# Safety assessment of coagulase-negative staphylococci used in food production

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*meiner Familie gewidmet*

# Contents

# *Chapter I*

# Scope and outline

## Scope

Coagulase-negative staphylococci (CNS) of species *S. carnosus* and *S. xylosus* are traditionally used in starter cultures and are purposely added in high numbers of $10^6$-$10^7$ cfu/g to produce fermented foods. In addition, CNS like *S. condimenti*, *S. equorum*, *S. piscifermentans* and *S. succinus* belonging to the microbiota of spontaneously fermented foods were shown to occur in numbers of $10^6$ to $10^7$ cfu/g and therefore, have the potential for future use in starter cultures. Thus, enormous amounts of living bacteria are incorporated into the human body. However, for the latter CNS species, long term experience in respect of their sanitary harmlessness in fermented food exists only with limitations.

Within the EU, only Denmark has a legal regulation for the use of cultures. In the other EU countries, the assessment of sanitary harmlessness concerning microorganisms in food production is not regulated by law up to now and is subjected to the producers of food which are able to orientate oneself if the organisms exhibiting a safe history in the fermented products. In the year 2004, the European Food Safety Authority (EFSA) has undertaken the task to establish a concept for the safety assessment of microorganisms used in food and feed production. The proposed “Qualified Presumption of Safety” (QPS) system applies to microorganisms in traditionally fermented foods having a history of safe use and is based on four pillars dealing with `establishing identity´, `body of knowledge´, `possible pathogenicity´ and `end use´. In respect to possible pathogenicity, it is interesting to note that clinical isolates or rather toxin formation of *S. carnosus, S. equorum, S. succinus* and *S. xylosus* have been described. This raises the question to the presence of virulence factors, or other potential

disease-causing threats in food associated CNS. Moreover, in CNS isolated from food, antibiotic resistance genes were detected and their importance for the spread of antibiotic resistances has been recognized. Therefore, horizontal transfer of antibiotic resistance determinants is considered as an important safety issue. However, the availability of sufficient safety relevant data in respect of CNS with use or future use in food production is to some extent poor. Indeed, sufficient data are required to draw consequences concerning sanitary harmlessness of microorganisms used in food production.

For this purpose, in the first part of the thesis (Chapter III-V) strains of the species *S. carnosus*, *S. condimenti*, *S. equorum*, *S. piscifermentans*, *S. succinus* and *S. xylosus* were investigated phenotypically and partly genotypically in respect of the incidence of antibiotic resistances, pathogenicity factors and other undesired properties like the formation of biogenic amines and binding of proteins to extracellular matrix proteins.
Based on these insights in the second part of the thesis (Chapter VI) a DNA microarray was developed to rapidly and simultaneously detect the potential for expression of safety relevant properties in CNS with future use in food production. To increase the application potential of this microarray, in addition technological relevant properties were considered in array design. This microarray was in the following used for the genotypic investigation of, in the first part of the thesis, phenotypically characterized CNS concerning the presence of safety relevant properties.

## Outline of the thesis

**Chapter II** gives an overview in food fermentations and staphylococci, with a special focus on coagulase-negative staphylococci (CNS) traditionally used as starter cultures and CNS involved in spontaneous fermentations and therefore, the potential for use in starter or protective cultures. In this context, undesired properties and possible pathogenicity factors in staphylococci are described and the assessment of microorganisms with use in food production is taken into consideration. In doing so, "Qualified Presumption of Safety" (QPS) system proposed by European Food Safety Authority (EFSA) is explained. Finally, detection of safety relevant properties in food associated CNS as well as microarray technique as a tool for detection of safety relevant properties in microorganisms are described.

**Chapter III** describes the application of disk diffusion method to investigate the resistance of CNS strains associated with food or used in starter cultures. For this, 330 strains of the species *S. carnosus*, *S. condimenti*, *S. piscifermentans*, *S. equorum*, *S. succinus* and *S. xylosus* has been used. The investigation against 21 antibiotics revealed that the incidence and number of resistances in CNS was depending on species and source of isolation. Resistance phenotypes have mostly been shown in species of *S. equorum*, *S. succinus* and *S. xylosus* isolated from cheese and sausage and/or meat starter cultures, predominantly to lincomycin, penicillin, fusidic acid, oxacillin, ampicillin and tetracycline. Finally, phenotypic resistances to β-lactam antibiotics, lincomycin and tetracycline were verified by PCR amplification and traced back to the genes *bla*Z, *lnu*A and *tet*K. This chapter gives a comprehensive insight into the incidence of antibiotic resistances in food associated CNS.

This chapter has been published in International Journal of Food Microbiology:
Resch, M., Nagel, V., Hertel, C., 2008. Antibiotic resistance of coagulase-negative staphylococci associated with food and used in starter cultures. International Journal of Food Microbiology 127, 99-104.

**Chapter IV** describes the investigation of 330 CNS of species *S. carnosus*, *S. condimenti*, *S. piscifermentans*, *S. equorum*, *S. succinus* and *S. xylosus* isolated from fermented food and starter cultures concerning their hemolytic activity of human and sheep blood agar plates. Moreover, 35 selected strains were tested by immunoblot analysis for their ability to produce toxins like the most known staphylococcal enterotoxins (SEs), the toxic shock syndrome

toxin 1 (TSST-1), and the exfoliative toxin A (ETA). In respect of hemolysis, after 48 h incubation, more than half of the strains exhibited weak to moderate hemolytic activity with human blood and about one third of the strains with sheep blood. Moreover, every second strains produced at least one of the investigated toxins with a preference of SED and SEH. The results of this chapter show that the use of CNS in food production demands a safety evaluation.

This chapter has been published in International Journal of Food Microbiology:
Zell[§], C., Resch[§], M., Rosenstein, R., Albrecht, T., Hertel, C., Götz, F., 2008. Characterization of toxin production of coagulase-negative staphylococci isolated from food and starter cultures. International Journal of Food Microbiology 127, 246-251.
[§] contributed equally to the work

**Chapter V** describes the phenotypically investigation of 32 strains of species *S. carnosus*, *S. condimenti*, *S. piscifermentans*, *S. equorum*, *S. succinus* and *S. xylosus* isolated from fermented food, starter cultures and patients in clinics in respect of the formation of binding proteins to extracellular matrix (ECM) proteins and biogenic amines (BA) by amino acid decarboxylases. The binding capacity to the ECM fibronectin and fibrinogen were investigated detecting defined grown, fluorescent labeled cells added to microtiter plates coated with ECM. HPLC (high performance liquid chromatography) was used to detect the formation of six BA of growing and resting cells CNS cells. The results showed only low binding capacities to ECM except of some strains of *S. equorum* and *S. succinus*. Formation of BA like tyramine, 2-phenylethylamine and tryptamine has been shown to occur mainly in strains of species *S. carnosus, S. condimenti* and *S. piscifermentans*. Compared to resting cells, higher amounts especially of 2-phenylethylamine and putrescine were observed for growing cells. This chapter gives an insight in the ECM binding and the formation of BA in respect of safety assessment of CNS used in the production of fermented foods.

This chapter has been published in International Journal of Food Microbiology:
Seitter (née Resch), M., Geng, B., Hertel, C., 2011. Binding to extracellular matrix proteins and formation of biogenic amines by food-associated coagulase-negative staphylococci. International Journal of Food Microbiology 145, 483-487.

**Chapter VI** describes the design of a DNA microarray for detection of safety and technological relevant properties in food associated CNS of species *S. carnosus*, *S. condimenti*, *S. piscifermentans*, *S. equorum*, *S. succinus* and *S. xylosus*. The microarray containing probes for genes encoding antibiotic resistances, toxins, decarboxylases (e.g. biogenic amine formation), nucleases, hemolysis, hydrolyses as well as binding proteins to ECM, lipases, proteases, stress response factors, or nitrate dissimilation. The results obtained by DNA-microarray study of 32 CNS were compared to them of phenotypically assessment (Chapter III, IV and V). The phenotypic formation of antibiotic resistances, biogenic amines and binding to ECM could be partially verified through detection of the responsible genes by DNA microarray hybridization. However, genetic background of phenotypically detected hemolytic activity and toxin formation could not be detected by microarray hybridization and is still unknown. Genes involved in catalase, superoxide dismutase and nitrate dissimilation, were well detected. The prevalence of genes involved in dissimilatory nitrate reduction in strains of *S. carnosus*, *S. condimenti* and *S. piscifermentans* compared to strains of *S. equorum*, *S. succinus* and *S. xylosus* was shown. The results of this chapter show the use of DNA microarray to detect safety and technological relevant properties in food associated CNS.

This chapter has been published in International Journal of Food Microbiology:
Seitter (née Resch), M., Nerz, C., Rosenstein, R., Götz, F., Hertel, C., 2011. DNA microarray based detection of genes involved in safety and technologically relevant properties of food associated coagulase-negative staphylococci. International Journal of Food Microbiology 145, 449-458.

## Co-authors

This thesis comprises studies that were carried out in collaboration with several researchers. The studies described in Chapter III and V concerning the detection of antibiotic resistances as well as the formation of binding proteins to extracellular matrix proteins and biogenic amines in food associated CNS were supervised by PD Dr. Christian Hertel. However, characterization of toxin formation in Chapter IV has been supervised by PD Dr. Christian Hertel (hemolytic activity of human and sheep blood agar plates) and Prof. Dr. Friedrich Götz (formation of staphylococcal enterotoxins, toxic shock syndrome toxin 1, and exfoliative toxin A). In addition, the work presented in Chapter VI was supervised by PD Dr. Christian Hertel and Prof. Dr. Friedrich Götz together.

**Chapter III:** Verena Nagel and Annika Schmidt assisted the investigation of antibiotic resistance genes by agar disc diffusion testing. Markus Kranz handled the sequencer during PCR amplification. Labor Enders & Partner provided advice and support in the performance of disk diffusion testing and Charles M.A.P. Franz was critically reading the manuscript.

**Chapter IV:** Marion Seitter carried out the investigations of hemolytic activity of human and sheep blood agar plates supervised by PD Dr. Christian Hertel. Christiane Zell, Till Albrecht and Ralf Rosenstein contributed to the formation of staphylococcal enterotoxins, toxic shock syndrome toxin 1, and exfoliative toxin A supervised by Prof. Dr. Friedrich Götz.
Marion Seitter and Christiane Zell contributed equally to this study.

**Chapter V:** Bettina Geng developed the strategy for investigation of binding proteins to ECM by microtiter plate assays with immobilized ECM proteins and crystal violet staining. Claudia Lis introduced in the method for the investigation of binding protein formation to ECM. Annika Schmidt assisted in both, the investigation of binding proteins to ECM and the formation of biogenic amines. Charles Franz was critically reading the manuscript.

**Chapter VI:** Dr. Ralf Rosenstein and Dr. Christiane Nerz assisted in the selection of probes spotted on microarray. Dr. Christiane Nerz contributed in the design of PCR primers for generation of microarray probes and the analysis of DNA microarray data. Eric Hüfner and Jens Pfannstiel provided helpful advice in performance of DNA microarray hybridization experiments and array scanning. Dr. Christiane Nerz, Prof. Dr. Friedrich Götz and PD Dr.

Christian Hertel contributed to the interpretation and discussion of the results. D. Edwards providing the R functions for the normalization and background correction of microarray data. Charles Franz was critically reading the manuscript.

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# *Chapter II*

# Introduction

## Food fermentations and staphylococci

Fermentation is a traditional technique for preservation and improvement of plant and animal raw materials (Bernardeau *et al.*, 2006; Buckenhueskes and Holzapfel, 2015; Lücke and Holzapfel, 2015). About 25% of the food products are produced by fermentation resulting in desirable effects like stability, hygienic safety thru (natural) preservation achieved by different hurdles in the ripening processes ($a_w$ and pH decrease), high sensory quality and nutritive value (Bourdichon *et al.*, 2012; Jofré *et al.*, 2009; Straub *et al.*, 1995). These positive effects are achieved by the formation of metabolites exhibiting antagonistic properties as well as reduction of natural available substances which might impair human health e.g. toxins, indigestive galactosides and protease inhibitors. The safety and beneficial property on health of fermented products is principally proven by long term experience. During a traditionally spontaneous fermentation a competitive microbial population containing various microorganisms is selected from the natural microflora controlling the fermentation process (Hammes, 1991; Nout, 2014). Today, the fermentation flora of different food products is widely established. In particular lactic acid bacteria of genera *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Pediococcus*, *Weissella* as well as staphylococci, micrococci, brevibacteria, *Arthrobacter*, yeast and moulds are of importance (Bernardeau *et al.*, 2006; Bourdichon *et al.*, 2012; Hammes, 2012; Place *et al.*, 2003). Starter cultures has been developed to standardize fermentation and ripening processes in industrial production of fermented food in order to maintain constant sensory quality and product safety and high value on human health. Typically starter organisms are added in high numbers ($10^6$–$10^7$ cfu/g) during production of

fermented food. Likewise CNS belonging to the microbiota of spontaneously fermented foods were shown to occur in numbers of $10^6$ to $10^7$ cfu/g (Blaiotta *et al.*, 2004b; Latorre-Moratalla *et al.*, 2010; Mauriello *et al.*, 2004; Oliveira *et al.*, 2010; Rantsiou *et al.*, 2005; Talon and Leroy, 2014; Talon *et al.*, 2008). Therefore food poisoning and food spoilage microorganisms are suppressed resulting in a more reliable and safe fermentation process (Rosenstein *et al.*, 2009).

The genus *Staphylococcus* (*S.*) contains currently 47 validly described species (DSMZ, 2015) which are frequently isolated from skin, skin glands and mucosa of humans and animals (Götz *et al.*, 2004). Further they have been sporadically isolated from other sources like soil, water, sand, air, plant surfaces, meat and poultry, milk and dairy products (Götz *et al.*, 2006; Irlinger, 2008; Kloos *et al.*, 1992; Kloos and Schleifer, 1986; Place *et al.*, 2002). Based on the phylogenetic marker 16S and/or 23S rRNA as well as DNA-DNA hybridization studies, the genus *Staphylococcus* could be divided into different groups (Götz *et al.*, 2006; Kloos *et al.*, 1992).

The most important are the following groups:

1. *S. epidermidis*-group (e.g. *S. capitis, S. caprae, S. epidermidis, S. haemolyticus, S. hominis, S. saccharolyticus* and *S. warneri*)
2. *S. simulans*-group (e.g. *S. carnosus* and *S. simulans*)
3. *S. saprophyticus*-group (e.g., *S. saprophyticus, S. cohnii, S. arlettae, S. gallinarum, S. kloosii, S. xylosus* and *S. equorum*)
4. *S. sciuri*-group (e.g., *S. lentus, S. sciuri* and *S. vitulus*)
5. *S. intermedius*-group (e.g., *S. delphini* and *S. intermedius*)
6. *S. aureus*-group (e.g., *S. aureus* and *S. aureus* subsp. *anaerobius*)

Generally, the *S. epidermidis*- and *S. simulans*-group containing coagulase-negative and novobiocin-susceptible species, the *S. saprophyticus*- and *S. sciuri*-group coagulase-negative and novobiocin-resistant species and the *S. intermedius*- and *S. aureus*-group coagulase-positive and novobiocin-susceptible species, respectively.

In the present thesis the species *S. carnosus, S. condimenti* and *S. piscifermentans*, belonging to the *S. simulans*-group, are designated as *S. carnosus*-group. Strains of species *S. equorum, S. succinus* and *S. xylosus*, closely related to the opportunistic pathogenic *S. saprophyticus*, belonging to *S. saprophyticus*-group and are designated as *S. xylosus*-group.

Traditionally, staphylococci are grouped into coagulase positive (CPS) and coagulase-negative staphylococci (CNS) by testing their ability to produce extracellular coagulase (Somerville and Proctor, 2009; Stutz *et al.*, 2011). The test was established based on the phenotypic properties of *S. aureus* which is able to produce hemolysin, protease, lipase and enterotoxin as well as extracellular coagulase. Coagulase is a *S. aureus* protein involved in blood coagulation and its secretion is tested by enzymatic conversion of fibrinogen in rabbit plasma to fibrin (Rivera *et al.*, 2007). The ability of *S. aureus* secreting coagulase offfers the possibility to identify *S. aureus* from other CNS species and therefore the basis of traditional differentiating method of staphylococci (Götz *et al.*, 2006).

*S. aureus* is often involved in nosocomial infections and plays an important role in food-borne diseases (Chiang *et al.*, 2012; de Moura *et al.*, 2012; Giammarinaro *et al.*, 2005; Le Loir *et al.*, 2003; Palavecino, 2004). The CPS *S. aureus* and *S. schleiferi* (Calvo *et al.*, 2000) as well as the coagulase variable *S. hyicus* (Aarestrup and Jensen, 2002; Casanova *et al.*, 2011) are part of pathogenic organisms which may cause food intoxications and/or serious infections in humans (Götz *et al.*, 2006; Le Loir *et al.*, 2003). But also the group of CNS are reported to be frequently involved in nosocomial infections and containing opportunistic pathogenic species like *S. saprophyticus*, *S. epidermidis* and *S. haemolyticus*, which are also of importance for human health (Chiang *et al.*, 2012; Götz *et al.*, 2006; Piette and Verschraegen, 2009; Witte, 1999). For example, *S. saprophyticus* is the most frequent causative organism of urgent urinary tract infections (Ferreira *et al.*, 2012; Kooken *et al.*, 2014; Martineau *et al.*, 2000). *S. epidermidis* plays a role in nosocomial bacteremia and other infections like prosthetic and natural valvular endocarditis (Duah, 2010; Giammarinaro *et al.*, 2005; Martín *et al.*, 2006; von Eiff *et al.*, 2002). Moreover, it is involved in chronic polymer-associated infection due to the ability of forming biofilms on catheters and implantation materials (Chen *et al.*, 2013; von Eiff *et al.*, 2002).

However, CNS with pathogenic potential has also been regularly isolated from spontaneous fermentation processes. For example, *S. epidermidis* has been isolated from raw cured ham (Landeta *et al.*, 2013; Marín *et al.*, 1992; Podkowik *et al.*, 2013; Rodríguez *et al.*, 1996) and fermented raw sausages (Aymerich *et al.*, 2003; Cachaldora *et al.*, 2013; Mauriello *et al.*, 2004) as well as *S. saprophyticus* from raw cured ham (Rodríguez *et al.*, 1996; Tu *et al.*, 2010), sausages (Cachaldora *et al.*, 2013; Fontán *et al.*, 2007; Samelis *et al.*, 1998) and cheese (Aydemir *et al.*, 2015; Ruaro *et al.*, 2013; Vernozy Rozand *et al.*, 1996). Possible reservoirs

are raw materials but also food handling staff where enterotoxin producing CNS has been detected (Rall *et al.*, 2010; Udo *et al.*, 1999).

On the other hand some CNS species are important in food fermentations because their technological importance in food processing (Blaiotta *et al.*, 2004b; Bonomo *et al.*, 2009; Even *et al.*, 2010; Irlinger, 2008; Jeong *et al.*, 2014). CNS are frequently isolated from fermented meat products (Hartmann *et al.*, 1995; Landeta *et al.*, 2013; Martín *et al.*, 2006; Marty *et al.*, 2012; Rossi *et al.*, 2001; Talon and Leroy, 2014). *S. xylosus* has been often described as dominating species in sausages and fermented meat products (Blaiotta *et al.*, 2004b; Cocolin *et al.*, 2001; Coton *et al.*, 2010; García-Varona *et al.*, 2000; Greppi *et al.*, 2015; Iacumin *et al.*, 2012; Marty *et al.*, 2012; Rossi *et al.*, 2001; Simonová *et al.*, 2006). *S. carnosus* and partly *S. saprophyticus* have been isolated as domination microflora of traditional Greek sausages, salami and other meat products (Blaiotta *et al.*, 2004b; Coppola *et al.*, 2000; Martín *et al.*, 2006; Marty *et al.*, 2012; Papamanoli *et al.*, 2002; Samelis *et al.*, 1998; Simonová *et al.*, 2006). Moreover, also other CNS species like *S. simulans*, *S. epidermidis*, *S. haemolyticus*, *S. warneri*, *S. equorum*, *S. cohnii*, *S. capitis* and *S. intermedius* have been isolated from fermented meat products and sausages (Aymerich *et al.*, 2003; Blaiotta *et al.*, 2004b; Cachaldora *et al.*, 2013; Chajęcka-Wierzchowska *et al.*, 2015; García-Varona *et al.*, 2000; Iacumin *et al.*, 2012; Lorenzo *et al.*, 2012; Marty *et al.*, 2012; Rossi *et al.*, 2001; Vilar *et al.*, 2000). In addition to fermented meat, *S. carnosus* was found together with species *S. piscifermentans* in the fermentation flora of soy sauce and fermented fish in Asia (Beddows, 1985; Hammes *et al.*, 1995; Hartmann *et al.*, 1995; Tanasupawat *et al.*, 1991; Tanasupawat *et al.*, 1992). Additionally, *S. piscifermentans* has been described isolated from tuna candy products (Hwang *et al.*, 2010).

The typical microflora of surface ripened cheeses like “red smear” cheeses including *inter alia* strains of *Brevibacterium linens*, *Debaryomyces hansenii* and *Geotrichum candidum*, *Corynebacterium* sp., *Arthrobacter* sp., *Staphylococcus* sp. (Bockelmann, 2011; Larpin-Laborde *et al.*, 2011; Mounier *et al.*, 2007; Place *et al.*, 2003)**.** In respect of staphylococci, cell counts of the species *S. equorum*, *S. xylosus*, *S. vitulus* and *S. succinus* subsp. *casei* has been described as 0.1 to 5% of the smear flora (Bockelmann, 2002; Brennan *et al.*, 2002; Cogan, 2011; Cogan, 2014; Hoppe-Seyler *et al.*, 2000; Irlinger *et al.*, 1997; Place *et al.*, 2003; Ruaro *et al.*, 2013). Thereof *S. equorum* is the predominant species on the surface of semi-soft smear cheeses and naturally occurring in cheese brines (Bockelmann, 2002; Hoppe-Seyler *et al.*,

2004; Schornsteiner *et al.*, 2014). Up to now this species has not been applied in commercial starter cultures. Although the species is present in aged cheese brines and due to the beneficial effects, *S. equorum* has been suggested for use in starter cultures in future (Hoppe-Seyler *et al.*, 2004; Jaeger *et al.*, 2002; Place *et al.*, 2003; Plé *et al.*, 2015). Moreover, food associated CNS have been described isolated from goat's milk associated with or without mastitis (De Buyser *et al.*, 1987; El-Jakee *et al.*, 2013; Harvey and Gilmour, 1988; Vernozy Rozand *et al.*, 1996), especially the species *S. equorum* and *S. xylosus* from goat's milk and cheese (Bockelmann and Hoppe-Seyler, 2001; Meugnier *et al.*, 1996; Ruaro *et al.*, 2013; Vernozy Rozand *et al.*, 1996)**.**

Regarding food associated CNS of *S. carnosus*- and *S. xylosus*-group, in fermented meat products the presence of *S. carnosus* has been shown (Götz *et al.*, 2006; Janssens *et al.*, 2012; Wagner *et al.*, 1998). This species has originally been isolated from fermented sausages. It was reclassified and described based on genotypical and physiological differences like DNA sequence homology, chemical composition of peptidoglycan, biochemical properties and its occurrence in meat products (car.nó sus. L. adj. *carnosus* pertaining to flesh), which gave the species the name (Schleifer and Fischer, 1982; Wagner *et al.*, 1998). Compared with other staphylococci, the species *S. carnosus* has only low DNA sequence homology with *S. aureus*. Usually, this species produces no pathogenicity factors (e.g. toxins, hemolysins, protein A, coagulase, clumping factors) which are typical for *S. aureus* strains (Becker *et al.*, 2007b; Rosenstein and Götz, 2010; Wagner *et al.*, 1998). Based on physiological and genotypical differences, *S. carnosus* can be divided into the subspecies *carnosus* and *utilis* (ut.ti'lis L. adj. *utilis* useful). For example, characteristic properties like hemolysis of blood or the formation of biogenic amines have been shown in *S. carnosus* subsp. *carnosus* while they could not be observed in strains of *S. carnosus* subsp. *utilis* (Hammes *et al.*, 1995; Probst *et al.*, 1998). Both subspecies of *S. carnosus* occur in commercial starter cultures (Probst *et al.*, 1998). This is not astonishing through to the fact, that *S. carnosus* has been used since 1950 as component of starter cultures for the production of fermented raw sausage (Rosenstein *et al.*, 2009; Wagner *et al.*, 1998). The species *S. condimenti* has firstly been described based on DNA-DNA similarity studies and physiological data in the year 1998 originating from *S. carnosus* isolated from soy sauce mash (Probst *et al.*, 1998). Derived from this origin, also the species *S. condimenti* (con.di.men'ti. L. n. *condimentum* spice; L. gen. n. *condimenti* of the spice) has been reported (Probst *et al.*, 1998). Besides the species *S. carnosus* (Tanasupawat *et al.*, 1991; Zaman *et al.*, 2014), the flora of fermented fish and soy sauce mash contains the species

*S. piscifermentans* (pis.ci.fer.men'tans. L. n. *piscis,* fish; M.L. part.adj. *fermentans,* fermenting; M.L. adj. *piscifermentans,* fish fermenting) (Tanasupawat *et al.*, 1992). DNA homology studies showing that within different strains of *S. piscifermentans*, a high degree of DNA relatedness (>79%) occurs compared to a low degree of relatedness (<43%) observed to strains belonging to other *Staphylococcus* species (Tanasupawat *et al.*, 1992).

Within species *S. equorum*, the subspecies *linens* (lin'ens. L. part. pres. of *linere*: smearing; named because the organism was isolated from the surface of a red smeared cheese) is of importance in food fermentations (Jeong *et al.*, 2013; Place *et al.*, 2003). *S. equorum* subsp. *linens* has been isolated from the microflora of a surface ripened Swiss mountain cheese made from raw milk and differentiated on the basis of DNA-DNA hybridization and phenotypic characteristics. By comparative sequence analysis the close relatedness of *S. equorum* subsp. *linens* to the species *S. succinus*, *S. xylosus*, *S. saprophyticus*, *S. gallinarum*, and *S. arlettae* has been shown (Place *et al.*, 2003). Further, the isolation of *S. equorum* from fermented sausages has been described (Fonseca *et al.*, 2013; Leroy *et al.*, 2010; Rebecchi *et al.*, 2015). *S. equorum* subsp. *linens* has been applied as starter culture for the production of surface ripened cheeses in combination with *Debaryomyces hansenii*, *Geotrichum candidum*, *Brevibacterium linens*, *Corynebacterium casei* showing a high performance in sensory and hygienic aspects (Bockelmann, 2002; Cogan, 2011; Gori *et al.*, 2013; Place *et al.*, 2003). Additionally to *S. equorum* subsp. *linens*, *S. succinus* subsp. *casei* (ca´se.i. L. gen. n. casei of cheese, named because the organism was isolated from cheese) isolated from a Swiss surface ripened cheese has been described on the basis of DNA-DNA hybridization studies, cell wall composition and phenotypic characteristics (Burri, 1999; Place *et al.*, 2002; Rea *et al.*, 2007). The species *S. xylosus* (xy.lo'sus. M.L. adj. *xylosus* xylose.) was isolated from human skin and classified based on cell wall composition, lactic acid configuration, morphological and physiological characters like the ability to ferment xylose (Schleifer and Kloos, 1975). The presence in animals and therefore in fresh and fermented meat products like sausages has been described (Cocolin *et al.*, 2001; de la Rosa *et al.*, 1990; García-Varona *et al.*, 2000; Greppi *et al.*, 2015; Iacumin *et al.*, 2012; Simonová *et al.*, 2006). Moreover, starter cultures contain *S. xylosus* (Gardini *et al.*, 2002; Labrie *et al.*, 2014; Lauková *et al.*, 2010; Stahnke, 1995).

Starter cultures have been applied for a long time in the production of fermented products like raw sausages (Hammes *et al.*, 1995; Hammes and Hertel, 1998; Lücke and Holzapfel, 2015;

Ordóñez *et al.*, 1999). In general, starter cultures are a combination of pure or mixed strains belonging to different lactic acid bacteria, *Micrococcaceae*, yeasts and/or mould species (Bassi *et al.*, 2015; Hammes and Knauf, 1994; Samelis *et al.*, 1998; Vogel *et al.*, 2011). The metabolic activity of starter culture, consisting preparations of living microorganisms or their resting forms, has desired effects in the fermentated food. They are used in order to obtain specific biochemical activities as well as reduction of hygienic risks (Samelis *et al.*, 1998; Vogel *et al.*, 2011). To achieve dedicated organoleptic characteristics, the addition of starter cultures with special regard to strain selection is of major importance (Ciuciu Simion *et al.*, 2014; Di Maria *et al.*, 2002; Garriga *et al.*, 2005).

Traditionally CNS strains of the species *S. carnosus* and *S. xylosus* are used alone or in combination with other microorganisms to produce fermented sausages or meat products like cured ham (Blaiotta *et al.*, 2004a; Corbiere Morot-Bizot *et al.*, 2007; Even *et al.*, 2010; Hammes and Hertel, 1998; Hammes and Knauf, 1994; Janssens *et al.*, 2012; Rantsiou and Cocolin, 2006; Samelis *et al.*, 1998; Simonová *et al.*, 2006; Talon *et al.*, 2002; Talon and Leroy, 2014; Wagner *et al.*, 1998). CNS are used in starter cultures of meat products due to their relevant properties in formation and stabilization of desired red color through the ability to reduce nitrate to nitrite (Hammes, 2012; Marco *et al.*, 2006; Neubauer and Götz, 1996; Rosenstein *et al.*, 2009). As a result of this the nitrate concentration is lowered and nitrite reacts with myoglobin to nitrosomyoglobin, which results in the typical red color development (Hammes, 2012; Mauriello *et al.*, 2004; Neubauer and Götz, 1996; Rosenstein *et al.*, 2009; Talon *et al.*, 2007; Talon *et al.*, 1999). The presence of nitrite additionally results in hygienic safety aspects by the control of food pathogenic and spoilage organisms (Hammes, 2012; Hammes *et al.*, 1995; Rossi *et al.*, 2001)**.** Other positive effects of starter cultures are the reduction of hydrogen peroxide produced by catalase-negative lactobacilli, through superoxide dismutase and catalase activity and therefore, the prevention of rancidity by lipid oxidation (Barrière *et al.*, 2001; Blaiotta *et al.*, 2004b; Essid and Hassouna, 2013; Mauriello *et al.*, 2004; Montel *et al.*, 1996; Rosenstein *et al.*, 2009; Rossi *et al.*, 2001). Further, they leading to a moderate lowering of the pH due to their ability of partial neutralize organic acids produced from lactic acid bacteria (Hammes and Knauf, 1994; Liepe, 1982; Rantsiou *et al.*, 2005; Tabanelli *et al.*, 2012; Wagner *et al.*, 1998). Another positive aspect, together with endogenous meat enzymes, is the contribution in the development of characteristic flavor and aroma or the formation of precursors of flavor compounds in fermented meat products (Hierro *et al.*, 1997; Martín *et al.*, 2007; Rossi *et al.*, 2001). Flavor development of fermented meat

and also cheese could be traced back to the formation of different non-volatile and volatile compounds like esters (Casaburi *et al.*, 2006; Hammes and Hertel, 1998; Irlinger *et al.*, 1997; Janssens *et al.*, 2012; Montel *et al.*, 1998; Montel *et al.*, 1996; Søndergaard and Stahnke, 2002). Additionally, the formation of peptides and amino acids by the proteolytic activity of CNS (Casaburi *et al.*, 2006; Jeong *et al.*, 2014; Martín *et al.*, 2006; Mauriello *et al.*, 2004; Montel *et al.*, 1996; Rossi *et al.*, 2001) as well as flavor active free fatty acids and aromatic compounds due lipolytic effects has been mentioned (Landeta *et al.*, 2013; Martín *et al.*, 2006; Massa and Turtura, 1989; Mauriello *et al.*, 2004; Montel *et al.*, 1996; Rantsiou *et al.*, 2005; Rossi *et al.*, 2001). There are further positive effects of CNS on the color and flavor of cheese during ripening (Hannon *et al.*, 2004; Hoppe-Seyler *et al.*, 2004; Ruaro *et al.*, 2013).

In addition to the species *S. carnosus* and *S. xylosus*, also other strains of *S. carnosus*- and *S. xylosus*-group exhibit potential for future application in starter or protective cultures. Among these species, *S. piscifermentans* and *S. condimenti* can be mentioned in the context of soy and fish sauces (Probst *et al.*, 1998; Tanasupawat *et al.*, 1992; Wei *et al.*, 2013), *S. equorum* subsp. *linens* and *S. succinus* subsp. *casei* with smear and/or surface ripened cheese (Place *et al.*, 2002; Place *et al.*, 2003) and *S. equorum* subsp. *equorum* with cured ham (Schlafmann *et al.*, 2002).

## Undesired properties and possible pathogenicity factors of staphylococci

In general, virulence factors and other undesired properties like antibiotic resistances are not as well established in CNS like in *S. aureus* (Huebner and Goldmann, 1999; Rosenstein and Götz, 2010; Tan *et al.*, 2014). Already at the end of the last century it has been described that CNS used in food fermentation should be considered with attention because the genus *Staphylococcus* contains species of established or potential pathogenicity (Hammes *et al.*, 1995; Jeljaszewicz, 1984). Although food associated CNS species are off less clinical importance than e.g. species *S. saprophyticus* (Even *et al.*, 2010; Huebner and Goldmann, 1999; Jarvis and Martone, 1992; Spencer, 1996) they also have been isolated from nosocomial infections (Couto *et al.*, 2001; Even *et al.*, 2010; Koksal *et al.*, 2009; Misawa *et al.*, 2015; Novakova *et al.*, 2006).

First of all deeper insight of pathogenic staphylococci has to be taken before looking after the safety relevant aspects of food associated staphylococci of *S. carnosus*- and *S. xylosus*-group. Compared to food associated CNS, pathogenic staphylococci are frequently responsible for community acquired and nosocomial infections. One of the most important problems is the constant increase of antibiotic resistances, especially multiple antibiotic resistances has become a serious problem (French, 2010; Palka-Santini *et al.*, 2007; Pasberg-Gauhl, 2014; Volokhov *et al.*, 2003). In particular, mainly enterococci and staphylococci constitute the highest treatment rates of gram-positve bacteria involved in infections (Arias *et al.*, 2003; Falcone *et al.*, 2015; Kresken *et al.*, 1999).

### Antibiotics and antibiotic resistances

Antibiotics are small molecules either naturally produced by bacteria and fungi or semi-synthetic and synthetic compounds with antimicrobial activity. They are used to treat and prevent disease in human and veterinary medicine as well as growth promoter in food animals (Allen, 2014; Berger-Bächi and McCallum, 2006; Phillips *et al.*, 2004; Witte, 1998a).

Based on the structure of the antibiotic or the mechanism they affect, antibiotics are classified in different groups (Neu, 1992; Walsh, 2003), e.g. the inhibition of 1) bacterial cell wall biosynthesis, 2) protein synthesis, and 3) DNA replication and 4) RNA synthesis.

***1) Inhibition of cell wall biosynthesis***

- **β-lactam antibiotics** (including penicillins, carbapenems and cephalosporins) containing e.g. methicillin, oxacillin, penicillin G, ampicillin and cefoxitin inhibiting the peptide binding and therefore the cross-linking of peptidoglycan units catalyzed by penicillin binding proteins (transpeptidases called PBP) (Bush and Jacoby, 2010; Hölltje, 1998; Kohanski *et al.*, 2010; Tipper and Strominger, 1965; Wise and Park, 1965).

- **Glycopeptide antibiotics** like vancomycin and teicoplanin inhibiting the elongation of the sugar backbone and crosslinking of the peptidoglycan by targeting the terminal D-Ala-D-Ala of the staphylococcal peptidoglycan stem peptide as well as blocking transglycosylase and transpeptidase activity (Berger-Bächi and McCallum, 2006; Kahne *et al.*, 2005; Kohanski *et al.*, 2010). Vancomycin is one of the last antibiotic which can be used against multiresistant MRSA (methicillin-resistant *Staphylococcus aureus*) if all other antibiotics fail and thus only used in practice to treat hospital-acquired infections by MRSA (Berger-Bächi and McCallum, 2006; Coimbra *et al.*, 2011; Simon and Stille, 1997).

***2) Inhibition of protein synthesis***

Antibiotics that inhibit the synthesis of protein can be differentiated into inhibitors of 50S and 30S subunit of the ribosome (Kohanski *et al.*, 2010).

- **Macrolide** (erythromycin), **lincosamide** (clindamycin and lincomycin), **strepto-gramin** (dalfopristin/quinupristin), **amphenicol** (chloramphenicol) and **oxazolidinone** (linezolid) antibiotics are of different classes with different chemical structures but the same mechanism of action. They inhibit the protein synthesis affecting 50S ribosome subunit (Katz and Ashley, 2005; Kohanski *et al.*, 2010; Mukhtar and Wright, 2005). Generally, the inhibition of 50S ribosome subunit may result in

  1) inhibiting formation of the initiation complex (binding of the N-formyl-methionyl-tRNA to the ribosome) and thus protein synthesis e.g. by oxazolidinones (Berger-Bächi and McCallum, 2006; Patel *et al.*, 2001; Patra and Shah, 2012).

  2) translocation of peptidyl-tRNAs and thus inhibiting the elongation of peptidyltransferase involved in formation of the peptide chain e.g. by macrolide, lincosamide and streptogramin B (MLS) antibiotics (Achard *et al.*, 2005; Berger-Bächi

and McCallum, 2006; Kohanski *et al.*, 2010; Vannuffel and Cocito, 1996). Further, resistance to lincosamides by dimethylation of adenine residue of the 23S rRNA molecule resulting in cross-resistance to other MLS antibiotics (Achard *et al.*, 2005; Leclercq, 2002; Wang *et al.*, 2008; Weisblum, 1995).

- **Fusidic acid** inhibiting the release of elongation factor G from the ribosome by its binding and thus stalling protein synthesis (Castanheira *et al.*, 2010; Farrell *et al.*, 2011; Kinoshita *et al.*, 1968).

- **Tetracyclines** and **aminoglycoside antibiotics** like kanamycin, gentamicin, neomycin and streptomycin inhibiting the protein synthesis affecting 30S ribosome subunit (Kohanski *et al.*, 2010). Tetracyclines inhibiting the bacterial protein synthesis by binding and thus blocking the access of aminoacyl-tRNAs to the ribosome (Chopra and Roberts, 2001; Kohanski *et al.*, 2010). Aminoglycoside inhibiting the protein synthesis by the binding of the 16S rRNA component of the 30S ribosome subunit. (Davis, 1987; Hancock, 1981; Kohanski *et al.*, 2010) and promotes protein mistranslation by incorporation of inappropriate amino acids into elongating peptide strands (Davies *et al.*, 1965; Kohanski *et al.*, 2010).

***3) Inhibition of DNA replication***

- ***Quinolones including fluoroquinolones*** like ofloxacin and ciprofloxacin targeting the enzymes DNA-gyrase (topoisomerase II) and DNA-topoisomerase IV at the DNA cleavage stage and prevent supercoiling (Chen *et al.*, 1996; Couzinet *et al.*, 2005b; Drlica *et al.*, 2008; Drlica and Zhao, 1997; Gubaev and Klostermeier, 2014; Kohanski *et al.*, 2010; Yamagishi *et al.*, 1996).

- **Coumarins** like novobiocin binding to the B subunit of DNA gyrase and inhibit supercoiling of the DNA by blocking the ATPase activity (Burlison *et al.*, 2006; Gilbert and Maxwell, 1994; Maxwell, 1993; Stieger *et al.*, 1996; Sugino *et al.*, 1978).

- **Sulphonamide** like sulphamethoxazole and **trimethoprim** showing bacteriostatic effects on bacterial metabolism by competitively inhibition of enzymes involved in two steps of folic acid biosynthesis. The sulphonamide component is competing with p-aminobenzoic acid, important for the synthesis of tetrahydrofolic acid. Trimethoprim competitively binds to dihydrofolate reductase and thus inhibiting the

conversion of dihydrofolic acid to tetrahydrofolic acid, a metabolically active cofactor for the synthesis of thymine. The stopped synthesis of DNA precursors like thymine and the interference with folic acid metabolism results in DNA synthesis inhibition (Drews, 1980; Gaylarde and Sarkany, 1972; Jerry and Smilack, 1999; Vouloumanou *et al.*, 2011).

***4) Inhibition of* RNA synthesis**

- ***Rifamycin antibiotics*** like rifampicin binding to the β-subunit of DNA-dependent RNA polymerase and thus inhibit the transcription (Campbell *et al.*, 2001; Hartmann *et al.*, 1967; Kohanski *et al.*, 2010; Naryshkina *et al.*, 2001; Tupin *et al.*, 2010).

A problem in respect of antibiotic resistances is that they already occur in natural environment to protect the antibiotic producers themselves from their own products (Phillips *et al.*, 2004; Teuber, 2000). Three different resistance mechanisms based on the following strategies has been described (Berger-Bächi and McCallum, 2006; Oliphant and Eroschenko, 2015).

- Inactivation of the antibiotic due to chemical/enzymatic modification
  e.g. the enzyme β-lactamase is breaking the ring structure of β-lactam-antibiotics like penicillin resulting in ineffectiveness of the antibiotic (Berger-Bächi and McCallum, 2006; Oliphant and Eroschenko, 2015).
- Prevention of the antibiotic from reaching its target.
  e.g. by closing the cell wall for antibiotics and/or due to efflux mechanisms like the efflux pump of tetracycline discharging the antibiotic (Berger-Bächi and McCallum, 2006; Noguchi *et al.*, 1986) or efflux mechanism encoded by *msrA* resulting in resistance to macrolides and type B streptogramins (Fiebelkorn *et al.*, 2003; Ross *et al.*, 1990; Ross *et al.*, 1989). Further, efflux pump modifications and mutations of gene targets has been mentioned involved in fluoroquinolone resistance (Hooper, 2000; Hooper, 2002). In this context also intrinstic resistances by the absence of the target area can be mentioned.
- Alteration of the target area of the antibiotic resulting in insensitivity to the drug.
  e.g. alteration of the target site by mutations like ribosomal target modification by 23S rRNA methylation due to *erm* genes leading to reduced binding by MLS agents to the ribosome (Fiebelkorn *et al.*, 2003; Roberts *et al.*, 1999). If multiple antibiotics have the same target and one microorganism is resistant to one antibiotic, it is also resistant

to other antibiotics with the same target. This is called cross-resistance (Berger-Bächi and McCallum, 2006).

Other effects which may be involved in the development of antibiotic resistance are complex intrinsic features such as cell wall structure or metabolic properties (Even *et al.*, 2010; Kastner *et al.*, 2006) as well as point mutations *inter alia* in the case of macrolide, quinolone, rifampicin and fusidic acid resistances (Even *et al.*, 2010; McManus, 1997; Prunier *et al.*, 2005; Tenover, 2006).

In addition to hospitals, the industrial animal breeding is an important reservoir for antibiotic resistances because there is an evolutionary pressure in the presence of antibiotics which promote the development and transfer of resistance genes (Allen, 2014; Amyes and Evans, 2015; Sommer and Dantas, 2011; Witte, 1998b)**.** Moreover it has been reported, that antibiotics used in human medicine are of the same classes as them which are used in animals (Phillips *et al.*, 2004). Thus it is expected, that extensive use of antimicrobial agents in animal husbandry contribute in the development of antibiotic-resistant microorganisms in animals and humans (Aarestrup and Wegener, 1999; Allen, 2014; Barber *et al.*, 2003; Martín *et al.*, 2006; Normanno *et al.*, 2007; Tenover and Hughes, 1996; Tenover and McGowan, 1996).

The resistance situation for all new antibiotics is comparable and the phenomenon often has been described in medicine (Kresken *et al.*, 1999). Use of antibiotic causes an enrichment of resistant organisms, if the genetic information of the resistance to one antibiotic is present (Allen, 2014; Teuber, 2000; Wendlandt *et al.*, 2015). Thus, antibiotic resistant organisms could also be present in food matrix (Gardini *et al.*, 2003; Jeong *et al.*, 2014; Kastner *et al.*, 2006; Mauriello *et al.*, 2000; Rebecchi *et al.*, 2015; Teuber *et al.*, 1996).

Several ways how resistant organisms reach the food matrix have been described.

- Resistant microorganisms already exist in raw material like raw milk products or raw meat (Talon *et al.*, 2007; Teuber *et al.*, 1999). Moreover, it has been shown, that the microorganisms derived from animals reflecting the antibiotics used in the treatment by their resistance spectrum (Teuber, 2000).
- Compared with other causative organisms like *E. coli* or *Salmonella*, staphylococci are able to survive the fermentation of food produced of raw basic materials if they are not

be subjected to any further heat treatment (Montanari *et al.*, 2015; Teuber *et al.*, 1996). Because of this, staphylococci have often been isolated from raw sausages and raw milk cheese (Iacumin *et al.*, 2012; Mauriello *et al.*, 2000; Schornsteiner *et al.*, 2014; Talon and Leroy, 2014; Teuber, 2000).

- Resistant microorganisms are able to enter the food matrix via faecal contaminations during slaughtering or milking, poor industrial hygiene, or contaminated drinking water during food production processes (Phillips *et al.*, 2004; Rebecchi *et al.*, 2015; Teuber *et al.*, 1999).
- Another possibility to enter the food matrix is through the use of starter or probiotic cultures in food fermentation (Bernardeau *et al.*, 2006; Verraes *et al.*, 2013). In this context microorganisms are consciously added in high microbial counts of about $10^6$-$10^7$ cfu/g (Bernardeau *et al.*, 2006; Talon and Leroy, 2014).

Based on these reservoirs, resistant strains or resistance genes may be distributed to humans through the food chain (WHO (World Health Organization), 2002; Martín *et al.*, 2006; Phillips *et al.*, 2004; Swartz, 2002; Verraes *et al.*, 2013) or are able to contribute to the resistance situation in medical environment (Teuber, 1999).

For this, it is necessary that resistance genes are located on so called transferable elements like conjugative plasmids (circular DNA strands), transposons or mobile DNA strands (Teuber, 2000; Teuber *et al.*, 1999; Verraes *et al.*, 2013). Plasmids are circular DNS structures which are together with the bacterial chromosome available in the cell. Transposons are mobile DNA elements, which are able to reach other places within the genome, but in contrast to plasmids they are not able to exist independent of the genome (Brown-Jaque *et al.*, 2015; Licht and Wilcks, 2005; Neu, 1992; Shapiro, 1983). In general, most staphylococci contain at least one plasmid (Huebner and Goldmann, 1999; Kloos *et al.*, 1981) which may be transferred via conjugation, the main mechanism of horizontal gene transfer of bacteria in natural environments, from one species to another (Clewell *et al.*, 1995; Forbes and Schaberg, 1983; Frost, 2014; Huebner and Goldmann, 1999; Teuber *et al.*, 1999). This phenomenon has been described as to be involved in the spread of antibiotic resistance determinants, e.g. aminoglycoside and β-lactam resistance (Huebner and Goldmann, 1999). Moreover, it has been shown that the underlying resistance genes as well as the mechanisms of gene transfer are the same in disease-causing organisms and organisms isolated from food (Teuber *et al.*, 1996). In this context the spread of antibiotic resistances via food like milk and meat has often

been shown (Chajęcka-Wierzchowska *et al.*, 2015; Klare *et al.*, 2003; Perreten *et al.*, 1998; Teuber *et al.*, 1999; Werner *et al.*, 1997; Witte, 1999). The possibility of genetic transfer from pathogenic to non pathogenic organisms in the food matrix has been demonstrated for instance by the transfer of multi-resistance plasmid pK214 of *Lactococcus lactis* subsp. *lactis* K214 isolated from raw milk soft cheese. This multi-resistance plasmid is carrying antibiotic resistance genes like chloramphenicol acetyltransferase *cat*, streptomycin adenylase *str*, tetracycline resistance gene *tet*(S) as well *mef214* a putative drug efflux gene (Perreten *et al.*, 1997; Perreten *et al.*, 2001; Teuber *et al.*, 1999). Efflux and membrane proteins are involved in transmembrane export of different substances such as heavy metals, organic solvents, dyes, disinfectants, and antibiotics (Lawrence and Barrett, 1998; Levy, 1992; Nikaido, 1996; Perreten *et al.*, 2001; Sutcliffe, 1999). The plasmid pK214 could be transferred to *Enterococcus faecalis* JH2-2 where the resistance has been phenotypically detectable (Perreten *et al.*, 1997; Perreten *et al.*, 2001; Teuber *et al.*, 1999). Following sequence analyses showed that the existence of genetical information of four different species was present on the plasmid. For instance, streptomycin adenylase gene conferring resistance to streptomycin showed 98.8% homology to adenylase of *S. aureus* as well as the tetracycline resistance gene 99.8% homology to gene *tet*(S) of *Listeria monocytogenes* (Teuber *et al.*, 1999). Regarding this, the possibility of genetic transfer in sausage has been shown (Hertel *et al.*, 1995; Rossi *et al.*, 2014) because staphylococci, *Listeria* as well as enterococci are able to survive the process of food production (Montanari *et al.*, 2015; Perreten *et al.*, 1997). In this way, food associated microorganisms may contribute to the resistance situation of pathogenic microorganisms.

In respect of staphylococci, methicillin-resistant *S. aureus* (MRSA) and methicillin-resistant *S. epidermidis* are of utmost importance and emerged as significant pathogens of communal and nosocomial infections during the last twenty years (Falcone *et al.*, 2015; Ippolito *et al.*, 2010; Kresken *et al.*, 2006; Kresken *et al.*, 1999; Schaberg *et al.*, 1991; Vannuffel *et al.*, 1995; von Eiff *et al.*, 2002; Witte, 1999). But also other antibiotics like lincomycin and erythromycin depending to the group of MLS antibiotics are of clinical importance (Lina *et al.*, 1999; Versporten *et al.*, 2014). Additionally, tetracycline and aminogycoside antibiotics containing streptomycin, gentamycin and kanamycin should be mentioned (Versporten *et al.*, 2014; Weigel *et al.*, 2003). MRSA are prominent hospital pathogens showing for a long period of time sensitivity to the glycopeptide antibiotic vancomycin. Therefore, vancomycin has been used very consciously and was only applied if all other antibiotics are ineffective

(Cunha, 2008; Falcone *et al.*, 2015; Simon and Stille, 1997). In regard to clinical CNS, resistance to methicillin is one of the most frequently observed (Kresken *et al.*, 1999; May *et al.*, 2014).

Increasing resistance of staphylococci to β-lactam antibiotics e.g. methicillin has been described as a major clinical problem (Falcone *et al.*, 2015; Vannuffel *et al.*, 1995). In this context serious morbidity and even death in healthy children and adults were reported (Furuya and Lowy, 2006; Kollef and Micek, 2006; Schnellmann *et al.*, 2006; Thampi *et al.*, 2015). In *S. aureus* and CNS, frequently methicillin resistance is mediated by the overproduction of penicillin-binding protein PBP2a with low affinities for β-lactam antibiotics (Hackbarth and Chambers, 1989; Normanno *et al.*, 2007; Oliphant and Eroschenko, 2015; Vannuffel *et al.*, 1995). PBP2a producing strains are resistant to all β-lactams (Chambers, 1997; Normanno *et al.*, 2007; Oliphant and Eroschenko, 2015). The gene encoding PBP2a is the *mecA* gene showing high levels of homology in MRSA and other methicillin-resistant CNS species like *S. epidermidis* (Monecke *et al.*, 2012; Shore and Coleman, 2013; Vannuffel *et al.*, 1995). Further, the *mecA* gene has been described as a molecular marker of methicillin resistance in staphylococci (Choi *et al.*, 2003; Okolie *et al.*, 2015; Vannuffel *et al.*, 1995) and all classes of β-lactam antibiotics (Schnellmann *et al.*, 2006). Moreover, in the US *S. aureus* strains has been isolated from clinical lethal infections, exhibiting resistances to methicillin (MRSA) as well as resistances to vancomycin (VRSA). In one of the investigated clinical isolate beside methicillin and vancomycin resistance, resistances to trimethoprim, β-lactames (gene *blaZ*), aminoglycosides (gene *aacA-aphD*) and quaternary ammonium compounds has been observed (Weigel *et al.*, 2003). In this context it should be mentioned, that genes coding for pathogenicity factors as well as antibiotic resistances can be transferred from one species to another by horizontal gene transfer (Baba *et al.*, 2002; Marty *et al.*, 2012; Novick *et al.*, 2001; Rossi *et al.*, 2014). By genetic analyses it has been shown that the vancomycin resistance gene was transferred from co-isolate *Enterococcus faecalis* to methicillin resistant *S. aureus* by inter-species transfer via transposon Tn*1546* (Clark *et al.*, 2005; Rosenberg Goldstein *et al.*, 2014). The coexistence of *Enterococcus faecalis* and staphylococci strains is comparatively frequent. By way of example, in 62% of the fecal samples isolated from vancomycin resistant enterococci colonized patients, *S. aureus* has also been isolated. Just as 87% of these *S. aureus* isolates exhibited methicillin resistance (Ray *et al.*, 2003). Moreover, homologous form of gene *mecA* causing resistance to methicillin is present in natural isolates of *S. sciuri* (Couto *et al.*, 1996) and phenotypic resistance to methicillin has been shown in *S. aureus* after transferring this gene (Wu *et al.*, 2001).

As a consequence of this, it is not surprising that resistances to one or more antibiotics are detected in food associated CNS (Coton *et al.*, 2010; de Moura *et al.*, 2012; Even *et al.*, 2010; Mauriello *et al.*, 2000; McKay, 2008; Perreten *et al.*, 1998; Rebecchi *et al.*, 2015; Witte *et al.*, 2008). Moreover the food matrix has been described as a reservoir for the spread of antibiotic resistances (Even *et al.*, 2010; Perreten *et al.*, 1997; Rebecchi *et al.*, 2015). Hence, the presence of transferable resistance genes in microorganisms occurring in food or used as starter cultures are undesired due to the possible high risk of resistance transfer between different bacteria (Talon and Leroy, 2011).

**Toxin formation, pathogenicity factors and undesired properties of staphylococci**

Besides antibiotic resistances other virulence factors should be considered which are originally identified and characterized in *S. aureus* and are involved in enterotoxigenicity and pathogenicity of staphylococci (Gemmell and Lang, 2015; Podkowik *et al.*, 2013). Numerous virulence factors of *S. aureus* are described to be involved in pathogenesis and diseases e.g. toxins, cell wall-associated proteins, and enzymes (Gemmell and Lang, 2015; Gundogan *et al.*, 2013; Hart *et al.*, 1993; Iandolo, 1990; Otto, 2004; Waldvogel, 1990).

***Formation of toxins***

Up to now, *S. aureus* enterotoxicoses are frequently of major concern in foodborne diseases (Argudín *et al.*, 2010; Cavadini *et al.*, 1998; Straub *et al.*, 1999; Zeleny *et al.*, 2015) and outbreaks connected with milk and dairy products (Carfora *et al.*, 2015; Hein *et al.*, 2001; Hummerjohann *et al.*, 2014), poultry, meat and meat products as well as cream-filled bakery products have been reported (U.S. Department of Agriculture, Food Safety and Inspection Service 1996; Cavadini *et al.*, 1998; Stewart *et al.*, 2003; Straub *et al.*, 1999; Zeaki *et al.*, 2014). In this context a major issue is the possibility that *S. aureus* persists for a long time in the food matrix as well as the ability to form heat stable toxins (Hein *et al.*, 2001; Hennekinne *et al.*, 2012).

Of utmost importance are pore forming toxins like α-toxin (Dahlberg *et al.*, 2015; Haslinger *et al.*, 2003; Kaneko and Kamio, 2004; Monecke *et al.*, 2008) as well as other two-component cytolysins like γ-hemolysin, leukocidin and panton-valentine leukocidin (Boussaud *et al.*, 2003; Gillet *et al.*, 2002; Kamio *et al.*, 1993; Kaneko and Kamio, 2004; Liu, 2015; Monecke

*et al.*, 2008; Nguyen *et al.*, 2002; Panton and Valentine, 1932; Supersac *et al.*, 1993). Further, β- and δ-hemolysins, super antigens like toxic-schock-syndrom toxin (TSST-1) (Liu, 2015; McCormick *et al.*, 2003; Monecke *et al.*, 2008; Schlievert *et al.*, 1981) as well as staphylococcal enterotoxins (SEs) frequently involved in food poisoning has to be mentioned (Le Loir *et al.*, 2003; Song *et al.*, 2015; Wang *et al.*, 2004). Exfoliative toxins A and B (ETA and ETB) causing staphylococcal scalded skin syndrome (SSSS) and/or bullous impetigo (Ahrens and Andresen, 2004; Imani Fooladi *et al.*, 2015; Ladhani *et al.*, 1999; Ladhani *et al.*, 2001; Monecke *et al.*, 2008; Yamaguchi *et al.*, 2002).

Hemolysins are exotoxins that cause lysis of erythrocytes either by pore formation (α-hemolysis) or cytolytic action by degrading sphingomyelin (β-hemolysis). They are distinguishable by the hemolysing reaction on blood agar plates (Essmann *et al.*, 2003; Smyth *et al.*, 1975; Stutz *et al.*, 2011). Moreover, α-toxin damages cell membranes and it is supposed, that this toxin therefore contribute to tissue damage in several infections (Berube and Bubeck Wardenburg, 2013; Bramley *et al.*, 1989; Callegan *et al.*, 1994; Dahlberg *et al.*, 2015; Gemmell *et al.*, 1991; O'Callaghan *et al.*, 1997; Patel *et al.*, 1987). The sphingomyelinase activity of the β-toxin has been described e.g. in combination with tissue necrosis during experimental murine mastitis (Bramley *et al.*, 1989; O'Callaghan *et al.*, 1997), inducing mild inflammatory changes in the bovine mammary gland (Calvinho *et al.*, 1993; O'Callaghan *et al.*, 1997) and the *in vitro* lyses of bovine epithelial cells (Cifrian *et al.*, 1996; O'Callaghan *et al.*, 1997; Scali *et al.*, 2015).

Staphylococcal enterotoxins (SEs) are emetic toxins and named based on their emetic activities after oral administration in a primate model (Podkowik *et al.*, 2013). Five major serological SE types discovered in the 60s, SEA to SEE have been described (Bergdoll *et al.*, 1973; Blaiotta *et al.*, 2004a; Dinges *et al.*, 2000; Morandi *et al.*, 2007; Zeleny *et al.*, 2015). Additionally to them new types of SEs as well as other related genes lacking emetic properties in monkey model or showing homology to enterotoxins called *S. aureus* enterotoxin like (SEl) are reported (Lina *et al.*, 2004; Park *et al.*, 2011). Among these, there are the serological types SEG to SEJ (U.S. Department of Agriculture, Food Safety and Inspection Service 1996; Bergdoll *et al.*, 1973; Blaiotta *et al.*, 2004a; Morandi *et al.*, 2007; Munson *et al.*, 1998; Ren *et al.*, 1994; Su and Wong, 1995; Zhang *et al.*, 1998; Zschöck *et al.*, 2005) encoded by the genes *ent*A to *ent*E as well as *ent*G to *ent*J (Fueyo *et al.*, 2001), as well as the SEls SEK-SElU, SElV2, SElX (Becker *et al.*, 2007a; Blaiotta *et al.*, 2004a; Fitzgerald *et al.*, 2001; Hennekinne *et al.*, 2010; Jarraud *et al.*, 2001; Kuroda *et al.*, 2001; Letertre *et al.*, 2003; Morandi *et al.*, 2007; Omoe *et al.*, 2003; Omoe *et al.*, 2002; Orwin *et al.*, 2003; Orwin *et al.*, 2001; Podkowik

*et al.*, 2013; Wilson *et al.*, 2011). They are showing superantigen nature, stimulating non-specific T-cell proliferation and are resistant to the inactivation thru gastrointestinal proteases like pepsin or heat (Balaban and Rasooly, 2000; Bergdoll, 1979; Blaiotta *et al.*, 2004a; Dinges *et al.*, 2000; Genigeorgis, 1989; Martín *et al.*, 2006; Nedelkov *et al.*, 2000; Niskanen, 1977; Tatini, 1976; Zeleny *et al.*, 2015). As a result of heat stability, SEs are very problematic in food production and therefore it is logical that most of the food based poisoning are of bacterial origin in the world and are caused by consumption of food contaminated with SEs (Hernandez *et al.*, 1993; Jaulhac *et al.*, 1991; Lee *et al.*, 1977; Sergeev *et al.*, 2004; Zeleny *et al.*, 2015). Thereby uptake of SEs may result in gastroenteritis, e.g. vomiting and diarrhea (de Freitas Guimarães *et al.*, 2013; Gustafson *et al.*, 2015; Nedelkov *et al.*, 2000). TSST-1 is of importance in staphylococcal toxic shock syndrome particularly in menstrual cases (Becker *et al.*, 1998; Liu, 2015; Stingley *et al.*, 2014). The presence of exfoliative toxins in the human body causes erythema and fever as well as subsequently formation of large fragile superficial blisters resulting in the rupturing and denuding of the skin surface, called staphylococcal scalded-skin syndrome (Becker *et al.*, 1998; Imani Fooladi *et al.*, 2015; Ladhani *et al.*, 1999; Ladhani *et al.*, 2001; Yamasaki *et al.*, 2005). But also enterotoxigenicity of CNS species *S. carnosus*, *S. xylosus* and *S. equorum* isolated from food matrices like goat and sheep milk, cheese, ham as well as black pudding has been described (Bautista *et al.*, 1988; Coton *et al.*, 2010; de Moura *et al.*, 2012; Even *et al.*, 2010; Marty *et al.*, 2012; Rodríguez *et al.*, 1996; Vernozy-Rozand *et al.*, 1996).

***Binding to extracellular matrix proteins***

Moreover cell wall proteins have been described as other important properties in staphylococci pathogenicity. In this context specific binding mediated by proteins to extracellular matrix (ECM) proteins like fibrinogen, fibronectin, vitronectin, laminin, and collagen can be mentioned (Gemmell and Lang, 2015; Huebner and Goldmann, 1999; Świtalski *et al.*, 1983; Wilkinson, 1997). The binding to fibrinogen, fibronectin and collagen has been reported in combination with colonization of bacteria and infections (Frees *et al.*, 2005; Gemmell and Lang, 2015; Höök and Foster, 2000; O´Brien *et al.*, 2002; Peacock *et al.*, 1999).

By example, fibronectin-binding protein A induces staphylococcal binding to the ECM fibronectin, and may be important in the attachment of *S. aureus* during infection and

therefore facilitating the colonization of injured tissue and the invasion into the host cells and/or implanted biomaterials (Brett Finlay and Caparon, 2000; Hos *et al.*, 2015; Palmqvist *et al.*, 2005; Preissner and Chatwal, 2000; Rivera *et al.*, 2007; Świtalski *et al.*, 1983; Wolz *et al.*, 2000). Binding proteins to fibrinogen build the so called "clumping factor" which is responsible for clumping of erythrocytes and may cause thrombosis (Edwards and Massey, 2011; Rivera *et al.*, 2007). In respect of binding to fibrinogen, clumping factors A and B are expressed on the surface of *S. aureus* mediating fibrinogen-depended adhesion and clumping of *S. aureus* cells (McDevitt *et al.*, 1997; Ní Eidhin *et al.*, 1998; Palmqvist *et al.*, 2005). Moreover, staphylococcal species isolated from soft tissue infections are frequently able to bind to ECM and therefore contributing to the virulence CPS and CNS (Gemmell and Lang, 2015; Świtalski *et al.*, 1983)**.** Even though, up to date binding to ECM has not been described for food associated strains of *S. carnosus*- and *S. xylosus*-group, binding properties of closely related species *S. simulans* and *S. saprophyticus* has been shown (Christensen *et al.*, 1985; Gemmell and Lang, 2015; Świtalski *et al.*, 1983).

***Formation of biogenic amines***

Another important safety relevant characteristic, to be considered in the selection of strains used as starter cultures, is the formation of biogenic amines by amino acid decarboxylation of staphylococci (EFSA, Panel on Biological Hazards (BIOHAZ), 2011a; Gardini *et al.*, 2002; Landeta *et al.*, 2013). Biogenic amines (BA) are compounds of low molecular weight and are commonly present in living organisms in which they are responsible for many essential functions like the brain activity and regulation of the body temperature (Ladero *et al.*, 2011; Silla Santos, 1996; Suzzi and Gardini, 2003; ten Brink *et al.*, 1990). They are naturally present in foods like fruits, vegetables, meat, fish, chocolate and milk (Bover-Cid *et al.*, 2014; Suzzi and Gardini, 2003)**.** High amounts of BA may be observed in spoiled food products (Bover-Cid *et al.*, 2014; Gardini *et al.*, 2002; Silla Santos, 1996; Tasić *et al.*, 2012). Equally they may be formed partly in high concentrations in fermented vegetables, wine, bear, fermented meat and fish products as well as cheeses or products with high protein content thru metabolic activity of microorganisms namely decarboxylation of free amino acids (Bover-Cid and Holzapfel, 1999; Bover-Cid *et al.*, 2001; Bover-Cid *et al.*, 2014; Gardini *et al.*, 2002; Garriga *et al.*, 2005; Halász *et al.*, 1994; Montel *et al.*, 1999; Parente *et al.*, 2001; Shalaby, 1996; Silla Santos, 1996; Suzzi and Gardini, 2003; ten Brink *et al.*, 1990).

The formation of BA in fermented food *inter alia* is affected by the availability of free amino acids, the presence of microorganisms from raw materials and/or added starter cultures showing enzymatic activity to form BA as well as conditions promoting growth for microorganisms or influencing enzyme activity like temperature and pH. During food fermentation these conditions are given including intensive microbial activity and thus the possibility of BA formation (EFSA, Panel on Biological Hazards (BIOHAZ), 2011a). In foods and meat products the BA cadaverine, 2-phenylethylamine, putrescine, tryptamine, tyramine, histamine, spermine and spermidine has been described (Bover-Cid *et al.*, 2014; Naila *et al.*, 2010; Shalaby, 1996; Suzzi and Gardini, 2003). BA are an indicator for food quality and hygiene during food processing (García-Moruno *et al.*, 2005) and thus of interest in respect of food safety aspects (EFSA, Panel on Biological Hazards (BIOHAZ), 2011a; Garriga *et al.*, 2005; Marino *et al.*, 2011; Naila *et al.*, 2010; Straub *et al.*, 1995; Suzzi and Gardini, 2003).

BA like cadaverine, 2-phenylethylamine, putrescine, tryptamine and tyramine are formed by food associated CNS species *S. carnosus*, *S. piscifermentans* and *S. xylosus* isolated from sausages and meat products (Ansorena *et al.*, 2002; Coton *et al.*, 2010; De Las Rivas *et al.*, 2008; EFSA, Panel on Biological Hazards (BIOHAZ), 2011a; Even *et al.*, 2010; Landeta *et al.*, 2007; Martín *et al.*, 2006; Martuscelli *et al.*, 2000; Silla Santos, 1998; Straub *et al.*, 1995; Suzzi and Gardini, 2003). In context with microbial food spoilage especially the BA cadaverine, putrescine, and histamine, has been reported due to their correlation with increased decarboxylase production (Garriga *et al.*, 2005; Halász *et al.*, 1994). Moreover, histamine has been described being associated with food poisoning caused by spoiled fish and partially with cheese (Rauscher-Gabernig *et al.*, 2009; Straub *et al.*, 1995; Taylor *et al.*, 1989) as well as the consumption of food containing high concentrations of BA, especially histamine and tyramine could have different pharmacological effects like vasoactive and/or psychoactive properties and therefore may affect consumer health (Bover-Cid and Holzapfel, 1999; Bover-Cid *et al.*, 2014; Garriga *et al.*, 2005; Mariné-Font *et al.*, 1995). By way of comparison they may affect the gastric, intestinal and nervous system. In this content the induction of headache or migraine seizures, difficulties of breathing, effects to the blood pressure, palpitations, hypertonia, hypotonia and different allergic reaction like scombroid poisoning has been mentioned (Alvarez and Moreno-Arribas, 2014; Anderson *et al.*, 1993; Calles-Enríquez *et al.*, 2010; Lonvaud-Funel, 2001; Rice *et al.*, 1976; Shalaby, 1996; Straub *et al.*, 1995; Suzzi and Gardini, 2003; Taylor, 1983; ten Brink *et al.*, 1990). In the year 2011,

the EFSA (European Food Safety Authority) Panel on Biological Hazards (BIOHAZ) performed qualitative risk assessment of BA in fermented foods and concluded that the present knowledge and data on toxicity of individually or in combinations occurring BA are limited and thus of low relevance. However, because of the described pharmacological effects of histamine and tyramine the BIOHAZ panel has considered histamine and tyramine as the most toxic BA and them with the most importance concerning food safety. Nevertheless, the BIOHAZ panel demands that the formation of BA by microorganisms during food fermentation is an undesired property and thus microorganisms intended to be used as starter cultures in fermented food should be confirmed as not producing BA (EFSA, Panel on Biological Hazards (BIOHAZ), 2011a).

As an overall view it should be mentioned that in context with undesired properties of staphylococci other possible virulence determinants existing. As an example, lipases contribute to the pathogenesis and infections like boils or abscesses, may be involved in the colonization and persistence of the organism to the skin or promoting adherence by releasing free fatty acids (Gemmell and Lang, 2015; Gribbon *et al.*, 1993; Longshaw *et al.*, 2000).

## Safety relevant aspects of staphylococci used as starter cultures

In principle, safety of fermented products is proven due long-term experience, e.g. the traditionally use of *S. carnosus* used as fermentation organism (Hammes, 2012; Liepe, 1982). Within the EU, only Denmark has a legal regulation for the use of cultures demanding notification and approval as well as containing documentation on safety (Wessels *et al.*, 2004). However, no specific legal regulation in respect of starter cultures used for the production of fermented food in other countries of Europe exists (Vogel *et al.*, 2011). On the one hand, they have to comply with legal requirements like Regulation (EC) No. 178/2002 or, in Germany, the Food and Feed Code (Lebensmittel- und Futtermittelgesetzbuch, LFGB) demanding safety of microbial cultures for the intended use (Vogel *et al.*, 2011). Due to the lack of legal regulations in the EU countries (except of Denmark), the recommendation of German Research Foundation (Deutsche Forschungsgemeinschaft, DFG) about starter cultures (DFG, 1987) can be considered as orientation guide for the use of microorganisms in food. Further, determination of long-term experience by the concept of "history of safe use" of DFGs Senate Commission on Food Safety (SKLM) is suggested (Bourdichon *et al.*, 2012; Engel *et al.*, 2011). For example, *S. carnosus* has a "history of safe use" thru its traditional use as fermentation organism over long time as well as the proven safety of fermented products (Hammes, 2012; Talon and Leroy, 2014). However, for other species with potential to be used in starter cultures e.g. *S. condimenti, S. equorum, S. piscifermentans* and *S. succinus* long-term experience in respect of sanitary harmlessness exists only limited. According to this, virulence factors and other potential disease-causing threads of strains used as starter cultures in food fermentation should be verified in respect of their safety and sanitary harmlessness (Borriello *et al.*, 2003; Talon and Leroy, 2014). Thus, it should be regulated which characteristics strains used in starter cultures exhibit or not.

In contrast to starter cultures used in food production, microorganisms used in animal nutrition have been regulated by the Scientific Committee on Animal Nutrition at EU level (SCAN, 2003). To handle this problem, in the year 2003 a working group consisting of members of Scientific Committee on Animal Nutrition, Scientific Committee on Food and the Scientific Committee on Plant of the European Commission has published a working paper and suggested a concept to harmonize the safety assessment of microorganisms used in feed/food production. This approach considers the ‘‘history of safe use", but also takes the different regulatory practices in Europe into account. Moreover, the evidence of antibiotic

resistances in microorganisms is an important property in Europe, where resistances are considered as undesired properties (European Commission, 2003).

In this concept concerning safety assessment of microorganisms used in food production, the implementation of a "Qualified Presumption of Safety" (QPS) approach and therefore QPS status for selected groups of microorganism has been suggested. In this case, no additional safety assessment of strains belonging to this group and with use in food production is necessary. The QPS system is based on four pillars of safety assessment dealing with `establishing identity´ (at taxonomic level), `body of knowledge´ or `familiarity´ (including history of use, scientific literature, clinical aspects, industrial applications and ecology), `possible pathogenicity´ (identification of safety concerns) and the `end use´ (EFSA, 2004; EFSA, 2005b; EFSA, 2007a; Leuschner *et al.,* 2010). This means in the first step the accurate taxonomic grouping of the microorganism at genus and (sub)species level for which QPS is requested. Second, if the suggested group (familiarity) contains adequate knowledge to draw the consequences in respect of their safety. Third, if the suggested group contains known pathogenic microorganisms. The last criterion is the intended end use, e.g. if the microorganisms are being viable in the end product and will be eaten or not like in the case of plant protection. Moreover, the microorganisms can be used during production and will be not present in the end product.

In 2004, scientific colloquium has been organized by European Food Safety Authority (EFSA) having an open scientific debate on the principles behind the QPS approach suggested in the working paper of DG SANCO Scientific Committees. The participants were asked to explore how the QPS may be further developed in regard to a possible implementation by the EFSA (EFSA, 2004). Based on the suggestion of the participants of the scientific colloquium and the comments in respect of the working paper, a proposal how the QPS approach may be implemented has been prepared. The implementation of QPS system has been supposed to simplify the safety assessment by focusing on microorganisms and microbial products used as food and feed additive revealing the utmost risk and unknown factors. However, the primary QPS concept has been modified as the product specific data should also be considered because microorganisms may exhibit different properties depending on the surrounding matrix (EFSA, 2005a). In the year 2007, a second public consultation on the QPS approach has been performed by the EFSA containing the most common used microorganisms in food production (EFSA, 2007a). By this, microorganisms have been selected where safety relevant

data is present in respect to the implementation of QPS status and four groupings were performed. Within the group of gram positive bacteria the species *S. carnosus* and *S. xylosus* has been suggested to obtain QPS status because of their traditional use as starter cultures (EFSA, 2007a). In the later published opinion concerning the QPS approach of selected microorganisms referred by EFSA, strains were selected for an initial assessment of their suitability for QPS status. It has been concluded by the Scientific Committee, that the data for 3 of the 4 groups are sufficient to ensure that QPS status provide similar to safety assessment thru case by case studies. Taxonomic units of microorganisms has been reviewed and recommended for the QPS list (EFSA, 2007b; EFSA, 2007c; Leuschner *et al.*, 2010). Since the year 2008, the list of biological agents recommended for QPS is been annually updated by the Biological Hazards (BIOHAZ) Panel (EFSA, 2008; EFSA, BIOHAZ Panel (EFSA Panel on Biological Hazards), 2013; EFSA, BIOHAZ Panel (EFSA Panel on Biological Hazards), 2014; EFSA, Panel on Biological Hazards (BIOHAZ), 2009; EFSA, Panel on Biological Hazards (BIOHAZ), 2010; EFSA, Panel on Biological Hazards (BIOHAZ), 2011b; EFSA, Panel on Biological Hazards (BIOHAZ), 2012; Leuschner *et al.*, 2010). Indeed, in the lists of QPS biological agents published in the scientific opinions since 2007, *Staphylococcus* strains are not been suggested to be suitable for QPS status. Although the absence of specific microorganisms from the QPS list does not necessarily imply a risk while using them as starter cultures. As an explanation for the absence of the list they may simply not been asked to asses by the EFSA or although individual strains of a taxonomic group may be safe it was not possible to asses the whole group as safe based on the existing knowledge (Bourdichon *et al.*, 2012). Thus, if CNS strains of species *S. carnosus* and *S. xylosus* are used in food and feed production, a case by case study as well as a full risk assessment of these strains at strain level is recommended. In respect of this, the absence of toxin or biogenic amine formation in the end-products and formation of acquired antibiotic resistances are an important safety issue (Marty *et al.*, 2012; Talon and Leroy, 2011; Talon and Leroy, 2014). Ensuring this demand, detection of genotypes *inter alia* of acquired antibiotic resistances for strains with use as commercial starter or protective cultures has been suggested (Jeong *et al.*, 2014; Kastner *et al.*, 2006).

Particularly for CNS with the potential for future use as starter cultures, data requested by the four pillars are only limited available. Although to some extent studies of safety relevant properties in CNS have been performed, there is still a research need to obtain sufficient knowledge about safety issues of food associated CNS.

**Safety relevant properties of *S. carnosus*- and *S. xylosus*-group**

Regarding the requested "body of knowledge" to reach the QPS status of CNS with use or future use in starter cultures, the history of utilization is an important source of knowledge influencing the safety assessment (EFSA, 2004; EFSA, 2005a; Leuschner *et al.*, 2010). For example, some CNS has a proven long-term experience in fermented food products and therefore a long history of apparent safe use. In this context, species used in starter cultures like *S. carnosus* and *S. xylosus* or being components of the dominating fermentation biota like *S. equorum*, *S. xylosus* and *S. succinus* can be mentioned (Coton *et al.*, 2010).

However, in few cases clinical isolates of species *S. carnosus*, *S. equorum*, *S. succinus* and *S. xylosus* have been described (Couto *et al.*, 2001; Domínguez *et al.*, 2002; Koksal *et al.*, 2009; Misawa *et al.*, 2015; Novakova *et al.*, 2006; Papapetropoulos *et al.*, 1981; Petinaki *et al.*, 2001; Rahman *et al.*, 2012). On the other hand, by the investigation of the biodiversity of different CNS, including 297 clinical isolates, any allocation to these species could not be found (Even *et al.*, 2010). In addition to this, other undesired properties like antibiotic resistances, toxin and BA formation have been described to occur in food associated CNS of species *S. carnosus*, *S. equorum*, *S. piscifermentans* and/or *S. xylosus.* The formation of enterotoxins *inter alia* has been shown in strains of the important starter organism *S. carnosus* and *S. xylosus* as well as food associated CNS species *S. equorum* (Bautista *et al.*, 1988; de Moura *et al.*, 2012; Orden *et al.*, 1992; Podkowik *et al.*, 2013; Rodríguez *et al.*, 1996; Soares *et al.*, 2011; Valle *et al.*, 1990; Vernozy-Rozand *et al.*, 1996). By annotation and analysis of *S. carnosus* genome, genes could be detected showing homologies in amino acid sequences of toxins and other pathogenicity factors of *S. aureus* or rather *S. epidermidis* (Rosenstein *et al.*, 2009). Moreover, the formation of BA cadaverine, 2-phenylethylamine, putrescine, tryptamine or tyramine has been described in different studies by food associated CNS species *S. carnosus*, *S. piscifermentans*, *S. xylosus* and *S. equorum* of fermented sausages (Ansorena *et al.*, 2002; Bermúdez *et al.*, 2012; Cachaldora *et al.*, 2013; Even *et al.*, 2010; Martín *et al.*, 2006; Martuscelli *et al.*, 2000; Straub *et al.*, 1995). Similar results have been shown for food associated staphylococci investigating antibiotic resistance phenotype and genotype. In these studies, resistances to antibiotics including some of therapeutic importance or antibiotic resistance genes in *S. carnosus*, *S. equorum*, *S. piscifermentans*, *S. succinus* and *S. xylosus* strains isolated from food has been detected (Chajęcka-Wierzchowska *et al.*, 2015; de Moura *et al.*, 2012; Even *et al.*, 2010; Gardini *et al.*, 2003; Holley and Blaszyk, 1997; Kastner *et al.*, 2006; Martín *et al.*, 2006; Marty *et al.*, 2012; Perreten *et al.*, 1997; Soares *et al.*, 2011; Talon

and Leroy, 2014; Teuber *et al.*, 1996). Considering genes for antibiotic resistances and pathogenicity factors it should be mentioned that they can be transferred from one species to another species via horizontal gene transfer. For example, homologous form of gene *mecA* responsible for methicillin resistance has been shown in natural isolates of *S. sciuri* (Couto *et al.*, 2001) and phenotypic methicillin resistance in *S. aureus* after transferring this gene (Wu *et al.*, 2001). In this context it is important to know, that horizontal transfer of antibiotic resistance genes has been shown to occur in food (Rossi *et al.*, 2014; Teuber *et al.*, 1999). Furthermore, investigation of horizontal gene transfer during sausage fermentation of pamβ1 between *L. curvatus* and *S. carnosus* showed efficient transfer by conditions prevailing in the meat matrix (Hertel *et al.*, 1995). As horizontal gene transfer may be facilitated by the food matrix it is supposed that the food matrix acts as reservoir for the spread of antibiotic resistances (Franz *et al.*, 2005; Rebecchi *et al.*, 2015; Teuber, 1999; Werner *et al.*, 1997; Witte, 1999). Therefore, horizontal transfer of antibiotic resistance determinants is considered as an important safety issue and the question to the presence of virulence factors or other potential disease-causing threads in food associated staphylococci with use or future use in starter cultures arises. Concerning formation of binding proteins to ECM it can be mentioned that up to date only binding protein formation of closely related species *S. simulans* and *S. saprophyticus* has been observed but not of food associated strains of *S. carnosus*- and *S. xylosus*-group (Chung *et al.*, 2005; Świtalski *et al.*, 1983).

In respect of safety assessment of new starter organisms it is possible to consider the degree of relatedness. Phylogenetic consideration shows, that species used in food production like *S. xylosus* as well as *S. equorum* are closely related to undesired species like *S. saprophyticus* (Kooken *et al.*, 2014; Place *et al.*, 2003; Shah *et al.*, 2007). Therefore, performing safety assessment it is important considering the undesired properties of closely related species.

## Detection of safety relevant properties

The implementation of QPS system and thus QPS status for selected groups of microorganisms implies no additional safety assessment of microorganism used in food production (Leuschner *et al.*, 2010). In context with the four pillars of QPS concept (`establishing identity´, `body of knowledge´, `possible pathogenicity´ and `end use´) adequate knowledge over the suggested microorganism group is required in order to answer the question if pathogenic microorganisms belonging to (Coton *et al.*, 2010; EFSA, 2004; EFSA, 2005b; EFSA, 2007a; Leuschner *et al.*, 2010; Sohier *et al.*, 2008). In pathogenic staphylococci amongst others resistances to different clinical important antibiotics, formation of toxins and binding to extracellular matrix proteins have been described (Gemmell and Lang, 2015; Götz *et al.*, 2006). Further, formation of biogenic amines by food associated CNS during food fermentation is an undesired property and thus it has been confirmed that the formation of BA by microorganism used as starter cultures should not be present (Alvarez and Moreno-Arribas, 2014; EFSA, Panel on Biological Hazards (BIOHAZ), 2011a; Gardini *et al.*, 2002). For this reasons it is of utmost importance to get knowledge of the presence or absence of such properties in CNS used in food production. Thus, the third pillar of the QPS-status proposed by the EFSA is dealing with the possible pathogenicity of the selected organisms (EFSA, 2007a; EFSA, 2007b). In order to evaluate this pathogenicity suitable approaches are necessary.

Phenotypic analysis such as disk susceptibility or broth dilution testing (CLSI, 2009a; CLSI, 2009b) as well as genotypic analysis e.g. PCR based methods are used for detection of antibiotic resistances (Aymerich *et al.*, 2003; Chajęcka-Wierzchowska *et al.*, 2015; Jeong *et al.*, 2014; Kastner *et al.*, 2006; Martineau *et al.*, 2000; Rebecchi *et al.*, 2015; Strommenger *et al.*, 2003). Toxins could be detected on two different approaches: 1. the demonstration of the toxin itself using immunological systems like serological typing of immunoblot analysis for the ability of toxin formation (Bautista *et al.*, 1988; Becker *et al.*, 1998; Podkowik *et al.*, 2013; Rodríguez *et al.*, 1996; Sospedra *et al.*, 2013) and 2. detection of the toxin gene e.g. using PCR based systems (Becker *et al.*, 2001; de Moura *et al.*, 2012; Letertre *et al.*, 2003; Martín *et al.*, 2006; Schumacher-Perdreau *et al.*, 1995; Soares *et al.*, 2011). Biogenic amines are generally proven using high performance liquid chromatography (HPLC) (Bover-Cid *et al.*, 2014; EFSA, Panel on Biological Hazards (BIOHAZ), 2011a; Gardini *et al.*, 2002; Straub *et al.*, 1993). For the detection of ECM binding microtiter plate assays with immobilized

ECM proteins and crystal violet staining have been reported (Štyriak *et al.*, 1999; Wolz *et al.*, 2000).

Phenotypic detection of metabolites is often time consuming and limited by a maximum number of samples. To overcome such limiting issues detection of genotypes by PCR and hybridization analysis becomes more and more important and plays a key role in rapid and reliable identification (Strauss *et al.*, 2015). In this context, DNA microarray techniques have been established as a useful tool for high throughput identification of safety relevant properties in CNS (Even *et al.*, 2010; Marty *et al.*, 2012). This technique allows simultaneous detection of gene probes saving time and resources (Monecke *et al.*, 2003; Strommenger *et al.*, 2007). Therefore, using one trial, the possibility is given to get information about the presence or absence of a large number of genes e.g. in context with toxins, antibiotic resistances, adhesins and binding proteins (may being involved in colonization) as well as exoenzymes like proteases and lipases or other virulence-associated genes (Call *et al.*, 2003a; Call *et al.*, 2003b; Cassone *et al.*, 2006; Even *et al.*, 2010; Monecke *et al.*, 2007; Otsuka *et al.*, 2008; Perreten *et al.*, 2005; Saunders *et al.*, 2004; Sergeev *et al.*, 2004; Spence *et al.*, 2008; Strauss *et al.*, 2015). DNA microarrays have been described offering an alternative method for the simultaneous screening of several genes or target DNA sequences on a single glass slide (Bruant *et al.*, 2009; Even *et al.*, 2010; Hamelin *et al.*, 2007; Otsuka *et al.*, 2008; Perreten *et al.*, 2005; Schena and Davis, 1999; Walther *et al.*, 2008; Wang, 2014). Such microarrays are assembled using single-stranded oligonucleotides (25- to 80-mers long and synthesized chemically) or doubled-stranded DNAs (200 to 800 bp long obtained by polymerase chain reaction (PCR)) of specific gene probes or target sequences which were spotted on e.g. (glass)slides with modified surfaces (Wang, 2014). In the following the microarray slide is hybridized with labeled DNA. The generated target-probe duplexes on the microarray are visualized using a fluorescence scanner followed by analyzing of the image (Call *et al.*, 2003a; Lockhart and Winzeler, 2000; Monecke *et al.*, 2003; Schena, 2000; Wang, 2014).

During the last years different microarray technology based assays have been developed to assess phenotypic properties e.g. antibiotic resistances (Call *et al.*, 2003a; Cassone *et al.*, 2006; Even *et al.*, 2010; Huber *et al.*, 2011; Perreten *et al.*, 2005; Strauss *et al.*, 2015; Strommenger *et al.*, 2007; Volokhov *et al.*, 2003; Zhu *et al.*, 2007) and/or toxins (Even *et al.*, 2010; Monecke *et al.*, 2007; Otsuka *et al.*, 2008; Sergeev *et al.*, 2004) combined with or without species identification (Call *et al.*, 2003a; Cleven *et al.*, 2006; Couzinet *et al.*, 2005a; Giammarinaro *et al.*, 2005; Perreten *et al.*, 2005; Spence *et al.*, 2008; Volokhov *et al.*, 2003).

More precisely tetracycline resistance could be detected using a DNA microarray containing PCR-generated products involved in tetracycline resistance about 550 bp length spotted on a glass slide (Call *et al.*, 2003a). Detection of macrolide resistance and other antibiotic resistance genes by oligonucleotide microarrays in *S. aureus*, *S. haemolyticus* and other staphylococcal isolates are reported (Cassone *et al.*, 2006; Volokhov *et al.*, 2003; Zhu *et al.*, 2007). Further, for the detection of up to 90 antibiotic resistance genes in gram-positive bacteria of species *S. haemolyticus*, *Lactococcus lactis*, *Enterococcus faecium* and others, a DNA microarray containing two specific oligonucleotide probes has been designed (Perreten *et al.*, 2005). This microarray has been improved by the addition, replacing and redesigning of oligonucleotide probes for the detection of 117 antibiotic resistance genes in gram-positive bacteria including *Staphylococcus* strains (Strauss *et al.*, 2015).

The use of DNA microarray techniques for detection of toxin genes has also been described, e.g. using a microarray based assay for simultaneous detection and identification of 16 staphylococcal enterotoxin genes (Sergeev *et al.*, 2004). But also microarrays are developed for the detection of antibiotic resistance genes in combination with toxin genes (Lin *et al.*, 2009; Monecke *et al.*, 2007). By example, identification and typing of clinical *S. aureus* isolates using a DNA macroarray with special interest to genes coding amongst others for *S. aureus* specific proteins, antibiotic resistances and putative virulence proteins has been described (Trad *et al.*, 2004). The DNA macroarray was desiged by comparative analysis of seven clinical *S. aureus* isolates. Probes spotted on DNA-macroarray were designed by selection of genes with nucleotide sequence similarity <80% and PCR amplification of a specific 400 – 500 bp fragment of each gene. By the use of DNA macroarray containing polynucleotide amplicons the authors aimed to detect not only common genes but also modified genes by horizontal gene transfer and unknown genes, similar to well described genes, which have not been described yet. Recently, a PCR-product microarray has also been developed for the identification of virulence factors and antibiotic resistances of *S. aureus* isolates (Palka-Santini *et al.*, 2007). Moreover, microarray containing PCR generated probes has been used to perform comparative genomics by identifying the presence or absence of *S. aureus* genes (Witney *et al.*, 2005). However, microarrays developed until now are mostly dealing with detection of safety relevant properties in pathogenic staphylococci but limited in respect of safety concerning CNS genes due to missing data.

Lately, microarray hybridization has also been established as useful technique for the detection (or screening) of safety relevant properties in food associated CNS. In this context, a diagnostic microarray has been developed for simultaneous detection of 268 genes coding for antibiotic resistance, toxin determinants and biogenic amine production (Even *et al.*, 2010). However, because of the lack of safety relevant information of food associated CNS, they cannot all be considered in the design of the array. To avoid this drawback, sequences of other CNS or species like lactococci, lactobacilli, *Bacillus cereus*, *Clostridium perfringens* or *Enterococcus faecalis* has been used to design the CNS microarray (Even *et al.*, 2010). With regard to formal classification of staphylococcal species and most subspecies, DNA similarity >70% has been described as the criterion to determine species boundaries (Götz *et al.*, 2006). Thus, the design of microarray probes as well as hybridization conditions have to be taken into consideration allowing detection of new unknown genes with sequence similarity between 70% and 100% to known genes. In addition to improve the further use of microarrays by food processing industry, the simultaneous detection of safety hazards together with technologically relevant properties for food processing industry using only one array should be considered (Even *et al.*, 2010)**.** Such technological relevant genes could include enzymatic properties like high nitrate reductase and catalase activities as well as proteases and lipases (Flores and Toldrá, 2011). Those properties are amongst others involved in formation of red color development and stability in meat products (Hammes, 2012; Marco *et al.*, 2006; Neubauer and Götz, 1996), the overall flavor of cured meat products (Casaburi *et al.*, 2006; Hierro *et al.*, 1997; Martín *et al.*, 2007; Rossi *et al.*, 2001) and the prevention of rancidity by lipid oxidation (Barrière *et al.*, 2001; Blaiotta *et al.*, 2004b; Essid and Hassouna, 2013; Montel *et al.*, 1996; Rosenstein *et al.*, 2009), respectively.

Overall considered, further research is needed if microarray based analyses are used to determine safety and technological relevant properties of CNS just as getting more knowledge about them being present in food associated CNS.

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# *Chapter III*

## Antibiotic resistance of coagulase-negative staphylococci associated with food and used in starter cultures

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## Abstract

The resistance of 330 coagulase-negative staphylococci (CNS) associated with food or used in starter cultures and belonging to the species *Staphylococcus carnosus*, *Staphylococcus condimenti*, *Staphylococcus piscifermentans*, *Staphylococcus equorum*, *Staphylococcus succinus* and *Staphylococcus xylosus*, against 21 antibiotics was determined using the disk diffusion method. The incidence and number of resistances was found to be species and source of isolation dependent. Most strains of *S. equorum* (63%), *S. succinus* (90%) and *S. xylosus* (95%) exhibited resistances against up to seven antibiotics, whereas only few strains of *S. carnosus* (12%) and *S. piscifermentans* (27%) were antibiotic resistant. Resistances to lincomycin, penicillin, fusidic acid, oxacillin, ampicillin and tetracycline were predominant. Among strains of *S. xylosus*, the incidence of resistance ranged from 22% for tetracycline up to 69% for penicillin. Concerning the source of isolation, resistances were often determined in strains of S *equorum*, *S. succinus* and *S. xylosus* isolated from cheese (87%) and sausage (83%), and strains of *S. xylosus* obtained from meat starter cultures (93%). Remarkably, all CNS were sensitive to the clinically important antibiotics chloramphenicol, clindamycin, cotrimoxazol, gentamicin, kanamycin, linezolid, neomycin, streptomycin, synercid and vancomycin. The phenotypic resistances to β-lactam antibiotics, lincomycin and tetracycline were verified by PCR amplification and could be traced back to the genes *bla*Z, *lnu*A and *tet*K, respectively. This study permitted a comprehensive insight into the incidence of antibiotic resistances in food-associated CNS.

## 1. Introduction

To date, the genus S*taphylococcus* contains 41 validly described species (DSMZ, 2008) that are traditionally grouped into coagulase-positive (CPS) and coagulase-negative staphylococci (CNS). Main habitats are skin, skin glands and mucous membranes of humans and animals, and only a few species are of special importance in foods and for human health. The CPS *Staphylococcus aureus* causes food intoxications and is involved in severe human infections, whereas the CNS *Staphylococcus epidermidis*, *Staphylococcus haemolyticus* and *Staphylococcus saprophyticus* are opportunistic pathogens (Götz et al., 2006; Le Loir et al., 2003). Among CNS, the species *S. carnosus* (ssp. *carnosus* and *utilis*), *S. condimenti*,

*S. equorum* (ssp. *equorum* and *linens*), *S. piscifermentans*, *S. succinus* (ssp. *casei* and *succinus*) and *S. xylosus* are either associated with foods or play a major role in the food processing industry. Strains of *S. carnosus* and *S. xylosus* are traditionally used in starter cultures for meat fermentations (Hammes and Hertel, 1998; Ordóñez et al., 1999). The other species are often isolated from fermented foods and therefore may have the potential for future application in starter or protective cultures (Bockelmann, 2002; Place et al., 2002 and 2003; Probst et al., 1998; Schlafmann et al., 2002; Tanasupawat et al., 1992).

Due to the intensive use of antibiotics in public health and animal husbandry, antibiotic resistance in pathogens including *S. aureus* has been an increasing medical problem during the last decades (Mazel and Davies, 1999). Research in recent years showed that resistances to antibiotics, including resistance to some antibiotics of therapeutic importance, also occur in strains of the important starter organism *S. carnosus* and *S. xylosus* (Gardini et al., 2003; Holley and Blaszyk, 1998; Kastner et al., 2006; Martín et al., 2006; Mauriello et al., 2000; Teuber et al., 1996), and in a few cases the underlying genes could be detected (Kastner et al., 2006; Perreten et al., 1997). Starter organisms are purposely added in high numbers ($10^7$-$10^8$ CFU/g) to produce fermented foods. In addition, the CNS belonging to the microbiota of spontaneously fermented foods were shown to occur in numbers of $10^6$ to $10^7$ CFU/g (Bockelmann and Hoppe-Seyler, 2001; Ercolini et al., 2003; Mauriello et al., 2004; Parente et al., 2001; Rantsiou et al., 2005; Rodriguez et al., 1996; Rossi et al., 2001; Teuber et al., 1996). Thus, enormous amounts of living bacteria are incorporated into the human body. Antibiotic resistances determinants contained in starter organisms or naturally occurring CNS may thus be transferred to commensals or pathogenic bacteria (Teuber et al., 1999). In addition, horizontal transfer of resistance gene was shown to occur in food (Teuber et al., 1999) and may be facilitated by the food matrix (Hertel et al., 1995). Therefore, the question arises regarding the contribution of food as a reservoir for the spread of antibiotic resistance (Franz et al. 2005; Teuber, 1999; Werner *et al*., 1997; Witte, 1999).

Recently, the European Food Safety Authority (EFSA) has undertaken the task to establish a concept for the safety assessment of microorganisms used in food and feed production. The proposed “qualified presumption of safety” (QPS) system (EFSA, 2004) is similar in concept and purpose to the GRAS system (Burdock and Carbin, 2004) in the USA. It is based on four pillars dealing with establishing identity, body of knowledge, possible pathogenicity and end use. With regard to possible pathogenicity, it is interesting to note that clinical isolates of

*S. carnosus*, *S. equorum*, *S. succinus* and *S. xylosus* have recently been described (Couto et al., 2001; Dominguez et al., 2002; Novakova et al., 2006; Petinaki et al., 2001). This raises the question to the presence of virulence factors, or other potential disease-causing threats. In addition, horizontal transfer of antibiotic resistance determinants is considered an important safety issue. Although food isolates of *S. xylosus* and *S. equorum* have been described to produce enterotoxins (Bautista et al., 1988; Rodriguez et al., 1996; Vernozy-Rozand et al., 1996) and studies about the incidence of antibiotic resistances have been published, there is still a need to obtain sufficient knowledge about safety issues of food-associated CNS.

In this study the incidence of resistance against 21 antibiotics among 330 strains of food-associated CNS was investigated using the disk diffusion test according to the NCCLS guidelines. Phenotypic resistances were confirmed using PCR detection systems for the known antibiotic resistance genes in staphylococci.

## 2. Materials and methods

### 2. 1. Bacterial strains and growth conditions

In this study 330 CNS and the type strains of *Staphylococcus carnosus*, *Staphylococcus condimenti*, *Staphylococcus equorum*, *Staphylococcus succinus*, *Staphylococcus piscifermentans* and *Staphylococcus xylosus* were used. The CNS were isolated from various foods, commercial starter cultures and from patients in clinics. *Staphylococcus aureus* ATCC 25923 and *Escherichia coli* ATCC 35218 served as reference strains for disk diffusion testing (NCCLS, 2003). *S. xylosus* VF5 (containing *tet*K gene, Perreten et al., 1998), *Enterococcus faecium* FAIR E 25 (containing *tet*L gene, Hummel et al., 2007), *E. faecium* 5749 (containing *tet*M gene, Weigel et al. 2007), *S. aureus* N315 (containing *bla*Z gene, Kuroda et al., 2001), *S. aureus* Mu50 (containing *mec*A gene, Kuroda et al., 2001) and *S. aureus* pBMSa1 (containing *lnu*A gene, Loeza-Lara et al., 2004) were used as positive control for PCR tests. All strains were cultured in Standard I nutrient broth (Merck) at 30°C or 37°C with shaking at 180 rpm.

**2. 2. DNA isolation**

Total DNA was isolated by suspending the cells of a single colony in 1 ml sterile PBS buffer (containing per liter: 8.0 g NaCl, 0.2 g KCl, 1.44 g $Na_2HPO_4$, and 0.24 g $KH_2PO_4$, pH 8.3) and using the GenElute Bacterial Genomic DNA Kit (Sigma-Aldrich). For cell lysis, lysozyme (Serva Electrophoresis, 20 mg/ml) and lysostaphin (Sigma-Aldrich, 55 U/ml) were added and the mixture was incubated at 37°C for 60 min.

**2. 3. Species identification by rRNA gene sequence analysis**

Amplifications were carried out in a Primus Thermocycler (MWG-Biotech). DNA solution or a bacterial suspension adjusted in saline-tryptone diluent to 0.5 McFarland standard (approximately 1.5 x $10^8$ cells/ml) were used as template. Amplification of 16S rRNA gene fragments (1550 bp) was carried out as described previously (Meroth et al., 2003). Partial 23S rRNA gene fragments (2900 bp) were amplified using primers 335IRjul97V (5´-GGT GGA TGC CYW GGC-3´) and 2896Rstaph700 (5´-GTC TTC GAT CGA TTA G-3´) (personal communication, W. Ludwig, TU München, Germany). Purification of PCR fragments was performed with the EXO-SAP method by adding Exonuclease I (Fermentas) and Shrimp Alkaline Phosphatase (Fermentas). DNA sequences of purified PCR fragments were determined by the dideoxy chain termination method using the Dye Terminator Cycle Sequencing Quick Start Kit (Beckman Coulter), the CEQ™ 8000 Genetic Analysis System (Beckmann Coulter) and primer 616V and 317modR700 (5´-ACC TGT GTC GGT TTG CGG TAC -3´) for the 16S and 23S rRNA gene fragments, respectively. To determine the closest relatives, a search of the Arb database (Ludwig et al., 2004) and GenBank database by using the BLAST algorithm (Altschul et al., 1990) was conducted.

**2. 4. Antimicrobial susceptibility testing**

Antibiotic resistances were tested by applying the disk diffusion assay according to the guidelines of the NCCLS (NCCLS, 2003) using Mueller Hinton agar (Becton Dickinson). The following antimicrobial susceptibility test disks (Becton Dickinson) were used: ampicillin (10 µg), cefoxitin (30 µg), chloramphenicol (30 µg), clindamycin (2 µg), erythromycin (15 µg), gentamicin (10 µg), kanamycin (30 µg), linezolid (30 µg), ofloxacin (5 µg), oxacillin (1 µg), penicillin G (10 units), rifampin (5 µg), tetracycline (30 µg), trimethoprim-sulfa-methoxazole (Cotrimoxazol: 1.25/23.75 µg), quinupristin-dalfopristin (Synercid: 15 µg), vancomycin (30 µg). Furthermore, strains were tested for susceptibility to neomycin (30 µg) and streptomycin (10 µg) as described by EFSA (2005) and to fusidic acid (10 µg),

lincomycin (2 µg) and novobiocin (30 µg) as described by Mauriello et al. (2000). *E. coli* ATCC 25922 and *S. aureus* ATCC 25923 served as controls to ensure the accuracy of testing. Inhibition zones were measured precisely to a millimeter and the resistance or susceptibility of the antibiotics were interpreted as suggested by the NCCLS standards (NCCLS, 2003), except for those antibiotics not included in the standard. For the antibiotics fusidic acid and lincomycin the zone diameter interpretive chart supplied from Oxoid (1993) was used.

**2. 5. Detection of antibiotic resistance genes by PCR**

Genes responsible for resistances towards β-lactam antibiotics (β-lactamase gene *bla*Z and *mec*A gene coding for methicillin/oxacillin resistance via penicillin binding protein 2a), lincomycin (lincosamide nucleotidyltransferase gene *lnu*A), and tetracycline (gene for tetracycline efflux protein *tet*K and *tet*L, ribosomal protection protein *tet*M) were amplified by PCR using already published primers targeted against *bla*Z (Martineau et al., 2000), *lnu*A (formerly known as *lin*A) (Lina et al., 1999), *mec*A (Martineau et al., 2000), and *tet*K, *tet*L, *tet*M (Gevers et al., 2003). Amplification was carried out in 50 µl volumes containing 20 pM of each specific primer, each deoxyribonucleotide triphosphate at a concentration of 0.2 mM, reaction buffer (final concentrations 10 mM Tris HCl [pH 9.0], 50 mM KCl, 1.5 mM magnesium chloride, 0.1% Tween 20), 1.5 U *Taq* polymerase (Genaxxon Bioscience), and 1 µl of DNA solution or a bacterial suspension whose turbidity was adjusted to a 0.5 McFarland standard which after lysis by heating (in the thermocycler) served as source of template DNA. PCR amplifications were carried out as described previously (Gevers et al., 2003; Lina et al., 1999; Martineau et al., 2000) with the following modifications. The initial denaturation step was performed at 94°C for 4 min and the final extension step at 72°C for 5 min. PCR products were separated by electrophoresis on a 1.2% agarose gel and visualized by staining with ethidium bromide. For the *bla*Z, *lnu*A and *tet*K positive isolates, the purified PCR products were directly sequenced with the appropriate forward primer to confirm the sequence authenticity. Sequencing was performed with Dye Terminator Cycle Sequencing Quick Start Kit (Beckmann Coulter) on the CEQ™ 8000 Genetic Analysis System (Beckmann Coulter). On-line similarity searches were performed using the BLAST algorithm and GenBank database as described previously.

## 3. Results

### 3. 1. Incidence of phenotypic antibiotic resistances and multiple resistances

Three-hundred-thirty food-associated CNS isolates were investigated for their susceptibility to antibiotics. The species affiliation of strains obtained from different working groups in Europe was verified by partial rRNA sequence analysis resulting in 106, 2, 11, 64, 10 and 137 strains of *S. carnosus, S. condimenti, S. piscifermentans, S. equorum, S. succinus* and *S. xylosus*, respectively. Twenty-one antibiotics were chosen according to the recommendations of NCCLS (2003) for testing clinical relevant staphylococci, recent publications about antibiotic resistance in food-associated staphylococci (e.g. Mauriello et al., 2000) and EFSA recommendations (2005) for assessment of antibiotic resistances in bacteria. As shown in Table 1, the incidence of antibiotics resistance strongly varied from species to species. Resistance was more common among isolates belonging to the species *S. equorum, S. succinus* and *S. xylosus*. More than 60% of the strains of *S. equorum* and 90% of the strains of *S. succinus* and *S. xylosus* exhibited resistances to antibiotics. With regard to multiple resistances, strains of *S. succinus* exhibited up to 4 antibiotic resistances per strain, whereas strains of *S. equorum* and *S. xylosus* were resistant against up to 6 and even up to 7 antibiotics. Multiple resistant strains even included the type strains of *S. xylosus* and *S. equorum*. Strains belonging to the species *S. carnosus, S. condimenti,* and *S. piscifermentans,* on the other hand showed substantially fewer resistances to antibiotics (Table 1). The average number of phenotypic resistances was either one or two among *S. carnosus* and *S. piscifermentans* strains. Actually, the two investigated *S. condimenti* strains which included the *S. condimenti* type strain exhibited no resistances to the antibiotics investigated.

**Table 1:** Incidence of (multiple) antibiotic resistances of 330 CNS strains belonging to the species *S. carnosus*, *S. piscifermentans*, *S. condimenti*, *S. equorum*, *S. succinus* and *S. xylosus*

| Species (number of strains investigated) | Resistant strains (%) | Number of strains resistant against number of antibiotic(s) | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | 1 | 2 | 3 | 4 | 5 | 6 | 7 |
| *S. carnosus* (106) | 12 | 12 | 1 | | | | | |
| *S. condimenti* (2) | 0 | | | | | | | |
| *S. piscifermentans* (11) | 27 | 1 | 2 | | | | | |
| *S. equorum* (64) | 63 | 24 | 10 | 3 | | | 3 | |
| *S. succinus* (10) | 90 | 2 | 1 | 5 | 1 | | | |
| *S. xylosus* (137) | 95 | 17 | 17 | 34 | 32 | 22 | 7 | 1 |

**3. 2. Species and strain specificity of antibiotic resistance phenotypes**

Regarding the 21 antibiotics used for investigation of antibiotic resistances in CNS, the highest percentage of strains (>30%) were resistant against lincomycin and penicillin (Table 2). Furthermore, resistances to fusidic acid, oxacillin and ampicillin were observed in more than 20% of the strains. Approximately 18% of the CNS showed resistances against tetracycline and low percentages (3% or less) exhibited resistances to erythromycin, cefoxitin, rifampin and ofloxacin. Interestingly, all strains were sensitive to the clinically important antibiotics chloramphenicol, clindamycin, cotrimoxazol, gentamicin, kanamycin, linezolid, neomycin, streptomycin and synercid, as well as vancomycin, all of which have application in human therapy.

Among *S. carnosus* strains there were only few which showed resistance to antibiotics of clinical importance, e.g. less than 3% of strains showed resistance against ampicillin, tetracycline and oxacillin. Remarkably, 18% of the *S. piscifermentans* strains exhibited resistances to tetracycline, while no further resistances towards other clinically important antibiotics could be observed. *S. carnosus* and *S. piscifermentans* strains most frequently exhibited resistances to lincomycin (Table 2), while the type strains of *S. carnosus* ssp. *carnosus* DSM 20501$^T$, *S. carnosus* ssp. *utilis* DSM 11676$^T$, *S. condimenti* DSM 11674$^T$ and *S. piscifermentans* DSM 7373$^T$ exhibited no resistances at all.

Antibiotic resistance to lincomycin was also the most frequently encountered resistance among *S. equorum*, *S. succinus* and *S. xylosus* strains, but in contrast to *S. carnosus* and *S. piscifermentans*, the percentage of resistant strains was notably higher (approx. 50% or more) (Table 2). When comparing with strains belonging to the *S. carnosus* and *S. piscifermentans* species, these strains also exhibited more resistances to antibiotics of clinical importance. Thus 14% and 19% of *S. equorum* strains were resistant to erythromycin and oxacillin, respectively. Furthermore, 20% of *S. succinus* strains exhibited resistances to oxacillin and tetracycline, whereas approximately the double of *S. xylosus* strains showed resistances towards these antibiotics. The highest percentages of resistant strains were found among *S. succinus* and *S. xylosus* species. Accordingly, almost 50% and 70% of these strains exhibited resistance against the β-lactam antibiotics ampicillin and penicillin, respectively. Finally, half of the investigated *S. xylosus* strains were resistant to fusidic acid, but the role of this resistance is not important as this antibiotic is not used in either human or veterinary medicine.

With the exception of *S. equorum* ssp. *linens* DSM 15097$^T$, which was not resistant to the antibiotics tested, the type strains of *S. equorum*, *S. succinus* and *S. xylosus* exhibited also more resistances than the type strains of *S. carnosus* and *S. piscifermentans*.

**Table 2:** Distribution of antibiotic resistances detected in food-associated CNS belonging to the species *S. carnosus*, *S. piscifermentans*, *S. equorum*, *S. succinus* and *S. xylosus*

| Antibiotic | Incidence of resistance in all CNS | | Incidence of resistance (%) in species[a] | | | | |
|---|---|---|---|---|---|---|---|
| | Total number | (%) | *S. carnosus* (106) | *S. piscifermentans* (11) | *S. equorum* (64) | *S. succinus* (10) | *S. xylosus* (137) |
| Lincomycin | 120 | 36,4 | 6 | 27 | 48 | 80 | 53 |
| Penicillin | 104 | 31,5 | | | 6 | 60 | 69 |
| Fusidic acid | 74 | 22,4 | 1 | | 6 | 10 | 50 |
| Oxacillin | 73 | 22,1 | 3 | | 19 | 20 | 41 |
| Ampicillin | 71 | 21,5 | 1 | | | 40 | 48 |
| Tetracycline | 59 | 17,9 | 1 | 18 | 6 | 20 | 36 |
| Novobiocin | 35 | 10,6 | | | 8 | | 22 |
| Erythromycin | 11 | 3,3 | | | 14 | | 1 |
| Cefoxitin | 6 | 1,8 | 2 | | | | 3 |
| Rifampin | 2 | 0,6 | | | 3 | | |
| Ofloxacin | 1 | 0,3 | | | 2 | | |

[a] All food-associated CNS strains were sensitive to the antibiotics chloramphenicol, clindamycin, cotrimoxazol, gentamicin, kanamycin, linezolid, neomycin, streptomycin, synercid, and vancomycin.

### 3. 3. Antibiotic resistances depending of the origin of isolation

Differences in the incidence of antibiotic resistances depending on the origin of isolation were noted to occur (Table 3). Strains from hard and soft cheese (87%), sausage (83%), and meat starter cultures (93% excluding the *S. carnosus* strains) exhibited similar high percentages of resistances. Lower percentages of resistant strains were shown for strains isolated from cured ham (50% of the *S. equorum* strains). This is noteworthy because in this study 63% of all *S. equorum* isolates exhibited resistances. In contrast, low percentages of antibiotic resistances were shown for strains isolated from fermented fish (19%) and *S. carnosus* strains isolated from meat starter cultures (14%). Remarkably, *S. carnosus* strains isolated from sausage and from soy beans, as well as isolates belonging to the *S. condimenti* species were susceptible to all antimicrobials tested.

**Table 3:** Relationship between the incidence of antibiotic resistances and the source of isolation of food-associated CNS belonging to the species *S. carnosus*, *S. piscifermentans*, *S. equorum*, *S. succinus* and *S. xylosus*

| Origin | Total strains of origin / Species (Number of strains investigated) | Resistant strains (%)[a] | Phenotypic resistances[b] (%) in disk diffusion testing | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | AM | E | FA | FOX | L | NB | OFX | OX | P | RA | TE |
| Cured ham | Total strains (36) | 53 | 8 | 8 | 3 | | 33 | 3 | 3 | 19 | 8 | | 6 |
| | *S. equorum* (30) | 50 | | 7 | | | 30 | 3 | 3 | 17 | | | |
| | *S. xylosus* (6) | 67 | 50 | 17 | 17 | | 50 | | | 33 | 50 | | 33 |
| Fermented fish | Total strains (26) | 19 | | | | | 15 | | | 4 | | | 8 |
| | *S. carnosus* (15) | 13 | | | | | 7 | | | 7 | | | |
| | *S. piscifermentans* (11) | 27 | | | | | 27 | | | | | | 18 |
| Hard and soft cheese | Total strains (31) | 87 | 32 | 16 | 16 | 3 | 65 | 6 | | 35 | 55 | 6 | 42 |
| | *S. equorum* (17) | 76 | | 29 | 18 | | 65 | 12 | | 35 | 18 | 12 | 18 |
| | *S. succinus* (4) | 100 | 100 | | | | 100 | | | 25 | 100 | | |
| | *S. xylosus* (10) | 100 | 60 | | 20 | 10 | 50 | | | 40 | 100 | | 100 |
| Meat starter culture[b] | Total strains (139) | 46 | 25 | | 19 | 3 | 24 | 9 | | 14 | 24 | | 14 |
| | *S. carnosus* (81) | 14 | 1 | | 1 | 2 | 6 | | | 2 | | | 1 |
| | *S. xylosus* (57) | 93 | 60 | | 44 | 4 | 49 | 23 | | 30 | 60 | | 32 |
| Sausage[b] | Total strains (87) | 83 | 23 | 2 | 47 | 1 | 49 | 22 | | 40 | 52 | | 23 |
| | *S. equorum* (15) | 67 | | 7 | 7 | | 60 | 13 | | 7 | | | 7 |
| | *S. succinus* (3) | 100 | | | 33 | | 67 | | | 33 | 67 | | 67 |
| | *S. xylosus* (60) | 98 | 33 | 2 | 65 | 2 | 53 | 28 | | 55 | 72 | | 28 |
| Water buffalo raw milk | *S. xylosus* (4) | 100 | 75 | | 25 | | 100 | | | | 100 | | 75 |
| Patients in clinics | Total strains (5) | 80 | | 20 | | | 80 | | | | 20 | | |
| | *S. equorum* (2) | 100 | | 50 | | | 100 | | | | 50 | | |
| | *S. succinus* (3) | 67 | | | | | 67 | | | | | | |

[a] *S. condimenti* isolated from meat starter culture (one strain), *S. carnosus* isolated from sausage (nine strains) and the isolates of soy bean (one *S. carnosus* and one *S. condimenti* strain) were sensitive to all antibiotics tested.

[b] Antibiotics: Am, ampicillin; E, erythromycin; FA, fusidic acid; FOX, cefoxitin; L, lincomycin; NB, novobiocin. OFX, ofloxacin; OX, oxacillin; P, penicillin; RA, rifampin; TE, tetracycline. All food-associated CNS strains were sensitive to the antibiotics chloramphenicol, clindamycin, cotrimoxazol, gentamicin, kanamycin, linezolid, neomycin, streptomycin, synercid, and vancomycin.

### 3. 4. Detection of antibiotic resistance genes

PCR amplification of genomic DNA of ampicillin, lincomycin, oxacillin, penicillin and tetracycline resistant strains were used to link the phenotype to known antibiotic resistance genes (data not shown). The widespread occurrence of the tetracycline resistance gene *tet*K was exemplified by its presence in 93% of the 59 strains investigated. Strains in which the *tet*K gene was detected belonged to the species *S. piscifermentans*, *S. equorum*, *S. succinus* and *S. xylosus*, and were obtained from all origins, except from soy bean and from patients in clinics. The *tet*K gene was, however, not detected in one tetracycline-resistant *S. carnosus* strain. In contrast to widespread occurrence of *tet*K, the genes *lnu*A and *bla*Z, encoding resistances towards lincomycin and the β-lactam antibiotics ampicillin and penicillin, could be detected at a far lower incidence among the CNS. The *bla*Z gene was observed in 10% of the 104 strains which were resistant to β-lactam antibiotics. The *bla*Z gene was found in strains isolated from different origins, e.g. cured hams, cheese and sausages. Interestingly, only isolates of the *S. xylosus* species exhibited the *bla*Z gene. Similarly, the *lnuA* gene was also found only in a low percentage (3%) of the 120 strains which showed phenotypical resistance to lincomycin. This gene could only be detected in three lincomycin-resistant *S. piscifermentans* strains, and one *S. xylosus* strain isolated from water buffalo raw milk. Remarkably, the strains which harboured the *lnu*A gene, showed no inhibition zone for lincomycin in the disk diffusion test indicating a high incidence of lincomycin resistance of these strains. Finally, the tetracycline resistance genes *tet*L and *tet*M and the methicillin/oxacillin resistance gene *mec*A could not be detected in any of the resistant strains using PCR and specific primers.

## 4. Discussion

In this study, we showed that among food-associated staphylococci the incidence of resistant strains and the number of antibiotic resistances within strains are notably higher for *S. xylosus*, *S. equorum* and *S. succinus* (in the following named the *S. xylosus*-group) when compared to *S. carnosus*, *S. condimenti* and *S. piscifermentans* (in the following named the *S. carnosus*-group). This finding is in agreement with previous studies showing that strains of *S. carnosus* exhibit markedly less antibiotic resistances than strains of *S. xylosus* (Gardini et al., 2003; Martín et al., 2006). In addition, our grouping defined on the basis of the incidence

of antibiotic resistances is consistent with the phylogenetic grouping of staphylococci based on *dnaJ* gene sequences and rRNA gene fragment analysis (Götz et al., 2006; Kloos et al., 1992; Shah et al., 2007; Takahashi et al., 1999). Notably, all species of the *S. carnosus*-group are members of the phylogenetic *S. simulans*-group, in which only non-pathogenic staphylococci are included. On the other hand, all species of the *S. xylosus*-group belong to the phylogenetic *S. saprophyticus*-group which contains *S. saprophyticus*, an important opportunistic pathogen in human urinary tract infections (Götz et al., 2006). Thus, it is tempting to speculate that there is a correlation between the incidence of antibiotic resistances in food-associated CNS and their relatedness to (opportunistic) pathogenic *Staphylococcus* species. Research on the incidence of antibiotic resistances in pathogens revealed that as a result of continuous challenge with antibiotics, pathogens more frequently acquire resistances and multiple resistances (Kapil, 2005; Kresken and Hafner, 1999; Livermore, 2007). This is consistent with the observation that most *S. saprophyticus* strains exhibited resistances against numerous antibiotics, including those of clinical importance (Price and Flournoy, 1982). In addition, there seems to be a correlation between the transfer rate of antibiotic resistance determinants and the relatedness of donor and recipient, as shown by Udo et al. (1997) for the transfer of the mupirocin resistance from *S. haemolyticus* to *S. aureus*, *S. epidermidis*, *S. saprophyticus* and *S. haemolyticus*. Thus, the increased incidence of (multiple) antibiotic resistances in strains of the *S. xylosus*-group may be explained by the close relatedness of the species to the opportunistic pathogen *S. saprophyticus*. This hypothesis is supported by our finding that species of the *S. xylosus*-group contain higher percentages of antibiotic resistant strains than the species of the *S. carnosus*-group when compared to *S. saprophyticus* strains, especially with regard to resistances against penicillin, oxacillin and tetracycline (Price and Flournoy, 1982). Furthermore, the highest percentage of resistant strains (27%) were shown to occur amongst *S. piscifermentans* strains, which in the *S. carnosus*-group is the closest relative of the pathogenic staphylococci (Shah et al., 2007). In contrast, only 12% of the *S. carnosus* and none of the *S. condimenti* strains were antibiotic resistant.

In general, we did not observe that the incidence of antibiotic resistances depends on the source of isolation of the CNS. Only strains of the *S. xylosus*-group isolated from cheese, meat starter cultures and sausage exhibited higher percentages of antibiotic resistances (above 83%), with exception of the *S. equorum* strains isolated from cured ham (50% of resistant strains). Kaszanyitzky et al. (2003) investigated the antibiotic susceptibility of staphylococci isolated from humans, food and different animal species, and also found that the origin may

be of secondary importance in determining antibiotic resistance profiles. Therefore, it is not surprising that the two *S. equorum* and three *S. succinus* strains isolated from patients in clinics (Novakova et al., 2006) exhibited a low incidence of resistance, and were only resistant to the antibiotics erythromycin, lincomycin and penicillin.

Regarding the clinical important antibiotics we could show that all food-associated CNS are quite sensitive to chloramphenicol, clindamycin, cotrimoxazol, gentamicin, kanamycin, linezolid, neomycin, streptomycin, synercid and vancomycin. This finding is in contrast to published data showing that strains of *S. carnosus* and/or *S. xylosus* can be resistant to gentamicin, kanamycin, neomycin and clindamycin, even at low incidence (Gardini et al. 2003; Mauriello et al. 2000; Perreten et al., 1997). Furthermore, in our study none of the CNS exhibited resistance against vancomycin. This observation is consistent with results of the studies of Kastner et al. (2006), Martín et al. (2006) and Mauriello et al. (2000), demonstrating the absence of vancomycin resistances in *S. carnosus* and *S. xylosus* isolates obtained from fermented sausage and meat starter cultures. In contrast, Holley and Blaszyk (1998) reported on three vancomycin-resistant *S. carnosus* strains which showed no growth inhibition in the presence of 256 µg/ml vancomycin. Remarkably, this concentration is eightfold higher than the MIC concentration described by the Centers for Disease Control and Prevention (2002) for vancomycin-resistant *S. aureus* (VRSA, MIC 32 µg/ml) with clinical importance.

Our investigation on the underlying genetic determinants revealed that the tetracycline resistance gene *tet*K appears to be widely distributed among the food-associated CNS (93% of cases). This is consistent with results obtained by hybridisation and PCR analysis of tetracycline-resistant and food-associated *S. xylosus* strains (Kastner et al., 2006; Perreten et al., 1998). On the other hand, we detected the *lnuA* gene which encodes lincomycin resistance and which was formerly described in *S. haemolyticus* as gene *lin*A (Brisson-Noël and Courvalin, 1986), in only about 3% of the strains exhibiting resistance to lincomycin. Detection of this gene was described by Lüthje et al. (2007) for nine CNS strains isolated from bovines and by Perreten et al. (1998) for one *S. haemolyticus* isolate. However Kastner et al. (2006) could not detect this gene in four *S. xylosus* strains isolated from meat starter cultures. A comparably low correlation of phenotypic and genotypic detection of antibiotic resistance was shown in this study for β-lactam antibiotics and the *bla*Z gene (10% correlation). Similarly, Olsen et al. (2006) showed that the *bla*Z gene could be detected in

only 6 of the 60 investigated CNS isolates. Moreover, we could not detect the *mec*A gene which mediates methicillin and oxacillin resistance in any of the phenotypically resistant CNS. Martín et al. (2006) detected *mec*A in only 3.6% of 194 *S. xylosus,* and in none of 11 *S. carnosus* strains. The discrepancy of phenotypic resistance against lincomycin or β-lactam antibiotics and detection of *lnu*A or *bla*Z and *mec*A leads to the conclusion that these resistances may constitute an intrinsic resistance, or that it may be the result of possible unknown resistance genes. Nevertheless, it can be concluded that the genetic determinants or mechanism of antibiotic resistances in food-associated CNS are different to that described in pathogenic staphylococci.

The incidence of antibiotic resistances in food-associated bacteria may cause problems due to the risk of transfer of antibiotic resistance determinants (Teuber et al., 1999). In this study, we investigated for the first time antibiotic resistance profiles in strains of the species *S. equorum*, *S. succinus* and *S. piscifermentans*, thus permitting conclusions on the occurrence of antibiotic resistances in the phylogenetic groups of CNS. Due to the large number of strains investigated, we conclude that especially strains of the *S. xylosus*-group exhibit resistances in high numbers, and that this high incidence is probably a reflection of the close phylogenetic relationship of these food-associated CNS with the pathogenic Staphylococcus species. Additional studies, especially genetic studies are needed to assess the contribution of the presence of antibiotic resistances in food-associated CNS to the distribution of antibiotic resistance determinants within the food chain.

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# *Chapter IV*

## Characterization of toxin production of coagulase-negative staphylococci isolated from food and starter cultures

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## Abstract

In this study a comprehensive analysis of toxin production of food associated coagulase-negative staphylococci (CNS) was investigated. The strains belong to the following staphylococcal species, *Staphylococcus carnosus, Staphylococcus condimenti*, *Staphylococcus equorum, Staphylococcus piscifermentans, Staphylococcus succinus,* and *Staphylococcus xylosus,* which were isolated from fermented food and starter cultures. A collection of 330 strains were analyzed with respect to their hemolytic activity. 59% of the strains exhibited weak to moderate hemolytic activity with human blood and 34% with sheep blood after 48 h incubation. A selection of 35 strains were tested by immunoblot analysis for their ability to produce toxins, such as the most common staphylococcal enterotoxins (SEs), the toxic shock syndrome toxin 1 (TSST-1), and the exfoliative toxin A (ETA). 18 of the 35 strains produced at least one of the toxins with the SED and SEH being the most common. These indicate that the use of CNS in food production demands a safety evaluation.

## 1. Introduction

The number of species of the genus *Staphylococcus* is steadily increasing. While 36 species are listed in the 2006 review (Götz *et al.*, 2006), currently, 41 species are described (http://www.dsmz.de/dsmz/). Most of the species are harmless and have never been associated with any kind of infection. However, some species of this genus cause a variety of diseases by production of a series of enzymes and toxins, invasion of host cells and tissues, and their ability to escape the immune system. The coagulase-positive *Staphylococcus aureus* produces a great number of toxins and is the best known staphylococcal pathogen. For long time *S. aureus* was believed to be the only pathogen in this genus, while the coagulase-negative staphylococci (CNS) were expected to be saprophytic or rarely pathogenic (Kloos and Bannerman, 1994). However, there are some CNS species such as *Staphylococcus epidermidis, Staphylococcus haemolyticus, Staphylococcus saprophyticus* that play a role in nosocomial and bloodstream infections (Jarvis and Martone, 1992; Pittet and Wenzel, 1995; Spencer, 1994, 1996). As essentially opportunistic microorganisms CNS can cause severe infections, especially among immunocompromised people, and are often difficult to treat because of the relatively high prevalence of multiresistant strains. In reports from different

parts of Europe the oxacillin resistence of CNS lies between 70% and 80% (Diekema *et al.*, 2001; Gardini *et al.*, 2003). Similar rates were observed in the USA, Canada and Latin America (Diekema *et al.*, 2001; Hanberger *et al.*, 2001; Kloos and Bannerman, 1994; Vincent *et al.*, 1995; Vincent, 2000). In some Brazilian health institutions, oxacillin resistance may be present in over 80% of isolates (Sader *et al.*, 2002). In a recent study Dar *et al.* (2006) showed that 22.5% of CNS isolates from 750 human subjects were resistant to methicillin. Antibiotic resistant strains were also found in food (Gardini *et al.*, 2003; Martin *et al.*, 2006) and genes for microbial resistance to tetracycline, erythromycin and β-lactams have been detected in CNS isolated from starter cultures, probiotic bacteria, fermented food and meat (Simeoni *et al.*, 2008).

Later reports even postulate that an antibiotic-induced SOS response promotes horizontal dissemination of pathogenicity island-encoded virulence factors in staphylococci (Ubeda *et al.*, 2005). These virulence factors, responsible for severe infections, include toxins such as hemolysins a, b, g and d, leukocidin, exfoliative toxins A and B, toxic shock syndrome toxin 1 (TSST-1), and a family of emetic pyrogenic superantigens, classified after their biological activities and structural relatedness (Balaban and Rasooly, 2000; Dinges *et al.*, 2000). Hemolysins are exotoxins that cause lysis of erythrocytes either by pore formation (α-hemolysis) or cytolytic action by degrading sphingomyelin (β-hemolysis). They are distinguishable by the hemolysing reaction on blood agar plates. *S. aureus* α-toxin, a protein of 34 kDa, leads to clear hemolysis zone and induces concentration dependent cell death via the apoptotic pathway (Essmann *et al.*, 2003). On the other hand, β-toxin has a molecular mass of 35 kDa and is also known as hot-cold toxin because hemolysis is absent or incomplete at 37 °C. Cooling the blood agar plates results in rapid hemolysis (Smyth *et al.*, 1975). These membrane damaging toxins possess cytotoxic, dermonecrotic and hemolytic properties and finally kill the target cells (Bernheimer, 1965; Thelestam and Blomqvist, 1988). At present there are 17 well-characterized, serologically distinct superantigens known in *S. aureus*: TSST-1, SE A, B, C (multiple minor variant forms exist), D, E, and I; and SE-like G, H, J, K, L, M, N, O, P, and Q (Avena and Bergdoll, 1967; Jarraud *et al.*, 2001; Munson *et al.*, 1998; Reiser *et al.*, 1984; Su and Wong, 1995; Vincent, 2000; Zhang *et al.*, 1998). In addition, SE-like proteins R, S, T, and U have been identified but are poorly characterized. Due to their superantigen nature, enterotoxins bind directly to the major histocompatibility complex class II molecule without undergoing the typical processing of normal antigens, which is caused by T cell stimulation and excessive production of cytokines such as

interleukin 1 (IL-1), IL-2, interferon gamma (IFN-g) and tumor necrosis factor alpha (TNF-a) (Huang *et al.*, 2007). Staphylococcal scalded-skin syndrome is an exfoliative dermatitis caused by cleavage of desmoglein 1, which is accompanied by separation of the epidermis at the stratum granulosum (Amagai *et al.*, 2002). This disruption is mediated by one of two *S. aureus* exotoxins, exfoliative toxins A and B (ETA and ETB). In contrast to earlier reports ETA and ETB are not superantigens (Plano *et al.*, 2000). Staphylococcal enterotoxins are water-soluble exoproteins with a molecular weight ranging from 26 to 30 kDa and are characterized by a disulfide loop close to the center of the molecule (Iandolo, 1989; Proft and Fraser, 2003). They are resistant to inactivation by gastrointestinal proteases such as pepsin as well as by heat. Particularly the heat stability of SEs is very problematic in food production (Hernandez *et al.*, 1993; Hilker *et al.*, 1968; Hoover *et al.*, 1983; Humber *et al.*, 1975; Lee *et al.*, 1977). Most of the outbreaks of food poisoning of bacterial origin in the world are caused by ingestion of food containing SEs (Jaulhac *et al.*, 1991). In many cases the origin of *S. aureus* infection is food associated and to date little attention has been paid to the toxigenic profile of CNS. However, there are several reports that suggest that more attention should be paid to this group of microorganisms. Enterotoxin production has been described in some CNS strains belonging to the following species: *S. capitis*, *S. cohnii*, S. *epidermidis*, S. *haemolyticus*, *S. hyicus subsp. hyicus*, or *S. xylosus* (Balaban and Rasooly, 2000; Breckinridge and Bergdoll, 1971; Hoover *et al.*, 1983; Olsvik *et al.*, 1982).

Strains of CNS such as *Staphylococcus carnosus* and *Staphylococcus xylosus* strains are traditionally used in meat fermentation. The species *Staphylococcus condimenti*, *Staphylococcus piscifermentans*, *Staphylococcus equorum* and *Staphylococcus succinus* are often isolated from fermented food and may therefore have the potential for future application as starter or protective cultures (Hoppe-Seyler *et al.*, 2004; Place *et al.*, 2002; Place *et al.*, 2003; Probst *et al.*, 1998; Tanasupawat *et al.*, 1992). The latest reports provide evidence that *S. xylosus* species isolated from ham contain enterotoxin producing strains. Vernozy-Rozand (Vernozy-Rozand *et al.*, 1996) reported enterotoxin E (SEE) as the most frequent enterotoxin found in *S. xylosus* and *S. equorum* isolated from food. There are also reports which emphasize that some CNS are able to produce TSST-1 alone or in combination with other enterotoxins (Crass and Bergdoll, 1986; Kahler *et al.*, 1986). In the study of Valle *et al.* (1991) 16% of the coagulase-negative strains were found to be TSST-1 producers. This data underscores the need for more in depth studies to better characterize the pathogenic potential of CNS species used in food production.

## 2. Materials and methods

### 2.1. Bacterial strains and growth conditions

In this study 330 CNS and the type strains of *S. carnosus*, *S. condimenti*, *S. equorum*, *S. succinus*, *S. piscifermentans* and *S. xylosus* were used. All CNS were isolated from various foods, commercial starter cultures and patients (see Table 1). The strains were identified to species level by 16S and/or 23S rDNA gene sequence analysis as described by Resch et al. (in press). All 330 strains were tested with respect to hemolytic activity. Of these strains, 35 were selected for investigation of the production of superantigens and ETA by Western blotting.

**Table 1:** Species and habitat of the CNS isolates examined in this study

| Species (no. of isolates) | Habitat (no. of isolates) |
|---|---|
| *S. carnosus* (106) | Fermented fish (15), meat starter culture (81[a]), sausage (9), soy (1) |
| *S. condimenti* (2) | Meat starter culture (1[a]), soy (1) |
| *S. equorum* (64) | Clinical isolate (2), cured ham (30), sausage (15), hard and soft cheese (17) |
| *S. piscifermentans* (11) | Fermented fish (11) |
| *S. succinus* (10) | Clinical isolate (3), sausage (3), hard and soft cheese (4) |
| *S. xylosus* (137) | Cured ham (6), hard and soft cheese (10), meat starter culture (57[a]), sausage (60), water buffalo raw milk (4) |

[a] Strains were isolated from commercial meat starter cultures.

### 2.2. Hemolysis test

All 330 CNS strains were cultivated first on Standard I nutrient agar (Merck) at 37 °C overnight. Blood agar plates had a volume of 20 ml agar and were prepared with Difco™ Tryptose Blood Agar Base (Becton Dickinson) and supplemented with 5% sterile defibrinated sheep blood (Biomerieux, Ca. No. 55822) or sterile defibrinated human blood (centre for blood donation Katharinenhospital Stuttgart: human erythrocyte concentrate leucocytes depleted). Defibrination of human blood was performed for 3 days at 4 °C by addition of Alsever-solution (20.5 g glucose, 8.0 g sodium citrate, 0.55 g citric acid, 4.2 g sodium chloride) at a ratio of 1:1. The blood agar plates were inoculated with the CNS, grown aerobically for 24 and 48 h at 37 °C, transferred to 4 °C overnight and examined for hemolytic activity. Quality control of the blood agar plates was performed with the test strains

*S. aureus* ATCC25923, *S. aureus* RN4220 (β-hemolysis) and *S. aureus* NCTC8325 (α-hemolysis). Strains with hemolysis zones of more than 1 mm width from the border of the colony were evaluated as moderate hemolysing, strains with small zones less than 1 mm as weak hemolysing.

### 2.3. Growth conditions for immunoblot analysis

For verification of toxins by immunoblot analysis the cellophane-over-agar (COA) cultivation method as recommended by Robbins et al. (1974) was used. With this cultivation method we obtained in general a higher content of toxins than in liquid culture. Strains were cultivated on Brain Heart Infusion (BHI, Roth) medium, pH 7.2. For solid BHI agar plates 15.0 g/l agar-agar was supplemented. Fresh BHI medium was inoculated with overnight cultures and grown to an $OD_{450nm}$ of 0.5. The cultures were diluted to a concentration of 5 x $10^6$ cells/ml and used for the cellophane-over-agar (COA) method. For cellophane cultures sterile NADIR dialysis membranes (cutoff of 10 to 20 kDa; Roth) were placed on BHI-agar in a 9 cm petri dish. The membranes were sterilized by autoclaving in aqua bidest for 20 min and then placed on agar plates. 0.2 ml of the diluted overnight cultures were plated on the COA and were cultivated for 16 h at 35 °C and for 40 h at 25 °C. The cultures and their secreted proteins were harvested by washing with 1 ml of sterile 20 mM Tris-HCl, pH 7.2 solution. The cells were pelleted by centrifugation for 10 min at 4500 x g, the supernatants filter sterilized using a 0.45 µm filter and stored at -20 °C.

### 2.4. Immunoblot analysis of superantigen production

A subset of 35 strains from the collection were selected for testing of superantigens. Selection criteria were that the six CNS species from various habitats are represented, and also a balance between antibiotic resistant and sensitive isolates (according to the study of Resch et al., in press) as well as hemolytic and nonhemolytic strains was considered. To assess superantigen production a 20 µl sample of the culture supernatants was separated on 12% SDS-PAGE according to Schagger and Jagow (Schagger *et al.*, 1988) and electrophoretically transferred (1.5 h, 300 mA) to nitrocellulose membranes (Protran nitrocellulose Schleicher and Schuell BA85) using a Semi-Dry-Blotting apparatus from Biorad. The semi-dry-transfer procedure was carried out according to the QIAexpress Detection and Assay Handbook 10/2002. For the washing of the membranes TBS buffer (10 mM Tris-HCl, pH 7.5/150 mM NaCl) and TBS-Tween/Triton buffer (20 mM Tris-HCl, pH 7.5, 500 mM NaCl, 0.05% (v/v) Tween20, and 0.2% (v/v) TritonX-100) were used. After blotting the membrane was washed

twice for 10 min with TBS buffer at room temperature (RT) and incubated overnight with 1x Roti-Block, blocking reagent (Roth, Karlsruhe, Germany) including goat anti-proteinA-polyclonal antibody diluted 1:10,000 (GeneTex, Inc. USA). The blocked membranes were washed twice for 10 min with TBS-Tween/Triton buffer and once for 10 min with TBS buffer. Then they were incubated with the specific anti-toxin antibodies diluted 1:1000 in 1x Roti-Block Buffer. Specific rabbit polyclonal antibodies against SEA, SEB, SEC, SED, SEH and TSST-1 and specific sheep polyclonal antibodies against SEE and ETA were used. The immuno blot was gently shaken for 1 h at room temperature and subsequently washed as described above. Then the membranes were incubated with the alkaline phosphatase labeled secondary antibodies, diluted 1**:**20,000 in 1x Roti-Block buffer. For the chemiluminescent detection NBT/BCIP-ready-to-use solution (Sigma-Aldrich) was used according to the manufacturer´s instructions.

## 3. Results

### 3.1. Hemolytic activity

There are six CNS species, *S. carnosus, S. condimenti, S. piscifermentans, S. xylosus, S. equorum,* and *S. succinus* which represent potential candidates for the use as a starter culture. In order to study the potential pathogenic risk of these species strains from different habitats were collected and classified in 330 strains that belong to one of the mentioned species. First of all the hemolytic activity on human and sheep blood agar was investigated (Table 2). Hemolysis was already seen after 24 h but increased after longer incubation. After 48 h incubation 34% of the 330 strains showed moderate and 25% weak hemolytic activity on human blood; 41% showed no hemolytic activity. In general, each species includes strains that exhibit hemolytic activity, and generally hemolysis was always stronger with human blood (Fig. 1).

**Table 2:** Hemolysis activity on human and sheep blood agar of food associated CNS strains

| Species (no. of strains tested) | Agar supplemented with | % of moderate (weak [a]) hemolysing isolates on human and sheep blood agar incubated for | |
|---|---|---|---|
| | | 24 h | 48 h |
| *S. carnosus* (106) | Human blood | 1.9 (2.8) | 3.8 (6.6) |
| | Sheep blood | - (-) | - (3.8) |
| *S. condimenti* [b] (2) | Human blood | 50.0 (-) | 50.0 (-) |
| *S. equorum* (64) | Human blood | 17.2 (37.5) | 43.8 (42.2) |
| | Sheep blood | - (15.6) | - (28.2) |
| *S. piscifermentans* (11) | Human blood | 36.4 (18.2) | 63.6 (-) |
| | Sheep blood | - (27.3) | 9.1 (54.5) |
| *S. succinus* (10) | Human blood | 40.0 (-) | 40.0 (-) |
| | Sheep blood | 20.0 (-) | 20.0 (20.0) |
| *S. xylosus* (137) | Human blood | 27.7 (19.0) | 49.6 (34.3) |
| | Sheep blood | 2.9 (12.4) | 6.6 (48.9) |
| Total strains (330) | Human blood | 18.2 (16.7) | 33.9 (24.5) |
| | Sheep blood | 1.8 (9.1) | 3.6 (29.4) |

[a] Weak hemolysis, small hemolysis zones less than 1 mm width; -, no hemolysis.
[b] No hemolysis was shown on sheep blood agar for *S. condimenti* isolates.

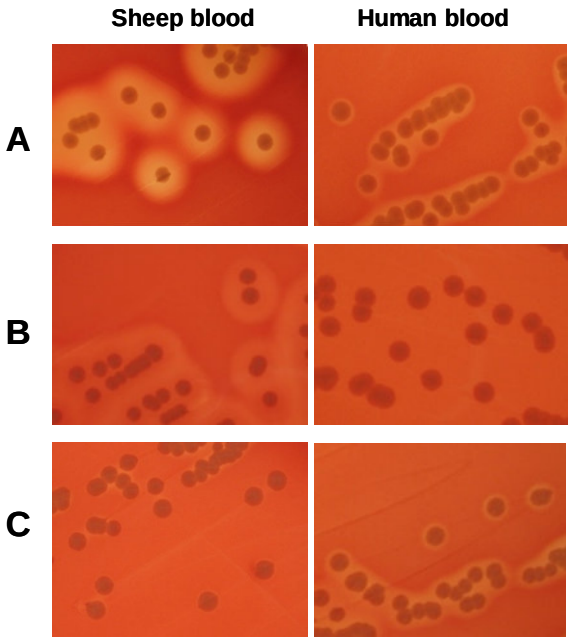


**Fig. 1:** a-hemolysing *S. aureus* NCTC 8325 (**A**), b-hemolysing *S. aureus* RN 4220 (**B**) and hemolysis of food associated *S. piscifermentans* isolate (**C**) on sheep and human blood agar after 24 h incubation.

Compared with the *S. aureus* control strains, most of the CNS isolates appeared to show b-hemolysis activity. A representative example of a/b-hemolysis by *S. aureus* and by a CNS strain on sheep *versus* human blood agar is shown in Fig. 1. Regarding the CNS species investigated, after 24 h incubation between 17 and 40% of the *S. equorum*, *S. piscifermentans*, *S. succinus* and *S. xylosus* strains exhibited moderate hemolysis with human blood and approximately 20% of the isolates showed weak hemolysis. For *S. carnosus* the lowest prevalence of hemolytic strains was observed. Of the 106 strains analyzed only approximately 4% exhibited moderate, and 6% weak hemolysis on human blood after 48 h. There is no real correlation between hemolysis and habitat (Table 3).

**Table 3:** Hemolysis activity on human and sheep blood agar of food associated CNS strains isolated from various habitats

| Habitat (no. of strains) | Agar supplemented with | % of moderate (weak [a]) hemolysing isolates on human and sheep blood agar incubated for | |
|---|---|---|---|
| | | 24 h | 48 h |
| Fermented fish (26) | Human blood | 23.1 (7.7) | 34.6 (3.8) |
| | Sheep blood | - (11.5) | 3.8 (34.6) |
| Hard and soft cheese (31) | Human blood | 19.4 (35.5) | 48.4 (22.6) |
| | Sheep blood | 6.5 (12.9) | 12.9 (19.4) |
| Clinical isolates (5) | Human blood | 40.0 (40.0) | 80.0 (-) |
| | Sheep blood | 20.0 (20.0) | 20.0 (40.0) |
| Cured ham (36) | Human blood | 19.4 (38.9) | 36.1 (52.8) |
| | Sheep blood | 2.8 (16.7) | 5.6 (36.1) |
| Soy (2) | Human blood | 50.0 (50.0) | 50.0 (50.0) |
| | Sheep blood | - | - |
| Meat starter cultures (139 [b]) | Human blood | 5.8 (7.9) | 12.2 (25.2) |
| | Sheep blood | - (2.2) | - (21.6) |
| Water buffalo raw milk (4) | Human blood | 50.0 (50.0) | 100.0 (-) |
| | Sheep blood | - (-) | - (50.0) |
| Sausage (87) | Human blood | 32.2 (13.8) | 56.3 (20.7) |
| | Sheep blood | 2.3 (14.9) | 4.6 (40.2) |

[a] Weak hemolysis, small hemolysis zones less than 1 mm width from the border of the colony, -, no hemolysis;
[b] Strains were isolated from commercial meat starter cultures.

However, an important question is whether strains currently used as starter culture show hemolytic activity. As mentioned in Table 1, among the 330 strains there were 81 *S. carnosus,* 57 *S. xylosus* strains, and 1 *S. condimenti* strain that were isolated from commercial meat starter cultures. Surprisingly, 12% of these meat starter cultures showed moderate hemolysis after 48 h incubation on human blood (Table 3).

The type strains *S. carnosus* ssp. *carnosus* DSM 20501, *S. carnosus* ssp. *utilis* DSM 11676$^{T}$, *S. equorum* ssp. *linens* DSM 15097, *S. succinus* ssp. *casei* DSM 15096, and *S. xylosus* DSM 20266 did not show hemolysis on human blood, while *S. condimenti* DSM 11674 and *S. piscifermentans* DSM 7373 revealed moderate hemolysis after 24 h. *S. equorum* ssp. *equorum* DSM 20674 and *S. succinus* ssp. *succinus* DSM 14617 showed weak hemolysis after 48 h.

**3.2. Production of superantigens and exfoliative toxins**

Since the analysis of superantigens and exfoliative toxins with 330 strains is time consuming, 35 CNS strains were selected. The CNS strains were analyzed with respect to production of superantigens such as enterotoxins and toxic shock syndrome toxin and proteolytic exfoliative toxin A. The most common enterotoxins (SEA — SEE, SHE, and ETA) were investigated in immunoblots using polyclonal antibodies specific for these toxins.

Most of these toxins are produced by *S. aureus* strains which were also used as reference strains (Table 4). According to the genome sequence, some strains contain a rather high number of annotated toxin genes such as the *S. aureus* strains N315, Mu50, NRS123. The annotation of an exotoxin gene is no guarantee that it is actually expressed. Therefore, the presence of exotoxins in the culture supernatant was verified by Western blot analysis. As it is well known that some enterotoxins are produced only at certain growth conditions, such as low temperature, growth on specific media, or on cellophane-over-agar (COA), various parameters were tested. The study found that BHI medium was superior to other media, and more exotoxins were produced by the COA method than by growth in liquid culture (data not shown).

**Table 4:** *S. aureus* strains used as positive control for toxin production

| Species | Strain | Exotoxin genes identified | Reference |
|---|---|---|---|
| *S. aureus* | N315 | sec , seg , sei , sel , sem , sen, seo , sep, tst-1, hla | Kuroda *et al.* (2001) |
| *S. aureus* | Mu50 | sea , sec , seg , sei , sel , sem , sen , seo, tst-1, hla | Kuroda *et al.* (2001) |
| *S. aureus* | Newman | seb | Schmidt *et al.* (2004) |
| *S. aureus* | COL | seb | Schmidt *et al.* (2004) |
| *S. aureus* | SA113 | No toxin gene published | Our strain collection |
| *S. aureus* | NRS 111 | sea , sec , see , tst-1 | C. Wolz |
| *S. aureus* | NRS 123 (MW2) | sea, sec, sed, seg2, seh, sek, sel, seo, seq, hla | Baba *et al.* (2002) |
| *S. aureus* | NRS 226 | sea , seh, sek | C. Wolz |

The strains always were cultivated at two different conditions: 16 h at 35 °C and 40 h at 25 °C and it was observed that temperature and incubation time had a strong influence on production of various toxins. In the Western blot purified TSST-1 was detectable as low as 0.83 ng (Fig. 2A). As shown with SEA it was possible to detect a positive signal in two of the 9 tested *S. carnosus* strains. The observed immuno bands were similar in size to the 27 kDa SEA of the *S. aureus* NRS226 control strain (Fig. 2B). The results of the exotoxin production in the 35 tested CNS strains are summarized in Table 5. With the exeption of *S. condimenti* and *S. succinus,* all other species include representatives which produce one or more of the tested enterotoxins; SEA, SED, SEE, and SEH are the most prevalent. ETA was only detectable in 2 strains and SEB, SEC and the TSST-1 were not detactable at all.

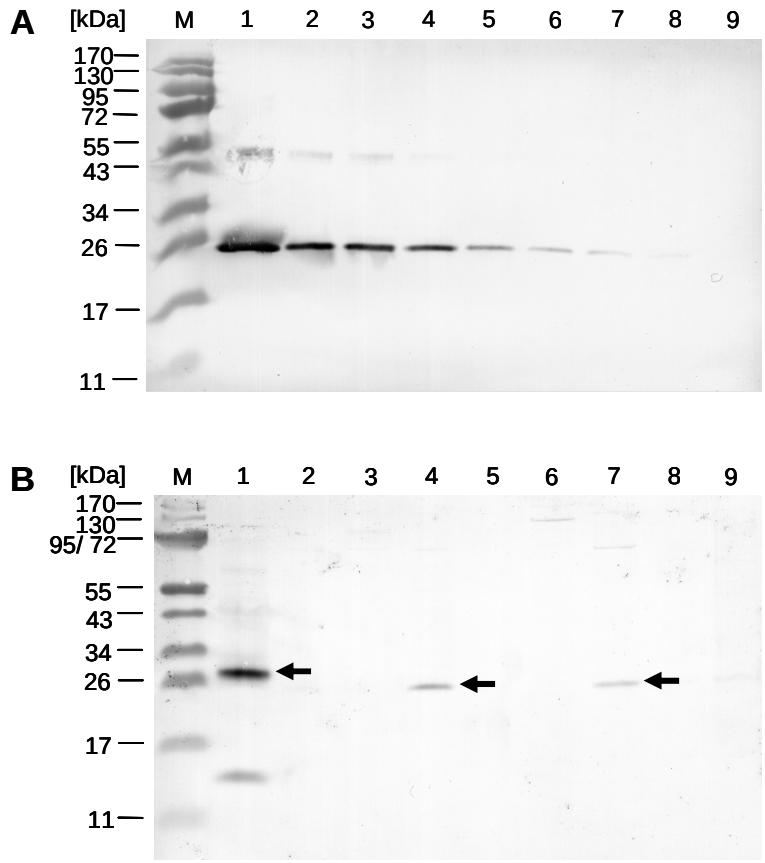


**Fig. 2:** Westernblot with TSST-1 antibody and purified TSST-1 (22 kDa) in following concentrations in lanes 1-9: 1, 83.33 ng; 2, 33.33 ng; 3, 16.66 ng; 4, 8.33 ng; 5, 3.33 ng; 6, 1.66 ng; 7, 0.83 ng; 8, 0.33 ng; 9, 0.00 ng (**A**). Westernblot with SEA antibody and culture supernatants of *S. aureus* NRS226 (positive control, lane 1, SEA = 27 kDa) and various *S. carnosus* strains (lanes 2-9). M, prestained marker proteins. Unspecific interactions with protein A in the *S. aureus* control strains was blocked with anti-protein A-antibody (**B**).

**Table 5:** Summary of exotoxin production in the 35 food associated CNS strains

| | No. of enterotoxin (SE) and exfoliative toxin A (ETA) positive strains[a] after 40 h at 25 °C | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Species (no. of isolates) | SEA | SEB | SEC | SED | SEE | SEH | TSST-1 | ETA |
| *S. carnosus* (9) | 2 | 0 | 0 | 0 | 1 | 1 | 0 | 1 |
| *S. condimenti* (2) | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| *S. equorum* (10) | 0 | 0 | 0 | 5 | 0 | 5 | 0 | 1 |
| *S. piscifermentans* (4) | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 |
| *S. succinus* (3) | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| *S. xylosus* (7) | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 |
| Total (35) | 2 | 0 | 0 | 5 | 1 | 8 | 0 | 2 |

[a] Cells were cultivated by the cellophane-over-agar method on BHI medium and incubated for 40 h at 25 °C. In *S. equorum* some strains produced more than one toxin.

## 4. Discussion

A large number of CNS species belong to the common microflora of fermented food. Due to their beneficial effects in food fermentation some of the species, such as *S. carnosus* (Schleifer and Fischer, 1982) or *S. xylosus* (Corbiere Morot-Bizot *et al.*, 2007; Dordet-Frisoni *et al.*, 2007; Schleifer and Kloos, 1975), are used as safe food starter cultures and feed additives. For almost 60 years *S. carnosus* has been used alone or in combination with other microorganisms, such as pediococci or lactobacilli, as a starter culture for the production of raw sausage. One of the main advantages of starter cultures in food processing is that the fermentation and the ripening process can be carried out under controlled conditions. Food poisoning and food spoilage of microorganisms can be suppressed, due to a more reliable fermentation process (Götz, 1990). During the ripening process of dry sausage *S. carnosus* exerts several desired functions. These include a gradual reduction of nitrate to nitrite, the development of a characteristic flavour, a moderate lowering of the pH, and the reduction of $H_2O_2$ produced by catalase-negative lactobacilli, thus preventing odours (Liepe and Porobic, 1983; Talon et al., 1999). Furthermore the use of *S. xylosus* as starter culture in sausages decreases the level of volatile compounds arising from lipid oxidation and so contributes to the aroma by avoiding rancidity (Barriere et al., 2001).

However, there are a number of other CNS species (*S. piscifermentans, S. condimenti, S. equorum,* and *S. succinus* subsp. *casei*) that are consistently found in great numbers in fermented food and which might play a role in the future as starter cultures in the food and feed sector. It is therefore desirable that there is a measure of national and international safety control. Examples of this are the "GRAS (generally recognized as safe)" status in USA, or the envisaged installation of "QPS" (Qualified Presumption of Safety) by the European Food Safety Authority, EFSA (EFSA, 2005). The QPS standard should guarantee that only safe (no health hazard) strains are used as starter cultures for food manufacturing.

A fairly large number (34%) of the 330 strains showed moderate hemolytic activity. It is difficult to say how risky β-hemolytic CNS strains are for the consumer. Huseby et al. (2007) found in *S. schleiferi* and *S. epidermidis* species specific differences in b-toxin whose protein sequence showed 72% and 52% homology to *S. aureus* b-toxin. It was also shown that the *S. intermedius* b-toxin exhibits a hemolysis activity on sheep erythrocytes which is five times higher than that of *S. aureus* b-toxin (Dziewanowska *et al.*, 1996). The authors suggest that the difference between the two b-toxins investigated could be a result of staphylococcal

adaption to the wide range of potential hosts. It should also be noted that the hemolytic activity can be influenced by synergistic effects of various hemolysins (Cifrian *et al.*, 1996) and therefore weak a- and b-hemolysis is difficult to distinguish on blood plates. One should use immunoblot to more reliably distinguish between weak a- and b-hemolysis. The tested CNS strains showed higher hemolysis activity on human than on sheep blood, while the *S. aureus* control strains exhibited a higher hemolysis activity on sheep blood, which was also described by Russell et al. (2006). Due to the generally higher sensitivity of CNS on human blood we recommend hemolysis testing not only on sheep but also on human blood. CNS strains used as starter culture should not express any hemolytic activity, as was also postulated by Franz et al. for enterococci with use in food production (Franz *et al.*, 1999).

A selection of 35 CNS strains were also investigated with respect to superantigen and exfoliative toxin A production (Table 5). PCR analysis was not a reliable tool, as the corresponding gene sequences varied too much from species to species. However, the immunoblot method provided evidence of enterotoxin and ETA production in the selected CNS strains. The sensitivity of this assay is fairly high; for example in *S. aureus* control strain the detection sensitivity of TSST-1 was 0.8 ng. For most of the toxins the optimal cultivation conditions were 40 h, 25 °C, and BHI, and COA. A fairly high number (18 of 35 tested strains) were toxin positive and some strains produced more than one toxin. The most prevalent enterotoxins were SED and SEH. Enterotoxin genes were previously detected in CNS strains isolated from dairy products (*S. xylosus, S. equorum, S. lentus, S. gallinarum, S. capitis*) (Vernozy-Rozand *et al.*, 1996) as well as meat (*S. xylosus, S. cohnii*) (Rodriguez *et al.*, 1994). SE and/or TSST-1 positive CNS strains were isolated from the hands of restaurant workers (Udo *et al.*, 1999).

This study shows that strains that are consistently found in great numbers in fermented food cannot necessarily be regarded as safe. Therefore, strains used in food production must be analyzed with respect of their toxigenic potential to avoid negative effects on the health of consumers.

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# *Chapter V*

## Binding to extracellular matrix proteins and formation of biogenic amines by food-associated coagulase-negative staphylococci

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## Abstract

In connection with a study on the DNA microarray based detection of genes involved in safety and technologically relevant properties (Seitter (née Resch) et al., 2011), food-associated coagulase-negative staphylococci (CNS) were investigated phenotypically with regard to their ability to bind to the extracellular matrix proteins (ECM) and to produce biogenic amines. The properties have been shown to be involved in the colonization of injured tissue and invasion into host cells as well as in pharmacologic effects on humans, respectively. The CNS exhibited a low, but nevertheless clearly measurable ECM binding capacity, except for strains of *Staphylococcus equorum* and *Staphylococcus succinus*, which show a comparable or even higher binding to fibrinogen and fibronectin than that of the control strain *Staphylococcus aureus* Cowan. Formation of biogenic amines could be often detected in *S. carnosus*, *S. condimenti* and *S. piscifermentans* strains, but rarely in *S. equorum* and not in *S. succinus* and *S. xylosus* strains. Mostly, 2-phenylethylamine, tyramine and tryptamine were formed by resting cells in amounts <25 mg/l, whereas growing cells formed high amounts (>100 mg/l) of 2-phenylethylamine and putrescine. This study confirmed the need of consideration of ECM binding and biogenic amine formation in the safety assessment of CNS used in the production of fermented foods.

## 1. Introduction

Coagulase-negative staphylococci (CNS) play an important role in food fermentation, especially in the production of fermented meat and milk products (Bockelmann, 2002; Hammes *et al.*, 1995; Hammes and Hertel, 1998; Place *et al.*, 2003; Schlafmann *et al.*, 2002). The most prominent species currently used or having the potential for use in starter cultures are *Staphylococcus carnosus*, *Staphylococcus condimenti*, *Staphylococcus equorum*, *Staphylococcus piscifermentans*, *Staphylococcus succinus* and *Staphylococcus xylosus*. Despite their usefulness in contributing to the organoleptic quality of the fermented product (Casaburi *et al.*, 2008), some concerns have in the recent past been raised with regard to their safety. Clinical isolates identified as *S. carnosus*, *S. equorum*, *S. succinus* and *S. xylosus* have been described (Couto *et al.*, 2001; Domínguez *et al.*, 2002; Novakova *et al.*, 2006; Petinaki *et al.*, 2001). However, Coton et al. (2010a) investigated 297 clinical isolates and showed that

the isolates belonged mainly to the species *S. epidermidis, S. capitis, S. hominis, S. warneri* and *S. haemolyticus*. None of the clinical isolates have been assigned to the food relevant species *S. carnosus, S. condimenti, S. equorum, S. piscifermentans, S. succinus* and *S. xylosus*.

Besides safety relevant properties like antibiotic resistances and toxin formation in food-associated CNS (Bautista *et al.*, 1988; Mauriello *et al.*, 2000; Perreten *et al.*, 1997; Resch *et al.*, 2008; Rodríguez *et al.*, 1996; Teuber *et al.*, 1996; Vernozy Rozand *et al.*, 1996; Zell *et al.*, 2008), the formation of the biogenic amines cadaverine, 2-phenylethylamine, putrescine, tryptamine and tyramine has been described to occur in strains of the species *S. carnosus, S. piscifermentans, S. equorum* and *S. xylosus* (Ansorena et al., 2002; Even et al., 2010; Martuscelli et al., 2000; Straub et al., 1995). However, for *S. condimenti* and *S. succinus* only little data on the biogenic amine formation is available. In general, the formation has been shown mostly for growing but also resting cells, in order to simulate the cell status which can occur during food fermentation. For example, in long ripened raw fermented sausages staphylococci grow only at the very beginning of fermentation and particularly on the surface of the sausages, thus resting cells are available in abundance (Santos, 1996; Straub et al., 1995). Ingestion of foods containing high concentrations of biogenic amines particularly histamine could have pharmacological effects. For example, the induction of headache, difficulties of breathing, palpitations, hypertonia, hypotonia and different allergic reaction like scombroid poisoning may be a result of the action of biogenic amines with the nervous system (Lonvaud-Funel, 2001; Shalaby, 1996).

The staphylococcal adherence to extracellular matrix (ECM) proteins by formation of binding proteins is considered as an important pathogenicity factor (Heilmann *et al.*, 1997; Świtalski *et al.*, 1983). For example, the binding to fibronectin may facilitate staphylococcal colonization of injured tissue and invasion into the host cells (Brett Finlay and Caparon, 2000; Preissner and Chatwal, 2000). Furthermore, the fibrinogen binding proteins are responsible for clumping of erythrocytes (clumping factor) and may be involved in thrombosis (Rivera *et al.*, 2007). For detection of ECM binding, microtiter plate assays with immobilized ECM proteins and crystal violet staining were used (Štyriak *et al.*, 1999). Although binding properties were detected for the CNS *S. simulans* and *S. saprophyticus* of non-food origin, binding to ECM by food-associated CNS was not investigated up to date (Christensen *et al.*, 1985; Świtalski *et al.*, 1983). Thus, in this study 32 food-associated CNS strains belonging to the species *S. carnosus, S. condimenti, S. piscifermentans, S. equorum, S. succinus* and

*S. xylosus* were investigated with regard to binding to the ECM proteins fibronectin and fibrinogen by applying a new microtiter plate assay. These strains have previously been well characterized regarding toxin formation (Zell *et al.*, 2008) and antibiotic resistances (Resch *et al.*, 2008). Here, their potential to form biogenic amines was investigated using resting and growing cells and HPLC based detection. This study is in connection with a study on the DNA microarray based detection of genes involved in safety and technologically relevant properties (Seitter (née Resch) et al., 2011).

## 2. Materials and methods

### 2.1. Binding to extracellular matrix proteins

For detection of binding to extracellular matrix proteins, 32 strains of the species *S. carnosus*, *S. condimenti*, *S. equorum*, *S. succinus*, *S. piscifermentans* and *S. xylosus* isolated from various foods, commercial starter cultures and patients in clinics were used. Staphylococci were aerobically cultured overnight at 37 °C on Standard I nutrient agar (Merck) and afterwards used for the investigation of their binding to ECM. The binding capacity of CNS to ECM proteins was investigated by detection of fluorescent labeled cells which were added to microtiter plates coated with fibronectin or fibrinogen. Strains inoculated from overnight grown Standard I nutrient agar were grown in brain heart infusion medium (Merck) at 37 °C, with shaking at 170 rpm. Cells were obtained either from the logarithmic ($OD_{600}$=0.5) or stationary phase and harvested by centrifugation (5000 x *g*, 4 °C, 10 min). In the latter case, the $OD_{600}$ was adjusted to 0.5 by dilution with phosphate buffered saline (PBS) containing per liter: 8 g NaCl, 0.2 g KCl, 1.15 g $Na_2HPO_4$ x 2 $H_2O$, 0.2 g $KH_2PO_4$, pH 7.2. The cells were washed twice with PBS and again resuspended in PBS to obtain an $OD_{600}$ of 0.5. Staining was performed for 1 h at 4 °C with 2 µl acridine orange solution (3.7 mg/ml PBS, Sigma-Aldrich) per ml cell suspension. Residues of acridine orange were removed by centrifugation for 10 min at 5000 x *g* (4 °C) and washing the cells twice with PBS. Afterwards, the fluorescent labeled cells were concentrated 10 fold ($OD_{600}$=5.0) by resuspending with appropriate volumes of PBS and subsequently given onto ECM coated and BSA blocked microtiter plates to perform binding assay.

For the binding assay, 96-well microtiter plates (MICROLON 600 high-binding plates, Greiner Bio-one) were used, containing a hydrophilic polystyrol surface with enhanced protein binding capacity. Plates were coated with fibrinogen (human plasma, Merck Biosciences, cat no. 341576) or fibronectin (human plasma, Sigma-Aldrich, cat no. F2006). Wells were filled (100 µl) with either 0.02% (approximately 21 µg/cm$^2$ of the microtiter plate) of fibrinogen or 0.0004% (approximately 0.4 µg/cm$^2$) of fibronectin dissolved in PBS, incubated overnight at 4 °C and then washed 3 times with PBS. After this, 100 µl of a 10% BSA solution (bovine serum albumin in PBS, approximately 11 mg BSA/cm$^2$) was added to each well to block unoccupied sites and to prevent nonspecific binding. The plates were incubated for 1 h at 4 °C and again washed for three times with PBS. 100 µl fluorescent labeled bacterial suspension (1x10$^8$ cfu/well) were added to 16 wells (2 rows of eight wells) coated with either fibrinogen or fibronectin. After incubation at ambient temperature for 1 h in the dark, the unbound cells were removed from one row (8 wells) by washing three times with 100 µl PBS. These washed wells were again filled with 100 µl PBS. The fluorescence was measured in the washed row (bound cells with 100 µl PBS) and the non-washed row (100 µl fluorescent labeled cells) using a Varian Cary Eclipse fluorescence spectrophotometer ($\lambda_{ex}$=485 nm and $\lambda_{em}$=515 nm). Binding to ECM was calculated by the fluorescence ratio of bound cells to labeled cells multiplied by 100. In addition, values were normalized using the values obtained with cells of *S. aureus* Cowan (Świtalski *et al.*, 1983) grown to the logarithmic growth phase, set to 100% (positive control). Results are based on three independent experiments.

**2.2. Biogenic amine formation**

The biogenic amine formation was investigated using the same strains. The staphylococci were cultured aerobically overnight at 37 °C on Standard I nutrient agar (Merck) and subsequently used for the inoculation of Standard I nutrient broth (Merck). Formation of biogenic amines was investigated by using either growing or resting cells. For both assays, strains were cultured in Standard I nutrient broth (Merck) with shaking at 180 rpm. For the investigation of growing cells, 100 µl of overnight culture were used to inoculate 10 ml Standard I nutrient broth, containing 0.1 g/l of L-ornithine (Sigma-Aldrich), L-lysine (Sigma-Aldrich), L-histidine (Serva), L-tyrosine (Sigma-Aldrich), L-tryptophan (Sigma-Aldrich) and L-phenylalanine (Sigma-Aldrich). Cultures were incubated at 37 °C for 24 h with shaking at 180 rpm. For the resting cells, overnight cultures were centrifuged at 2000 x *g* for 15 min and washed with phosphate buffer (0.07 mol/l, pH 6.0). After washing, cells were resuspended in

10 ml of the same buffer supplemented with 0.1 g/l of L-ornithine, L-lysine, L-histidine, L-tyrosine, L-tryptophan and L-phenylalanine, as well as 1 g/l D-glucose. Incubation was performed at 37 °C for 24 h without shaking. After incubation, samples of growing or resting cells were centrifuged for 10 min at 6000 x *g* to remove cell residues. For protein precipitation, the supernatants were mixed (20:1) with perchloric acid (70%) and stored overnight at 4 °C. After centrifugation at 13,000 x *g* (4 °C) for 10 min, the supernatants were filtered using a 0.45 µm filter (Schleicher und Schuell) and stored at -18 °C until analysis. Quantification of biogenic amines was performed by HPLC as described previously (Straub *et al.*, 1993) with the following modifications. Eluent A consisted of 2.16 g/l sodium salt of octansulfonic acid and 8.03 g/l sodium acetate in water adjusted to pH 4.5 with acetic acid. Eluent B contained 2.16 g/l sodium salt of octansulfonic acid, 12.73 g/l sodium acetate and 230 ml/l acetonitril (pH 4.5 with acetic acid). The flow rate was 0.9 ml/min and the gradient for eluent B was $t_0$ = 20%, $t_{30}$ = 40%, $t_{40}$ = 65%, $t_{44}$ = 65%, $t_{44.1}$ = 100%, $t_{60}$ = 100%, $t_{60.1}$ = 20%, $t_{75}$ = 20%. Biogenic amines fluorescent derivatives were obtained by addition of a solution of borate buffer (61.8 g/l boric acid and 40.0 g/l potassium hydroxide dissolved in water) containing 1.0 g/l o-phthaldialdehyde, 10.0 ml/l methanol and 3.0 ml/l β-mecaptoethanol. The flow rate for derivative reagent was 0.63 ml/min. HPLC detection of biogenic amines was performed in duplicates.

## 3. Results and discussion

Preliminary tests with cells of the positive control strain *S. aureus* Cowan showed that cells harvested in logarithmic growth phase revealed higher binding capacities (about twice) than cells obtained from the stationary phase. This is consistent with the findings of Kerdudou et al. (2006). Therefore, in this study we always used logarithmic growing cultures of *S. aureus* Cowan. In contrast to this, the investigation of the food-associated CNS was performed by using stationary grown cells, as they occur during raw sausage fermentation (Santos, 1996; Straub *et al.*, 1995) and showed in preliminary tests higher or comparable binding capacities than cells obtained from the logarithmic phase (data not shown). Moreover, the determination of binding to fibrinogen and fibronectin revealed a generally lower binding capacity for the food-associated CNS than that observed for *S. aureus* Cowan (Table 1). Remarkably, 2 out of 8 *S. equorum* strains and 1 out of 4 *S. succinus* strains exhibited high binding capacities which

were comparable to that observed for *S. aureus* Cowan. Furthermore, 3 *S. equorum* and 1 *S. succinus* strains showed binding capacities up to 64% higher than the capacities obtained with *S. aureus* Cowan. For *S. xylosus* strains, binding capacities of up to 62% were determined (Table 1). This is in contrast to the findings of (Świtalski *et al.*, 1983), who did not observe any binding of *S. xylosus* isolates to fibronectin. However, the assays used by these investigators was based on the quantification of $^{125}$I-labeled fibronectin binding to staphylococci and, in addition, they observed non-specific binding of the $^{125}$I-labeled fibronectin. More recently, a microtiter plate assay based on crystal violet staining of cells was successfully used to detect *S. aureus* binding to fibronectin (Wolz *et al.*, 2000). In our study, we also made use of such an assay, but labeled the staphylococcal cells with the fluorescent dye acridine orange. This assay could show for the first time that prominent, food-associated CNS was able to bind to fibrinogen and fibronectin. However, the observation of generally low binding capacities for some CNS, especially for those of the species *S. carnosus*, *S. condimenti* and *S. piscifermentans*, raises the question as to the authenticity of the binding, as adherence of staphylococci to smooth surfaces of plastic microtiter plates has also been described (Christensen *et al.*, 1985). To avoid false positive results by non-specific binding, adherence to the surface of the microtiter plates without fibronectin or fibrinogen and BSA used as agent to block unoccupied sites of microtiter plates was determined and served as control. It has been shown that a concentration of 10% BSA is suitable to block free unoccupied binding sites and thus this concentration was chosen for the blocking. The verification of an effective coating of microtiter plates with ECM and the authenticity of binding could be demonstrated as the test organisms exhibited a higher adherence to uncoated and unblocked microtiter plates than to microtiter plates coated with ECM. Nevertheless, because of some very low values obtained for binding of some CNS and the possibility that these may be due to some degree as a result of non-specific binding to the microtiter plate surface, we only considered those CNS as positive for ECM binding, which showed binding capacities values corresponding to more than 90% binding of the control *S. aureus* Cowan strain.

**Table 1:** Binding capacity (%) of food-associated CNS measured in microtiter plates coated with fibrinogen and fibronectin.

| Species (no. of strains tested) | No. of strains: % of cells (standard deviation) binding to[a] | | | | | |
|---|---|---|---|---|---|---|
| | Fibrinogen | | | Fibronectin | | |
| *S. aureus* Cowan (1) | 1: | 100 | (± 8) | 1: | 100 | (± 31) |
| *S. carnosus* (7) | 7: | 15 - 38 | (± 3 - 12) | 7: | 33 - 73 | (± 2 - 9) |
| *S. condimenti* (2) | 2: | 24 - 33 | (± 4 - 9) | 2: | 34 - 83 | (± 7 - 12) |
| *S. piscifermentans* (4) | 4: | 27 - 41 | (± 1 - 13) | 4: | 41 - 71 | (± 1 - 7) |
| *S. equorum* (8) | 6: | 14 - 22 | (± 0 - 9) | 3: | 27 - 67 | (± 2 - 19) |
| | 2: | 95 - 98 | (± 7 - 9) | 2: | 80 - 91 | (± 5 - 23) |
| | | | | 3: | 104 - 164 | (± 10 - 13) |
| *S. succinus* (4) | 3: | 19 - 65 | (± 6 - 14) | 3: | 31 - 65 | (± 5 - 8) |
| | 1: | 104 | ± 20 | 1: | 147 | ± 4 |
| *S. xylosus* (7) | 7: | 7 - 52 | (± 2 - 9) | 7: | 14 - 62 | (± 7 - 29) |

[a] Binding was normalized to the value obtained with *S. aureus* Cowan (100%).

**Table 2:** Formation of biogenic amines by food-associated CNS determined by HPLC.

| Species (no. of strains tested) | No. of positive strains: formation of biogenic amine[a] (mg/l[b]) by using | | | | | |
|---|---|---|---|---|---|---|
| | Resting cells | | | Growing cells | | |
| *S. carnosus* (7) | 3: | PHE | 2.8-10.5 | 3: | PHE | 16.2-94.9 |
| | | TRP | 1.6-11.1 | | TRP | 1.6-3.1 |
| | | TYR | 0.6-4.6 | | | |
| | 2: | PHE | 5.8-6.1 | 1: | CAD | 6.7 |
| | | PUT | 6.7-32.7 | | PHE | 57.9 |
| | | TRP | 3.2 | | PUT | 126.2 |
| | | TYR | 7.8-10.3 | | TRP | 1.1 |
| *S. condimenti* (2) | 2: | PHE | 5.0-14.8 | 1: | PHE | 23.1 |
| | | TRP | 3.0-6.5 | | TRP | 1.8 |
| | | TYR | 2.6-14.0 | | | |
| *S. piscifermentans* (4) | 4: | PHE | 1.8-17.7 | 3: | PHE | 26.5-87.6 |
| | | TRP | 1.8-7.7 | | TRP | 1.2-2.8 |
| | | TYR | 5.0-21.3 | | | |
| *S. equorum* (8) | 0: | - | - | 1: | PHE | 52.7 |
| | | | | | TRP | 1.7 |
| *S. succinus* (4) | 0: | - | - | 0: | - | - |
| *S. xylosus* (7) | 0: | - | - | 0: | - | - |

[a] CAD, cadaverine; PHE, 2-phenylethylamine; PUT, putrescine; TRP, tryptamine; TYR, tyramine; and -, no formation. Formation of histamine was not detected.

[b] Cell density ranged from $1.0 \times 10^8$ to $2.9 \times 10^9$ cfu/ml broth.

In the present study, the ability of CNS to form biogenic amines has been demonstrated by the investigation of resting and growing cells because it has been described that resting cells exist during raw sausage fermentation and are able to form biogenic amines in the stationary phase (Santos, 1996; Straub *et al.*, 1995). Moreover, their formation was also investigated using growing cells to show also the general potential of CNS to produce biogenic amines. Production of biogenic amine could be shown for *S. carnosus, S. condimenti* and *S. piscifermentans* strains, but rarely for *S. equorum* and not at all for *S. succinus* and *S. xylosus* strains (Table 2). The biogenic amines 2-phenylethylamine, tryptamine, and/or tyramine were often detected whereas, cadaverine and putrescine were only rarely detected and histamine was not produced at all. This finding is generally in agreement with previously published data (Ansorena *et al.*, 2002; Even *et al.*, 2010; Montel *et al.*, 1999; Straub *et al.*, 1995). For *S. carnosus* and *S. piscifermentans* strains, high amino acid decarboxylase activity has been described by Montel *et al.* (1999), and investigation of biogenic amine formation of resting *S. carnosus* cells in phosphate buffer demonstrated a high potential with regard to putrescine, cadaverine and especially 2-phenylethylamine and tyramine. The formation of biogenic amines 2-phenylethylamine and tyramine has been shown in *S. piscifermentans* isolates to a lesser extent, and for *S. xylosus* strains the production of biogenic amine was not detected (Straub *et al.*, 1995). Furthermore, formation of 2-phenylethylamine and tyramine by growing cells of *S. carnosus* and *S. equorum* has also been shown previously (Ansorena *et al.*, 2002; Even *et al.*, 2010).

Interestingly, in our study the CNS tended to form more biogenic amines when the cells were in the resting state. For 2-phenylethylamine and putrescine, we could observe a higher concentration (approximately 5 to 10 fold increase) when using growing cells. These observations are generally consistent with the results of Straub et al. (1995), showing that resting cells of *S. carnosus,* and to a lesser extent of *S. piscifermentans,* are able to form biogenic amines (Straub *et al.*, 1995). Furthermore, in previous studies the formation of higher amounts of biogenic amines by growing cells was also observed (Ansorena *et al.*, 2002; Even *et al.*, 2010; Martuscelli *et al.*, 2000). As an explanation, it was argued that decarboxylase activities may depend on the availability of nutrients and need to be induced by high substrate concentrations (Maijala and Eerola, 2002). Moreover, the effect of ecological conditions like pH, temperature, $a_w$, nutrients and food composition on decarboxylase activities, and therefore on the formation of biogenic amines during food fermentation, was also shown (Beutling, 1996; Gardini *et al.*, 2001). In our study, the experimental conditions

used to investigate biogenic amine formation by growing cells varied considerably from that used for the resting cells. Compared to Standard I nutrient broth (pH 7.5) used for the growing cells, the pH value of phosphate buffer used for the resting cells was lower (pH 6.0). However, biogenic amines were described to be formed as a kind of protection against acidic environments (Maijala and Eerola, 2002; Masson *et al.*, 1996). As an optimum for histamine, tyramine and tryptamine formation, a pH of 5.0 has been previously reported (Maijala and Eerola, 2002). Thus, we expected higher biogenic amine concentrations for the resting than for the growing cells, due to the lower pH in the phosphate buffer. This, however was found to apply to the formation of tryptamine only (Table 2). Other factors affecting biogenic amine formation might also include the NaCl and/or glucose contained in the Standard I nutrient broth. For example, the biogenic amine formation by *Lactobacillus delbrueckii* ssp. *bulgaricus* was shown to be reduced in the presence of 2.0% NaCl (Maijala and Eerola, 2002). Furthermore, the glucose content in the fermentation broth could also play a role in lowering the pH by enhanced metabolic activity, thus affecting the biogenic amine formation via alteration of the pH (Bover-Cid and Holzapfel, 1999).

Both properties, the ECM binding capacity as well as the formation of biogenic amines were found to be strain specific, especially with regard to the species *S. equorum* and *S. succinus* or rather *S. carnosus* and *S. equorum*. This observation may be explained by taking the possibility of horizontal gene transfer into consideration. For instance, variation of putrescine-production in the *S. epidermidis* species was shown to depend on the presence of a plasmid harboring a putative ornithine decarboxylase gene (Coton *et al.*, 2010b). The authors concluded that the putrescine-producing pathway in *S. epidermis* has been acquired through horizontal gene transfer. Thus, it is tempting to speculate that the strain variability observed in our study is attributed to the location of the corresponding genes on mobile genetic elements. Further research could be needed to elucidate the role of horizontal gene transfer in the acquisition of safety relevant properties by CNS.

In conclusion, this study demonstrates the potential of food-associated CNS to exhibit undesired properties like the formation of biogenic amines or the binding to ECM proteins. On the one hand, undoubtedly such properties would have to be considered in the safety assessment of any strain intended to be used in the production of fermented foods. On the other hand, further research is needed to evaluate the importance of the presence of such properties, with regard to the potential risks which may arise from the use of CNS in food

production. For example, a question which needs to be addressed is whether the presence of a weak ECM binding capacity, e.g. half of that observed for *S. aureus* that determined under laboratory conditions, is really of importance in the safety discussion. This is especially a difficult question when this weak binding capacity would be the sole safety concern associated with the strain and other concerns, such as biogenic amine production, are absent.

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# *Chapter VI*

## DNA microarray based detection of genes involved in safety and technologically relevant properties of food associated coagulase-negative staphylococci

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## Abstract

Aim of the work was to design a polynucleotide based DNA microarray as screening tool to detect genes in food associated coagulase-negative staphylococci (CNS). A focus was laid on genes with potential health concern and technological relevance. The microarray contained 220 probes for genes encoding antibiotic resistances, hemolysins, toxins, amino acid decarboxylases (e.g. biogenic amine formation), binding proteins to extracellular matrix (ECM), lipases, proteases, stress response factors, or nitrate dissimilation. Hybridization of genomic DNA isolated from 32 phenotypically characterized CNS permitted to detect numerous genes, corresponding with the phenotype. However, numerous hybridization signals were obtained for genes without any detectable phenotype. The antibiotic resistance genes *blaZ*, *lnuA*, and *tetK* involved in β-lactam, lincomycin and tetracycline resistance, respectively, were rarely identified in CNS, however, all species contained some strains with such resistance genes. Decarboxylase genes involved in biogenic amine formation were detected regularly in *Staphylococcus carnosus*, *S. condimenti*, *S. piscifermentans* and *S. equorum*, but was rarely correlated with the phenotype. The same applied for the fibrinogen (*clf*) and fibronectin (*fbp*) binding protein genes, whose phenotype (binding assay) was only correlated in *S. equorum* and *Staphylococcus succinus*. Although some CNS showed hemolytic activity and enterotoxin production (Immunoblot) the corresponding genes could not be verified. Technological relevant genes such as proteases or lipases revealed good hybridization signals. In addition, genes involved in nitrate dissimilation (*nre*, *nar*, *nir*), catalase (*kat*), or superoxide dismutase (*sod*) were well detected. Interestingly, genes involved in dissimilatory nitrate reduction were more prevalent in strains of *S. carnosus, S. condimenti* and *S. piscifermentans* than of *S. equorum, S. succinus* and *S. xylosus*.


## 1. Introduction

The genus *Staphylococcus* comprises 42 validly described species (DSMZ, 2009; DSMZ, 2010) which are grouped traditionally into coagulase-positive (CPS) and coagulase-negative staphylococci (CNS). It has been shown that the CPS *Staphylococcus aureus* and *Staphylococcus schleiferi* (Calvo *et al.*, 2000) as well as the coagulase-variable *Staphylococcus hyicus* (Aarestrup and Jensen, 2002) are pathogenic and/or toxigenic causing

serious infections and food intoxications in humans (Götz *et al.*, 2006; Le Loir *et al.*, 2003). The coagulase-negative species *Staphylococcus epidermidis*, *Staphylococcus haemolyticus*, *Staphylococcus saprophyticus* and *Staphylococcus warneri* are described as opportunistic pathogens (Götz *et al.*, 2006). On the other hand, the CNS *Staphylococcus carnosus* and *Staphylococcus xylosus* are traditionally used in starter cultures for the processing of fermented meat products (Hammes and Hertel, 1998; Ordóñez *et al.*, 1999). Furthermore, the CNS species *Staphylococcus condimenti*, *Staphylococcus equorum*, *Staphylococcus piscifermentans*, *Staphylococcus succinus* are associated with food and/or involved in spontaneous fermentation. Strains of *S. equorum* and *S. succinus* subsp. *casei* have been isolated from smear and/or surface ripened cheese (Bockelmann, 2002; Place *et al.*, 2002; Place *et al.*, 2003), *S. condimenti* and *S. piscifermentans* from soy sauce mash and fermented fish (Probst *et al.*, 1998; Tanasupawat *et al.*, 1992) and *S. equorum* from cured ham (Schlafmann *et al.*, 2002). Therefore, strains of these species may have the potential for future application as starter or protective cultures.

The European Food Safety Authority (EFSA) has undertaken the task to establish the Qualified Presumption of Safety (QPS) approach for the safety assessment of microorganisms used in food and feed production (EFSA, 2007). The history of use is an important source of knowledge forming the so-called body of knowledge, one of the four pillars of the safety assessments (EFSA, 2004; EFSA, 2005). Some CNS have a long history of apparent safe use, as proven by long-term experience with the fermented food products. The CNS are either components of the dominating fermentation biota, e.g. *S. equorum*, *S. xylosus* and *S. succinus* (Coton *et al.*, 2010) or used in starter cultures, e.g. *S. carnosus* and *S. xylosus*. On the other hand, concerns were raised questioning the presumption of safety of some CNS. Clinical isolates of the species *S. carnosus*, *S. equorum*, *S. succinus* and *S. xylosus* appeared in the literature (Couto *et al.*, 2001; Domínguez *et al.*, 2002; Novakova *et al.*, 2006; Petinaki *et al.*, 2001). However, Even et al. (2010) investigated the biodiversity of CNS, including 297 clinical isolates, but could not found any allocation to these species. Furthermore, undesired properties like toxin formation (Bautista *et al.*, 1988; Rodríguez *et al.*, 1996; Valle *et al.*, 1990; Vernozy-Rozand *et al.*, 1996; Zell *et al.*, 2008), antibiotic resistances (Even *et al.*, 2010; Gardini *et al.*, 2003; Holley and Blaszyk, 1997; Kastner *et al.*, 2006; Marín *et al.*, 1992; Mauriello *et al.*, 2000; Perreten *et al.*, 1997; Resch *et al.*, 2008; Teuber *et al.*, 1996) and biogenic amine formation (Ansorena *et al.*, 2002; Even *et al.*, 2010; Martuscelli *et al.*, 2000;

Straub *et al.*, 1995) have been described to occur in food associated CNS, e.g. *S. carnosus, S. equorum, S. piscifermentans* and/or *S. xylosus.*

Toxin formation is considered as an important pathogenicity factor in staphylococci, comprising alpha-toxin (Haslinger *et al.*, 2003), two-component cytolysins like γ-hemolysin, leukocidin and panton-valentine leukocidin (Boussaud *et al.*, 2003; Gillet *et al.*, 2002), super antigens like toxic-schock-syndrom toxin (TSST-1) (McCormick *et al.*, 2003), exfoliative toxins (Ahrens and Andresen, 2004; Yamaguchi *et al.*, 2002) and staphylococcal enterotoxins A-I involved in food poisoning (Le Loir *et al.*, 2003; Wang *et al.*, 2004). Antibiotic resistances have been shown to occur not only in pathogenic staphylococci but also in food associated CNS (Even *et al.*, 2010; Gardini *et al.*, 2003; Holley and Blaszyk, 1997; Kastner *et al.*, 2006; Marín *et al.*, 1992; Mauriello *et al.*, 2000; Perreten *et al.*, 1997; Resch *et al.*, 2008; Teuber *et al.*, 1996). Here resistances against (β-lactam antibiotics, lincomycin, tetracycline and erythromycin) were described to be predominating. Regarding food as a potential reservoir for the spread of antibiotic resistances (Franz *et al.*, 2005; Teuber, 1999; Werner *et al.*, 1997; Witte, 1999), the horizontally transferable resistance determinants are of special interest in the safety assessment (Borriello *et al.*, 2003). Also, the decarboxylation of amino acids leading to formation of biogenic amines is a property with special relevance to food safety. Strains of *S. carnosus*, *S. piscifermentans* and *S. xylosus* were described to form cadaverine, 2-phenylethylamine, putrescine, tryptamine and/or tyramine (Ansorena *et al.*, 2002; Martuscelli *et al.*, 2000; Straub *et al.*, 1995). Moreover, the ability of adherence to extracellular matrix (ECM) proteins via binding proteins is considered as pathogenicity factor (Świtalski *et al.*, 1983). Interestingly, besides the formation of biogenic amines, amino acid decarboxylases may also be involved in aspects of technological relevance like alpha-acetolactate decarboxylase which is e.g. responsible for the production of flavour active component acetoin via the 2,3 butanediol-pathway (Goupil-Feuillerat *et al.*, 1997; Parente *et al.*, 2004; Ravyts *et al.*, 2010). Binding to fibronectin may facilitate colonization of injured tissue and invasion into the host cells (Brett Finlay and Caparon, 2000; Preissner and Chatwal, 2000). Fibrinogen binding proteins constitute the so-called “clumping factor” which is responsible for clumping of erythrocytes and may be involved in thrombosis (Rivera *et al.*, 2007). Very recently, we could demonstrate that binding to ECM proteins is a property also detectable in food associated CNS, especially in *S. equorum* and *S. succinus* (Seitter (née Resch) *et al.*, 2011). Despite these data and additional safety studies (Bautista *et al.*, 1988; Rodríguez *et al.*, 1996; Vernozy-Rozand *et al.*, 1996; Zell *et al.*, 2008), there is still a need to

acquire further knowledge in order to make use of the QPS based approach in the safety assessment of food associated CNS.

To detect safety relevant properties in CNS, phenotypic investigations are routinely used, e.g. immunoblot analysis for toxin formation (Zell *et al.*, 2008), high performance liquid chromatography for detection of biogenic amines (Straub *et al.*, 1995) and disk susceptibility or broth dilution testing for antibiotic resistances (CLSI, 2009a; CLSI, 2009b). As the phenotypic detection is extensive and time consuming, the detection of genotypes by e.g. PCR and hybridization analysis plays a key role for rapid and reliable identification of the corresponding genotypes. Especially, DNA microarrays are useful tools, offering the possibility to simultaneously detect various genes involved in safety relevant properties, e.g. staphylococcal enterotoxin genes (Sergeev *et al.*, 2004) and antibiotic resistance genes (Perreten *et al.*, 2005). Recently, a PCR-product microarray has been developed for the identification of virulence factors and antibiotic resistances in *S. aureus* isolates (Palka-Santini *et al.*, 2007). However, the microarrays were mostly developed to detect safety relevant properties in pathogenic staphylococci or were limited in the number of genes or properties to be detected. Quite recently, an oligonucleotide based microarray has been developed to evaluate the safety of food associated CNS by detecting genes involved in antibiotic resistances, toxin production and biogenic amine formation (Even *et al.*, 2010). However, the array does not allow detecting technologically relevant properties in CNS and thus a microarray-based detection of safety hazards together with technological function is needed.

In this study, a polynucleotide based DNA microarray as screening tool for the combined detection of safety and technologically relevant properties in CNS associated with fermented food or used in starter cultures was designed. The recently published genome sequence of *S. carnosus* TM300 allows the inclusion of a number of technological relevant genes (Rosenstein *et al.*, 2009; Rosenstein and Götz, 2010). The DNA of strains phenotypically characterized in respect of toxin formation (Zell *et al.*, 2008), antibiotic resistances (Resch *et al.*, 2008), ECM binding and formation of biogenic amines (Seitter (née Resch) *et al.*, 2011) were subjected to hybridization. The results demonstrate that this microarray is useful to reveal genotypic background of numerous safety and technologically relevant properties in food associated CNS.

## 2. Materials and methods

### 2.1. Bacterial strains and growth conditions

Strains listed in Table A1 (supplementary data) were used for construction of microarray probes. *S. aureus* strains N315 (Kuroda *et al.*, 2001), Mu50 (Kuroda *et al.*, 2001), COL (Gill *et al.*, 2005), MW2 (Baba *et al.*, 2002), NRS111 (Hovde *et al.*, 1994), and NCTC10656 (Sergeev *et al.*, 2004) as well as *S. carnosus* TM300 (Rosenstein *et al.*, 2009) served as reference to evaluate the specificity and sensitivity of hybridizations. For detection of safety and technologically relevant properties, 32 strains of the species *S. carnosus*, *S. condimenti*, *S. equorum*, *S. succinus*, *S. piscifermentans* and *S. xylosus* isolated from various foods, commercial starter cultures, and patients in clinics were used. Staphylococci and *Escherichia coli* strains were aerobically cultured overnight at 37 °C (shaking at 180 rpm) in Standard I nutrient broth (Merck) and LB broth (Merck), respectively. Lactobacilli and enterococci were grown in MRS broth (Oxoid) at 30 °C or 37 °C.

### 2.2. DNA microarray design

A polynucleotide based DNA microarray was designed to detect genes coding for or being involved in safety and technologically relevant properties. For selection of genes a search of the GenBank database including genome and/or gene sequences of *S. carnosus* TM300 (Rosenstein *et al.*, 2009), *S. aureus* strains (Baba *et al.*, 2002; Gill *et al.*, 2005; Hovde *et al.*, 1994; Kuroda *et al.*, 2001; Sergeev *et al.*, 2004) and other staphylococci (see Table A1, supplementary data) was conducted, focusing on staphylococcal genes encoding antibiotic resistances, toxins, decarboxylases, nucleases, hydrolyses, ECM binding proteins, lipases, and proteases and being involved in hemolysis, dissimilatory nitrate reduction, and salt and oxidative stress tolerance. In addition, genes encoding transferable antibiotic resistances of other species like *Enterococcus faecium*, *Lactococcus lactis* and *Lactobacillus reuteri* were included (see Table A1, supplementary data). To avoid selection of orthologous or highly similar genes, for each sequence a search of the GenBank database by using the BLAST algorithm (Altschul *et al.*, 1990) was conducted. In case of similarities of >80%, one of the gene sequences was selected as representative. To permit the detection of genes sharing sequence similarities of >70%, polynucleotide probes of approximately 200 up to 1100 bp were constructed. Specific oligonucleotide primers (forward, reverse, and one semi-nested primer) were designed using the Fast PCR program (Jena, Germany) and avoiding cross

homologies, formation of hairpins, GC-content, binding quality primarily in respect of 3´-end and optimal PCR amplification conditions.

Semi-nested PCR amplifications were performed using genomic DNA, purified, concentrated via gel filtration and printed by BF-BIOlabs (Germany) using an Omnigrid 100 (Genemachines) on an epoxy-modified glass slide (Schott). The probes were spotted in duplicate per each slide. Selected genes, GeneBank accession numbers, sources, primer sequences and size of the probes spotted on the DNA microarray are shown in Table A1 (supplementary data).

**2.3. DNA extraction and labeling reactions**

Total genomic DNA was prepared using the method described by Marmur (1961). The concentration and quality of genomic DNA were assessed spectrophotometrically (GeneQuant 1300, Bio-Sciences AB) and by agarose gel electrophoresis (Sambrook and Russell, 2001). The DNA (4 µg) was labeled with biotin by using the Bioprime DNA Labeling System (Invitrogen Life Technologies) according to the manufacturer's protocol with the following modifications. After denaturation, mixtures containing DNA and random primers were immediately cooled on ice ethanol. Reactions were performed at 37°C for 2 h in the dark and stopped by addition of 1/10 volume stop buffer (pH 8.0). The labeled DNA was purified by repeated ethanol precipitation. To check the labeling efficiency, the amount of biotin-labeled product was determined using the direct detection method of BF-BIOlabs (Germany). Briefly, serial dilutions were prepared from the biotin-labeled DNA and spotted on Whatman membrane filters (ME 25, Schleicher & Schuell). The incorporated biotin became visible as blue colored spots after addition of Streptavidin-AP-conjugate (Streptavidin-Alkaline phosphatase-conjugate for nucleic acid detection, Roche Bioscience), NBT (50 mg/ml, Nitroblue tetrazolium, Roth) in 100% dimethylformamide and BCIP (5-bromo-4-chloro-3-indolylphosphate, 75 mg/ml, Roth) in 70% dimethylformamide.

**2.4. Hybridization experiments**

According to the protocol of BF-BIOlabs (Germany) hybridizations were performed using 2 Geneframes (1.7 x 2.8 cm, 250 µl, ABgene House) placed on the slide and a coverslip on top. An aliquot (20 µl) of the biotin-labeled DNA was mixed with 230 µl hybridization solution containing 5 x SSC (pH 7.0), 0.5% SDS and 10% Arrayblock (BF-BIOlabs), heated at 95 °C for 5 min and then cooled on ice before bringing up to the slides. Slides were hybridized overnight rotating in a hybridization oven (Gesellschaft für Labortechnik mbH). To permit

detection of sequences with >70% similarity, for each species the hybridization temperature was calculated based on the formulas $T_m$ = 81.5 + 16.6 (log M ($Na^+$)) + 0.41 (% GC) and % homology = ($T_m$ – $T_h$) / l.4, whereby $T_m$ was the melting temperature, M ($Na^+$) the monovalent $Na^+$ molarity, % GC the GC content of the species, $T_h$ the hybridization temperature and % homology was set to 70% nucleotide identity (Gibbs *et al.*, 1984). After hybridization the frames were removed and the slides were washed twice with 2 x SSC-0.1% SDS and once with 1 x SSC-0.2% Tween20 under agitation. Microarrays were stained with Cy5-streptavidin (GE Healthcare), washed with 1 x SSC-0.2% Tween20 and dried under a stream of nitrogen. The hybridization analyses were performed in four technical replicates, resulting in 8 replicates of each spot (gene). Arrays were scanned for fluorescence at 635 nm on a GenePix 4000B microarray scanner (Axon Instruments). Acquisition of fluorescent spots and quantification of fluorescent spot intensities were performed using Gene Pix Pro software version 6.0 (Axon Instruments). Non-linear normalization and background correction were performed as described by Edwards (2003). The R functions for the normalization and background correction were kindly provided by D. Edwards (Department of Biostatistics, Novo Nordisk, Denmark). Hybridization signals with intensities below half of background were considered as negative, whereas with similar or higher intensities than that obtained for the topoisomerase genes *parC* and *parE* (for *S. carnosus, S. condimenti, S. equorum, S. succinus* and *S. xylosus*) and DNA gyrase gene *gyrA* and *gyrB* (for *S. piscifermentans*) as positive. Signals with intermediate intensities were considered as ambiguous.

## 3. Results

### 3.1. Design of the DNA microarray

To detect genes being involved in safety and technologically important properties in CNS, a polynucleotide based microarray covering 220 genes was designed. The sequences originated mainly from pathogenic species *S. aureus* and *S. epidermidis* and the food associated species *S. carnosus* and *S. xylosus* (see Table A1, supplementary data). To detect antibiotic resistance genes acquired by horizontal gene transfer (Even *et al.*, 2010), sequences of mobile genetic elements originating from *Staphylococcus*, *Lactobacillus*, *Lactococcus* and *Enterococcus* species were included. As shown in Table 1, among the selected 157 genes with a potential health risk, 51 encode antibiotic resistances and murein biosynthesis, 33 toxins, 7 hemolysins,

51 encode decarboxylation of amino acids, and 15 encode other risk factors such as formation of ECM binding proteins, heat stable nucleases, hydrolases, hyaluronate lyase, staphylokinase, leukotoxin and leukocidin. Further, 63 genes with technological relevance, such as 8 lipases, 34 proteases, 21 stress response genes (catalase, superoxide dismutase, genes for arsenic efflux) as well as nitrate and nitrite dissimilation has been selected. All genes represented on the microarray including accession number, origin (strain), primer sequences used for amplification, size of the probe and position (spot) numbers on the microarray are compiled in Table A1 (supplementary data).

**Table 1:** Number of strains used as source for DNA sequences to construct the microarray probes for detection of properties of safety and technological relevance in food associated CNS.

| Properties of | No. of strains of the species | | | | | |
|---|---|---|---|---|---|---|
| | *S. aureus* | *S. epidermidis* | *S. carnosus* | *S. xylosus* | Other staphylococcal species [a] | Other species [b] |
| Safety relevance | | | | | | |
| Antibiotic resistances | 37 | 4 | | | 2 | 8 |
| Toxin formation | 32 | 1 | | | | |
| Hemolysis | 7 | | | | | |
| Decarboxylation | 20 | 12 | 19 | | | |
| Others [c] | 15 | | | | | |
| Technological relevance | | | | | | |
| Lipolysis | 3 | 2 | | 1 | 2 | |
| Proteolysis | 25 | | 9 | | | |
| Others [d] | | | 15 | 6 | | |

[a] *S. equorum*, *S. hyicus*, *S. sciuri*, and *S. warneri*.
[b] *Enterococcus faecium*, *Lactococcus lactis* and *Lactobacillus reuteri*.
[c] Formation of ECM binding proteins, heat stable nucleases, hydrolases, hyaluronate lyase, staphylokinase, leukotoxin and leukocidin.
[d] Stress response (catalase, superoxide dismutase, genes involved in arsenic efflux), nitrate dissimilation.

### 3.2. Evaluation of the DNA microarray

To evaluate the functionality of the microarray, several hybridizations were performed using genomic DNA of *S. aureus* N315, *S. aureus* Mu50 and *S. carnosus* TM300, which harbor the corresponding genes as most of the probes were derived from these strains. For evaluation of probes selected to detect toxin genes in CNS, the genomic DNA of the *S. aureus* strains COL, MW2, NRS111, and NTCT10656 harboring these genes were included into the experiments.

Remarkably, with the mentioned *S. aureus* strains positive hybridization signals were obtained for all tested toxin and hemolysis genes as well as genes encoding ECM binding proteins, heat stable nucleases, hydrolases, hyaluronate lyase, staphylokinase, leukotoxin and leukocidin (Table 2). On the other hand, with regard to the 51 antibiotic resistance and murein biosynthesis genes, as well as 51 amino acid decarboxylation genes, only 26 and 29, respectively, showed positive hybridization signals when using the genomic DNA of *S. aureus* MU50 and *S. aureus* N315. A similar low detection level was obtained for technological relevant genes involved in lipolysis, stress response, nitrate and nitrite dissimilation when using genomic DNA of *S. carnosus* TM300 (Table 2). For example, only 28 of the 34 probes used to detect protease genes showed positive hybridization signals when using genomic DNA of *S. aureus* strains COL, MW2, NRS111, NTCT10656.

**Table 2:** Evaluation of the functionality of the DNA microarray by hybridizations using the DNA of *S. aureus* strains and *S. carnosus* TM300.

| Properties of | No. of genes spotted on the microarray | No. of positive hybridization signals obtained with DNA of | | | |
|---|---|---|---|---|---|
| | | *S. aureus* N315 | *S. aureus* MU50 | Other *S aureus* strains [a] | *S. carnosus* TM300 |
| Safety relevance | | | | | |
| Antibiotic resistances | 51 | 22 | 26 | 22 | 5 |
| Toxin formation | 33 | 30 | 31 | 33 | 3 |
| Hemolysis | 7 | 6 | 7 | 7 | 1 |
| Decarboxylation | 51 | 29 | 25 | 24 | 27 |
| Others [b] | 15 | 11 | 15 | 15 | |
| Technological relevance | | | | | |
| Lipolysis | 8 | 5 | 4 | 4 | 5 |
| Proteolysis | 34 | 26 | 27 | 28 | 16 |
| Others [c] | 21 | 11 | 8 | 9 | 19 |

[a] Strains COL, MW2, NRS111, and NTCT10656.
[b] Formation of ECM binding proteins, heat stable nucleases, hydrolases, hyaluronate lyase, staphylokinase, leukotoxin and leukocidin.
[c] Stress response (catalase, superoxide dismutase, genes involved in arsenic efflux), nitrate dissimilation.

### 3.3. Detection of genes with safety and technological relevance

Using genomic DNA of *S. carnosus*, *S. condimenti*, *S. equorum*, *S. piscifermentans*, *S. succinus* and *S. xylosus* genes coding for house-keeping genes such as DNA gyrase, catalase, superoxide dismutase, ClpB chaperon homologue protease, ATP-dependent Clp protease, and FtsH ATP-dependent metalloprotease were detected in all strains by microarray

hybridization. Moreover, several safety and technological relevant genes showed positive hybridization signals. These genes were compared to the previously detected phenotypes of the investigated CNS strains (Resch *et al.*, 2008; Seitter (née Resch) *et al.*, 2011; Zell *et al.*, 2008) (Table 3). Among the 32 tested CNS strains 63 antibiotic resistance phenotypes were detectable, indicating that many strains showed multiple resistance phenotypes. However, the phenotypes correlated only with 29 antibiotic resistance or murein biosynthesis genotypes by microarray hybridization. On the other hand, 20 genotypes did not correlate with an antibiotic resistance phenotype. An even lower correlation was obtained with hemolysins and toxins. However, the formation of biogenic amines and binding proteins correlated quite well with the corresponding genotypes. In addition, many other decarboxylase genes could be identified.

**Table 3:** Overview about the safety relevant properties in food associated CNS detected by microarray hybridization and comparison with the detected phenotypes.

| Properties | No. of CNS subjected to hybridization | No. of detected | | | |
|---|---|---|---|---|---|
| | | Phenotypes | Genes with phenotype | Phenotypes without genes | Genes without phenotype |
| Antibiotic resistances | 32 | 63 | 29 | 34 | 20 |
| Toxin formation | 15 | 7 | | 7 | |
| Hemolysis [a] | 15 | 11 | 2 | 9 | |
| Decarboxylation [b] | 32 | 20 | 18 | 2 | 50 |
| Binding proteins [c] | 15 | 8 | 7 | 1 | 35 |

[a] Observed after 48 h on human blood agar.
[b] Biogenic amines formed by growing cells.
[c] Binding to fibrinogen and fibronectin.

### 3.4. Genes associated with a potential health risk

A detailed overview about the results obtained by microarray hybridization in comparison to the 63 detected antibiotic resistance phenotypes (Resch *et al.*, 2008) is provided in Table 4. Remarkably, positive hybridization signals were obtained with the probes of *blaZ*, *pbp2*, *lnuA*, and *tetK*, whose presence correlates with the corresponding phenotypes, β-lactam, lincomycin and tetracycline resistance, respectively. In nearly all CNS species, the presence of the antibiotic resistance genes correlated with the resistance phenotype. Beside strong positive signals for *blaZ* and *pbp2* (murein biosynthesis gene), in 12 cases (20%) ambiguous weak secondary signals were found, which were, however, allotted with the corresponding phenotype. Ambiguous weak signals were also obtained with the other probes that

nevertheless could be allotted to the phenotype in at least 6 cases (10%). This applies to the antibiotic resistance ofloxacin (*norA*), tetracycline (*tetM* and gene products similar to tetracycline resistance protein spot no. 141), as well as to the murein biosynthesis genes (*pbp3* and *pbp4*) in *S. carnosus*, *S. piscifermentans* and *S. equorum*. However, in 22 cases the ambiguous weak signals remained without any allocation to a phenotype. This applies to the genes *norA* and *vanA* as well as to two genes whose gene products are similar to a chloramphenicol resistance protein (spot no. 125).

**Table 4:** Comparison of the presence of antibiotic resistance genes detected in food associated CNS with the phenotypically detected properties.

| Species (no. of strains tested) | Phenotypic resistance[a] (no. of positive tested strains) | No of strains with positive/ambiguous hybridization signal | Gene or gene function (spot no.) |
|---|---|---|---|
| *S. carnosus* (7) | b-lactams (3): | | |
| | Ampicillin (1) | -/1 | *blaZ* (136) |
| | Oxacillin (2) | -/1 | *pbp3* (87) |
| | Penicillin (1) | 3/4 | *pbp*2 (134) |
| | | -/1 | *pbp*4 (144) |
| | Erythromycin (1) | -/- | |
| | Lincomycin (2) | -/- | |
| | Tetracycline (1) | -/1 | Similar to tetracycline resistance (141) |
| | | -/1 | *nor*A (147) |
| | | -/1 | Similar to chloramphenicol resistance protein (125) |
| | | -/1 | Similar to bicyclomycin resistance protein TcaB (150) |
| *S. condimenti* (2) | | -/1 | *pbp*3 (87) |
| | | -/2 | *pbp*2 (134) |
| *S. piscifermentans* (4) | Lincomycin (1) | 1/- | *lnu*A (24) |
| | Tetracycline (1) | 1/- | *tet*K (28 and 6) |
| | | -/1 | *tet*M (142) |
| | | -/3 | *pbp*2 (134) |
| | | -/1 | *pbp*4 (144) |
| | | -/1 | *nor*A (147) |
| | | -/1 | *van*A (212) |
| *S. equorum* (8) | b-lactams (5): | | |
| | Ampicillin (1) | 1/- | *blaZ* (136) |
| | Oxacillin (4) | -/1 | *pbp*3 (87) |
| | Penicillin (3) | 1/2 | *pbp*2 (134) |
| | Erythromycin (3) | -/- | |
| | Lincomycin (5) | 1/- | *lnu*A (24) |
| | Ofloxacin (1) | -/1 | *nor*A (147) |
| | Tetracycline (2) | 1/- | *tet*K (28 and 6) |
| | | -/2 | Similar to chloramphenicol resistance protein (125) |
| | | 2/2 | Similar to bicyclomycin resistance protein TcaB (150) |
| *S. succinus* (4) | b-lactams (1): | | |
| | Ampicillin (1) | -/- | |
| | Oxacillin (1) | -/- | |
| | Penicillin (1) | -/- | |
| | Lincomycin (4) | -/- | |
| | Tetracycline (1) | 1/- | *tet*K (28 and 6) |
| | | -/1 | *nor*A (147) |
| | | -/1 | Similar to chloramphenicol resistance protein (125) |
| *S. xylosus* (7) | b-lactams (6): | | |
| | Ampicillin (5) | 1/- | *blaZ* (136) |
| | Oxacillin (5) | -/5 | *pbp*2 (134) |
| | Erythromycin (1) | -/- | |
| | Lincomycin (6) | -/- | |
| | Tetracycline (4) | 3/- | *tet*K (28 and 6) |
| | | -/7 | similar to chloramphenicol resistance protein (125) |

[a] Data from (Resch *et al.*, 2008).

Although various CNS species representatives showed hemolytic activity and in some cases toxin production (e.g. enterotoxins) (Zell *et al.*, 2008), ambiguous weak hybridization signals were only obtained with 2 *S. carnosus* and *S. piscifermentans* strains for *hlb* gene (sphingomyelinase) (Table 5). On the other hand, for all species the microarray hybridization revealed mostly positive hybridization signals for *sepA* coding for an extracellular elastase precursor that is putatively involved in toxin formation. Despite numerous additional probes on the microarray coding for genes involved in hemolysis and toxin formation no further hybridization signals were obtained. Thus, the phenotypes described recently (Zell *et al.*, 2008) could not be correlated with a genotype by microarray hybridization.

**Table 5:** Comparison of the presence of genes involved in toxin formation detected in food associated CNS with phenotypically detected properties.

| Species (no. of strains tested) | Phenotype/formation of [a] (no. of positive tested strains) | No of strains with positive/ambiguous hybridization signal | Gene or gene function (spot no.) |
|---|---|---|---|
| *S. carnosus* (5) | Hemolysis: Human blood (3) Sheep blood (1) | -/1 | *hlb* (216) |
| | SEA (2) | -/- | |
| | | 5/- | *sep*A (209) |
| *S. piscifermentans* (1) | Hemolysis: Human blood (1) Sheep blood (1) | -/1 | *hlb* (216) |
| | | -/1 | *sep*A (209) |
| *S. equorum* (6) | Hemolysis: Human blood (5) Sheep blood (2) | -/- | |
| | SED (5) | -/- | |
| | SEH (3) | -/- | |
| | | 6/- | *sep*A (209) |
| *S. succinus* (2) | Hemolysis: Human blood (1) Sheep blood (1) | -/- | |
| | | -/2 | *sep*A (209) |
| *S. xylosus* (1) | Hemolysis: Human blood (1) Sheep blood (1) | -/- | |
| | | 1/- | *sep*A (209) |

[a] Data from (Zell *et al.*, 2008). Hemolysis was detected after 48 h and exotoxin production after 40 h of incubation at 25 °C. SEA, SED, and SHH, staphycoccal enterotxoin A, D and H.

Fibrinogen (*clfA* and *clfB*) and fibronectin binding protein encoding genes (*fbpA* and *fbpB*) were detected by microarray hybridization in all CNS strains except *S. condimenti* (Table 6). However, only for two species, *S. equorum* and *S. succinus*, the genotype could be connected with the corresponding phenotype (Seitter (née Resch) *et al.*, 2011). Again, ambiguous weak hybridization signals could be obtained for some strains showing no adequate phenotype in the binding assay.

**Table 6:** Comparison of the presence of genes coding for binding proteins to the ECM fibrinogen and fibronectin detected in food associated CNS with phenotypically detected properties.

| Species (no. of strains tested) | Binding to (no. of positive tested strains) [a] | No of strains with positive/ambiguous hybridization signal [b] | Gene or gene function (spot no.) |
|---|---|---|---|
| *S. carnosus* (5) | | 2/2 | *clf*A (219) |
| | | 2/3 | *clf*B (220) |
| | | 5/- | *fbp*A (217) |
| | | 2/2 | *fbp*B (218) |
| *S. piscifermentans* (1) | | -/1 | *clf*A (219) |
| | | -/1 | *clf*B (220) |
| | | -/1 | *fbp*A (217) |
| | | -/1 | *fbp*B (218) |
| *S. equorum* (6) | Fibrinogen (2) | 2/3 | *clf*A (219) |
| | | 2/2 | *clf*B (220) |
| | Fibronectin (4) | 6/- | *fbp*A (217) |
| | | -/1 | *fbp*B (218) |
| *S. succinus* (2) | Fibrinogen (1) | -/1 | *clf*A (219) |
| | | -/- | *clf*B (220) |
| | Fibronectin (1) | -/2 | *fbp*A (217) |
| | | -/- | *fbp*B (218) |
| *S. xylosus* (1) | | -/1 | *clf*A (219) |
| | | -/1 | *clf*B (220) |
| | | 1/- | *fbp*A (217) |
| | | -/1 | *fbp*B (218) |

[a] Data from (Seitter (née Resch) *et al.*, 2011). Binding of CNS was >90% of the control (*S. aureus* Cowan).

[b] No hybridization signals were obtained with strains of *S. condimenti.*

Genes encoding amino acid decarboxylases could be detected in all species (Table 7). However, in respect of biogenic amine formation only in case of *S. carnosus* the genes could be allocated with the corresponding phenotype such as formation of cadaverin and putrescin (Seitter (née Resch) *et al.*, 2011). Although we see the production of 2-phenylethylamine and tryptamine in many CNS strains, however, the corresponding genes could not be annotated.

On the other hand, genes encoding for hypothetical decarboxylases as well as alpha-aceto-lactate decarboxylase, aspartate decarboxylase, diaminopimelate decarboxylase, mevalonate decarboxylase could be identified (spot no. 127, 197, 55, 194 in Table A2, supplementary data).

**Table 7:** Comparison of the presence of genes involved in biogenic amine formation in food associated CNS with phenotypically detected properties.

| Species (no. of strains tested) | Formation of [a] (no. of positive tested strains) | No of strains with positive/ambiguous hybridization signal | Gene or gene function (spot no.) |
|---|---|---|---|
| *S. carnosus* (7) | CAD (1), PUT (1) | 7/- | Arginine/lysine/ornithine decarboxylase (154 ) |
| | | 7/- | Similar to ornithine decarboxylase (162) |
| | | 5/- | Ornithine/lysine/arginine decarboxylase (207) |
| | PHE (4), TRP (4) | | |
| *S. condimenti* (2) | | 2/- | Arginine/lysine/ornithine decarboxylase (154 ) |
| | | 2/- | Similar to ornithine decarboxylase (162) |
| | | -/1 | Similar to lysine decarboxylase family (145) |
| | PHE (1), TRP (1) | | |
| *S. piscifermentans* (4) | | -/2 | Arginine/lysine/ornithine decarboxylase (154 ) |
| | | -/2 | Ornithine/lysine/arginine decarboxylase (207) |
| | PHE (3), TRP (3) | | |
| *S. equorum* (8) | | 6/- | Ornithine/lysine/arginine decarboxylase (207) |
| | | -/5 | Similar to lysine decarboxylase family (145) |
| | PHE (1), TRP (1) | | |
| *S. succinus* (4) | | -/2 | Ornithine/lysine/arginine decarboxylase (207) |
| | | -/2 | Similar to lysine decarboxylase family (145) |
| *S. xylosus* (7) | | 1/- | Ornithine/lysine/arginine decarboxylase (207) |
| | | -/5 | Similar to lysine decarboxylase family (145) |

[a] Data from (Seitter (née Resch) *et al.*, 2011). Growing cells were used to detect biogenic amine formation. CAD, cadaverine, PHE, 2-phenylethylamine, PUT, putrescine, TRP, tryptamine.

### 3.5. Genes of technological relevance

The hybridizations also revealed the presence of genes coding for technologically relevant properties (see Tables A3 and A4, supplementary data). The lipase gene, *geh*, coding for glycerol ester hydrolase, which is able to hydrolyze emulsions of lipids containing short chain fatty acids, was detected in all CNS species except *S. equorum*. Furthermore, positive hybridization signals were obtained for genes encoding Clp proteases, which were described to be involved in virulence, oxidative and heat shock response, as well as for genes encoding metallo-proteases and other putative proteases (Table A3, supplementary data). Moreover, the potential for detection of other genes with technological relevance like those involved in

nitrate and nitrite dissimilation and oxidative stress response was demonstrated. As compiled in Table A4 (supplementary data) probes of *nre*, *nar*, *nir*, *kat* and *sod* involved in nitrogen regulation, dissimilatory nitrate reduction, catalase and superoxide dismutase showed positive hybridization signals. However, the genes involved in nitrogen regulation and nitrate dissimilation were more prevalent in strains of *S. carnosus*, *S. condimenti* and *S. piscifermentans* than in *S. equorum*, *S. succinus* and *S. xylosus*. Finally, genes of the arsenate resistance operon, which may be involved in osmotic stress tolerance, were detected in all CNS species except *S. condimenti*.

## 4. Discussion

In this study, a polynucleotide based DNA microarray for the detection of safety and technologically relevant properties of food associated CNS was designed. In contrast to oligonucleotide based microarrays, this array permits to simultaneously detect genes with sequence similarities down to approximately 70%. Hybridizations with the genomic DNA of 32 CNS, several *S. aureus* and *S. carnosus* strains used to generate the probes, demonstrated the functionality of the microarray as well as its sensitivity and specificity. With regard to technological properties, numerous genes were detected in all CNS species, although the probes have been derived from sequences of strains of the species *S. aureus*, *S. carnosus*, *S. epidermidis* and *S. xylosus* only. Thus, this microarray allows to detect genes involved in food (especially meat) fermentation, e.g. nitrate dissimilation as well as catalase, proteases, and lipases, beyond the species borders within the food associated CNS. Moreover, use of this microarray revealed the presence of numerous genes involved in properties of safety relevance, e.g. antibiotic resistances, biogenic amine formation and ECM binding. However, in many cases the detected genotype could not be connected with a phenotype and vice verse, questioning in principle the usefulness of the microarray as tool to detect safety relevant properties. However, there are numerous conclusive explanations why a corresponding genotype or phenotype is missing. Nevertheless, especially due to the combination with the detection of genes of technological relevance, this microarray constitutes a useful tool for rapid and large-scale screening of CNS strains for their genetic potential with regard to safety and technological relevant properties.

A comparison of the phenotypically determined antibiotic resistance profiles (Resch *et al.*, 2008) with the genotypic data obtained by microarray hybridization revealed that resistances against β-lactam antibiotics (e.g. ampicillin, penicillin), lincomycin and tetracycline can be traced back to the presence of the genes *blaZ, pbp2, lnuA* and *tetK*, respectively. This finding is consistent with published data about the occurrence and genetic background of antibiotic resistances in food associated CNS (Even *et al.*, 2010; Kastner *et al.*, 2006; Luthje *et al.*, 2007; Perreten *et al.*, 1998; Resch *et al.*, 2008). On the other hand, a rather high discrepancy between phenotypes and genotypes has been observed (Table 3). This observation is consistent with the findings of (Zhu *et al.*, 2007), describing the microarray based detection of antibiotic resistance genes in clinical isolates of *S. aureus* and numerous CNS. While for *S. aureus* isolates the authors found a high correlation (>90%) between the genotypes and phenotypes, for CNS the correlation ranged from 62 to 75% only. Such discrepancies between phenotypically and genotypically detected antibiotic resistances were also observed for food associated CNS (Even *et al.*, 2010; Perreten *et al.*, 1998). Often discussed reasons are the presence of silent genes which might be turned on *in vivo* or defined regulation of the antibiotic resistance genes which remain undetected under the standardized *in vitro* conditions used to determine the antibiotic resistance profiles of staphylococci (Perreten *et al.*, 2005; Zhu *et al.*, 2007). Nevertheless, it should be interesting to know whether antibiotic susceptible starter organisms harbor silent antibiotic resistance genes, because they may reach consumers through the food chain and might be expressed *in vivo* or the food ecosystem (Perreten *et al.*, 2005).

Furthermore, in this study numerous ambiguous weak hybridization signals were obtained, especially for the murein biosynthesis *pbp* genes, and for several strains with positive phenotype the corresponding genes could not be detected (Resch *et al.*, 2008). Beside the general possibility of the presence of up to now unknown genes or intrinsic resistances, the ambiguous weak or missing detection of the genotype may be explained by large sequence divergences. Most sequences used to design the probes were derived from *S. aureus* strains. Taking the house keeping gene and phylogenetic marker *dnaJ* as example, the sequence similarity between *S. aureus* and food associated CNS ranges from 77 to 78% (Shah *et al.*, 2007), which is close to the detectable threshold of the polynucleotide probes on the microarray. Thus, small sequence similarities may explain the absence of clearly positive hybridization signals, at least in case of non-mobile antibiotic resistance determinants. In such

cases microarrays based on oligonucleotide probes targeted against conserved regions in antibiotic resistance genes (Zhu *et al.*, 2007) might be of advantage.

Microarray hybridizations using genomic DNA of selected CNS revealed no unambiguously detectable genes involved in toxins and hemolysins formation, except gene *sepA* coding for a putative extracellular elastase precursor (Table 5). This result is in agreement with the missing of detection of enterotoxin genes in CNS isolated from fermented food (Even *et al.*, 2010). However, in our previous study 7 of the 32 CNS strains were found to form enterotoxins, as shown by Western blot analysis (Zell *et al.*, 2008). Several microarrays based on oligonucleotide probes (Monecke *et al.*, 2007; Saunders *et al.*, 2004; Spence *et al.*, 2008) or gene segments (Palka-Santini *et al.*, 2007) have already been described for the reliable detection of toxins and/or hemolysin genes in *S. aureus* isolates, demonstrating in principal the microarray based verifiability of such genes. In addition, in this study PCR-generated polynucleotide probes to increase the specificity and sensitivity of detection has been chosen, as shown for long oligonucleotide and polynucleotide probes (Chung *et al.*, 2005; Kane *et al.*, 2000; Taroncher-Oldenburg *et al.*, 2003). In general, tolerance for nucleotide polymorphisms or variations in GC-contents increases with the length of gene probes and therefore, genes with altered sequences can still be detected without loss of hybridization signals (Southern *et al.*, 1999). Due to our chosen hybridization conditions, genes with similarities of 70% and higher should in general be detectable. As the control hybridization revealed excellent and highly specific signals, the question arises why the microarray failed to detect the toxin and hemolysin genes in food associated CNS. One explanation could be that the hemolysin core genes were derived from sequenced *S. aureus* strains. Similar gene sequences in CNS strains are most likely to diverge to be detectable by PCR or the microarray technology. At protein level the proteins are more related and can be detectable by immunoblot with cross-reacting antibodies as has been shown previously (Zell *et al.*, 2008). This problem can be overcome by sequencing more genomes from food associated CNS and to annotate the relevant toxin genes.

With regard to amino acid decarboxylases (e.g. biogenic amine formation) and ECM binding, the comparison of genotypes with phenotypes (Seitter (née Resch) *et al.*, 2011) revealed numerous putative genes for the observed phenotypes (Table 6 and 7). Especially in *S. carnosus*, the decarboxylase genes could unambiguously be identified by microarray hybridization, whereas for the other CNS species often ambiguous weak signals were obtained (Table 7). As the polynucleotide probes for these properties were mainly derived

from *S. carnosus*, the sequence divergence, which increases with the decrease of the evolutionary relationship of the organisms, seems to be responsible for the preferred detection of decarboxylase genes in *S. carnosus*. Interestingly, a gene encoding a conserved hypothetical decarboxylase protein (spot no. 194) could be detected only in strains of *S. carnosus*, *S. condimenti* and *S. piscifermentans*. As this gene is not detectable in the none-biogenic amine forming *S. xylosus*, *S. succinus* and to some extent *S. equorum* strains, there might be a correlation between the presence of this gene and formation of biogenic amines in some CNS. Moreover, numerous putative genes involved in ECM binding were detected without detection of the phenotype (Table 7). One possible explanation would be the presence of silent genes, which might have been detected in the microarray hybridization experiments. On the other hand, it has been described that due to structural variations the fibronectin-binding protein of CNS does not promote cell internalization (Shinji *et al.*, 2003). As structural changes may not necessarily be connected with large sequence modifications, one cannot rule out that the sequence similarity is high enough to permit detection by microarray hybridization but the changes in the sequences altered the function of the ECM binding proteins.

The knowledge about the presence of safety relevant properties like antibiotic resistances, toxin formation, biogenic amine formation and binding to ECM is indispensable for a thorough safety assessment of food associated CNS (Even *et al.*, 2010). From our results of genotypic (this study) and phenotypic investigations (Resch *et al.*, 2008; Seitter (née Resch) *et al.*, 2011; Zell *et al.*, 2008), it can be concluded that except for biogenic amine formation, especially strains of the species *S. equorum*, *S. succinus*, and *S. xylosus* exhibit antibiotic resistances, formation of toxins and binding to ECM. This incidence is probably a reflection of the close phylogenetic relationship of these food associated CNS with pathogenic *Staphylococcus* species (Resch et al., 2008). Furthermore, as the detection of the phenotype plays finally the crucial role in the case-by-case based assessment, microarrays can rather be considered as screening tool, as detected genes may be silent or down regulated. Thus, phenotypic expression of the genes needs to be investigated, at best in the food matrix to demonstrate their importance in food technology applications. Moreover, our microarray was comprehensively designed by including probes for genes coding for (pre)proteins with no or only hypothetically technological function. Therefore, the microarray constitutes a useful tool for further studies like transcriptome and/or mutant studies to explore the involvement of genes in one or other technological property of CNS, as already demonstrated for genes *nre*,

*nar*, *nir*, *kat* and *sod* involved in nitrogen regulation, nitrate reduction, nitrite reduction, catalase and superoxide dismutase activity, respectively. In addition, when the genetic potential of strains intended to be applied in starter cultures should be screened, it is highly convenient to include technologically important properties involved in nitrate dissimilation, control of oxidative damage (catalase), flavor formation (proteases, lipases), as has been demonstrated in this study.

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**Appendix A. Supplementary Data**

Supplementary data to this article can be found online at doi:10.1016/j.ijfoodmicro.2011.01.021.

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# *Chapter VII*

## Summary

Coagulase-negative staphylococci (CNS) are used in starter cultures for the production of fermented meat products due to their involvement in the development of desired red color, characteristic flavor as well as ensuring stability (Hammes, 2012; Marco *et al.*, 2006; Martín *et al.*, 2007). Starter cultures traditionally contain CNS of species *S. carnosus* and *S. xylosus* (Hammes and Hertel, 1998; Talon and Leroy, 2014). But also other CNS species like *S. condimenti*, *S. piscifermentans*, *S. equorum* and *S. succinus* have a potential for future use in starter cultures. The safety of fermented food products is principally proven by long-term experience as traditional methods are considered safe based on their long "history of safe use" (Vogel *et al.*, 2011). However, for the last mentioned species long-term experience concerning sanitary harmlessness exists only with limitations.

To get an insight in safety relevant properties of food associated CNS in Chapter III-V strains of the species *S. carnosus*, *S. condimenti* and *S. piscifermentans* (*S. carnosus*-group) as well as *S. equorum*, *S. succinus* and *S. xylosus* (*S. xylosus*-group) were phenotypically and partly genotypically investigated. Based on these insights in Chapter VI a DNA microarray was developed for rapid and simultaneous detection of various safety relevant properties in CNS with future use in the food production. To increase the application potential of this microarray, additionally technological relevant properties were considered in the array design. Subsequently, the designed microarray was used for the genotypic investigation of phenotypically characterized CNS concerning the presence of safety relevant properties.

In Chapter III, antibiotic resistances of 330 CNS belonging to *S. carnosus*- and *S. xylosus*-group isolated from food and starter cultures were examined. Resistances to 21 antibiotics were phenotypically determined and resistance genes *bla*Z, *lnu*A and *tet*K were detected in strains showing phenotypic resistances to b-lactam antibiotics, lincomycin and tetracycline.

Antibiotic resistance profiles in strains of the species *S. equorum*, *S. succinus* and *S. piscifermentans* are described and due to the high number of investigated strains an insight regarding the occurrence of antibiotic resistances in food associated CNS is given.

In Chapter IV toxin production of food associated CNS belonging to *S. carnosus*- and *S. xylosus*-group was investigated. First, 330 strains isolated from food, starter cultures and clinical isolates have been analyzed to hemolytic activity on human and sheep blood agar plates. Secondly, the ability of 35 selected strains to produce staphylococcal enterotoxins, toxic shock syndrome toxin 1 and exfoliative toxin A has been examined by immunoblot analysis. The chapter demonstrates that CNS strains present in high numbers in fermented food cannot necessarily be regarded as safe. Thus, strains used in the production of fermented food should be analyzed with respect of their toxigenic potential to avoid negative effects on human health.

Chapter V is dealing with the formation of binding proteins to extracellular matrix proteins (ECM) and the production of biogenic amines (BA) by 32 CNS of *S. carnosus*- and *S. xylosus*-group. Binding capacity of CNS to the ECM fibronectin and fibrinogen was investigated by detection of fluorescent labeled cells which were added to microtiter plates coated with ECM. The formation of six important BA was examined by HPLC using growing and resting cells. By the results of this chapter the ability of food associated CNS to develop undesired properties like the formation of binding proteins to ECM and BA was demonstrated. Thus, further research is needed concerning potential risks and the importance on human health if strains with these properties are used in the production of fermented food.

In Chapter VI, the design of a polynucleotide based DNA microarray as screening tool to detect genes of potential health concern and technological relevance in food associated CNS is described. The array considered 220 genes encoding for antibiotic resistances, hemolysins, toxins, amino acid decarboxylases (involved in the formation of BA), binding proteins to ECM, lipases, proteases, stress response factors, and nitrate dissimilation. Hybridization experiments were performed using genomic DNA isolated of 32 in Chapter III-V phenotypically characterized CNS allowing the detection of e.g. antibiotic resistance genes *blaZ*, *lnuA*, and *tetK*. Genes coding for decarboxylases as well as fibronectin and fibrinogen binding proteins were rarely correlated with the phenotype. Toxin genes could not be detected, whereas technological relevant genes like genes coding for proteases, lipases, catalase, superoxide dismutase or genes involved in dissimilatory nitrate reduction resulted in hybridization signals.

The present thesis provides data concerning safety relevant properties in food associated CNS which are important for accurate safety assessment (Even *et al.*, 2010). Comparison of the results of Chapter III-V with them of Chapter VI showed that antibiotic resistances, formation of toxins and binding proteins to ECM are more present in strains of *S. xylosus*- than in *S. carnosus*-group. In context with safety assessment of food associated CNS, the designed microarray can be used as screening tool for the detection of safety relevant combined with technologically important properties (nitrate dissimilation, control of oxidative damage by catalase, flavor formation by proteases and lipases). Summarizing, the array is able to make a contribution in enhancing the selection criteria of CNS used as starter organisms in respect to food safety as well as technologically relevant properties.

# Zusammenfassung

Koagulase negative Staphylokokken (KNS) werden in Starterkulturen für die Herstellung von fermentierten Fleischprodukten zur Umrötung, charakteristischen Aromabildung sowie zur Gewährleistung der Produktstabilität eingesetzt (Hammes, 2012; Marco *et al.*, 2006; Martín *et al.*, 2007). Traditionell enthalten Starterkulturen KNS der Spezies *S. carnosus* und *S. xylosus* (Hammes and Hertel, 1998; Talon and Leroy, 2014), aber auch andere KNS Spezies wie *S. condimenti, S. piscifermentans, S. equorum* und *S. succinus* haben ein Potential für den zukünftigen Einsatz in Starterkulturen. Die Sicherheit von fermentierten Lebensmitteln basiert zumeist auf Langzeiterfahrung infolge der sicheren Historie von traditionellen Methoden (Vogel *et al.*, 2011). Zuletzt genannte Spezies haben jedoch nur eine eingeschränkte Langzeiterfahrung hinsichtlich der gesundheitlichen Unbedenklichkeit.

Zur Bestimmung sicherheitsrelevanter Eigenschaften von lebensmittelassoziierten KNS wurden im Kapitel III-V Stämme der Spezies *S. carnosus, S. condimenti* und *S. piscifermentans* (*S. carnosus*-Gruppe) sowie *S. equorum, S. succinus* und *S. xylosus* (*S. xylosus*-Gruppe) phänotypisch und teils genotypisch untersucht. Weiterführend wurde im Kapitel VI ein DNA-Chip zum schnellen und simultanen Nachweis sicherheitsrelevanter Eigenschaften in KNS mit zukünftigem Einsatz in der Lebensmittelherstellung entwickelt. Um das Anwendungspotential des DNA-Chips zu erhöhen wurden bei der Konzeption des Chips technologisch relevante Eigenschaften mitberücksichtigt. Anschließend wurden phänotypisch charakterisierte KNS mit dem entwickelten Chip genotypisch auf sicherheitsrelevante Eigenschaften untersucht.

In Kapitel III wurden 330 KNS der *S. carnosus*- und *S. xylosus*-Gruppe isoliert aus Lebensmitteln und Starterkulturen phänotypisch hinsichtlich der Resistenzen gegenüber 21 Antibiotika charakterisiert sowie die Resistenzgene *bla*Z, *lnu*A und *tet*K in Stämmen mit phänotypischer b-Lactam-, Lincomycin- und Tetracyclin-Resistenz bestimmt. Die Studie beschreibt Antibiotikaresistenzprofile von Stämmen der Spezies *S. equorum*, *S. succinus* und *S. piscifermentans* und gibt durch die hohe Anzahl an untersuchten Stämmen einen Einblick in das Vorkommen von Antibiotikaresistenzen in lebensmittelassoziierten KNS.

In Kapitel IV wird die Bildung von Toxinen in lebensmittelassoziierten KNS der *S. carnosus*- und *S. xylosus*-Gruppe untersucht. Die hämolytische Aktivität auf Human- und Schafblutagar wurde von 330 Stämmen aus Lebensmitteln, Starterkulturen und klinischen Isolaten bestimmt sowie die Bildung von Staphylokokken-Enterotoxinen, Toxischen Schocksyndrom Toxin 1 und Exfoliativen Toxin A von 35 Stämmen mittels Immunoblot-Verfahrens. Das Kapitel

zeigt, dass KNS die vielfach in fermentierten Lebensmitteln vorkommen, nicht generell als sicher betrachtet werden können. Um negative Effekte auf die humane Gesundheit zu vermeiden sollte das toxigene Potential von in der Lebensmittelherstellung eingesetzten Stämmen bestimmt werden.

Kapitel V befasst sich mit der Bildung von Bindeproteinen an Extrazelluläre Matrixproteine (EZM) und von biogenen Aminen (BA) in 32 KNS Stämmen der *S. carnosus*- und *S. xylosus*-Gruppe. Die Bindekapazitäten an die EZM Fibronektin und Fibrinogen wurden mittels Mikrotiterplatten-Assays durch Fluoreszenzdetektion markierter Bakterienzellen an mit EZM beschichtete Mikrotiterplatten bestimmt. Die Bildung sechs wichtiger BA wurde mit HPLC sowie wachsenden und ruhenden Zellen untersucht. Dieses Kapitel demonstriert, das lebensmittelassoziierte KNS in der Lage sind unerwünschte Eigenschaften wie Bindeproteine an EZM und BA zu bilden. Daher ist weiterer Forschungsbedarf bezüglich potentieller Risiken und der Bedeutung in der menschlichen Gesundheit erforderlich, wenn Stämme mit diesen Eigenschaften zur Herstellung fermentierter Lebensmittel eingesetzt werden.

Kapitel VI beschreibt die Konzeption eines Polynukleotid-basierenden DNA-Chips als Hilfsmittel zum Screenen von Genen mit möglicher gesundheitlicher und technologischer Relevanz in lebensmittelassoziierten KNS. Auf dem DNA-Chip sind 220 Gene für Antibiotikaresistenzen, Hämolysine, Toxine, Aminosäuredecarboxylasen (BA Bildung), EZM Bindeproteine, Lipasen, Proteasen, Nitratatmung, Salz- und oxidative Stresstoleranz abgelegt. Die Hybridisierungen wurden mit genomischer DNA von 32, in Kapitel III-V phänotypisch untersuchten KNS, durchgeführt und können z.B. die Antibiotikaresistenzgene *blaZ*, *lnuA*, und *tetK* nachweisen. Gene, die für Decarboxylasen sowie Fibronektin- und Fibrinogen-Bindeproteine kodieren zeigten selten Übereinstimmung mit dem Phänotyp. Toxingene wurden nicht nachgewiesen. Technologisch relevante Gene (Proteasen, Lipasen, Katalase, Superoxiddismutase und Gene der dissimilatorischen Nitratreduktion) resultierten in Hybridisierungssignalen.

Die Ergebnisse tragen zum Wissen über sicherheitsrelevante Eigenschaften lebensmittelassoziierter KNS bei, welches für eine präzise Sicherheitsbewertung erforderlich ist (Even *et al.*, 2010). Ein Vergleich der Ergebnisse aus Kapitel III-V und Kapitel VI zeigt, dass in der *S. xylosus*-Gruppe Antibiotikaresistenzen sowie die Bildung von Toxinen und Bindeproteinen an EZM häufiger vorkommen als in der *S. carnosus*-Gruppe. Bei der Sicherheitsbewertung lebensmittelassoziierter KNS kann der konzipierte DNA-Chip als Hilfsmittel für ein kombiniertes Screening sicherheits- und technologisch relevanter Eigenschaften (Nitratreduktion, Kontrolle oxidativer Abbau durch Katalase, Geschmack- und Aromaverbesserung durch

Proteasen und Lipasen) herangezogen werden. Zusammenfassend trägt der DNA-Chip dazu bei die Auswahlkriterien von KNS, die als Starterorganismen verwendet werden, hinsichtlich der Lebensmittelsicherheit und technologisch relevanter Eigenschaften zu verbessern.

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# Appendix

This appendix contains supplementary data of ***Chapter VI*** which can be found online at doi:10.1016/j.ijfoodmicro.2011.01.021.

## Supplementary A

Table A1: Genes used for the design of the polynucleotide based DNA microarray and oligonucleotide sequences used for the construction of the probes to detect genes in food associated CNS involved in properties of safety and technological concern

| Property | Description of gene product | Gene | GeneBank accession no. | Locus_tag / gi | Strain | Primer [a] forward reverse nested | Size[b] | Spot no. |
|---|---|---|---|---|---|---|---|---|
| Resistance to ß-lactame antibiotics | Methicillin resistance protein | *mecR1* | NC_002758 | SAV0042 | *S. aureus* Mu50 | actaccaactgtcgtagtcg cgtttcaacataacctacgaacc tcacgattcctgttccag | 1052 | 140 |
| | Penicillin-binding protein 4 | *pbp4* | NC_002758 | SAV0642 | *S. aureus* Mu50 | catacgaaccgacgagtg gctaatccagcgacaagg tcttggatagtccgcgtg | 965 | 144 |
| | Oxacillin resistance-related FmtC protein | *fmtC* | NC_002745 | SA1193 | *S. aureus* N315 | actttaggtgtccctgagg gtcgctctaaatccacgg tcacctaacggatctcca | 944 | 83 |
| | Penicillin binding protein 2 prime | *mecA* | NC_002745 | SA0038 | *S. aureus* N315 | agtagaaatgactgaacgtccg cgttgtaaccaccccaag ggaacttgttgagcagagg | 1010 | 35 |
| | Penicillin-binding protein 2 | *pbp2* | NC_002951 | SACOL1490 | *S. aureus* COL | tcttagcgtatggacctgc gattgtgcagtaccactgc accacttactgagctagg | 806 | 134 |
| | Penicillin-binding protein 3 | *pbp3* | NC_002745 | SA1381 | *S. aureus* N315 | gaacacttcagcggtacc ggagatgtatcgacacctg acgcaatgatcgtgttgg | 1078 | 87 |
| | Penicillin-binding protein 1 | *pbpA* | NC_002745 | SA1024 | *S. aureus* N315 | cgtaaaaatgatgtctgaggcc tgtgtaatcggtctaccgac gactacaatatgcgtcgaacc | 352 | 74 |
| | ß-Lactamase | *blaZ* | NC_003140 | SAP010 | *S. aureus* N315 | acagttcacatgccaaagag tcattacactcttggcgg gttcagattggcccttagg | 687 | 136 |
| Resistance to tetracycline | Tetracycline resistance protein | *tetS* | X92946 | gi\|2467210; 27974-26034 | *Lactoc. lactis* (*pK214*) | agataaggcagagcctgg gttaaacagacactgcgtcc ctcttgaatacaacgggctg | 850 | 20 |

| Property | Description of gene product | Gene | GeneBank accession no. | Locus_tag / gi | Strain | Primer[a] forward reverse nested | Size[b] | Spot no. |
|---|---|---|---|---|---|---|---|---|
| | Similar to tetracyclin resistance | | NC_002758 | SAV0137 | *S. aureus* Mu50 | ctgatgccattagtatacgcac<br>aatcgattagagccgcag<br>acctacagataccgccac | 983 | 141 |
| | Tetracycline resistance protein | *tetM* | NC_002758 | SAV0398 | *S. aureus* Mu50 | acatcatagacacgccag<br>caacggaagcggtgatac<br>atcgtagaagcggatcac | 918 | 142 |
| | Tetracycline efflux protein | *tetK* | NC_006871 | gi\|60677337; 1493-114 | *S. epidermidis* ATCC12228 | acaaggagtaggatctgctg<br>agcaaactcattccagaagc<br>caataacactcatggttccagg | 587 | 28 |
| | Tetracycline efflux protein | *tet(K)* | M16217 | gi\|150832; 305-1684 | *S. epidermidis* ATCC12228 | acaaggagtaggatctgctg<br>agcaaactcattccagaagc<br>caataacactcatggttccagg | 587 | 6 |
| Resistance to quinolones | DNA gyrase subunit A | *gyrA* | NC_002758 | SAV0006 | *S. aureus* Mu50 | gcgttatactgaagcgcg<br>tgtagataccggcaaacg<br>catcaccgaaacgatctcg | 1085 | 138 |
| | DNA gyrase subunit B | *gyrB* | NC_002758 | SAV0005 | *S. aureus* Mu50 | tggtgactgcattgtcag<br>ctgtacgtgcgacttgtg<br>tcgttcaaagtgctctgag | 1109 | 137 |
| | Topoisomerase IV subunit A | *parC* (*grlA*) | NC_002758 | SAV1355 | *S. aureus* Mu50 | ttcaagagcgtgcattgc<br>acccatcaattttggacgac<br>catcactaatagcgaccatgt | 930 | 149 |
| | Topoisomerase IV subunit B | *parE* (*grlE*) | NC_002758 | SAV1354 | *S. aureus* Mu50 | acgactacaagagtctgcg<br>acgttgtaacgtgaagcc<br>tgcttgaacaagcggtttc | 1018 | 148 |
| | Quinolone resistance protein | *norA* | NC_002758 | SAV0695 | *S. aureus* Mu50 | tatatcgccgtttggtgg<br>caaataacgcacctgcga<br>gttcaatccgcctgcaaa | 838 | 147 |
| Resistance to lincomycin, clindamycin | Lincomycin resistance protein | *linA* | AY541446 | gi\|44985410; 721-236 | *S. aureus* (*pBMSa1*) | gttagatggaggttgggg<br>tcattttgaaaaacgtaattgcc<br>cgtaattgccaccttcgg | 260 | 24 |
| Resistance to macrolide-lincosamide-streptogramin B | ermB methylase | *ermB* | AF516335 | gi\|21886737; 7963-8700 | *E. faecium* (*pUW786*) | agaaaccgataccgtttacg<br>acgatattctcgattgaccca<br>atctggaacatctgtggt | 469 | 16 |

| Property | Description of gene product | Gene | GeneBank accession no. | Locus_tag / gi | Strain | Primer[a] forward reverse nested | Size[b] | Spot no. |
|---|---|---|---|---|---|---|---|---|
| | Adenine methylase | *ermC* | X82668 | gi\|1638843; 188-922 | *S. equorum* (*pE194*) | tctttgaaatcggctcagg<br>aagtaatgccaatgagcg<br>atcttttagcaaacccgt | 303 | 14 |
| | rRNA adenine N-6-methyltransferase | *ermA* | NC_002745 | SA0048 | *S. aureus* N315 | tatgtgcaccagtatcgc<br>tgggaagaataaagcgtcct<br>agaataaagcgtcctcttgtga | 999 | 36 |
| | Macrolide efflux protein | *mdt(A)* | X92946 | gi\|2467210; 10534-11790 | *Lactoc. Lactis* (*pK214*) | atgaacacatcagtcccg<br>agattagcccaactggtc<br>cagggcatacggtcctta | 863 | 18 |
| | Methylase | *ermGT* | M64090 | gi\|148726; 168-902 | *Lactob. reuteri 100-233* (*pGT633*) | ttgagattggttcaggga<br>agagacaagaattgttcaaagc<br>gtgtaattatgtaaccgccattga * | 73 | 5 |
| | Phosphorylase | *mphBM* | AF167161 | gi\|9801975; 5665-6565 | *S. aureus* (*pSR1*) | cgaactatggcctcgaca<br>catcgtttcatacgccga<br>ctgattccatagcaaactcagc | 293 | 32 |
| Resistance to pristinamycin | Pristinamycin resistance protein | *vgaB* | U82085 | gi\|15718667; 629-2288 | *S. aureus* BM12235 (*pIP1633*) | aggtaagaatggagttggca<br>acaaatagcttggcaaaagc<br>tgtttcatcttgaatggaggt | 1152 | 13 |
| Resistance to streptogamin A | Streptogramin A acetyltransferase | *satG* | AF229200. | gi\|7595743; 4684-5328 | *E. faecium* (*pUW1965*) | ttgttctatcgccagtgg<br>tgtttatcaattcaagtgtgggtg<br>ggttcaaatcttggtccgatg | 315 | 30 |
| | Streptogramin A acetyltransferase | *vatC* | AF015628 | gi\|3660655; 1307-1945 | *S. aureus* BM12292 (*pIP1714*) | tctattggacccggaacg<br>tcaccattcaggatgcaatc<br>tctatctctaggtcccacca | 359 | 21 |
| | Vga-A variant (putative ABC protein) | | AF186237 | gi\|22096304; 5065-6639 | *S. aureus* BM3327 | ctcagtattcacggtgcg<br>tcatcatgattcgggtagc<br>acctacgatcatgggagac | 1143 | 17 |
| Resistance to streptogramin B | Streptogramin B lactonase | *vgbB* | AF015628 | gi\|3660655; 399-1286 | *S. aureus* BM12292 (*pIP1714*) | ccgattcaggtccatacg<br>tgcaaaccatacggatcc<br>aggtaataccatgaggttccg | 783 | 22 |
| Resistance to virginiamycin A-like antibiotics | Acetyltransferase | *vatB* | U19459 | gi\|1181626; 67-705 | *S. aureus* BM12235 (*pIP1633*) | gaaatatggccctgatcc<br>tttgtgctgaccaatccc<br>acaactgaattcgcagcaac | 444 | 4 |

| Property | Description of gene product | Gene | GeneBank accession no. | Locus_tag / gi | Strain | Primer [a] forward reverse nested | Size[b] | Spot no. |
|---|---|---|---|---|---|---|---|---|
| | ATP-binding protein | *vga* | M90056 | gi\|153124;909-2477 | *S. aureus* BM3093 (*pIP680*) | actatattcggcaagcgct<br>tgtcattattcgagtggctact<br>tggttcatctagtaccaacg | 971 | 8 |
| | Acetyltransferase | *vatA* | L07778 | gi\|398084; 258-917 | *S. aureus* BM3093 (*pIP680*) | tgaccatggacctgatcc<br>agggatttccaccgacaa<br>agcaacattctttgtgacaacag | 454 | 23 |
| Resistance to virginiamycin B-antibiotics | Hydrolase | *vgh* | M20129 | gi\|153076; 641-1540 | *S. aureus* BM3093 (*pIP680*) | cagagtacccactaccgac<br>tcgaaacaaatgccatgtgg<br>cagtaattgcatgaggtcg | 552 | 7 |
| Resistance to chloramphenicol | Chloramphenicol acetyltransferase | *cat* | AF229200 | gi\|7595743; 1234-584 | *E. faecium* (*pUW1965*) | tacaatagcgacggagag<br>aagccagtcattaggcct<br>tcctgcatgataaccatcaca | 384 | 31 |
| | Chloramphenicol acetyltransferase | *cat* | X92946 | gi\|2467210; 22648-21916 | *Lactoc. lactis* (*pK214*) | aggatatgaactgtatcctgc<br>tggtaaccatcacataccg<br>ccgcatgatgaacttgca | 437 | 19 |
| | Chloramphenicol acetyltransferase | *cat* | NC_002013 | gi\|10956139; 1260-1910 | *S. aureus* (*pC194*) | tacaatagcgacggagag<br>aagccagtcattaggcct<br>tcctgcatgataaccatcaca | 384 | 1 |
| | Similar to chloramphenicol resistance protein | | NC_002745 | SA2241 | *S. aureus* N315 | acatgtatcagaagctgtcgt<br>ccaatgatggcactcgta<br>gtgtcatgaccaagcctc | 974 | 125 |
| Resistance to florfenicol and chloramphenicol | Florfenicol resistance protein | *cfr* | AJ249217 | gi\|9909977; 570-1619 | *S. sciuri* (*pSCFS1*) | catgaagtataaagcaggttggg<br>acatggataccagcagact<br>catacatctcaggtgcact | 626 | 33 |
| Resistance to aminoglycosides | Bifunctional Aac/Aph (2'-aminoglycoside phosphotransferase, 6'-aminoglycoside N-acetyltransferase) | *aacA* | NC_002774 | SAVP026 | *S. aureus* Mu50 | acattatacagagccttgggaag<br>acactatcataaccactaccga<br>cctcgtgtaattcatgttctgg | 356 | 151 |
| Resistance to kanamycin | Aminoglycoside 3'-phosphotransferase | *aphA* | NC_006663 | SEA0010 | *S. epidermidis* RP62A | aggaatgtctcctgctaagg<br>aggcttgatccccagtaag<br>gatgttgctgtctcccag | 517 | 208 |
| Resistance to kanamycin, neomycin | Nucleotidyltransferase | *aadD* | NC_002745. | SA0033 | *S. aureus* N315 | ctcttggtcgtcagactg<br>tccagaagtttctcagagtcg<br>cagacattacgaactggcac | 528 | 34 |

| Property | Description of gene product | Gene | GeneBank accession no. | Locus_tag / gi | Strain | Primer [a] forward reverse nested | Size[b] | Spot no. |
|---|---|---|---|---|---|---|---|---|
| Resistance to streptomycin, spectomycin | O-nucleotidylltransferase(9) | *ant(9)* | NC_002745 | SA0765 | *S. aureus* N315 | aacgctaacggtcagctt<br>tctaatttcacaagaggacgct<br>acgaggtgaaatcatgagca * | 119 | 59 |
| Resistance to vancomycin, teicoplanin | Vancomycin/teicoplanin A-type resistance protein | *van(A)* | NC_005054 | VRA0040 | *S. aureus* (*pLW1043*) | tgcaatactgtttggggg<br>cgctaatacgatcaagcgg<br>ggtcaatcagttcgggaa | 904 | 212 |
| Resistance to teicoplanin | Teicoplanin resistance protein | *vanZ* | M97297 | gi\|155036; 10116-10601 | *S. aureus* (*pLW1043*) | tgactccatttactgctactgg<br>tcttaaatgggtacggtaaacg<br>tcagtccaagaaagcctc | 234 | 9 |
| Resistance to bacitracin | Bacitracin resistance protein homolog | *bacA* | NC_002758 | SAV0683 | *S. aureus* Mu50 | gtcatccaattaggatccgtc<br>atgtgaagtccgatgctg<br>attgttgagcctgaacgg | 404 | 146 |
| Resistance to bleomycin | Bleomycin resistance protein | *bleO* | NC_002758 | SAV0034 | *S. aureus* Mu50 | ttgccagtcggggatatt<br>tcaaattgttgtcgggatc<br>tgatgtattggggtgcaa | 288 | 139 |
| Resistance to streptothricin | Streptothricin acetyltransferase | *sat4* | AF516335 | gi\|21886737; 15805-16347 | *E. faecium* (*pUW786*) | cgataaacccagcgaacc<br>acatagtatcgacggagc<br>gctataaggttattgtcctgggt | 393 | 15 |
| Resistance to bicyclomycin | Similar to bicyclomycin resistance protein TcaB | | NC_002758 | SAV2434 | *S. aureus* Mu50 | gctaattacgttaggcgtcatg<br>ccaagtggtccaattgacg<br>tgagtaagacccaaagtgg | 896 | 150 |
| Resistance to fosfomycin | Fosfomycin resistance protein | *fosB* | NC_002745 | SA2124 | *S. aureus* N315 | ttgagcttgcaggcctat<br>ctgttctcaagtgtgccag<br>agcttatgaccatcagggtc | 223 | 120 |
| Resistance to trimethoprim | Trimethoprim resistance protein | *dfrA* | NC_004461 | SE1119 | *S. epidermidis* ATCC12228 | gtcgtactcactaaccaagc<br>gcactaaatgtaagaatgtatgcgg<br>actgaagattcgacttcccag | 233 | 203 |
| Toxin formation | hypothetical protein, similar to enterotoxin A precursor | | NC_002745 | SA1430 | *S. aureus* N315 | attcagcgagtgcaatcg<br>accaccataactacattgcg<br>tgcgttttattggttgctcc | 304 | 88 |
| | Enterotoxin P (equal to Enterotoxin A) | *sep* (*sea*) | NC_002758 | SAV1948 | *S. aureus* Mu50 | tgaatactgtccttgagcac<br>cctaacgttgacaacaagtcc<br>ccacttgtaaatggtagcgag | 633 | 213 |

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|---|---|---|---|---|---|---|---|---|
| | Staphylococcal enterotoxin B | *seb* | NC_002951.2 | SACOL0907 | *S. aureus* COL | ccaacgttttagcagagagtc<br>gtcaaatttatctcctggtgcag<br>cccgtttcataaggcgag | 568 | 214 |
| | Enterotoxin typeC3 | *sec3* | NC_002745 | SA1817 | *S. aureus* N315 | ccaacgttttagcagagagtc<br>gtcaaacttatcgcctggt<br>tcataccaaaaagtattgccg | 610 | 113 |
| | Enterotoxin D (mature form) | *entD* | AY518388 | GI:41207521 | *S. aureus* NCTC 10656 | gcttgtacatatggaggtgtc<br>tagatgtcaatatgaaggtgctctg<br>tcataagagactttagacccatcag | 278 | 215 |
| | Enterotoxin SEM | *sem* | NC_002745 | SA1647 | *S. aureus* N315 | gctgatgtcggagttttg<br>agcttgtcctgttccagt<br>ctgctaatgtaactccaccg | 256 | 103 |
| | Enterotoxin SeN | *sen* | NC_002745 | SA1643 | *S. aureus* N315 | tgagattgttctacatagctgc<br>aaaactctgctcccactg<br>accttcttgttggacacc | 473 | 99 |
| | Enterotoxin SeO | *seo* | NC_002745 | SA1648 | *S. aureus* N315 | agtcaagtgtagaccctattgc<br>tttcatgaggtgttaccc<br>caccatatgtacaggcagt | 297 | 104 |
| | Enterotoxin P | *sep* | NC_002745 | SA1761 | *S. aureus* N315 | tgaattgcagggaactgc<br>ggatattgtccttgagcacc<br>tgactgaaggtctagctcc | 430 | 108 |
| | Enterotoxin Yent1 | *yent1* | NC_002745 | SA1645 | *S. aureus* N315 | agtctctagggctaatgtg<br>catcatgctcggtcacac<br>tgtgttaagtcttgcagcttact * | 131 | 101 |
| | Enterotoxin YENT2 | *yent2* | NC_002745 | SA1644 | *S. aureus* N315 | acgtagatttgtttgggac<br>ttaccagattcaggcatc<br>ccagtctcataaggcgaac | 329 | 100 |
| | Toxic shock syndrome toxin-1 | *tst* | NC_002745 | SA1819 | *S. aureus* N315 | tgttgcttgcgacaatcg<br>tcactttgatatgtggatccg<br>ccgttttatcgcttgaacg | 528 | 114 |
| | Hypothetical protein | | NC_002745 | SA1016 | *S. aureus* N315 | cacctctagtaacgtcaggac<br>acatatgctgcagcgact<br>ggtttgattgacgtctgc | 550 | 72 |

| Property | Description of gene product | Gene | GeneBank accession no. | Locus_tag / gi | Strain | Primer[a] forward<br>reverse<br>nested | Size[b] | Spot no. |
|---|---|---|---|---|---|---|---|---|
| | Extracellular enterotoxin type G precursor | *seg* | NC_002745 | SA1642 | *S. aureus* N315 | tgctcaacccgatcctaa<br>tctttagtgagccagtgtc<br>ccaccatacatacaacaacctc | 339 | 98 |
| | Extracellular enterotoxin type I precursor | *sei* | NC_002745 | SA1646 | *S. aureus* N315 | agatcttacgtatgctcaagg<br>acttacaggcagtccatc<br>gtcctgataaagtggccc | 275 | 102 |
| | Extracellular enterotoxin L | *sel* | NC_002745 | SA1816 | *S. aureus* N315 | tcatacagtcttatctaacggcg<br>agctttctggaagaccgt<br>agttgtaactgtttgatgcttgc | 590 | 112 |
| | Hypothetical protein, similar to exotoxin 1 | | NC_002745 | SA1009 | *S. aureus* N315 | tctttatggggcgctgaa<br>tcaatagtgtagttattaccgcca<br>cttctcgtgcacggaaatc | 280 | 69 |
| | Hypothetical protein, similar to exotoxin 2 | | NC_002745 | SA0357 | *S. aureus* N315 | tatccaaaagcagacgcg<br>tgtagctctttatgcgactc<br>actctatcagcaactgtg | 245 | 41 |
| | Hypothetical protein, similar to exotoxin 3 | | NC_002745 | SA1011 | *S. aureus* N315 | gcaacattgcaaacgtgg<br>tcgtgtaattatttccatcagcagt<br>tgtaaaattcatgtccaccga | 262 | 71 |
| | Hypothetical protein, similar to exotoxin 4 | | NC_002745 | SA1010 | *S. aureus* N315 | agataacggtacgattcatgc<br>catcagttgatggcaacct<br>ccttcataaactggaaatccgc | 285 | 70 |
| | Exotoxin 6 | *set6* | NC_002745 | SA0382 | *S. aureus* N315 | actcaattggtggcgtaac<br>tgtatttgtctgccgtcga<br>gtcgatagaatcacccatacg | 302 | 42 |
| | Exotoxin 7 | *set7* | NC_002745 | SA0383 | *S. aureus* N315 | agcaacattatcatacgggtg<br>acattgataacatcggacatacg<br>cctgtactcatttcaccgtaaag | 292 | 43 |
| | Exotonin 8 | *set8* | NC_002745 | SA0384 | *S. aureus* N315 | acagtaacgacgcaatcg<br>ccatacgatgctcttgcag<br>acgattgttcctgaaccc | 887 | 44 |
| | Exotonin 9 | *set9* | NC_002745 | SA0385 | *S. aureus* N315 | cgggtgtaatcacaacgac<br>agtgccatctatgacgtctg<br>cctgaaccaacgttaccg | 697 | 45 |

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|---|---|---|---|---|---|---|---|---|
| | Exotoxin 10 | *set10* | NC_002745 | SA0386 | *S. aureus* N315 | atagcaaaggtggcaagc<br>gtcaatgacatcactcatgcg<br>tgtagtgtactctaccgtacg | 282 | 46 |
| | Exotoxin 11 | *set11* | NC_002745 | SA0387 | *S. aureus* N315 | catcagaaggccaagcag<br>tgaatatccttactattcaacacatcacc<br>tgatcttaccgtatttagtcgtacct | 504 | 47 |
| | Exotoxin 12 | *set12* | NC_002745 | SA0388 | *S. aureus* N315 | agatgtctttgcggtacca<br>atctgccatacgctcgaa<br>aattctacctttatcagctgaccc | 274 | 48 |
| | Exotoxin 13 | *set13* | NC_002745 | SA0389 | *S. aureus* N315 | agtaaagctgttcacgcga<br>tctacctttatctgacgcgc<br>tgttataccgccaacgct | 297 | 49 |
| | Exotoxin 14 | *set14* | NC_002745 | SA0390 | *S. aureus* N315 | cagaagttcattcaggtcatgc<br>gtgcactgacaactccag<br>tctgtattacaccaccaac | 297 | 50 |
| | Exotoxin 15 | *set15* | NC_002745 | SA0393 | *S. aureus* N315 | caactactgctcagccag<br>cgtatcacccatgcgatg<br>agtcacagaagtagtgtgttgac | 380 | 51 |
| | Hypothetical protein | | NC_002745 | SA2323 | *S. aureus* N315 | acgattatctagagctaagcgtc<br>gttcaaagcatctgggtacag<br>tctaaaggtgtgtcccctg | 624 | 126 |
| | Hypothetical protein, similar to esterase | | NC_002745 | SA2140 | *S. aureus* N315 | catcaccgttccattggag<br>tgttaatggtaggccattcg<br>gttcgattaaggcgtccg | 324 | 121 |
| | Extracellular elastase precursor | *sepA* | NC_002976 | SERP2252 | *S. epidermidis* RP62A | caacacgtatgcaccagac<br>gtatgtacgcctccgttg<br>ttgtccaaaacgctctgg | 1038 | 209 |
| Hemolysis | Phospholipase C | *hlb* | NC_002951.2 | SACOL2003 | *S. aureus* COL | cttactgacaatagtgccaaagc<br>gaacgcatatacatcccatgg<br>ttgattgagggtcccatg | 709 | 216 |
| | Alpha-hemolysin precursor | | NC_002745 | SA1007 | *S. aureus* N315 | acacgtatagtcagctcag<br>tgcaattggtagtcatcacg<br>actgtagcgaagtctggtg | 848 | 68 |

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|---|---|---|---|---|---|---|---|---|
| | Hypothetical protein, similar to hemolysin | | NC_002745 | SA0780 | *S. aureus* N315 | cttttcaggaagcgagacg<br>tatcgcttcggtaccacc<br>accaaattcgtccaacac | 850 | 60 |
| | Hypothetical protein, similar to hemolysin III | | NC_002745 | SA1973 | *S. aureus* N315 | ttggtaatgcggcatctc<br>ccaattgtataagacacaccacc<br>gtaacaagataatacctacccagc | 420 | 117 |
| | Gamma-hemolysin chain II precursor | *hlgA* | NC_002745 | SA2207 | *S. aureus* N315 | agatagaagatatcggtcaagg<br>cggctaaacgatgtcttg<br>gatgtcttgtcacgtaagca | 723 | 122 |
| | Gamma-hemolysin component C | *hlgC* | NC_002745 | SA2208 | *S. aureus* N315 | taaagctatgcgatggcc<br>gagtcttccaattgacctcg<br>tggcatgagtgacatcca | 512 | 123 |
| | Gamma-hemolysin component B | *hlgB* | NC_002745 | SA2209 | *S. aureus* N315 | ggtaaaataacaccagtcagcg<br>gcccagtagaagccattc<br>ctatgaagttttggccagc | 622 | 124 |
| Other of safety concern | Fibronectin-binding protein A N-terminus | *fbpA* | BA000018.3 | SA1051 | *S. aureus* N315 | gctaacatagccgcttcc<br>tggtaatgatcgtcgcattg<br>cgatactatttaccgcactgtc | 1197 | 217 |
| | Fibronectin-binding protein homolog | *fbpB* | BA000018.3 | SA2290 | *S. aureus* N315 | tgtttagttggtgccactg<br>gtgcagaaggtcatgcag<br>ggatacaaacccaggtgg | 1017 | 218 |
| | Fibrinogen-binding protein A, clumping factor | *clfA* | BA000018.3 | SA0742 | *S. aureus* N315 | gtagcgttagtgctgcac<br>ttggtcatcgtccgtagg<br>caacgttatctccgcttgg | 1044 | 219 |
| | Clumping factor B | *clfB* | BA000018.3 | SA2423 | *S. aureus* N315 | tgtaactcttgaatcggagtcac<br>tggtgtagatacagcttcagg<br>tcagatagctactatgcagatcc | 1095 | 220 |
| | Cell wall hydrolase | *lytN* | NC_002745 | SA1090 | *S. aureus* N315 | ttggcattgggtatgtcg<br>tgaacaactttatgtgcaacctc<br>ctgctttacgccatccac | 967 | 77 |
| | Hypothetical protein | | NC_002745 | SA1018 | *S. aureus* N315 | ggaagttgatgggcatcg<br>caagtagctatacccgcattg<br>tccaactataatcgagtgctgtc | 304 | 73 |

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|---|---|---|---|---|---|---|---|---|
| | Hyaluronate lyase precursor | *hysA* | NC_002745 | SA2003 | *S. aureus* N315 | atcaaatcgctgtggctg<br>ctttcgtaacgtgcgacg<br>cagatgctgagtgactcg | 1020 | 118 |
| | Thermonuclease | *nuc* | NC_002745 | SA1160 | *S. aureus* N315 | gcttgctatgattgtggtagc<br>ccaatgttctaccatagcga<br>gttgtacaggcgtattcgg | 251 | 82 |
| | Staphylokinase precursor | *sak* | NC_002745 | SA1758 | *S. aureus* N315 | ttttgaaccaacaggccc<br>tgctctgataaatctgggac<br>agtgacttcgatctttgcg | 220 | 107 |
| | Staphylococcal nuclease | | NC_002745 | SA0746 | *S. aureus* N315 | agtttcaagtctaagtagctcagc<br>gacctgaatcagcgttgtc<br>ctttagccaagccttgacg | 419 | 58 |
| | Staphylococcal protein A spa | *spa* | NC_002745 | SA0107 | *S. aureus* N315 | tcttaaagacgacccaagc<br>tatagttcgcgacgacgtc<br>gtccagctaataacgctgc | 953 | 37 |
| | Leukotoxin D | *lukD* | NC_002745 | SA1637 | *S. aureus* N315 | tagtacttaaggcagccgg<br>agtatggttttgccagtcaac<br>accaatccagtgtagtcg | 638 | 96 |
| | Leukotoxin E | *lukE* | NC_002745 | SA1638 | *S. aureus* N315 | cagtaggactgattgcgc<br>aaatgagccattgccacc<br>ctatagatggtgctgactgg | 426 | 97 |
| | Leukocidin F | | NC_002745 | SA1812 | *S. aureus* N315 | atcgtggaggcttaactg<br>acatggttttcaccagacc<br>accaatagccccagaaac | 432 | 110 |
| | Leukocidin M | | NC_002745 | SA1813 | *S. aureus* N315 | tgttatcagcagcaacgac<br>tgcataatgtattccaggtctgt<br>tcgcaataactgaccagtg | 612 | 111 |
| Decarboxylation | Arginine/lysine/ornithine decarboxylases | | NC_012121 | Sca_0122 | *S. carnosus* TM300 | cacaatcaactgagcctatgc<br>tcttaactgtcgttgcgg<br>tgctagtcgtttaccgacag | 923 | 154 |
| | Orn/Lys/Arg decarboxylase | | NC_002758 | SAV0481 | *S. aureus* Mu50 | ctgtgttacatgcgctcg<br>taataggaatccctggcgg<br>caatatgtcgcgccagaa | 876 | 143 |

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|---|---|---|---|---|---|---|---|---|
| | Orn/Lys/Arg decarboxylase | | NC_004461 | SE2302 | *S. epidermidis* ATCC12228 | tagttaacggaacgacttcagg<br>tcatatgagacgtcacgac<br>aggttttatgtcgcgcgta | 1029 | 207 |
| | Hypothetical protein, similar to (P11926) Ornithine decarboxylase | | NC_012121 | Sca_0786 | *S. carnosus* TM300 | actatgaagcgcgatacag<br>ctgcataaacactggcag<br>caggctctacatcagcac | 369 | 162 |
| | Similar to lysine decarboxylase family | | NC_002758 | SAV0680 | *S. aureus* Mu50 | gcagtttattgtggtgcaag<br>tcgtatgaacgagtaccc<br>tccttagtgtcgcataacgg | 497 | 145 |
| | Hypothetical protein, similar to lysine decarboxylase | | NC_002745 | SA0439 | *S. aureus* N315 | agctttaacgatgggctc<br>taataggaatccctggcgg<br>gacaatatgtcgcgccag | 559 | 52 |
| | Hypothetical protein | | NC_002745 | SA2327 | *S. aureus* N315 | taagtatcggtggccctg<br>gctgtagcgaagtcttgc<br>ccatagctcctggaagac | 1041 | 127 |
| | Hypothetical protein | | NC_004461 | SE0450 | *S. epidermidis* ATCC12228 | tgtattcggagcaggatcag<br>cgattaagtgttgtagtggctc<br>acacctataggcttttggtg | 332 | 197 |
| | Hypothetical protein | | NC_002745 | SA0635 | *S. aureus* N315 | aagctattggcgtcatgc<br>tcgtatgaacgagtacccag<br>tccttagtgtcgcataacg | 334 | 55 |
| | Conserved hypothetical protein | | NC_012121 | Sca_2446 | *S. carnosus* TM300 | tcccgtttatcatggctagg<br>gtgtagttaatgggttcgtcg<br>tactgtcgtataggcaatcgc | 1090 | 194 |
| | Acetolactate synthase large subunit | *ilvB* | NC_002745 | SA1859 | *S. aureus* N315 | tgtagttacaagcggtccag<br>tcatttggaaaccaccgtc<br>gatgttgtcccacgtctg | 1127 | 116 |
| | Alpha-acetolactate decarboxylase | | NC_004461 | SE2143 | *S. epidermidis* ATCC12228 | aggtattgctacgcttacagg<br>tgatttagcatctactgggaagt<br>agtgaatatggaaacctgcag | 425 | 206 |
| | Hypothetical protein, similar to alpha-acetolactate decarboxylase | | NC_002745 | SA2007 | *S. aureus* N315 | caggttcaaacggtgagg<br>gaaatcctgctgatccga<br>agaaaccaacgactgaacc | 357 | 119 |

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|---|---|---|---|---|---|---|---|---|
| | Hypothetical protein, similar to alpha-acetolactate decarboxylase | | NC_002745 | SA2394 | *S. aureus* N315 | gcaacgttaacagggtctg<br>acgtctcgttatttaccggg<br>tgtatctcaacgacaacgtc | 491 | 130 |
| | Putative alpha-acetolactate decarboxylase | | NC_012121 | Sca_1958 | *S. carnosus* TM300 | actttgatggccggtatg<br>cttcatcgtgtactgggaagt<br>agtgcagatggaatccag | 493 | 188 |
| | Putative acetoacetate decarboxylase | | NC_012121 | Sca_2369 | *S. carnosus* TM300 | ccgattaacgcacctgtc<br>caagtaatcgtaaacggggc<br>ggaaatctgctagcggtg | 693 | 193 |
| | Aspartate 1-decarboxylase | *panD* | NC_002745 | SA2390 | *S. aureus* N315 | agagcaagagtgactgagtc<br>ctgctactttaggagcatgg<br>tcatgattatgactacatcgcc | 232 | 129 |
| | Aspartate 1-decarboxylase | | NC_004461 | SE2139 | *S. epidermidis* ATCC12228 | tgaactcaaaaatccatagagct<br>cacagctacttttggtgagtg<br>tcaactagtcttgaagccg | 223 | 205 |
| | Putative aspartate 1-decarboxylase | | NC_012121 | Sca_2074 | *S. carnosus* TM300 | ctaagattcaccgcgcac<br>catgacagctactttaggtgc<br>catccacttcaacaagacgtg | 225 | 190 |
| | Diaminopimelate decarboxylase | | NC_004461 | SE1080 | *S. epidermidis* ATCC12228 | tagtggatacgctcgagg<br>agggtaatggggcatctc<br>caacgcctcatactgtgc | 714 | 202 |
| | Diaminopimelate decarboxylase | *lysA* | NC_002745 | SA1232 | *S. aureus* N315 | ttagagaacagatgcgcc<br>agcaagatagtctccacg<br>cctacaattgatcgtcccg | 748 | 84 |
| | Putative diaminopimelate decarboxylase | | NC_012121 | Sca_0970 | *S. carnosus* TM300 | ttggagactctgtaggcg<br>tgagataagatgtcgctaacagg<br>tcaactactaccgctgctg | 340 | 167 |
| | Similar to diaminopimelate decarboxylase | | NC_002745 | SA0119 | *S. aureus* N315 | tcattgaagctgcgctag<br>tagaccatccgtatgcacc<br>ataacgtcgcctgtactg | 589 | 38 |
| | Glycine dehydrogenase subunit 1 | | NC_002745 | SA1366 | *S. aureus* N315 | gatcattacgcgccatcag<br>tcgctttatcgcgtcgaa<br>caggaattccgaatggct | 577 | 86 |

| Property | Description of gene product | Gene | GeneBank accession no. | Locus_tag / gi | Strain | Primer [a] forward reverse nested | Size [b] | Spot no. |
|---|---|---|---|---|---|---|---|---|
| | Probable glycine dehydrogenase | | NC_012121 | Sca_1160 | *S. carnosus* TM300 | agattaagccgtattgcg<br>gcaatgtcttgaaggcct<br>agtgcattaagagcctggt | 848 | 169 |
| | Histidinol-phosphate/aromatic aminotransferase and cobyric acid decarboxylase | | NC_012121 | Sca_0371 | *S. carnosus* TM300 | accaggattatctccaagagc<br>ccaatcgttatacggacacc<br>ttgatcttcgaaagccgc | 969 | 157 |
| | Hypothetical protein, similar to histidinol-phosphate aminotransferase | | NC_002745 | SA0679 | *S. aureus* N315 | actatcagcatatcagcctgg<br>tatcctacacgtaaacccgc<br>agcaagtgtatccgggtag | 595 | 56 |
| | Putative histidinol-phosphate aminotransferase | | NC_004461 | SE0504 | *S. epidermidis* ATCC12228 | agccgtattatcaccaggag<br>agtaattctaacaccgttaggg<br>cttctaaggcagcgattgc | 703 | 198 |
| | Hypothetical protein, similar to indole-3-pyruvate decarboxylase | | NC_002745 | SA0182 | *S. aureus* N315 | cgtcttaatggactcgctg<br>agttgcaatgagccatcac<br>cgatgatacgcagggaac | 842 | 39 |
| | Putative indole-3-pyruvate decarboxylase | | NC_012121 | Sca_2166 | *S. carnosus* TM300 | caggttcttatgctgaacgtg<br>taaattgcggcgtttcgg<br>tttatgcggcgcatcatg | 1022 | 191 |
| | Menaquinone biosynthesis protein | *menD* | NC_002745 | SA0896 | *S. aureus* N315 | tggatacaccccgatgag<br>accacgattcgcatagac<br>cttacgcgattgatggtcg | 875 | 64 |
| | Putative menaquinone biosynthesis protein MenD | | NC_012121 | Sca_0652 | *S. carnosus* TM300 | catgagttacgcggtatcg<br>attccaaatgcgtcggtg<br>ctcgaagtattgtgctgctg | 1128 | 161 |
| | Mevalonate diphosphate decarboxylase | | NC_004461 | SE0362 | *S. epidermidis* ATCC12228 | ggtgaaaagtggcaaagc<br>ttttacattgggacccgc<br>cgacactgttccacaatcg | 865 | 196 |
| | Mevalonate diphosphate decarboxylase | *mvaD* | NC_002745 | SA0548 | *S. aureus* N315 | cagaaatagagctggcatcg<br>tcacattaggacccgcat<br>cacaagataagtgaacggcg | 535 | 53 |
| | Mevalonate pyrophosphate decarboxylase | | NC_012121 | Sca_0245 | *S. carnosus* TM300 | gtggcgaaaactggcaaag<br>ctgtaccgatgttgctgg<br>taggacctgcatccattg | 859 | 156 |

| Property | Description of gene product | Gene | GeneBank accession no. | Locus_tag / gi | Strain | Primer [a] forward reverse nested | Size[b] | Spot no. |
|---|---|---|---|---|---|---|---|---|
| | Orotidine-5-phosphate decarboxylase | | NC_012121 | Sca_0820 | *S. carnosus* TM300 | agctatggcagggttagg taacttgcgacaggatcg tgtgtaattggacgtccc | 429 | 163 |
| | Orotidine-5-phosphate decarboxylase | | NC_004461 | SE0880 | *S. epidermidis* ATCC12228 | aatggaaggcttagcacg actgtcaattggatgctcac gatatgtgttgaccccaagg | 403 | 200 |
| | Orotidine-5-phosphate decarboxylase | *pyrF* | NC_002745 | SA1047 | *S. aureus* N315 | cagttggtaaggcgatgg tgtgtaatcggtctaccgac ccgactacaatatgcgtcg | 424 | 75 |
| | Pantothenate metabolism flavoprotein | | NC_004461 | SE0887 | *S. epidermidis* ATCC12228 | cgattatagtcgcgccag tcttgtttcgcaaagcgt aagcgttcggttacagct | 559 | 201 |
| | Pantothenate metabolism flavoprotein homolog | | NC_002745 | SA1054 | *S. aureus* N315 | gctagcaacagagacacc tagtcagatacggctgctg tcgtcaaatcggcttgtc | 483 | 76 |
| | Coenzyme A biosynthesis bifunctional protein coaBC (DNA/pantothenate metabolism flavoprotein) | | NC_012121 | Sca_0829 | *S. carnosus* TM300 | ggctatgaagtgcgtgtc tccgattgacgtatcacgg catcttgtgtctcagccg | 922 | 164 |
| | Uroporphyrinogen decarboxylase | *hemE* | NC_002745 | SA1652 | *S. aureus* N315 | tctcaaatagaccccgaacg gtaacgtctctggttgcac ctgggaaaacacgtgtc | 643 | 105 |
| | Uroporphyrinogen decarboxylase | | NC_004461 | SE1513 | *S. epidermidis* ATCC12228 | tatgagacaggctggacg tggaaatacaccgtgacct agtcgtgattctatgacatccc | 901 | 204 |
| | Uroporphyrinogen decarboxylase homolog | | NC_012121 | Sca_1408 | *S. carnosus* TM300 | agatcacagccggaatacc tgtttcaacgtatccgga tggttgtattacatcccatgg | 810 | 172 |
| | Putative pyruvate oxidase | | NC_012121 | Sca_1991 | *S. carnosus* TM300 | aaaaatggacggcgttcc ccaattacttggcggttagg acgttagatgccattgcac | 1002 | 189 |
| | Pyridoxal-deC | | NC_004461 | SE0112 | *S. epidermidis* ATCC12228 | ctatcgttgcggctagag acataaacgaatgacacgttgg tggcataaccagaatctgc | 831 | 195 |

| Property | Description of gene product | Gene | GeneBank accession no. | Locus_tag / gi | Strain | Primer [a] forward reverse nested | Size[b] | Spot no. |
|---|---|---|---|---|---|---|---|---|
| | 5-oxo-1,2,5-tricarboxilic-3-penten acid decarboxylase | | NC_004461 | SE0665 | *S. epidermidis* ATCC12228 | tcgaaaagcagttgtcgc gtaaaccagcacccacac tgcaatgatatcgcctgga | 604 | 199 |
| | Hypothetical protein, similar to 5-oxo-1,2,5-tricarboxilic-3-penten acid decarboxylase | | NC_002745 | SA0829 | *S. aureus* N315 | gcgaagatgctgtatggg agcgatataagttgtcagcg tgcaataatatctcccggatgtag | 725 | 61 |
| | 2-amino-3-carboxymuconate-6-semialdehyde decarboxylase | | NC_012121 | Sca_0567 | *S. carnosus* TM300 | taggcattgaagtcggtatcg tcaatcgccgcaatttcg actttctggcgcataatgc | 707 | 159 |
| | Similar to (Q8TDX5) 2-amino-3-carboxymuconate-6-semialdehyde decarboxylase (EC 4.1.1.45) | | NC_012121 | Sca_2226 | *S. carnosus* TM300 | acattcaatactacacgtgctg ccatctatcaggtgcaaagtg gcaaagtgcgcatctgaa | 428 | 192 |
| | Hypothetical protein, InterPro;Carboxymuconolactone decarboxylase | | NC_012121 | Sca_0034 | *S. carnosus* TM300 | cacgaactttagtggatgcg tgaaatagaaaggcgattccac aatgtaatggcctctgtc | 393 | 153 |
| Lipolysis | Lipase | *geh-2* | NC_002976 | SERP2388 | *S. epidermidis* RP62A | aacttactgcgcaagctc gcttatatcagtagtatctaagccgac caatcatattgtacgggtctcac | 1092 | 211 |
| | Lipase | | NC_002745 | SA0309 | *S. aureus* N315 | agtaactatgatcgcgctg cgtttgaaatccaggaagtcc agatacctctgcgtgtgg | 832 | 40 |
| | Hypothetical protein, similar to lipase LipA | | NC_002745 | SA0610 | *S. aureus* N315 | tttttggatgcacggtgg ctgatgatgtaaatgatgcgtacc tgactttcgaatggatcgc | 546 | 54 |
| | Glycerol esther hydrolase | *geh* | NC_002745 | SA2463 | *S. aureus* N315 | agtaaagtggcgtcacag acacaatgttagggttcaacg gctaaatctgatgcgtgtgtac | 968 | 131 |
| | Lipase precursor | *geh-A* | AF208033 | gi\|11493966; 304-2502 | *S. warneri* | cgaataaggtagcgcctc gacaaagtcgccatggtc gtcccaatcgtgttggac | 1052 | 2 |
| | Lipase precursor | *geh-1* | NC_002976 | SERP2297 | *S. epidermidis* RP62A | tactacgagcaacaggtc cgttacctaataagtctgacgca accattatgtggtgtaccgag | 864 | 210 |

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|---|---|---|---|---|---|---|---|---|
| | Lipase precursor | *lip* | P04635 | gi\|47135; 229-2154 | *S. hyicus* | tccgtaagtcggcttatgg taccataacgctcgtgacc acgttcatgattgctcgc | 920 | 27 |
| | Lipase precursor GehM | *gehM* | AF208229 | gi\|11493972; 466-2685 | *S. xylosus* DSM20266 | acgtaaatttgctgtcggc atgagctaataccgctgg gccattgaaaccatgcac | 1096 | 3 |
| Proteolysis | ClpB chaperone homologue | *clpB* | NC_002745 | SA0835 | *S. aureus* N315 | ccaatgttagcacgaggag agtatggattacgacgaaccg actccagttggacctagg | 941 | 62 |
| | ClpL putative ATP-dependent Clp proteinase chain clpL | | NC_012121 | Sca_1953 | *S. carnosus* TM300 | gcattaagccgtggtgag ccatcgaaacggttgagg agttaagatttgtgggttcgc | 951 | 187 |
| | ClpQ (hslV) homolog of ATP-dependent protease HslV | | NC_012121 | Sca_0887 | *S. carnosus* TM300 | ggtcaagtaacacttggcg cgttttaatgcacggcca agcacttaaagcgtagttgc | 348 | 165 |
| | Protease ClpX | *clpX* | NC_002745 | SA1498 | *S. aureus* N315 | tcttagaaggtacgactgc aaattgccttctgcgtcg cgtcgtatagttctggttcag | 588 | 89 |
| | Putative Clp protease, phage associated | | NC_012121 | Sca_0495 | *S. carnosus* TM300 | tcaaagttgtaggcgttgc tgcaatttgttccacgtc tgacctgtgttagcgact | 337 | 158 |
| | ATP-dependent Clp protease proteolyticsubunit homologue | *clpP* | NC_002745 | SA0723 | *S. aureus* N315 | tcttacaagcgcaagactc ctttgaccagtacgctctg cttgtccttgagcaccac | 249 | 57 |
| | ATP-dependent Clp proteinase chain clpL | | NC_002745 | SA2336 | *S. aureus* N315 | ctgcattaagtcgtggtgag tcaacgatgccgttgaag tgagtatggattacggcgtac | 1038 | 128 |
| | Similar to ATP-dependent Clp protease ATP-binding | | NC_012121 | Sca_1280 | *S. carnosus* TM300 | aacttgttgctggaagcg agcatcatataattccggattagct gattgagcgtaatccacgtg | 1034 | 171 |
| | Cysteine protease | *sspC* | NC_002745 | SA0899 | *S. aureus* N315 | tcaatttgtatacgtcatggcg tcataaacgattgggcgc tgcatgaggatatgctgtgta | 155 | 65 |

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|---|---|---|---|---|---|---|---|---|
| | Cysteine protease precursor | *sspB* | NC_002745 | SA0900 | *S. aureus* N315 | atcactaggcgcatttgc cattaccaacaactgctaggg tgttcatatgatggtacgcct | 864 | 66 |
| | Staphopain, Cysteine Proteinase | | NC_002745 | SA1725 | *S. aureus* N315 | attgcaaacgctgagagc taccatcgataatggtcgcc tagcgttcccaacaactg | 1082 | 106 |
| | Heat shock protein HslU | *clpY* | NC_002745 | SA1097 | *S. aureus* N315 | agattagtcaaggcgcag ggcatacttggtgcttcg gtatgaagtcgacgtgcac | 926 | 79 |
| | Heat shock protein HslV | *clpQ* | NC_002745 | SA1096 | *S. aureus* N315 | ggaaagcagctatggctg gacacaaatatcagccgctac gtttcaatgcacgtcctg | 387 | 78 |
| | Phage protease | | NC_002951 | SACOL0369 | *S. aureus* COL | attcatctggaggcagtg tcttctgggacgttctcg tcttctgcagtaagccaag | 442 | 132 |
| | Prophage phiN315 | | NC_002745 | SA1776 | *S. aureus* N315 | gagctgtcgctcaaagtc tcacctaacataatccgcac gcttcagcatattgaagtggg | 913 | 109 |
| | Serine protease | *htrA* | NC_002951 | SACOL1028 | *S. aureus* COL | agtaccgacgtaagcgtc ctacgatgagatgcagttgac cgtttcagacgagtctgc | 1169 | 133 |
| | Serine protease HtrA | *htrA* | NC_002745 | SA0879 | *S. aureus* N315 | agtaccgacgtaagcgtc ctacgatgagatgcagttgac cgtttcagacgagtctgc | 971 | 63 |
| | Serine protease HtrA, putative | | NC_002951 | SACOL1777 | *S. aureus* COL | ctggcgatagcgtattcg cttggccaggtgattga cgttctgatcggctttgac | 640 | 135 |
| | Serine protease SplA | *splA* | NC_002745 | SA1631 | *S. aureus* N315 | caccatacaattcagtggtagc gaattacctggttgtgcatacg catcaaattctataaacgttccactg | 402 | 95 |
| | Serine protease SplB | *splB* | NC_002745 | SA1630 | *S. aureus* N315 | ttacaaagtgggcgatcg tctggtgtaaagtagacgcc aggtgatccagagtttccg | 352 | 94 |

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|---|---|---|---|---|---|---|---|---|
| | Serine protease SplC | *splC* | NC_002745 | SA1629 | *S. aureus* N315 | gctgcagtcgttgaagag<br>ggcgtaaagtatacggcac<br>tccaataccgccatacac | 570 | 93 |
| | Serine protease SplD | *splD* | NC_002745 | SA1628 | *S. aureus* N315 | caataactggtgtcggcac<br>tataggtgaaccagagctgc<br>ttggacaaccgcatcaga | 512 | 92 |
| | Serine protease SplF | *splF* | NC_002745 | SA1627 | *S. aureus* N315 | taacatcagtgactggcg<br>gatggcttattaccggcatag<br>agattacaccaatagcttcgtg | 576 | 91 |
| | Serine protease; V8 protease; glutamyl endopeptidase | *sspA* | NC_002745 | SA0901 | *S. aureus* N315 | tgacaacagcgacacttg<br>ttatgcagcgtcagggttg<br>tgttatcgccattgtctgg | 954 | 67 |
| | Hypothetical protein, similar to serine proteinase Do, heat-shock protein htrA | *htrA* | NC_002745 | SA1549 | *S. aureus* N315 | cgatagcgtattcgcaatgg<br>cttggccaggtgattgac<br>cgttctgatcggctttgac | 636 | 90 |
| | Zinc metaloprotease | | NC_002745 | SA1105 | *S. aureus* N315 | gcttagaagagccaccag<br>tcgtcgaatatcattccacg<br>tacgaccaccgtctagtg | 924 | 80 |
| | Putative PDZ_metalloprotease family protein | | NC_012121 | Sca_0897 | *S. carnosus* TM300 | gttcatgagtacggccac<br>tgacgaatacagcacctg<br>acacttaaaagcgctgtcc | 1034 | 166 |
| | ftsH ATP-dependent metalloprotease FtsH homolog | | NC_012121 | Sca_0162 | *S. carnosus* TM300 | actttagagatccagcctg<br>tcttaccgtcacgaaccg<br>tctaaatcggcaccagag | 1021 | 155 |
| | Putative protease | | NC_012121 | Sca_0625 | *S. carnosus* TM300 | cagaatttgctactgcagagtc<br>gccttgtttttacactgctagg<br>agttaccaggattgactggtg | 951 | 160 |
| | Putative protease | | NC_012121 | Sca_1064 | *S. carnosus* TM300 | agtagaatctgcgtacgg<br>tgcagcaattttaacgtcagg<br>tctttcattgccccagtga | 958 | 168 |
| | Putative surface associated protein with similarity to proteases | | NC_012121 | Sca_0020 | *S. carnosus* TM300 | aatgcattgcctcgaagc<br>cggattaacagtaggcgg<br>acggtactattgggctcac | 1016 | 152 |

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|---|---|---|---|---|---|---|---|---|
| | Hypothetical protein, similar to O-sialoglycoprotein endopeptidase | | NC_002745 | SA1854 | *S. aureus* N315 | aaacgatttggcggtgtc<br>tgctaaatcagcaaatcgacc<br>gctgatacaaagagtggcc | 835 | 115 |
| | Hypothetical protein, similar to precessing proteinase | | NC_002745 | SA1122 | *S. aureus* N315 | tgtacctgatggagttgctc<br>acgactatcgacttgctg<br>tgtcaaacacgctaacacc | 1090 | 81 |
| | Probable carboxy-terminal processing proteinase ctpA | *ctpA* | NC_002745 | SA1253 | *S. aureus* N315 | gttctccagcagaacgtg<br>gtgtgtcaatagtaacgtctgg<br>gtgaatataatgaccatctggcg | 686 | 85 |
| Other of technological concern | Nitrate and nitrite reduction | *narT* | NC_012121 | Sca_1887 | *S. carnosus* TM300 | ggctttatggtgtggagc<br>tgccacatcgttacaagg<br>tgaaacaataccgtttgcgac | 942 | 173 |
| | Nitrate and nitrite reduction | *nreC* | NC_012121 | Sca_1888 | *S. carnosus* TM300 | taagtatgcctccaggtg<br>agccttttgcaatcaacg<br>tcaatatacgtatcgccgc | 217 | 174 |
| | Nitrate and nitrite reduction | *nreB* | NC_012121 | Sca_1889 | *S. carnosus* TM300 | tgcagtttgtcgcagatg<br>atgttacaatggtgccgc<br>ccgtttaaacccgacacc | 739 | 175 |
| | Nitrate and nitrite reduction | *nreA* | NC_012121 | Sca_1890 | *S. carnosus* TM300 | ggattgcatagtgcggtc<br>tgattaatacgaaacgtcgtgc<br>ttccggtaaaggacgacc | 291 | 176 |
| | Nitrate and nitrite reduction | *narI* | NC_012121 | Sca_1891 | *S. carnosus* TM300 | gtcactttgtcggactcg<br>aatggaacactccacacg<br>actaaacgtgtgaatggcc | 421 | 177 |
| | Nitrate and nitrite reduction | *narJ* | NC_012121 | Sca_1892 | *S. carnosus* TM300 | caaaccacccagcatacg<br>taagcatgatggctcacc<br>ttaaaggggcatacggac | 390 | 178 |
| | Nitrate and nitrite reduction | *narH* | NC_012121 | Sca_1893 | *S. carnosus* TM300 | ccagatatgccggtcatc<br>ccgaatgtataaccttgactgc<br>cgctcagttaaaccaagacg | 1040 | 179 |
| | Nitrate and nitrite reduction | *narG* | NC_012121 | Sca_1894 | *S. carnosus* TM300 | agtagatcctgcgttgtcag<br>agtcagaacgagactccc<br>cataccatgtcgcagcag | 1112 | 180 |

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|---|---|---|---|---|---|---|---|---|
| | Nitrate and nitrite reduction | *sirB* | NC_012121 | Sca_1895 | *S. carnosus* TM300 | tgtctgtaagtccaaccgg<br>tgataatggtcatcgctg<br>tctatcatacaccagccgc | 859 | 181 |
| | Nitrate and nitrite reduction | *nirD* | NC_012121 | Sca_1896 | *S. carnosus* TM300 | ggtacaaaagttctcgtacgc<br>atgtttcaacgcaacccg<br>gttcttgaacaaggcctg | 190 | 182 |
| | Nitrate and nitrite reduction | *nirB* | NC_012121 | Sca_1897 | *S. carnosus* TM300 | agtgtaatcggttggcgt<br>accacaaattgtctcgctg<br>agtgtatagtcttcaatcggttcac | 882 | 183 |
| | Nitrate and nitrite reduction | *sirA* | NC_012121 | Sca_1898 | *S. carnosus* TM300 | tgcagtttgtcgcagatg<br>atgttacaatggtgccgc<br>ccgtttaaacccgacacc | 739 | 184 |
| | Nitrate and nitrite reduction | *nirR* | NC_012121 | Sca_1899 | *S. carnosus* TM300 | tggtatgagacgtggtaagc<br>atttaatcgcgctgccaa<br>gcaatcgtcatatctgagttgc | 621 | 185 |
| | Nitrate and nitrite reduction | *nirC* | NC_012121 | Sca_1900 | *S. carnosus* TM300 | tgaagatacatatgcttcacgtgag<br>agaaagcatataacagacctacg<br>ccaacaaatgcccaaacg | 717 | 186 |
| | Catalase A | *katA* | AY571727 | gi\|45861337; 1-1394 | *S. xylosus* AS141 | caactgtagcaggcgaac<br>gaccatatgctggatcgg<br>tgatcttcgaatgcaccg | 916 | 26 |
| | Catalase B | *katB* | AY702101 | gi\|68137815; 264-1751 | *S. xylosus* DSM 20266 | agaaaatagtatgactgcaggacc<br>catatgagaataagcgaccttgc<br>agaaatctatgccaggcaca | 923 | 29 |
| | Superoxide dismutase | *sod* | NC_012121 | Sca_1174 | *S. carnosus* TM300 | agacagcgtaccatctgac<br>agttaacaacgttccagaatgc<br>tgatatttaagatagtaagcgtgttccc | 339 | 170 |
| | Superoxide dismutase | *sodA* | AY571681 | gi\|45861259; 17-616 | *S. xylosus* 41M06 | ttcaccatggcaaacacc<br>actacgttccagaatgcac<br>ataagcgtgttcccaaac | 425 | 25 |
| | Arsenic efflux pump protein | *arsB* | M80565 | gi\|155343; 664-1953 | *S. xylosus* (*pSX267*) | gctatcattacgggagtcgt<br>tgtgttaagacatgtagccac<br>tcagaacctatgacattcgc | 1007 | 11 |

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|---|---|---|---|---|---|---|---|---|
| | Arsenate reductase | *arsC* | M80565 | gi\|155343; 1971-2366 | *S. xylosus* (*pSX267*) | gtcgtagccaaatggctg<br>gatcctctaaaccccaatgc<br>ggacaattatcgtctgcatcac | 226 | 12 |
| | Repressor protein | *arsR* | M80565 | gi\|155343; 350-664 | *S. xylosus* (*pSX267*) | tcagatccaagtaggttgga<br>ttacaagcacagccttgg<br>acagccttggtcagatgt | 251 | 10 |

[a] In general, nested primer were designed as reverse primer, nested primer designed as forward primer are marked with a star (*).
[b] Predicted size of the microarray probe.

# Supplementary B

Table A2: Detection of further decarboxylase genes in food associated CNS by DNA microarray hybridization.

| Decarboxylase gene or gene function (Spot No.) | No. of strains with positive / ambigious hybridization signal (No. of strains investigated) | | | | | |
|---|---|---|---|---|---|---|
| | *S. carnosus* (7) | *S. condimenti* (2) | *S. piscifermentans* (4) | *S. equorum* (8) | *S. succinus* (4) | *S. xylosus* (7) |
| Hypothetical protein: decarboxylase (127) | 2/2 | -/1 | -/2 | -/1 | | -/7 |
| Hypothetical protein: decarboxylase (197) | | | -/1 | 3/4 | 2/1 | -/6 |
| Hypothetical protein: decarboxylase (55) | | | | -/4 | | -/2 |
| Conserved hypothetical protein: decarboxylase (194) | 6/- | 2/- | 4/- | | | |
| *ilvB* (116) | 7/- | 1/1 | 1/3 | 6/1 | -/3 | 1/6 |
| Alpha-acetolactate decarboxylase (206) | -/1 | | | 2/- | -/2 | 3/2 |
| Hypothetical protein: similar to alpha-acetolactate decarboxylase (119) | -/1 | | | 1/1 | | -/4 |
| Putative alpha-acetolactate decarboxylase (188) | 7/- | 2/- | 3/1 | | | -/3 |
| Putative acetoacetate decarboxylase (193) | 7/- | 2/- | | | | |
| *panD* (129) | -/1 | | -/1 | | -/1 | -/6 |
| Aspartate 1-decarboxylase (205) | | | -/1 | | -/1 | -/6 |
| Putative aspartate 1-decarboxylase (190) | 7/- | 2/- | 4/- | | | -/1 |
| *lysA* (84) | -/1 | -/1 | | -/2 | -/2 | -/6 |
| Diaminopimelate decarboxylase (202) | 4/1 | | -/1 | 5/2 | -/1 | -/7 |
| Putative diaminopimelate decarboxylase (167) | 7/- | 2/- | 3/1 | | -/2 | -/6 |
| Glycine dehydrogenase subunit 1 (86) | -/3 | | -/1 | -/1 | | -/1 |
| Probable glycine dehydrogenase (169) | 7/- | 2/- | 2/2 | -/3 | | -/7 |
| Histidinol-phosphate/aromatic aminotransferase and cobyric acid decarboxylase (157) | 7/- | 2/- | 3/1 | | | |
| Hypothetical protein, similar to indole-3-pyruvate decarboxylase (39) | | | -/1 | -/1 | | -/2 |
| Putative indole-3-pyruvate decarboxylase (191) | 7/- | 2/- | 4/- | 2/3 | | -/3 |
| Putative menaquinone biosynthesis protein MenD (161) | 7/- | 2/- | -/2 | | | |
| *mvaD* (53) | | | | -/1 | -/1 | -/2 |
| Mevalonate diphosphate decarboxylase (196) | | -/1 | -/1 | -/1 | -/2 | -/6 |
| Mevalonate pyrophosphate decarboxylase (156) | 7/- | 2/- | 4/- | | | |
| Orotidine-5-phosphate decarboxylase (163) | 7/- | 2/- | 2/2 | | | |
| Coenzyme A biosynthesis bifunctional protein coaBC (164) | 7/- | 2/- | 4/- | | | |
| Pantothenate metabolism flavoprotein (201) | | | | | | -/6 |
| *hemE* (105) | -/3 | | | | | |
| Uroporphyrinogen decarboxylase (204) | 5/- | -/1 | -/2 | 4/2 | -/2 | -/3 |
| Uroporphyrinogen decarboxylase homolog (172) | 7/- | 2/- | 4/- | -/4 | | |
| Putative pyruvate oxidase (189) | 7/- | 2/- | 4/- | -/1 | -/2 | -/2 |
| 5-oxo-1,2,5-tricarboxilic-3-penten acid decarboxylase (199) | | | -/1 | | | |
| Similar to 5-oxo-1,2,5-tricarboxilic-3-penten acid decarboxylase (61) | 4/2 | -/1 | -/2 | 3/3 | -/3 | -/7 |
| 2-amino-3-carboxymuconate-6-semialdehyde decarboxylase (159) | 7/- | 2/- | 1/1 | | | |
| Similar to 2-amino-3-carboxymuconate-6-semialdehyde decarboxylase (192) | 7/- | 2/- | -/2 | | | |
| Hypothetical protein: InterPro:Carboxymuconolactone decarboxylase (153) | 7/- | 2/- | | | | |

Table A3: Detection of lipase and protease genes in food associated CNS by DNA microarray hybridization.

| Lipase and protease gene or gene function (Spot No.) | No. of strains with positive / ambigious hybridization signal (No. of strains investigated) | | | | | |
|---|---|---|---|---|---|---|
| | *S. carnosus* (7) | *S. condimenti* (2) | *S. piscifermentans* (4) | *S. equorum* (8) | *S. succinus* (4) | *S. xylosus* (7) |
| *geh2* (211) | -/1 | -/1 | -/1 | -/- | -/- | -/2 |
| *geh* (131) | 7/- | 2/- | 1/1 | -/- | -/2 | 2/5 |
| *gehA* (2) | 1/1 | -/1 | -/1 | -/- | -/- | -/7 |
| *geh1* (210) | 1/3 | -/2 | -/1 | -/- | -/2 | -/7 |
| *gehM* (3) | -/- | -/- | -/- | -/- | -/- | 7/- |
| *clpB* (62) | 7/- | 2/- | 2/2 | 8/- | -/4 | -/7 |
| *clpL* (187) | 7/- | 2/- | 4/- | 1/- | -/- | -/- |
| *clpQ* (165) | 7/- | 2/- | 3/1 | -/- | 2/1 | -/- |
| *clpX* (89) | -/4 | -/- | -/1 | -/2 | -/1 | -/3 |
| Putative Clp protease, phage associated (158) | 4/- | 1/- | 1/- | -/- | -/- | -/1 |
| *clpP* (57) | 7/- | 2/- | 2/2 | 3/2 | -/4 | 1/6 |
| *clpL* (128) | 7/- | 1/1 | 1/2 | 2/1 | -/- | -/- |
| similar to ATP-dependent Clp protease ATP-binding (171) | 7/- | 2/- | 4/- | 5/2 | -/4 | 7/- |
| *sspB* (66) | 4/- | -/- | -/- | -/- | -/- | -/- |
| Staphopain, Cysteine Proteinase (106) | -/1 | -/- | -/1 | -/- | -/- | -/- |
| *clpY* (79) | 5/2 | 2/- | -/2 | 2/3 | -/3 | -/7 |
| *clpQ* (78) | -/- | -/- | -/- | -/- | -/1 | -/6 |
| Phage protease (132) | -/1 | -/- | -/- | -/- | -/- | -/1 |
| *htrA* (90) | 1/1 | -/- | -/- | -/- | -/1 | -/5 |
| Zinc metaloprotease (80) | -/2 | -/- | -/- | -/- | -/- | -/- |
| Putative PDZ_metalloprotease family protein (166) | 7/- | 2/- | 4/- | -/- | -/- | -/5 |
| ftsH ATP-dependent metalloprotease FtsH homolog (155) | 7/- | 2/- | 4/- | 7/1 | 2/2 | 7/- |
| Putative protease (160) | 7/- | 2/- | 3/1 | -/- | -/- | -/1 |
| Putative protease (168) | 7/- | 2/- | 4/- | 5/- | -/2 | -/1 |
| Putative surface associated protein with similarity to proteases (152) | 7/- | 2/- | 2/- | -/- | -/- | -/- |
| Hypothetical protein, similar to O-sialoglycoprotein endopeptidase (115) | -/2 | -/- | -/4 | -/2 | -/2 | -/6 |
| Hypothetical protein, similar to precessing proteinase (81) | 4/2 | -/1 | -/2 | 5/2 | -/2 | -/5 |
| *ctpA* (85) | -/1 | -/1 | -/- | -/- | -/- | -/1 |

Table A4: Detection of technologically relevant genes in food associated CNS by DNA microarray hybridization.

| Technological relevant gene or gene function (Spot No.) | No. of strains with positive / ambigious hybridization signal (No. of strains investigated) | | | | | |
|---|---|---|---|---|---|---|
| | *S. carnosus* (7) | *S. condimenti* (2) | *S. piscifermentans* (4) | *S. equorum* (8) | *S. succinus* (4) | *S. xylosus* (7) |
| *narT* (173) | 7/- | 2/- | 4/- | -/2 | -/- | -/6 |
| *nreC* (174) | 7/- | 2/- | 1/3 | -/- | -/- | -/- |
| *nreB* (175) | 7/- | 2/- | 3/1 | -/- | -/- | -/6 |
| *nreA* (176) | 7/- | 2/- | -/- | -/- | -/- | -/- |
| *narI* (177) | 7/- | 2/- | 3/1 | -/- | -/- | -/- |
| *narJ* (178) | 7/- | 2/- | 1/3 | -/2 | -/- | -/- |
| *narH* (179) | 7/- | 2/- | 4/- | 7/1 | -/1 | 4/3 |
| *narG* (180) | 7/- | 2/- | 4/- | -/1 | -/- | -/6 |
| *sirB* (181) | 7/- | 2/- | 4/- | -/- | -/- | -/- |
| *nirD* (182) | 6/1 | 2/- | -/1 | -/- | -/- | -/- |
| *nirB* (183) | 7/- | 2/- | 4/- | -/5 | -/- | -/6 |
| *sirA* (184) | 7/- | 2/- | 2/2 | -/- | -/- | -/2 |
| *nirR* (185) | 7/- | 2/- | -/2 | -/- | -/- | -/- |
| *nirC* (186) | 7/- | 2/- | 4/- | -/- | -/- | -/- |
| *katA* (26) | 6/1 | 2/- | -/2 | 8/- | -/4 | 6/1 |
| *katB* (29) | 5/2 | 2/- | 2/2 | 8/- | 4/- | 7/- |
| *sod* (170) | 7/- | 2/- | 4/- | -/4 | -/2 | -/6 |
| *sodA* (25) | 4/3 | -/- | -/2 | 7/- | 4/- | 7/- |
| *arsB* (11) | 2/- | -/- | 3/- | 8/- | 3/1 | 7/- |
| *arsC* (12) | 1/1 | -/- | 3/- | 3/1 | 4/- | 7/- |
| *arsR* (10) | 4/1 | -/- | -/3 | 6/- | -/2 | 2/5 |

**Eidesstattliche Versicherung gemäß § 7 Absatz 7 der Promotionsordnung der Universität Hohenheim zum Dr. rer. nat.**

1. Bei der eingereichten Dissertation zum Thema

   "Safety assessment of coagulase-negative staphylococci used in food production"

   handelt es sich um meine eigenständig erbrachte Leistung.

2. Ich habe nur die angegebenen Quellen und Hilfsmittel benutzt und mich keiner unzulässigen Hilfe Dritter bedient. Insbesondere habe ich wörtlich oder sinngemäß aus anderen Werken übernommene Inhalte als solche kenntlich gemacht.

3. Ich habe nicht die Hilfe einer kommerziellen Promotionsvermittlung oder -beratung in Anspruch genommen.

4. Die Bedeutung der eidesstattlichen Versicherung und der strafrechtlichen Folgen einer unrichtigen oder unvollständigen eidesstattlichen Versicherung sind mir bekannt.

Die Richtigkeit der vorstehenden Erklärung bestätige ich. Ich versichere an Eides Statt, dass ich nach bestem Wissen die reine Wahrheit erklärt und nichts verschwiegen habe.

_____ _____

Ort und Datum Unterschrift

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