a decrease in intensity of the bands at 45 and 25 kDa. The electrophoretic profile resulting from the action of *S. simulans* (Fig. 1, lanes e and f for strains Ssm12 and Ssm21, respectively) and *S. carnosus* strains (Fig. 1, lanes g and h for strains SC28 and SC54, respectively) showed a complete disappearance of protein bands of about 60 kDa. Furthermore, the electrophoretic profile resulting from the action of strains Ssm12 and Ssm21 showed a decrease in intensity of protein band of 38 kDa and a complete disappearance of protein bands in the range 75-100 kDa. Finally, all the staphylococcal stains assayed did not show amino acid decarboxylase activity in fact, histamine, cadaverine and putrescine were never produced (the limits of determination for all the standards were 10 ppm).

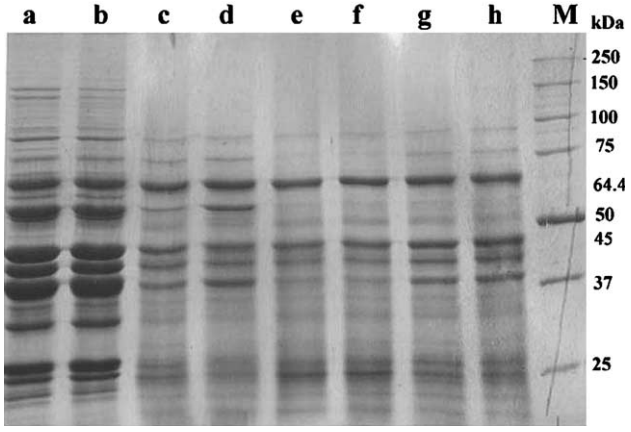

Fig. 1. SDS–PAGE of sarcoplasmic proteins hydrolysis by *S. carnosus* and *S. simulans* strains. Lane a: uninoculated control at 0 h of incubation; lane b: uninoculated control at 192 h of incubation; lane c–h: samples containing different strains at 192 h of incubation; lane c: *S. carnosus* SC31; lane d: *S. carnosus* SC55; lane e: *S. simulans* Ssm12; lane f: *S. simulans* Ssm21; lane g: *S. carnosus* SC28; lane h: *S. carnosus* SC54; lane M: perfect proteins marker.

4. Discussion

S. carnosus and *S. simulans* are two of the three species of the genus *Staphylococcus* whose employment as starter cultures for fermented sausages is permitted by Italian law (Repubblica Italiana, 1995) and knowledge of their technological properties is a matter of considerable interest. Nitrate reductase and catalase activities are considered to be the most important properties of staphylococci to be used as starter cultures for fermented sausages production (Weber, 1994). Results obtained previously (Mauriello et al., 2004) reported that all staphylococcal strains belonged to *S. xylosus*, *S. equorum* and *S. lentus* species were able to reduce nitrate to nitrite at 30 °C by agar plate method and a similar result was reported by Miralles et al. (1996) who found three strains of *S. xylosus* and one strain of *S. epidermidis* able to reduce nitrates to nitrites. Our results obtained by agar plate method at 30 °C are in agreement with those and are confirmed by spectrophotometric method. Instead, contrasting results were obtained applying the two methods at 15 and 20 °C. In fact, all the strains showed nitrate reductase activity by spectrophotometric method, whereas by the agar plate method several strains did not produce nitrite from nitrate. The capability of staphylococcal strains to reduce nitrate in nitrate broth, at the temperature adopted during the ripening of the sausages are reported also by Coppola, Iorizzo, Saotta, Sorrentino, and Grazia (1997) who found different strains belonging to the species of *S. xylosus*, *S. equorum* and *S. simulans* able to reduce nitrates to nitrite at 18 and 30 °C. So, the different results obtained at 15 and 20 °C indicate the importance of finding a suitable method for a correct detection of positivity and intensity of properties under examination. Confronting the results obtained by spectrophotometric and agar-plate methods, the latter show a low sensitivity. In fact, all the strains unable to reduce nitrate by this method were able to reduce nitrate by the spectrophotometric method releasing from 3.75 to 7.02 mM of nitrite/mg of dry matter. Therefore the results obtained suggest that the use of spectrophotometric method could be more suitable for the detection of low nitrate reductase activity. As already reported in a previous study (Mauriello et al., 2004), in which all staphylococcal species studied showed antioxidant activities strain dependent, the antioxidant activities of the strains assayed in this work were quite variable. In agreement with Talon, Walter, Chartier, Barriére, and Montel (1999) we found strains of staphylococci with a cell-bound catalase activity.

Kenneally, Leuschner, and Arendt (1998) examining the lipolytic activity of meat starter cultures by agar plate method and measuring the FFAs development in broth, found that the method employed in broth system was the more sensitive. Our results obtained using the "copper method" for the detection of free fatty acids showed a greater sensitivity of the method indicating its meaningful application for evaluation of FFAs in meat systems as also reported by Leuschner et al. (1997). In fact, this assay gives a quantitative estimation of the lipolytic activity of the assayed microrganism, contrary to the titration method that allows to have only a qualitative estimation. Our results showed that all the strains were unable to hydrolyse pork fat releasing less than 0.5 mg/ml of palmitic acid as also reported by Kenneally et al. (1998). In relation to the proteolytic abilities of strains studied, the agar plate assay showed that all the strains tested were able to hydrolyse the sarcoplasmic proteins but none were capable to use myofibrillar proteins. These results were not in line with those obtained in previous studies (Mauriello et al., 2002; Mauriello et al., 2004) in which we found that all the species studied (*S. xylosus*, *S. saprophyticus*, *S. equorum*, *S. lentus*, *S. warneri* and *S. succinus*) showed greater proteolysis against myofibrillar than sarcoplasmic proteins. This may be due to the fact that this is the first time we consider *S. carnosus* and *S. simulans* for the evaluation of the proteolytic activity.

In the present work, the proteolytic activity on sarcoplasmic proteins determined by agar plate method, was confirmed by electrophoretic and spectrophotometric assays for all the strains. The sarcoplasmic protein pattern of the control samples (uninoculated) did not reflect major proteolytic changes as result of the possible endogenous activity, showing that *S. carnosus* and *S. simulans* possessed interesting proteinases. These results were obtained also in a previous study (Mauriello et al., 2002) in which three *S. xylosus* strains able to hydrolyse sarcoplasmic proteins showed a decrease in intensity of several protein bands at low molecular weight (below 50 kDa). Finally, all the strains studied utilized the different nitrogen sources of the soluble meat extract releasing a low level of amino acids. A similar result was reported by Rodriguez, Nunez, Cordoba, Bermudez, and Asensio (1998) who found no increase in free amino acids concentration from inoculated meat pork slices despite an extensive hydrolysis of sarcoplasmic proteins.

In conclusion, all strains studied possessed antioxidant, nitrate reductase activities, growth characteristics (temperature, pH and NaCl) and the inability to form BA that would make them eligible as a good starter cultures for different technological conditions and sausage type manufacture. Considering the reactions that could be taking in place in the meat mixture and the involvement of endogenous meat enzymes and complex microflora, further studies are necessary to evaluate the technological properties of microbial strains in sausage production and to evaluate their effects on product quality.

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