Article

Diversity and Safety Aspects of Coagulase Negative Staphylococci in Ventricina Del Vastese Italian Dry Fermented Sausage

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Abstract: Ventricina del Vastese is a traditional dry fermented sausage from Central Italy not yet characterized for occurrence, identity and safety of coagulase negative staphylococci (CNS), a bacterial group technologically important for this kind of products.

Therefore, in this study, 98 CNS isolates from four manufacturers were differentiated by Repetitive element palindromic PCR (Rep-PCR) and identified by 16S rRNA gene sequencing. These were examined for genes encoding biogenic amine (BA) production, resistance to aminoglycosides, β-lactams and tetracyclines and staphylococcal enterotoxins (SEs).

Staphylococcus succinus (55%) predominated, followed by *S. xylosus* (30%), *S. epidermidis* (7.4%), *S. equorum* (3.1%), *S. saprophyticus* (3.1%) and *S. warneri* (1%). One *S. succinus* subsp. *casei* isolate was slightly β-hemolytic. SEs and the histidine decarboxylase gene hdcA were not detected, while the tyrosine decarboxylase gene *tdc*A was detected in four S. xylosus isolates. A *bla*Z beta-lactamase gene and tetracycline resistance genes *tet*K (six isolates) and *tet*A (one isolate also bearing tetK) were found, respectively.

However, fewer hazardous and AR traits compared to CNS examined in other studies were found, as a probable consequence of using meat from animals reared in conditions that allow to minimize pathogen circulation and antibiotic use. Therefore, appropriate production conditions can reduce these threats.

Keywords: fermented sausage; Ventricina del Vastese; traditional production; coagulase negative staphylococci; hazardous genetic traits; antibiotic resistance

1. Introduction

Dry fermented sausages are produced from raw meat fermented and ripened by a composite microbiota. Microbial groups with the most relevant roles in the ripening process are lactic acid bacteria (LAB) and coagulase negative staphylococci (CNS), which concur in making these products safe and tasteful. The main role of LAB is product acidification at different extents, according to the technological process, with inhibition of most pathogenic and deteriorating microorganisms, while the most relevant role of CNS is favoring the development of an optimal red color through the formation of nitroso-myoglobin after stepwise nitrate reduction to nitric oxide and its combination with myoglobin. Both microbial groups are involved in the formation of flavors and aromatic substances [1].

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The traditional versions of dry fermented sausages are manufactured according to ancient regional processes and exploiting a naturally occurring microbiota. Among these, Ventricina del Vastese is a fermented dry sausage listed among traditional products by the Italian Ministry of Agriculture (https://www.politicheagricole.it/flex/cm/pages/ServeBLOB.php/L/IT/IDPagina/17979 accessed on 22 october 2022) and typical of a territory close to the Adriatic Coast including parts of Abruzzo e Molise Italian regions. Its recipe dates back to the 18th century and its peculiarities are that it is made of knife cut cubes of pork meat of about 2 - 4 cm side mixed with fat 20-30% (w/w), salted and abundantly spiced with sweet and hot chili pepper powder (15-30 g/kg), filled in natural casings. These are usually pig bladder or cecum and confer to the sausage a diameter of 9 - 20 cm. The product is aged for 100 - 150 days at temperatures not exceeding 13°C. According to the production specifications fixed by an official document with legal value as per the Italian traditional food safeguard system, the "Disciplinare di Produzione" approved by the international Slow Food association (https://www.fondazioneslowfood.com/it/presidi-slow-food/ventricina-del-vastese/ accessed on 22 october 2022) and proprietary for manufacturers belonging to the association "Associazione di Promozione e Tutela della Ventricina del Vastese". These producers are committed to using meats from animals, also of autochthnous swine races, raised outdoors or in pens with not less than five square meters space per head. Animals are fed exclusively with cereals, legumes, fruits and acorns produced locally. Use of preservatives, including nitrate, is not allowed.

Ventricina del Vastese was little characterized for CNS species composition and identity, with just one study carried out for a single producer to compare the effects of ripening in natural conditions or in a ripening chamber and the safety of isolates was not evaluated [2].

Coagulase negative staphylococci (CNS) are naturally associated to dry fermented sausages, since they normally colonize human and animal skin [3]. This bacterial group includes strains able to develop desired flavor and aroma compounds from proteolysis and lipolysis [1]. CNS starters for meat products are commercially available [4], but safety of these bacteria must be ascertained at the strain level since risk characters, such as presence of transferable antibiotic resistance (AR) genes and staphylococcal enterotoxins (SEs) were shown to occur rather frequently [5,6]. In addition, the sausage associated CNS species *Staphylococcus equorum* and *S. succinus* were isolated also from human clinical specimens [3], so the safety characteristics of individual strains naturally present or used as starter cultures in fermented meats must be carefully evaluated. As a consequence, CNS species are not included in the updated list of biological agents with a qualified presumption of safety (QPS) status by the European Food Safety Authority [7].

As for other traditional naturally fermented and ripened products, also for Ventricina del Vastese sausage the characterization of the dominant microbiological consortia is required for a knowledge of the bacterial species and strains involved in product transformation and for a selection of the technologically best suited strains

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devoid of risk characters.

Therefore, in this study the product was characterized with respect to presence, identity and safety status of CNS by analyzing the products of four artisanal manufacturers adhering to the association " Associazione di Promozione e Tutela della Ventricina del Vastese".

2. Materials and Methods

2.1. Bacterial strains and culture conditions

Bacterial strains used in this study were all new isolates from Ventricina del Vastese sausages. The sausage samples were analyzed by homogenizing 10 g in 90 ml of sterile physiological solution (NaCl 9 g/l), serially diluting the homogenate and inoculating plates of Mannitol Salt Agar (MSA) medium (Biolife Italiana, Milan, Italy) incubated aerobically at 37°C for 48 h. Ventricina del Vastese samples were collected at 0, 20, 50 and 150 days of ripening from four manufacturers of the production area that use meat provided by local farms that raise no more than 15 pigs at a time in the period January – May 2021. Pure cultures of the isolates were obtained by double streaking single colonies from the count plates on the same medium. One isolate colony was grown in Brain Heart Infusion (BHI) broth (Biolife Italiana) in the above conditions prior to DNA extraction. Broth cultures from single colonies were stored at -80°C in the same medium added with 20% (v/v) glycerol for long term maintenance.

The determination of hemolytic activity was carried out and interpreted as described by Zell et al. [8].

2.2. DNA isolation

DNA was extracted from 1 mL of fresh culture using the Genomic DNA Extraction Kit RBC Bioscience (Diatech Labline, Jesi, AN, Italy), according to the manufacturer instructions. The quantity and integrity of the extracted DNA were checked by comparison with known amounts of Lambda DNA (ThermoFisher Scientific, Rodano, MI, Italy) on 1.5% w/v agarose gels in 1 × TAE buffer (80 mM Tris-acetate, 2 mM EDTA, pH 8.0) at 120 V stained with 1:10,000 diluted GelRed (Biotium, Società Italiana Chimici, Rome, Italy).

2.3. PCR assays

All of the PCR tests were carried out with the EmeraldAmp GT PCR Master Mix Takara Clontech (Diatech, Jesi, Italy). Repetitive element palindromic PCR (Rep-PCR) was carried out with the GTG_5 primer as described by [9]. Primers 27f/1492r [10] were used to amplify a 1494 bp region of the16S rRNA gene. Screenings for tyrosine decarboxylase *tdc*A and histidine decarboxylase *hdc*A genes were carried out according to Lagioia et al. [11] and Rossi et al. [12], respectively. Primers used to detect tetracycline efflux genes *tet*A/C, *tet*G and ribosomal protection proteins for tetracycline resistance genes *tet*M, O, P, Q, S, T, W are those designed by Yu et al. [13]. All other antibiotic resistance genes, i.e. *bla*Z, *mec*A, *tet*K, *tet*L, *aac*(6')-Ie+*aph*(2'), *ant*(4)-Ia, *aph*(3')-IIIa, *erm*A, *erm*B, *erm*C and *msr*A, were sought as referenced by Rebecchi et al. [6]. In addition, primer pairs aac6f (5'-CCTTGCGATGCTCTATG-3')/aac6r (5'-TCCCCGCTTCCAAGAG-3'), amplifying a fragment of 204 bp from genes of families

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*aac*6 and *aac*4 found in staphylococci, and ant6f (5′-GCGCAAATATTAATATACCTAAA-3′)/
ant6r (5′-GGGCAATAAGGTAAGATCA-3′), amplifying a fragment of 157 bp from *ant*6 (*aad*E) family genes ever found in CNS, were designed in this study by NCBI database search (https://www.ncbi.nlm.nih.gov/nucleotide/) and verification of correct annealing by Blastn (https://blast.ncbi.nlm.nih.gov/). SE genes were sought according to Omoe et al. [14].

Since positive control bacterial strains were not available for all the genes screened the DNA suitability for amplification was constantly checked by running parallel PCR reactions for the 16S rRNA gene. PCR products were separated by electrophoresis as described above.

2.4. Sequencing

Sequencing of the amplification products was carried out on both strands by Eurofins Genomics on amplicons purified with the Wizard® SV Gel and PCR Clean-Up System (Promega Italia Srl, Milan, Italy) with primers 27f/1492r for 16S rRNA gene and the same primers used for amplification for all other genes. All genes found in screenings were sequenced for identity confirmation.

2.5. Data analyses

Data of CNS counts for the different producers were compared by Unweighted Pair Group Method, using Arithmetic Averages (UPGMA) and correlation similarity index using PAST 4.03 free statistical software downloaded from https://past.en.lo4d.com/windows. Rep-PCR profiles were compared by the BioNumerics V5.10 software (Applied-Maths, Belgium), using the Dice coefficient for pairwise comparison and UPGMA clustering.

3. Results and discussion

3.1. CNS microbiota composition

The analysis of Ventricina del Vastese samples for CNS content at different times resulted in the numerical trends shown in Figure 1A, where it is possible to observe that a similar evolution of this bacterial group took place in the products of the four manufacturers examined. Though for manufacturer B lower numbers were most often present, positive correlations were determined among the count data series (Figure 1B).

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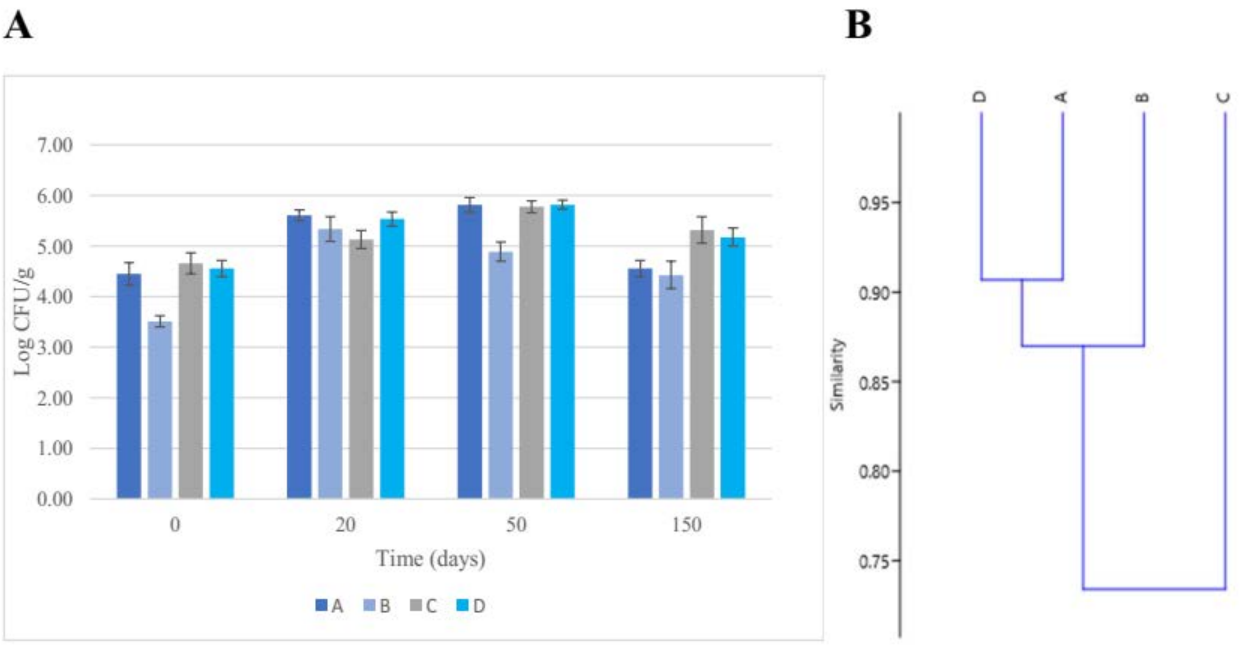

Figure 1. CNS evolution in Ventricina del Vastese sausage of four manufacturers A, B, C and D (A) and correlation among the evolution trends for the different manufacturers (B).

Maximum count data approached those reported for other Italian dry sausage types [15,16] but the persistence of the highest count values until day 50 can be a consequence of slow drying due to the large diameter of the product.

The isolates obtained from each manufacturer of Ventricina del Vastese listed in Table 1 according to the time of isolation.

Table 1. CNS isolates from manufacturers A, B, C and D with time of isolation.

Manufacturer	A	B	C	D
Day 0	A1, A3, A4, A5, A6, A7, A8	B1, B2	C1, C2, C3, C4, C5, C6, C7, C8	D1, D2, D3, D4, D5, D6, D7
Day 20	A9, A10, A11, A12, A14, A16, A17	B4, B6, B8	C9, C10, C11, C12, C13, C14, C15, C16, C17	D8, D9, D10, D11, D12, D13, D14, D15, D16
Day 50	A22, A23, A24, A26, A27, A28	B10, B11	C18, C19, C20, C21, C22, C23, C24	D17, D18, D19, D20, D21, D22, D23
Day 150	A29, A31, A32, A33, A34	B12, B15	C25, C26, C27, C28, C29, C30	D24, D25, D26, D27, D28, D29, D30

The number of isolates per manufacturer depended on the number of production lots analyzed, two for manufacturers A, C and D and one for manufacturer B, and on the number of colony morphologies observed, that ranged between two and seven.

The Rep-PCR genotypes of the isolates are shown in Figure 2, where they are clustered according to the similarity percentage and with species indication for the isolates identified by sequencing of the 16S rRNA gene.

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Figure 2. Clustered Rep-PCR profiles of CNS isolated from Ventricina del Vastese sausage. Identification based on 16S rRNA gene is shown for isolates representing clusters separated above 90% similarity.

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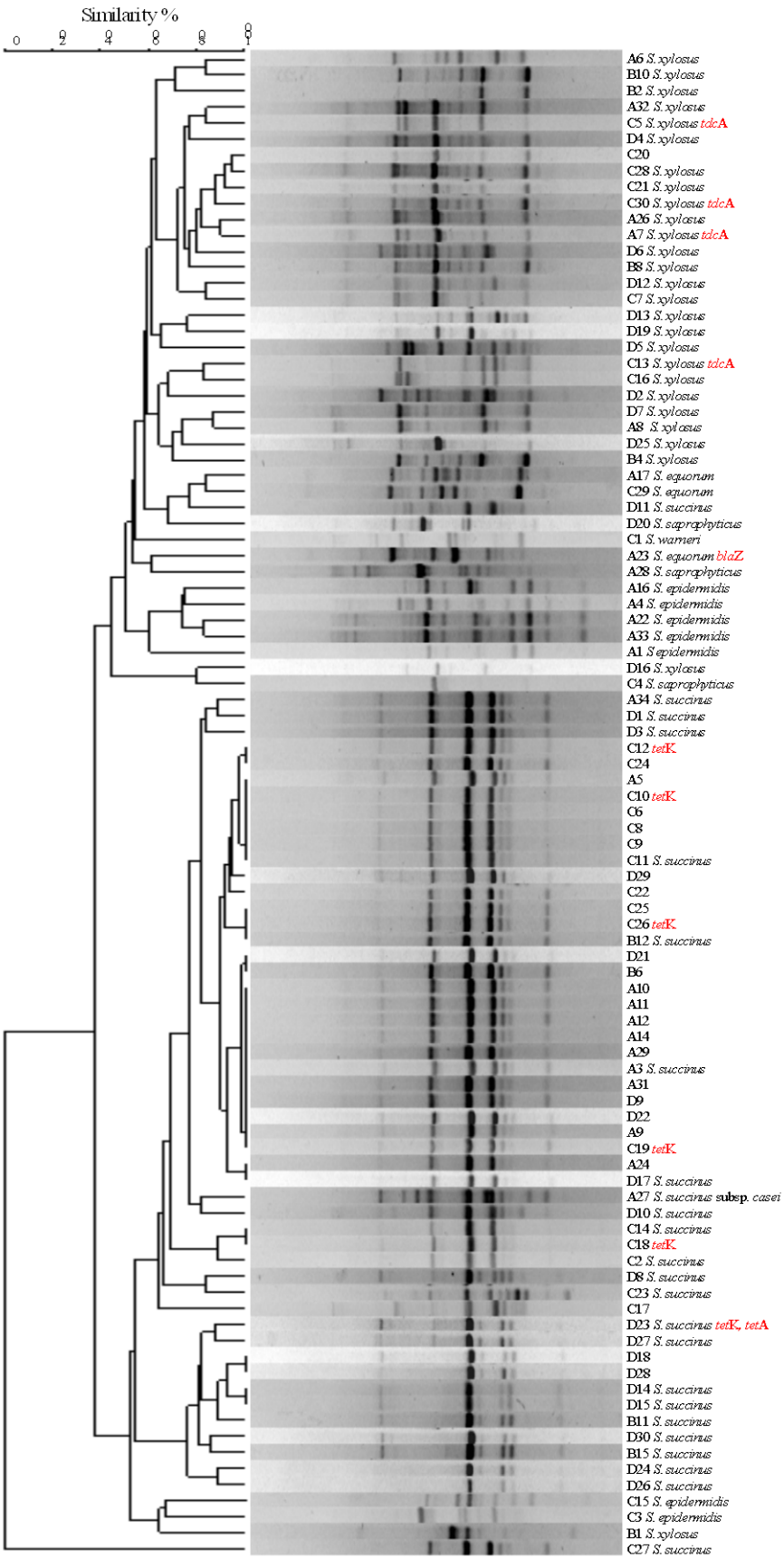

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For clusters of isolates sharing more than 90% profile similarity only one component was sequenced. As it can be observed, the most numerous group was that of *S. succinus* isolates, representing 55% of the total, while 30% isolates were assigned to the species *S. xylosus*, 7.4% to *S. epidermidis*, 3.1% to *S. equorum*, and 3.1% to *S. saprophyticus*. Only one isolate was identified as *S. warneri*. This species association is typical of Southern-European type of fermented dry sausages with low acidity levels in which 3-methyl-1-butanol, acetoin and diacetyl represent distinctive aroma compounds formed by *S. succinus* and *S. xylosus* [17].

All the species identified, including those mostly implicated in human infections, i.e. *S. epidermidis* [18] and *S. saprophyticus* [19] were detected at the end of ripening (Table 1, Figure 2). *S. epidermidis* isolates were retrieved from manufacturers A and C, while *S. saprophyticus* was isolated also from manufacturer D. It can be mentioned that two isolates from manufacturer C grown on MSA, omitted from Figure 2, were identified as *Bacillus velezensis*, a bacterial species under consideration as starter for fermented foods [20] whose role in fermented sausages should be examined.

In Ventricina del Vastese sausage the species *S. succinus* predominated among CNS and was isolated from all manufacturers. The occurrence of *S. succinus* in dry-fermented sausages from Italy was reported previously but its frequency of isolation was at most 14.7% in sausages from the Campania region [21]. In a screening of CNS species present in fermented sausages of different European countries *S. succinus* was identified only in Belgian products as a minor component of the CNS population [22]. Therefore, in Ventricina del Vastese this species is exceptionally predominating, possibly selected in consequence of the manufacturing method that creates high salt concentrations on the surface of the meat cubes of which this sausage is constituted. Indeed, this species can be isolated from fermented products with high salt content, such as doenjang, a traditional fermented soybean Korean food [23]. Strains of *S. succinus* are able to colonize the surfaces of the manufacturing plants, as found in a study aimed to the characterization of the CNS population in a small establishment producing French traditional dry fermented sausages [24].

Similarly to what observed in this study, *S. succinus* strains from fermented soybean products were susceptible to all antibiotics tested, did not form biogenic amines and were not hemolytic [23]. Among them, strain 14BME20 was selected as a starter candidate and its genomic analysis confirmed that it is devoid of virulence factor-encoding genes and possesses genes for lipid degradation that can lead to the formation of volatile compounds [25]. *S. succinus* isolates can bear a lysine decarboxylase-encoding gene that confers the capacity to form cadaverine at low levels [23].

In the *S. succinus* 14BME20 genome (Acc. N. NZ_CP018199.1) a nitric oxide synthase (NOS) gene is found, indicating the possible production of nitric oxide (NO) from L-arginine in the presence of oxygen and NADPH [26], which could contribute to meat red color though this species does not reduce nitrate with exception of the subsp. *S. suc-*

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cinus subsp. *casei* [27]. Since in Ventricina del Vastese addition of nitrate is not allowed, NOS activity of *S. succinus* could compensate for red color maintenance.

The *S. succinus* isolates exhibited a lower diversity compared to the other CNS species in Ventricina del Vastese, with some clusters joined at similarity above 90% that comprised isolates from different manufacturers. This bacterial group could therefore represent a typical component of CNS population in the production area to be characterized for its influence on the quality and distinctness of the product. Indeed, *S. succinus* was reported to produce species-specific volatile compounds during soybean fermentation when used as a starter culture [28].

3.2.Safety assessment of Ventricina del Vastese CNS

Among the CNS isolates examined a minority were found to bear hazardous traits, with four strains of *S. xylosus* potentially able to form tyramine for the presence of a *tdc*A gene and seven strains harboring AR determinants. The AR genes found in this study *bla*Z and *tet*K were reported to be frequent in CNS [5,6], while the *mec*A conferring methicillin resistance and *mrs*A encoding a macrolide efflux protein previously found in some of the species identified in this study [5,6] were absent in isolates from Ventricina del Vastese sausage. In addition, the gene *bla*Z, reported by Rebecchi et al. [6] to be the most prevalent in sausage associated CNS, in this study was infrequent. Since a strong correlation was found between presence of *bla*Z and *tet*K and phenotypic resistance to penicillins and tetracycline, respectively [6], the CNS isolates carrying these genes found in this study are likely phenotypically resistant as well.

Importantly, no multidrug resistance MDR genetic profiles were observed, with just one strain possessing two AR genes, *tet*K and *tet*A. The gene *tet*K is plasmid encoded in *S. aureus* [29] and therefore prone to be transferred. On the other hand, the gene *tet*A found in this study was not detected previously in CNS from food and, by a database search, it was found to be chromosomally encoded in a *S. cohnii* clinical isolate (Acc. N. Accession: UHEC01000001.1) among CNS and in coagulase positive staphylococci.

Notably, the AR gene harboring isolates found in this study were mostly associated to a single manufacturer, with only one exception, suggesting a localized selection of AR CNS. Therefore, examining bacterial isolates for AR might be useful to identify the sources of the AR bacteria to revert risk trends at the farm level.

Staphylococcal enterotoxins were absent in the isolates from this study, differently than found by Soares Nunes et al. [30] who isolated *S. epidermidis*, *S. hominis*, *S. succinus*, *S. xylosus* and *S. saprophyticus* strains that harbored most often *seb*/*sec* and *sea*, followed by *sed*/*seh*/*selm* and *sei*/*seln*, also expressed *in vitro*. Finally, weak hemolytic activity was found only in the isolate *S. succinus* subsp. *casei* A27.

The rather low frequency of antibiotic resistance genes on Ventricina del Vastese sausage and the absence of MDR are indicative of a production process in which selective pressure for AR is low, possibly because of little need for antibiotic usage in animal raising in a small farm. This hypothesis is supported by the results of Fontana et al. [31], who observed that extensive farming and small manufacturing plants determined a lower occurrence of AR in meat-associated CNS compared to production on industrial

level. However, the presence of a few AR CNS in Ventricina del Vastese sausage might be indicative of AR arising even in a virtuous production system and needs to be monitored in order to adopt measures that avoid a further spread of the AR determinants.

Characterizing traditional products in terms of specific microbiota and its safety status is a first step toward exploitation of autochthonous bacterial strains to supplant those with hazardous traits that may naturally occur. For example, it was shown that use of autochthonous starter cultures including CNC can reduce the formation of BAs in fermented sausages [32]. Therefore, evaluation of single strains for their potential use as starter cultures must follow.

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