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Determination of enterotoxin genes in coagulase - negative staphylococci from autochthonous Croatian fermented sausages

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ABSTRACT

The aim of this study was to determine the presence of enterotoxin genes in coagulase - negative staphylococci from traditional Croatian fermented sausages. A total of 39 isolates were collected from the dry sausage "kulen" and Slavonian homemade sausages as presumptive CNS, and were subjected to PCR identification to the genus level. All isolates belonged to the *Staphylococcus* genus. Strains were tested for the presence of 13 enterotoxin genes; *se*A, *se*B, *se*C, *se*G, *se*I, *tsst*1, *se*D, *se*E, *se*H, *se*J, *se*M, *se*N and *se*O by the PCR method. All strains were negative to all enterotoxin genes. The observed results indicate the absence of risk related to enterotoxogenic potential of coagulase - negative staphylococci as indigenous microbiota from autochthonous Croatian traditional sausages.

Key words: enterotoxin genes, coagulase - negative staphylococci, fermented sausages

Introduction

Standardization of dry sausage quality should include the implementation of technologically and hygienically acceptable starter cultures. Dominant autochthonous strains of lactic acid bacteria (LAB) or coagulase - negative staphylococci (CNS) which are isolated from a specific fermented sausage should be considered as potential starter cultures for the same product. These strains are most adapted to the specific intrinsic

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ecology of specified fermented sausages and the technological procedures implemented (ZDOLEC et al., 2013). Coagulase - negative staphylococci are usually present in lower numbers compared to LAB in fermented sausages, but their lipolitic, proteolitic and nitrite - reducing activity is of particular importance. Research of different kinds on Croatian traditionally fermented sausages has showed that CNS counts ranged from 3 to 4 $\log_{10}$ CFU/g and main species identified were *S. xylosus* and *S. carnosus* (KOZAČINSKI et al., 2006; ZDOLEC et al., 2007; ZDOLEC et al., 2008). Characterization of autochthonous CNS strains employed biochemical and molecular tools, in order to select potential functional starter cultures (BABIĆ et al., 2011). On the other hand, there are limited data on the presence of risk determinants in that microbial group, such as antimicrobial resistance or the ability to produce enterotoxins. The latter is mainly detected in coagulase - positive staphylococci (LE LOIR et al., 2003), while the enterotoxic potential of food - related CNS has not been thoroughly evaluated. Several studies have reported the presence of enterotoxin genes in CNS isolated from food of animal origin (VERNOZY - ROZAND et al., 1996; ZELL et al., 2008), but there is a lack of data related to Croatian traditional food products. Thus, the aim of this research was to assess the riskiness of indigenous CNS isolated from two Croatian traditional fermented sausages - kulen and Slavonian homemade dry sausage - in terms of determination of the molecular markers of enterotoxin production.

Materials and methods

Strains. A total of 70 isolates were collected from kulen and homemade Slavonian dry sausages at the end of the maturation period (120 and 40 days, respectively). For microbiological analysis, 25 grams of the product was diluted in 225 mL of salt peptone water and homogenized for 2 minutes (Stomacher 400 Circulator, Seward, UK). Further serial decimal dilutions were prepared, and 0.1 mL of appropriate dilution was spread on Manitol Salt Agar (MSA, bioMerieux, France) and incubated at 30 °C for 48 hours. Isolates were subjected to gram staining, catalase (Kovacs reagens, Merck, Germany) and the coagulase test (Bactident Coagulase, Merck). For further analyses, the isolates were kept at - 20 °C in Brain Hearth Infusion (BHI) broth, supplemented with 20% of glycerol.

Molecular analyses

Isolation of DNA from cultures. Isolates were grown in BHI broth for 24 hours at 37 °C, streaked on MSA and incubated at 37 °C. Separated colonies were resuspended in 200 µl of distilled water (UltraPure™ DNase⁄RNase - Free Distilled Water, Invitrogen, UK), heated for 20 minutes at 95 ºC, and centrifuged for 1 minute at 14000 rpm. The resulting supernatant was used as the DNA template for the PCR.

Table 1. Primers used in this study

Primer	Sequence (5' - 3')	Gene	Product size (bp)	Reference
SEA - 1	GAAAAAAGTCTGAATTGCAGGGAACA	*se*A	560	JARRAUD et al. (2002)
SEA - 2	CAAATAAATCGTAATTAACCGAAGGTTC			
SEB - 1	ATTCTATTAAGGACACTAAGTTAGGGA	*se*B	404	JARRAUD et al. (2002)
SEB - 2	ATCCCGTTTCATAAGGCGAGT			
SEC - 1	GTAAAGTTACAGGTGGCAAAACTTG	*se*C	297	JARRAUD et al. (2002)
SEC - 2	CATATCATACCAAAAAGTATTGCCGT			
SED1	CTAGTTTGGTAATATCTCCT	*se*D	317	BLAIOTTA et al. (2004)
SED2	TAATGCTATATCTTATAGGG			
SEE1	TAGATAAAGTTAAAACAAGC	*se*E	170	BLAIOTTA et al. (2004)
SEE2	TAACTTACCGTGGACCCTTC			
SEG - 1	AATTATGTGAATGCTCAACCCGATC	*se*G	642	JARRAUD et al. (2002)
SEG - 2	AAACTTATATGGAACAAAAGGTACTAGTTC			
SEH1	CGAAAGCAGAAGATTTACACG	*se*H	495	BLAIOTTA et al. (2004)
SEH2	GACCTTTACTTATTTCGCTGTC			
SEI - 1	CTCAAGGTGATATTGGTGTAGG	*se*I	576	JARRAUD et al. (2002)
SEI - 2	AAAAAACTTACAGGCAGTCCATCTC			
ESJ1	CAGCGATAGCAAAAATGAAACA	*se*J	426	BLAIOTTA et al. (2004)
ESJ2	TCTAGCGGAACAACAGTTCTGA			
SEM1	CCAATTGAAGACCACCAAAG	*se*M	517	BLAIOTTA et al. (2004)
SEM2	CTTGTCCTGTTCCAGTATCA			
SEN1	ATTGTTCTACATAGCTGCAA	*se*N	682	BLAIOTTA et al. (2004)
SEN2	TTGAAAAAACTCTGCTCCCA			
SEO1	AGTCAAGTGTAGACCCTATT	*se*O	534	BLAIOTTA et al. (2004)
SEO2	TATGCTCCGAATGAGAATGA			
TST - 1	TTCACTATTTGTAAAAGTGTCAGACCCACT	*tsst*1	180	JARRAUD et al. (2002)
TST - 2	TACTAATGAATTTTTTTATCGTAAGCCCTT			

Identification of strains by PCR to the genus level. For identification of the strains to the genus level, part of the 16S rRNA *Staphylococcus* gene was amplified (MASON et al., 2001). The PCR reaction mixture of 20 µL contained 10 µl of HotStarTaq Master Mix (Qiagen, Germany), 6 µL water (RNase - free Water, Qiagen, Germany), 1 µL of primer Staph 16S - 1 (CCT ATA AGA CTG GGA TAA CTT CGG G), 1 µL of primer Staph 16S - 2 (CTT TGA GTT TCA ACC TTG CGG TCG) and 2 µL of DNA template. The final concentration of each primer (Invitrogen, UK) in the reaction mixture was 0.5 µM. Amplifications were carried out in a Veriti 96 Well Thermal Cycler (Applied Biosystems, SAD) with a polymerase activation step (95 ºC/15 min), followed by 35 denaturation cycles (95 ºC/1 min 30 sec), annealing (55 ºC/1 min) and extension (72 ºC/1 min), and a final extension step (72 ºC/7 min). The amplification product size (expected, 791 bp) was determined by means of QIAxcel (Qiagen, Germany) and the computer program QIAxcel BioCalculator.

Detection of enterotoxin genes by PCR. The presence of enterotoxin genes was evaluated using the primers listed in Table 1. The PCR reaction mixture of 20 µL contained 10 µL of HotStarTaq Master Mix (Qiagen, Germany), 6 µL water (RNase - free Water, Qiagen, Germany), 1 µL of both primers (initial concentration 10 µM) and 2 µL of DNA template. The PCR protocol was followed as described above.

Results

Among the 70 selected colonies, a total of 39 were Gram positive, catalase positive and coagulase - negative cocci. As presumptive CNS, they were further characterized by molecular tools in order to determine which of them belongs to the genus *Staphylococcus* and whether they are carriers of enterotoxin genes. Identification of the strains to the genus level (*Staphylococcus*) was carried out by amplification of the gene part 16S rRNA characteristic for the genus *Staphylococcus,* according to MASON et al. (2001). All isolates tested (n = 39) belonged to the genus *Staphylococcus*. Fig. 1 presents the results of PCR reaction for the first 9 isolates.

The presence of genes for the enterotoxins *se*A, *se*B, *se*C, *se*G, *se*I and *tsst*1 were determined using primers according to JARRAUD et al. (2002), and *se*D, *se*E, *se*H, *se*J, *se*M, *se*N i *se*O according to BLAIOTTA et al. (2004). The enterotoxin genes were not found in any of the isolates tested (n = 39). As an example, we showed the results of the detection of the *se*A gene by the PCR method for 9 isolates (Fig. 2).

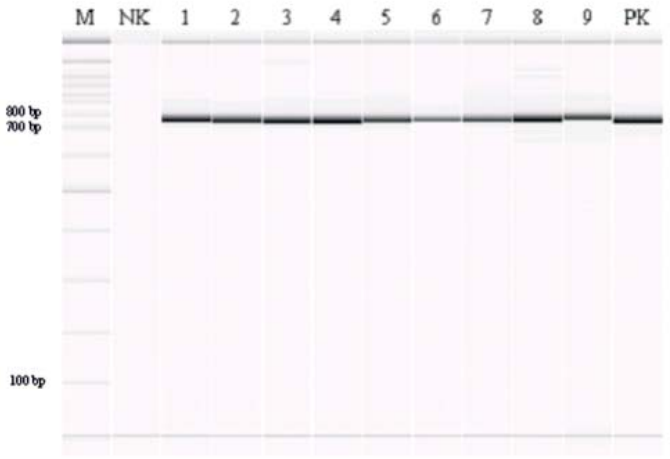

Fig. 1. PCR identification of strains to the genus level (*Staphylococcus*). M - marker with amplification products sizes 15, 100, 200, 300, 400, 500, 600, 700, 800, 900, 1000, 1200, 1500, 2000 and 3000 bp; NK - negative control; PK - positive control, *Staphylococcus aureus* ATCC 25923; 1 - 9 - isolates.

Fig. 2. PCR results of detection of *se*A gene. M - marker with amplification products sizes 15, 100, 200, 300, 400, 500, 600, 700, 800, 900, 1000, 1200, 1500, 2000 and 3000 bp; NK - negative control; PK - positive control *Staphaylococcus aureus se*A positive (Health Protection Agency, UK); 1 - 9 - isolates.

Discussion

The results show that standard Gramm staining, catalase and coagulase tests were appropriate tools for selecting MSA colonies as presumptive CNS for genetic studies. Further phenotypic characterization should be followed in order to test the technological suitability of strains as potential starter cultures for fermented sausage application. Also, biochemical identification of the strain should be confirmed by species - specific PCR, because some CNS species could not be properly identified by the phenotypic method alone (IRLINGER, 2008). Our results show that there is no risk from enterotoxins in the selected indigenous CNS of Croatian traditional fermented sausages, which is one of the required criteria for selection of autochthonous starter cultures. In general, the presence of enterotoxin genes in food - related CNS is rare, but still a systematic survey for enterotoxin genes in food CNS is requested (BLAIOTTA et al., 2004). Several studies have reported positive findings of enterotoxin genes in CNS from food, indicating that a specific risk for consumers is present (ZELL et al., 2008; VERAS et al., 2008). DA CUNHA et al. (2006) found four positive CNS strains (n = 20) for *se*A and *se*C genes, but the enterotoxins were not found in a specified food, meaning that the genes were not expressed or enterotoxins were present in small quantities. However, the authors suggest that more data are needed for evaluating the interaction of enterotoxin - positive CNS and the food matrix, but also the conditions which could support enterotoxin production *in situ*. Our results support the findings of EVEN et al. (2010) who clearly demonstrated the low occurrence of safety hazards (especially enterotoxins) in CNS from fermented foodstuffs. Among 129 CNS tested, they detected only the *se*C gene in one single strain. Results reported by BLAIOTTA et al. (2004) are also in accordance with our findings, regarding the absence of enterotoxin genes in CNS from meat and dairy products.

In conclusion, the presented results showed that indigenous CNS from Croatian traditionally fermented sausages are not carriers of enterotoxin genes, which is one of the important criteria for selecting potential starter cultures. However, more safety hazards of the selected technologically applicable strains should be evaluated, including antimicrobial resistance and biogenic amine production.

References

BABIĆ, I., M. MARKOV, D. KOVAČEVIĆ, A. TRONTEL, A. SLAVICA, J. ĐUGUM, D. ČVEK, I. K. SVETEC, S. POSAVEC, J. FRECE (2011): Identification and characterization of potential autochthonous starter cultures from a Croatian "brand" product "slavonski kulen". Meat Sci. 88, 517 - 524.

BLAIOTTA, G., D. ERCOLINI, C. PENNACCHIA, V. FUSCO, A. CASABURI, O. PEPE, F. VILLANI (2004): PCR detection of staphylococcal enterotoxin genes in *Staphylococcus* spp. strains isolated from meat and dairy products. Evidence for new variants of seG and seI in *S. aureus* AB - 8802. J. Appl. Microbiol. 97, 719 - 730.

DA CUNHA, M. L. R. S., E. PERESI, R. A. O. CALSOLARI, J. P. A. JÚNIOR (2006): Detection of enterotoxins genes in coagulase - negative staphylococci isolated from foods. Braz. J. Microbiol. 37, 70 - 74.

EVEN, S., S. LEROY, C. CHARLIER, N. B. ZAKOUR, J. - P. CHACORNAC, I. LEBERT, E. JAMET, M. - H. DESMONTS, E. COTON, S. POCHET, P. - Y. DONNIO, M. GAUTIER, R. TALON, Y. LE LOIR (2010): Low occurrence of safety hazards in coagulase negative staphylococci isolated from fermented foodstuffs. Int. J. Food Microbiol. 139, 87 - 95.

IRLINGER, F. (2008): Safety assesment of dairy microorganisms: Coagulase - negative staphylococci. Int. J. Food Microbiol. 126, 302 - 310.

JARRAUD, S., C. MOUGEL, J. THIOULOUSE, G. LINA, H. MEUGNIER, F. FOREY, X. NESME, J. ETIENNE, F. VANDENESCH (2002): Relationships between *Staphylococcus aureus* genetic background, virulence factors, agr groups (alleles), and human disease. Infect. Immun. 70, 631 - 641.

KOZAČINSKI, L., N. ZDOLEC, M. HADŽIOSMANOVIĆ, Ž. CVRTILA, I. FILIPOVIĆ, T. MAJIĆ (2006): Microbial flora of the Croatian traditionally fermented sausage. Archiv Lebensmittelhyg. 57, 141 - 147.

LE LOIR, Y., F. BARON, M. GAUTIER (2003): *Staphylococcus aureus* and food poisoning. Genet. Mol. Res. 2, 63 - 76.

MASON, W. J., J. S. BLEVINS, K. BEENKEN, N. WIBOWO, N. OJHA, M. S. SMELTZER (2001): Multiplex PCR protocol for the diagnosis of staphylococcal infection. J. Clin. Microbiol. 39, 3332 - 3338.

VERAS, J. F., L. S. DO CARMO, L. C. TONG, J. W. SHUPP, C. CUMMINGS, D. A. DOS SANTOS, M. M. O. P. CERQUEIRA, A. CANTINI, J. R. NICOLI, M. JETT (2008): A study of the enterotoxigenicity of coagulase negative and coagulase - positive staphylococcal isolates from food poisoning outbreaks in Minas Gerais, Brazil. Int. J. Infect. Dis. 12, 410 - 415.

VERNOZY - ROZAND, C., C. MAZUY, G. PREVOST, C. LAPEYRE, M. BES, Y. BRUN, J. FLEURETTE (1996): Enterotoxin production by coagulase - negative staphylococci isolated from goats' milk and cheese. Int. J. Food Microbiol. 30, 271 - 280.

ZDOLEC, N., M. HADŽIOSMANOVIĆ, L. KOZAČINSKI, Ž. CVRTILA, I. FILIPOVIĆ, K. LESKOVAR, N. VRAGOVIĆ, D. BUDIMIR (2007): Fermented sausages produced in the household - microbiological quality (In Croatian). Meso IX, 6, 318 - 324.

ZDOLEC, N., M. HADŽIOSMANOVIĆ, L. KOZAČINSKI, Ž. CVRTILA, I. FILIPOVIĆ, M. ŠKRIVANKO, K. LESKOVAR (2008): Microbial and physicochemical succession in fermented sausages produced with bacteriocinogenic culture of *Lactobacillus sakei* and semi - purified bacteriocin mesenterocin Y. Meat Sci. 80, 480 - 487.

ZDOLEC, N., V. DOBRANIĆ, A. HORVATIĆ, S. VUČINIĆ (2013): Selection and application of autochthonous functional starter cultures in traditional Croatian fermented sausages. Int. Food Res. J. 20, 1-6.

ZELL, C., M. RESCH, R. ROSENSTEIN, T. ALBRECHT, C. HERTEL, F. GÖTZ (2008): Characterization of toxin production of coagulase - negative staphylococci isolated from food and starter cultures. Int. J. Food Microbiol. 127, 246 - 251.

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SAŽETAK

Cilj ovog rada bio je utvrditi prisutnost gena za enterotoksine u koagulaza - negativnih stafilokoka iz tradicionalnih hrvatskih fermentiranih kobasica. Ukupno je prikupljeno 39 izolata iz kulena i domaće slavonske kobasice te identificirano PCR - om do razine roda. Svi izolati pripadali su rodu *Staphylococcus.* PCR - om je određivana prisutnost 13 gena za enterotoksine: *se*A, *se*B, *se*C, *se*G, *se*I, *tsst*1, *se*D, *se*E, *se*H, *se*J, *se*M, *se*N i *se*O. Ni u jednom izolatu nije utvrđena prisutnost gena za enterotoksine. Dobiveni rezultati upućuju na odsutnost rizika povezanog s toksogenim potencijalom koagulaza-negativnih stafilokoka kao dijela autohtone mikroflore hrvatskih fermentiranih kobasica.

Ključne riječi: geni za enterotoksine, koagulaza-negativni stafilokoki, fermentirane kobasice