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# Phenotypic and Molecular Characterization of *Staphylococcus xylosus*: Technological Potential for Use in Fermented Sausage

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## ABSTRACT

Micrococcaceae *strains are applied to fermented sausage as starter cultures, where several members of this family are naturally found. The aim of the present work was to isolate and characterize* Staphylococcus xylosus *from artisanal sausages produced in South Region of Brazil. From 89 isolates presenting catalase positive and coagulase negative activities, 25 strains were selected for phenotypic characterization. Nine strains identified as* Staphylococcus xylosus *by API-STAPH were evaluated for their nitrate reduction capacity, which showed satisfactory growth of the strains in the presence of nitrite and sodium chloride, demonstrating their potential for use as starter cultures in fermented sausage. The strains were also evaluated through genus and species-specific PCR, which showed only two as S. xylosus, differing from results found in phenotypic characterization.*

**Key words:** *Staphylococcus xylosus*, fermented sausage, starter cultures, PCR

## INTRODUCTION

Lactic acid bacteria (LAB) and Gram-positive catalase positive cocci (GCC+) (*Micrococcaceae*) are the most important bacteria found in meat sausages, for the manufacturing technology. They are used as starter cultures in meat products where LAB are responsible for producing lactic acid and then preventing the growth of pathogens. *Micrococcaceae* family, mainly *Staphylococcus* and *Kocuria* strains, are known for ensuring colour stability and preventing lipid oxidation in fermented meat products. Besides, these strains also influence the level of aromatic compounds due to their lipolytic and proteolytic activities (Talon et al., 2002; Aymerich et al., 2003; Spricigo and Pianovsky, 2005).

Several staphylococcal species have been isolated from fermented sausages. *S. xylosus* and *S. saprophyticus* strains were dominant in Greek fermented sausages (Samelis et al., 1998; Papamanoli et al., 2002; Drosinos et al., 2005). In

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Spanish sausages, *S. xylosus* was the dominant species (Garcia-Varona et al., 2000). Aymerich et al (2003) identified *S. xylosus*, *S. carnosus* and *S. epidermidis* in low-acid chorizos. Staphylococcal flora was more diversified in Italian sausages, in which species such as *S. xylosus* , *S. saprophyticus*, *S. equorum* followed by *S. warneri*, *S. epidermidis* and *S. lentus* were identified (Coppola et al., 2000; Cocolin et al., 2001a, Rossi et al., 2001). In traditional French dry fermented sausages, dominant species were *S. xylosus*, *S. carnosus*, *S. warneri* and *S. saprophyticus* (Montel et al., 1992, 1996).

*S. xylosus* is used in meat industry as a starter culture for fermented sausages, as it ensures colour formation and contributes to aroma development (Martín et al., 2005). The most promising starter strains are those isolated from naturally fermented meat products, where they are well adapted and are the dominant population (Drosinos et al., 2005).

Isolation, identification and characterization of *Micrococcaceae* from artisanal meat products ensure its application as starter culture in sausages keeping its artisanal characteristic with controlled fermentation and ripening processes. It is an alternative of processing because allows a stable product at room temperature and the enzymatic reactions carried out by *Micrococcaceae* influence the typical flavour of naturally fermented sausages. However, characterization of such microorganisms is needed to ensure their safe using as starter cultures.

Identification of microflora from meat sausages using only phenotypic methods is insufficient for characterizing a microorganism as it may be uncertain (Blaiotta et al, 2003), due to a subjective and ambiguous interpretation of the colorimetric profile resulting from biochemical (sugar fermentation) tests (Quere et al, 1997). Due to this, molecular methods have been increasingly used in characterization procedures: randomly amplified polymorphic DNA-PCR analysis (Rossi et al., 2001; Martín et al., 2005), species-specific PCR (Aymerich et al., 2003; Morot-Bizot et al., 2003; Rantsiou et al., 2005), multiplex PCR (Morot-Bizot et al., 2003). Many typing methods have also been used to characterize staphylococci, such as pulse-field gel electrophoresis (PFGE) (Snopkova et al., 1994), denaturing gradient gel electrophoresis (DGGE) of 16S rRNA gene fragments (Cocolin, et al., 2001b; Rantsiou, et al., 2005) and amplification of the 16S-23S intergenic spacer region (Rossi et al., 2001; Blaiotta et al., 2003).

The aim of the present work was to isolate and characterize *Staphylococcus xylosus* from artisanal sausages produced in South Region of Brazil by means of phenotypic and molecular methods, as well as to characterize their technological potencial for use as starter cultures in fermented sausage.

## MATERIALS AND METHODS

### Isolation of Micrococcaceae

Wild strains of *Micrococcaceae* were isolated from thirty eight (38) samples of artisanal sausages collected in twenty one cities of South Region of Brazil. After removing the casing, 25 g of each sample were homogenized in 225 mL of 0.1% peptone water. Decimal dilutions were prepared and plated in BHI Agar (Brain Heart Infusion, Merck), incubated at 35ºC for 48 h and then purified by streaking onto BHI Agar (Merck). Isolated strains were studied by cell morphology, Gram staining and catalase test for assuring their classification in Micrococcaceae family. Selected strains were stored at –50ºC in BHI broth (Merck) containing 20 % glycerol (Merck).

### Phenotypic Characterization

The isolates were subjected to coagulase production test using rabbit plasma in EDTA (Merck). API-STAPH (BioMerieux) galleries were also used. This system provides the following tests: urea production, arginine, n-acetyl-glucosamine, reduction of nitrate, acetoin and sugar fermentation (maltose, d-mannitol, d-mannose, lactose, sucrose, raffinose, d-trehalose, d-xylose, d-glucose, d-fructose). As recommended by the manufacturer, the bacterial suspension was inoculated into the galleries and incubated at 36ºC for 18-24 h.

### Effect of temperature, pH and sodium chloride (NaCl) concentrations

The effect of pH was tested by growth in BHI broth (Merck) adjusted for pH values of 5.0 and 5.5. Effect of different concentrations of NaCl on growth was evaluated in BHI broth (Merck) supplemented with 10 and 15% NaCl. Both testes were evaluated for growth ability at different temperatures: 15 and 45ºC.

**Tolerance to NaCl and sodium nitrite ($NaNO_2$)**

Tolerance to NaCl and $NaNO_2$ was tested by growth on BHI agar supplemented with different NaCl (1.5, 2.0, 2.5, and 3.0%) and $NaNO_2$ (80, 100, 120, 150 and 200 μg/g) concentrations and incubated at 35ºC for 48h.

The simultaneous tolerance of strains to NaCl and $NaNO_2$ was examined on BHI agar (Merck) supplemented with NaCl (3%) and $NaNO_2$ (200 μg/g) incubated at 35°C for 48h.

**Capacity to reduce nitrites**

The ability of strains to reduce nitrites was tested in BHI broth (Merck) pH 5.0, supplemented with 156 and 300 μg/g of $NaNO_2$ and incubated at 35°C for 48h. The level of residual nitrite was measured by spectrophotometry at 474 nm (Harrigan and McCance, 1976).

**Lipolytic activity**

The lipolytic activity of strains was tested according to Kouker and Jaeger (1987) and Haba et al. (2000). The medium contained: 0.8% of nutrient broth (BBL); 0.4% NaCl; 1% of agar–agar, was adjusted to pH 7, autoclaved (121ºC for 15 min) and cooled at 60ºC. Then, a 2.5% final concentration of sterilized olive oil and 10 mL of Rhodamine B (1 mg/mL) dissolved in distilled water sterilized by filtration was added. After sonication, used by means of correct emulsification, the medium was spread onto Petri dishes. Lipase activity was identified on the plate as an orange fluorescent halo under UV light at 350 nm after 24–26 h of incubation at 37°C.

**Molecular Characterization**

Isolated strains phenotypically characterized as *Staphylococcus xylosus* and reference strain ATCC 29971 were grown overnight in BHI broth at 35ºC. One milliliter (mL) of each culture was centrifuged at 13,000 *g* for 2 minutes and DNA isolation was carried out from pellet cells by using the Wizard® Genomic DNA Purification Kit (Promega).

Amplifications were performed with the primers TstaG422/Tstag765 (Martineau *et al.*, 2001) and XYLF/XYLR (Morot-Bizot *et al*., 2003; Corbiére Morot-Bizot et al., 2004) allowing the identification of the *Staphylococcus* genus and *S. xylosus* species, respectively. The TstaG422/Tstag765 and XYLF/XYLR amplify fragments of 370 bp and 539 bp, respectively (Morot-Bizot et al., 2003). Besides, a reference strain of *Lactobacillus plantarum* (ATCC 8014) was used as negative control in all PCR reactions.

For molecular identification at genus level, PCR reactions were carried out in a final volume of 25 μL containing 2 μL of extracted DNA, 2.5 mM $MgCl_2$, 0.2 μM of each TstaG422/Tstag765 primers, 200 μM of each dNTP and 1.25 U of *Taq* DNA polymerase in 1 x buffer according to the manufacturer's instructions (Promega). Amplifications were performed in a Minicycler™ (MJ Research, Inc. Watertown, MA) under following conditions: 3 min at 96ºC, then 40 cycles of 30 s at 95ºC, 60 s at 55ºC, 30 s at 72ºC and a final extension of 3 min at 72ºC (Morot-Bizot et al., 2003; Martineau *et al.*, 2001).

For molecular identification at species level, PCR reactions were carried out in a final volume of 25 μL containing 2 μL of extracted DNA, 1.5 mM $MgCl_2$, 0.2 μM of each XYLF/XYLR primer, 200 μM of each dNTP and 1.25 U of *Taq* DNA polymerase in 1X buffer according to the manufacturer's instructions (Promega). Amplifications were performed in a Minicycler™ (MJ Research, Inc. Watertown, MA) under following conditions: 5 min at 94ºC, then 40 cycles of 60 s at 94ºC, 60 s at 55ºC, 3 min at 72ºC and a final extension of 6 min at 72ºC (Morot-Bizot et al, 2003; Corbiére Morot-Bizot et al., 2004).

The PCR products (10 μL reaction + 2 μL loading buffer) were separated through electrophoresis at 400mA and 80V for 50 min in 2.5 % agarose gel, 1X TBE buffer and stained with ethidium bromide. The visualization was performed in UV-transilluminator and the images photographed with a digital camera (Canon Powershot A70).

## RESULTS AND DISCUSSION

From a total of 175 *Micrococcaceae* strains isolated from naturally fermented sausages, 89 (50.8%) were catalase-positive and coagulase-negative. API-STAPH system was used for phenotypic characterization of the isolated strains (Tables 1 and 2). Twenty-one (84 %) out of twenty-five strains were identified as *Staphylococcus* spp, one strain (4 %) was identified as *Kocuria varians* and three (12 %) presented doubtful profile. Concerning to *Staphylococcus* identified strains, *S. xylosus* was

the dominant species (42.8 %), followed by *S. saprophyticus* (28.5 %), *S. lentus* (19 %), *S. epidermidis* (4.7 %) and *S. warneri* (4.7 %). Papamanoli et al. (2002) found *S. saprophyticus* as the dominant species, followed by *S. xylosus* and *S. carnosus* in dry fermented sausage. Samelis et al. (1998) found that *S. saprophyticus* and *S. xylosus* dominated the *Micrococcaceae* populations in traditional Greek salami. These species occur in several regions of Europe and have been reported in some studies about isolation and phenotypic characterization of micrococci from meat products: *S. saprophyticus* (Seager *et al*, 1986; Coppola *et al*, 2000), *S. epidermidis* (Kotzekidou, 1992; Coppola *et al*, 2000) and *S. xylosus* (Miralles *et al*, 1996; Coppola *et al*, 2000). *S. xylosus* is the dominant species in most dry fermented sausages, being used as starter cultures because of its contribution to aroma and taste formation. Otherwise, *S. saprophyticus* and *S. epidermidis* are the dominant species found on human skin and are occasionally isolated from the skin of domestic animals. These species are probably acquired from the skin of pork or from human manipulation during manufacture of sausages. Since *S. saprophyticus* and *S. epidermidis* are opportunistic pathogens isolated from human urinary tract, they are not recommended for using as starter cultures.

The majority of strains identified as *S. xylosus* presented the typical characteristics of the species (Tables 1 and 2), such as nitrate reduction (88.8 %), acetoin production (77.7 %) and acid production from xylose (77.7 %) and mannose (88.8 %) but not from raffinose (11.1 %). Some strains identified as *S. xylosus* did not ferment xylose and mannose, as also reported by Drosinos et al. (2005) and Samelis et al. (1998). Few strains identified as *S. saprophyticus* were able to reduce nitrate (33.3 %) whereas 83.3 % produced acetoin. Majority of strains identified as *S. saprophyticus* did not produce acid from mannose and xylose, but were able to ferment maltose and trehalose.

**Table 1 -** Biochemical tests for identification of *Staphylococcus* isolated from naturally fermented sausages.

| Identification | Isolates | Nitrate Reduction | Urea | Acetoin | Acetyl-glucosamine | Arginine | Catalase | Coagulase |
|---|---|---|---|---|---|---|---|---|
| *S. xylosus* | 9(36) | 8(88,8) | 4(44,4) | 7(77,7) | 8(88,8) | 8(88,8) | 9(100) | 0 |
| *S. saprophyticus* | 6(24) | 2(33,3) | 5(83,3) | 5(83,3) | 4(66,6) | 0 | 6(100) | 0 |
| *S. lentus* | 4(16) | 3(75) | 0 | 0 | 4(100) | 1(25) | 4(100) | 0 |
| *S. epidermidis* | 1(4) | 1(100) | 1(100) | 0 | 1(100) | 1(100) | 1(100) | 0 |
| *S. warneri* | 1(4) | 1(100) | 1(100) | 0 | 1(100) | 0 | 1(100) | 0 |
| *Doubtful profile* | 3(12) | 2(66,6) | 1(33,3) | 0 | 0 | 2(66,6) | 3(100) | 0 |

Values in parenthesis represent % of positive isolates.

**Table 2 -** Sugar fermentation profile of *Staphylococcus* isolated from naturally fermented sausages.

| Identification | Glucose | Mannose | Maltose | Lactose | Trehalose | Mannitol | Xylose | Sucrose | Raffinose |
|---|---|---|---|---|---|---|---|---|---|
| *S. xylosus* | 9(100) | 8(88,8) | 9(100) | 8(88,8) | 9(100) | 9(100) | 7(77,7) | 8(88,8) | 1(11,1) |
| *S. saprophyticus* | 6(100) | 3(50) | 6(100) | 5(83,3) | 6(100) | 4(66,6) | 1(16,6) | 6(100) | 0 |
| *S. lentus* | 4(100) | 4(100) | 4(100) | 4(100) | 4(100) | 4(100) | 3(75) | 4(100) | 4(100) |
| *S. epidermidis* | 1(100) | 1(100) | 1(100) | 0 | 0 | 1(100) | 0 | 1(100) | 0 |
| *S. warneri* | 1(100) | 1(100) | 1(100) | 1(100) | 1(100) | 1(100) | 0 | 1(100) | 0 |
| *Doubtful profile* | 3(100) | 2(66,6) | 3(100) | 1(33,3) | 2(66,6) | 3(100) | 1(33,3) | 2(66,6) | 1(33,3) |

Values in parenthesis represent % of positive isolates.

The selection of strains presenting desirable characteristics is necessary in order to achieve fermented sausages with appropriate technological and sensory properties. The first rule in selecting strains for using as starter cultures is nitrate reduction, as it influences the colour formation (Garcia-Verona et al., 2000). As secondary criteria, acetoin production and urease activity should be also evaluated. In the present work, strains identified as *S. xylosus* presented desirable characteristics also reported by Drosinos et al. (2005): nitrate reductase and urease activities and acetoin production. According to Smith and Palumbo (1983), tolerance to NaCl and $NaNO_2$ and growth between 27 and 43°C are important characteristics. Strains tested in the present work

showed positive catalase and negative coagulase activities, growth at 15 and 45°C and at two NaCl concentrations (10 and 15 %) and pH values (5.0 and 5.5) in BHI broth (Table 3).

**Table 3 -** Characterization of *Staphylococcus* xylosus (BHI broth) isolated from naturally fermented sausages.

| | AB1 | AC3 | R1 | S4 | AD5 | AD1 | Q3 | M4 | U5 |
|---|---|---|---|---|---|---|---|---|---|
| *pH* | | | | | | | | | |
| *5.5* | + | + | + | + | + | + | + | + | + |
| *5.0* | + | + | + | + | + | + | + | + | + |
| *Temperature* | | | | | | | | | |
| *$15^0$* | + | + | + | + | + | + | + | + | + |
| *$45^0$* | + | + | + | + | + | + | + | + | + |
| *NaCl* | | | | | | | | | |
| *10%* | + | + | + | + | + | + | + | + | + |
| *20%* | + | + | + | + | + | + | + | + | + |

+ represent positive growth.

Growth of strains was not inhibited on solid substrate (BHI agar) under different NaCl concentrations, showing tolerance to a concentration of 3 % for this salt (Fig. 1). Strain AD1 showed the most accentuated decrease in growth, from $10^{8.7}$ CFU/mL to $10^8$ CFU/mL. Seven out of nine strains tolerated a concentration of 200 □g/g of $NaNO_2$ and its growth was not changed in the absence of $NaNO_2$ (Fig. 2).

Strains AB1 and AC3 showed a decrease in growth in presence of 200 μg/g of $NaNO_2$, from $10^8$ CFU/mL to $10^3$ CFU/mL. The same seven out of nine strains did not present differences in growth when NaCl and $NaNO_2$ were used simultaneously, showing an appropriated tolerance (Fig. 3).

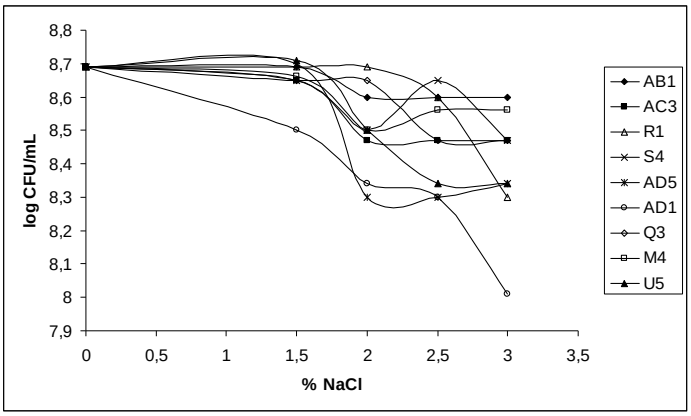


**Figure 1** - Growth (log CFU/mL) of *Staphylococcus xylosus* strains isolated from naturally fermented sausages, in different concentrations of NaCl.

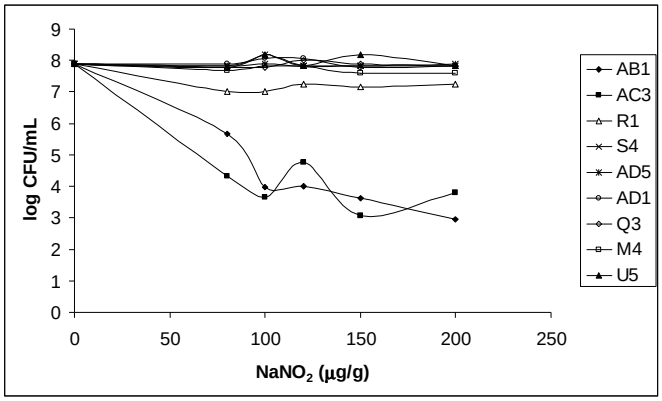


**Figure 2** - Growth (log CFU/mL) of *Staphylococcus xylosus* strains isolated from naturally fermented sausages in different concentrations of $NaNO_2$.

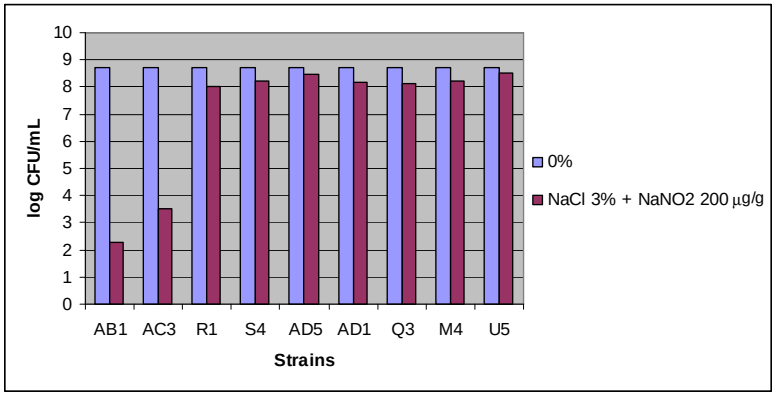


**Figure 3** - Growth (log CFU/mL) of *Staphylococcus xylosus* strains isolated from naturally fermented sausages, in broth added of NaCl and $NaNO_2$.

The presence of curing salts, such as sodium nitrite and nitrate that improve the colour of the product, has an important bacteriostatic action against *Clostridium botulinum* and pathogenic microorganisms. Since as nitrite reduction ensures the colour formation in fermented sausages, it is necessary that the final product presents low levels of residual nitrites decreasing the risk of nitrosamine formation (Hugas and Monfort, 1997). The ability of starter cultures in reducing nitrites can be due to enzymatic activity of nitrite reductase or by lowering of pH. Spectrophotometric measures did not present detectable levels of residual nitrites in tests carried out and control did not present change in $NaNO_2$ initial concentration. Considering that pH was adjusted to 5.0 and nitrite reductase activity was not evaluated, apparently the nitrite reduction was carried out by *S. xylosus* strains.

*S. xylosus* also presented lipolytic and proteolytic activities (Hames and Hertel, 1998). In the present work, lipolytic activity was tested in the nine isolated strains and all of them showed lipolytic activity identified on plate assay, as an orange fluorescent halo under UV light (Table 4). Martín et al (2005) observed lipolytic activity in 99 out of 194 isolates of *S. xylosus* from slightly fermented sausages.

**Table 4** - Lipolytic activity of *Staphylococcus* xylosus isolated from naturally fermented sausages.

| **Lypolitic Activity** | **AB1** | **AC3** | **R1** | **S4** | **AD5** | **AD1** | **Q3** | **M4** | **U5** |
|---|---|---|---|---|---|---|---|---|---|
| | + | + | + | + | + | + | + | + | + |

+ represent positive for lipolytic activity (orange fluorescent halo under UV light).

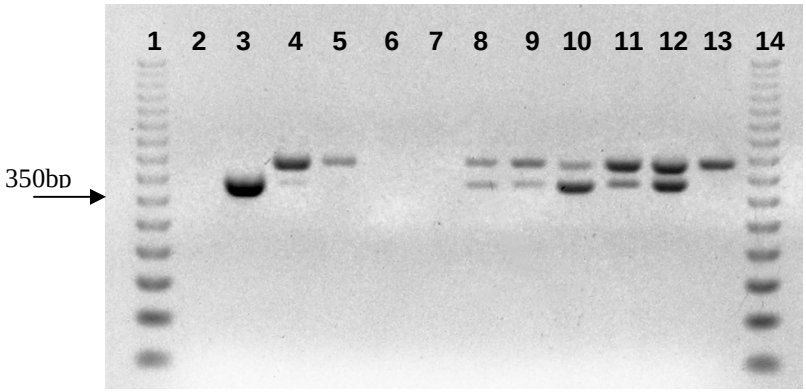


**Figure 4** - PCR amplification with genus-specific primers TstaG422/Tstag765, using 50 ng of template DNA. Lanes 1 and 14: ladder 50 bp (Promega); lane 2: control (*S. xylosus* ATCC 29971); lane 3: control (water); lanes 4 – 13: wild strains of GCC+ isolated from naturally fermented sausages; lane 14: ladder 50 bp (Promega).

In order to perform the molecular characterization of *S. xylosus* strains isolated from naturally fermented sausages, primers amplifying specific fragments of *Staphylococcus* genus and *S. xylosus* species were used. Nine strains identified as *S. xylosus* and one strain identified as *S. epidermidis* were selected for PCR analysis, as the identification by phenotypical methods has limitations and could result in misidentifications (Rhoden and Miller, 1995; Blaiotta et al., 2003, Morot-Bizot *et al*, 2006). Nine out of ten strains presented the expected fragment when primers TstaG422/Tstag765 were used, confirming that the strains belonged to *Staphylococcus* genus (Fig. 4 and 5). Figure 4 shows a reaction in with 50 ng of template DNA were used. As some strains did not present the expected fragment, the reaction was repeated with 2 μL of template DNA without dilution (Fig. 5). Concentration of template DNA seemed to influence the reaction with primers TstaG422/Tstag765. Such influence was not observed in reactions where primers XYLF/XYLR were used (Fig. 6).

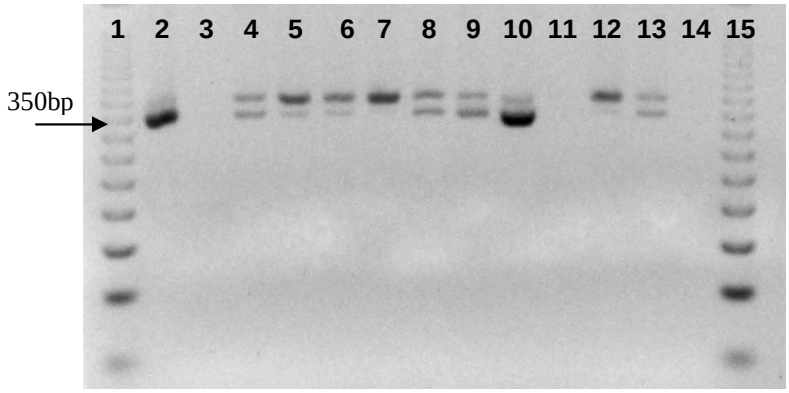


**Figure 5** - PCR amplification with genus-specific primers TstaG422/Tstag765 using 2 μL of template DNA (about 215 ng). Lanes 1 and 15: ladder 50 bp (Promega); lane 2: positive control (S. xylosus ATCC 29971); lane 3: negative control (water); lanes 4 – 13: wild strains of GCC+ isolated from naturally fermented sausages; lane 14: negative control (L. plantarum ATCC).

Only two strains confirmed to belong to *S. xylosus* species, as these were the only strains to give an expected PCR product of 539 bp when using *S. xylosus* species-specific primers (Fig. 6). The other strains did not present any fragment, suggesting that they belonged to other *Staphylococcus* species and were in disagreement with phenotypic characterization. Similar results were reported by Morot-Bizot et al., (2003) where only 7 out of 27 strains isolated from food environments and identified as *S. xylosus* by API STAPH system, were identified to this species by specific PCR. In contrast, Di Maria et al. (2002) obtained agreement between phenotypic and genotypic characterization.

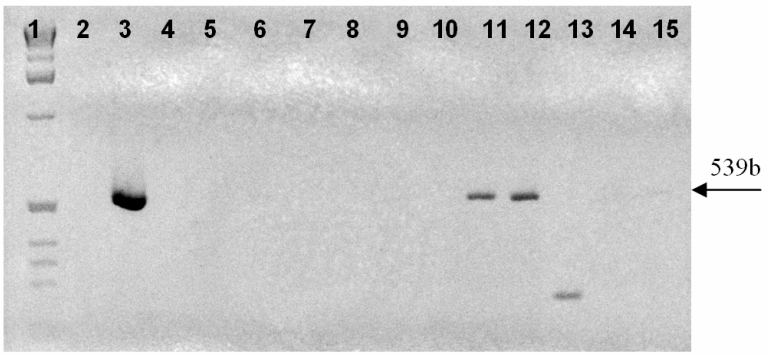


**Figure 6** - PCR amplification with species-specific primers XYLF/XYLR. Lane 1: ladder 1 kb (Invitrogen); lane 2: negative control (water); lane 3: positive control (S. xylosus ATCC 29971); lanes 4 – 13: wild strains of GCC+ isolated from naturally fermented sausages.

## CONCLUSION

These results demonstrated that molecular methods were significantly important for identification at species level, as it allowed an accurate identification of *S. xylosus* strains. The presence of this species among the indigenous flora of naturally fermented sausages produced in South Region of Brazil indicated its good adaptation capacity in this kind of product. The selected *S. xylosus* strains showed nitrate reductase, catalase and lipase activity, satisfactory growth in the presence of NaCl and $NaNO_2$ and reducing nitrites ability, hence could be recommended as starter cultures and used in the production of fermented sausages.

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## RESUMO

Cepas de *Micrococcaceae* são aplicadas em embutidos cárneos fermentados como culturas iniciadoras, onde vários membros desta família são naturalmente encontrados. O objetivo deste trabalho foi isolar e caracterizar *Staphylococcus xylosus* de embutidos cárneos artesanais produzidos na região Sul do Brasil. Dos 89 isolados que apresentaram atividades positiva para catalase e negativa para coagulase, 25 cepas foram selecionadas para caracterização fenotípica. Nove cepas identificadas como *Staphylococcus xylosus* por API-STAPH foram avaliadas para capacidade de redução de nitratos e o crescimento satidfatório das cepas foi verificado na presença de nitrito e NaCl, demonstrando seu potencial para utilização como culturas iniciadoras em embutidos cárneos fermentados. As cepas foram ainda avaliadas quanto ao gênero e espécie através da reação em cadeia da polimerase e apenas duas cepas foram identificadas como *S. xylosus*, diferindo dos resultados encontrados na caracterização fenotípica.

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