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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 the occurrence, identity and safety of coagulase-negative staphylococci (CNS), a bacterial group technologically important for this kind of product. Therefore, in this study, 98 CNS isolates from four manufacturers were differentiated using repetitive element palindromic PCR (Rep-PCR) and identified using 16S rRNA gene sequencing. These were examined for genes encoding biogenic amine (BA) production, resistance to aminoglycosides, β-lactams, 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 *hdc*A were not detected, whereas the tyrosine decarboxylase gene *tdc*A was detected in four *S. xylosus* isolates. The *bla*Z beta-lactamase gene in an *S. equorum* isolate, tetracycline resistance genes *tet*K in six *S. succinus* isolates and *tet*A in one *S. succinus* isolate also bearing *tet*K were found. The product examined is characterized by a peculiar CNS species ratio and a low occurrence and diversity of AR transferable genes than found in other studies, as a probable consequence of production only with meat from animals raised in small farms with extensive rearing systems in which antibiotic usage is infrequent.

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

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1. Introduction

Dry fermented sausages are produced from raw meat which is 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 to different extents, according to the technological process, with inhibition of the most pathogenic and deteriorating microorganisms, whereas the most relevant role of CNS favors the development of an optimal red color through the formation of nitrosomyoglobin 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].

The traditional versions of dry fermented sausages are manufactured according to ancient regional processes and exploit a naturally occurring microbiota. Among these,

Ventricina del Vastese is a fermented dry sausage listed among traditional products by the Italian Ministry of Agriculture [2] and typical of a territory close to the Adriatic Coast, including parts of the Abruzzo and 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 sides, mixed with 20–30% of fat (w/w), which are salted and abundantly spiced with sweet and hot chili pepper powder (15–30 g/kg) and fill in natural casings. These casings 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" is approved by the international Slow Food Association [3] 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 autochthonous swine races, raised outdoors or in pens with no less than five square meters of 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, in which the safety of isolates was not evaluated [4].

Coagulase-negative staphylococci (CNS) are naturally associated with dry fermented sausages as they normally colonize human and animal skin [5]. This bacterial group includes strains which are able to develop the desired flavors and aroma compounds from proteolysis and lipolysis [1]. CNS starters for meat products are commercially available [6], but the safety of this bacteria must be ascertained at the strain level as risk characters, such as the presence of transferable antibiotic resistance (AR) genes and staphylococcal enterotoxins (SEs), were shown to occur rather frequently [7,8]. In addition, the sausage-associated CNS species *Staphylococcus equorum* and *S. succinus* were also isolated from human clinical specimens [5], so the safety characteristics of the 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 [9].

As for other traditional naturally fermented and ripened products, the characterization of the dominant microbiological consortia for Ventricina del Vastese sausage is also required to gain knowledge of the bacterial species and strains involved in product transformation and for a selection of the technologically best-suited strains, devoid of risk characters, to be used as autochthonous starter cultures for quality and safety improvement. Therefore, in this study, the product was characterized with respect to the presence, identity and safety status of CNS by analyzing samples from four artisanal manufacturers adhering to the association " Associazione di Promozione e Tutela della Ventricina del Vastese" who promoted this research to obtain an evaluation of the uniqueness, authenticity and safety of their product on a scientific basis.

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. For their isolation, Ventricina del Vastese samples were collected in the period January–May 2021 at 0, 20, 50 and 150 days of ripening from four manufacturers in the production area who use meat from local farms that raise no more than 15 pigs at a time. Sausage samples of 10 g were homogenized in 90 mL of sterile physiological solution (NaCl 9 g/L). The homogenates were serially diluted and inoculated in plates of mannitol salt agar (MSA) medium (Biolife Italiana, Milan, Italy) and incubated aerobically at 37 °C for 48 h. Pure cultures of the isolates were obtained by double streaking single colonies

from the count plates on the same medium. A single colony of each isolate 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 with 20% (*v*/*v*) glycerol added for long term maintenance.

The determination of hemolytic activity was carried out and interpreted as described by Zell et al. [10].

2.2. DNA Isolation

DNA was extracted from 1 mL of fresh culture using the genomic RBC Bioscience DNA extraction kit (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) stained with 1:10,000 diluted GelRed (Biotium, Società Italiana Chimici, Rome, Italy) and run at 120 V.

2.3. PCR Assays

All of the PCR tests were carried out using the EmeraldAmp GT PCR Master Mix Takara Clontech (Diatech, Jesi, Italy). Repetitive element palindromic PCR (Rep-PCR) was carried out using the GTG_5 primer, as described by Versalovich et al. [11]. Primers 27f/1492r [12] were used to amplify a 1494 bp region of the 16S rRNA gene. Screenings for tyrosine decarboxylase *tdc*A and histidine decarboxylase *hdc*A genes were carried out according to Lagioia et al. [13] and Rossi et al. [14], respectively. Primers used to detect the tetracycline efflux genes *tet*A/C, *tet*G and the ribosomal protection proteins for tetracycline resistance genes *tet*M, O, P, Q, S, T and W are those designed by Yu et al. [15]. Other antibiotic resistance genes, i.e., *bla*Z, *mec*A, *tet*K, *tet*L, *aac*(6′)-Ie+*aph*(2′), *aph*(3′)-IIIa, *erm*A, *erm*B, *erm*C and *msr*A, were sought, as referenced by Rebecchi et al. [8]. In addition, other primer pairs were designed in this study using search of AR genes found in staphylococci in the NCBI database (https://www.ncbi.nlm.nih.gov/nucleotide/ (accessed on 22 October 2022) and verification of correct annealing and specificity by Blastn (https://blast.ncbi.nlm.nih.gov/ (accessed on 22 October 2022). These primer pairs are aac6f (5′-CCTTGCGATGCTCTATG-3′)/aac6r (5′-TCCCCGCTTCCAAGAG-3′) and ant6f (5′-GCGCAAATATTAATATACCTAAA-3′)/ant6r (5′-GGGCAATAAGGTAAGATCA-3′), respectively, amplifying fragments of *aac*6 (204 bp) and *ant*6 (157 bp) families of aminoglycoside resistance encoding genes. SE genes were sought according to Omoe et al. [16].

As positive control bacterial strains were not available for 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 using electrophoresis, as described above. In addition, PCR tests were repeated three times to ensure the reliability of the results.

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 of the other genes sequenced. All genes detected in the screenings were sequenced for identity confirmation.

2.5. Data Analyses

Data of CNS counts for the different producers were compared using unweighted pair group method with arithmetic averages (UPGMA) and correlation similarity index using PAST 4.03 free statistical software downloaded from https://past.en.lo4d.com/windows (accessed on 22 October 2022). Rep-PCR profiles were compared using BioNumerics V5.10 software (Applied-Maths, Sint-Martens-Latem, Belgium), with the dice coefficient for pairwise comparison and UPGMA clustering.

3. Results and Discussion

3.1. CNS Microbiota Composition

The analysis of the 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).

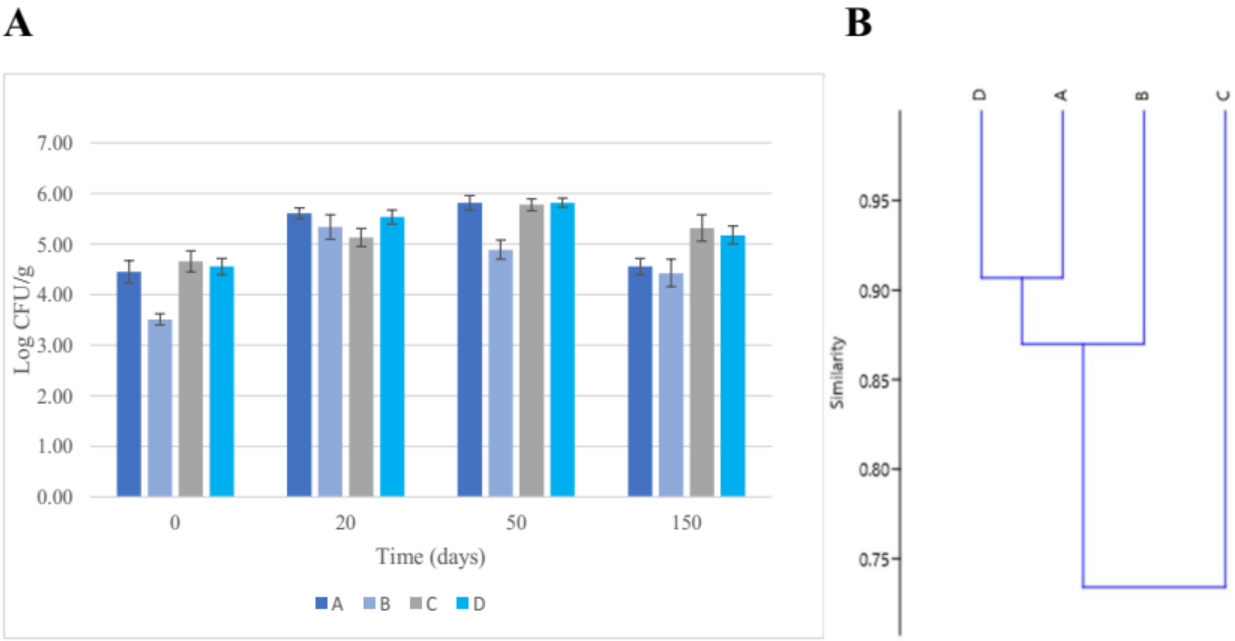

Figure 1. CNS evolution in Ventricina del Vastese sausage of four manufacturers A, B, C and D (A) and correlation among the evolution trends for the different manufacturers (B).

The maximum count data approached those reported for other dry sausage types from Southern Italy [17,18], 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 are 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, which 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 the sequencing of the 16S rRNA gene.

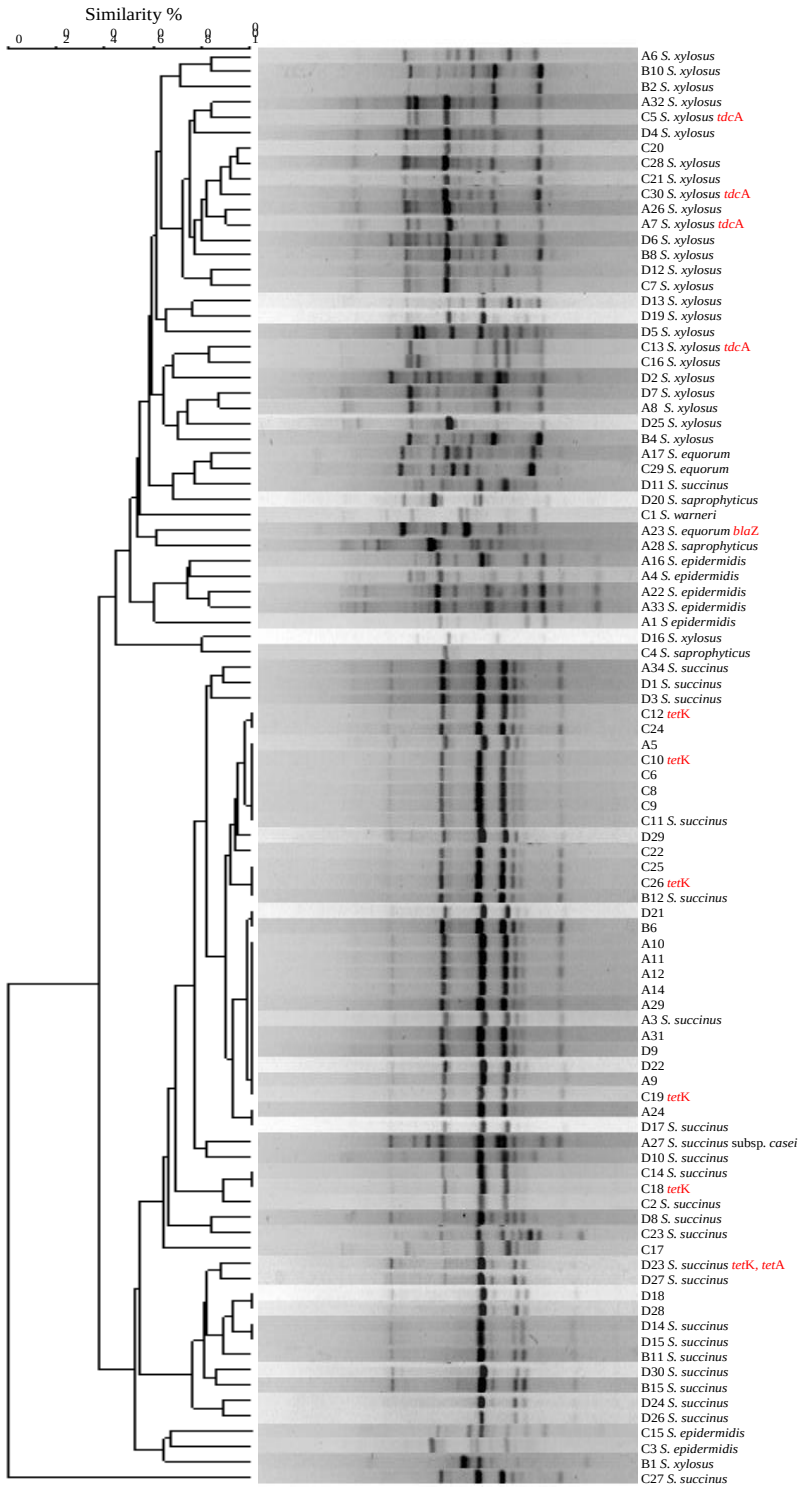

Figure 2. Clustered Rep-PCR profiles of CNS isolated from Ventricina del Vastese sausage. Identification based on 16S rRNA gene is shown for isolates representing clusters separated above 90% similarity.

For clusters of isolates sharing more than a 90% profile similarity, only one component was sequenced. As it can be observed, the most numerous group was that of the *S. succinus* isolates, representing 55% of the total, whereas 30% of the 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 the 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* [19].

All the species identified, including those mostly implicated in human infections, i.e. *S. epidermidis* [20] and *S. saprophyticus* [21], were detected at the end of ripening (Table 1, Figure 2). The *S. epidermidis* isolates were retrieved from manufacturers A and C, whereas *S. saprophyticus* was also isolated 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 a starter for fermented foods [22] 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 previously reported, but its frequency of isolation was at most 14.7% in sausages from the Campania region [23]. In a screening of CNS species present in fermented sausages from different European countries, *S. succinus* was identified only in Belgian products as a minor component of the CNS population [24]. Therefore, in Ventricina del Vastese, this species is exceptionally predominating, possibly as a consequence of the manufacturing method that creates high salt concentrations on the surface of the meat cubes constituting the sausage. Indeed, this species can be isolated from fermented products with high salt content, such as doenjang, a traditional fermented soybean Korean food [25]. Strains of *S. succinus* are able to colonize the surfaces of the manufacturing plants, as found in a study aimed at the characterization of the CNS population in a small establishment producing French traditional dry fermented sausages [26].

The *S. succinus* strains from fermented soybean products did not show hazardous traits, as they were susceptible to all of the antibiotics tested, did not form biogenic amines and were not hemolytic [25]. Among them, the 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 [27]. In addition, in this study, the *S. succinus* isolates had a low frequency of AR determinants and did not bear genes for the production of the most dangerous BAs, histamine and tyramine [13,14]. The only BA formed by *S. succinus* is cadaverine, as a lysine decarboxylase-encoding gene was reported to be present in the isolates producing low levels of this compound [25].

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) by this bacterial species from L-arginine in the presence of oxygen and NADPH [28], which could contribute to the meat's red color, though *S. succinus* does not reduce nitrate with the exception of the subspecies *S. succinus* subsp. *casei* [29]. As the addition of nitrate is not allowed in Ventricina del Vastese, the NOS activity of *S. succinus* could compensate for the 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 a similarity above 90% that comprised isolates from different manufacturers. This bacterial group could, therefore, represent a typical component of the 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 when used as a starter culture [30].

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 due to the presence of a *tdc*A gene and seven strains harboring AR determinants (Figure 2). The AR genes found in this study, *bla*Z and *tet*K, were reported to be frequent in CNS [7,8], whereas the *mec*A conferring methicillin resistance and the *mrs*A encoding a macrolide efflux protein previously found in some of the species identified in this study [7,8] were absent in the isolates from Ventricina del Vastese sausage. In addition, the gene *blaZ*, reported by Rebecchi et al. [8] to be the most prevalent in sausage-associated CNS, was infrequent in this study. As a strong correlation was found between the presence of *blaZ* and *tet*K and a phenotypic resistance to penicillin and tetracycline, respectively [8], 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 only one strain possessing two AR genes, *tet*K and *tet*A. The gene *tet*K is plasmid-encoded in *S. aureus* [31] and, therefore, is prone to be transferred. On the other hand, the gene *tet*A found in this study was not previously detected in CNS from food, and, through a database search, it was found to be chromosomally encoded in an *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 with 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 trends of AR bacteria selection at a farm level.

Staphylococcal enterotoxins were absent in the isolates obtained in this study, which is different than the findings by Soares Nunes et al. [32] who isolated *S. epidermidis*, *S. succinus*, *S. xylosus* and *S. saprophyticus* strains which harbored most often *seb*/*sec* and *sea*, followed by *sed*/*seh*/*selm* and *sei*/*seln* and were also expressed in vitro. Finally, weak hemolytic activity was only found in the isolate *S. succinus* subsp. *casei* A27.

The low frequency of the AR genes in CNS from Ventricina del Vastese sausage and the absence of MDR are indicative of a production process in which selective pressure for AR is weak, possibly because of the little need for antibiotic usage in animals raised in small farms with extensive rearing systems. Indeed, Fontana et al. [33] observed that extensive farming for artisanal sausage production determines a low occurrence of AR genes in CNS compared to production on an industrial scale with animals farmed intensively. However, even a low level of AR gene occurrence in CNS from Ventricina del Vastese indicates the need to monitor the spread of these genes and adopt measures that reduce their presence.

The effectiveness of autochthonous starter cultures including CNC in improving the safety of fermented sausages was demonstrated by the reduction in BA formation [34]. Therefore, the isolation and characterization of CNS present in Ventricina del Vastese, as well as in other similar products, are indispensable to allow the selection of strains to be tested as autochthonous starters eventually able to reduce BA formation and the occurrence of strains with risk factors such as transferable AR genes.

4. Conclusions

The characterization of the CNS microbiota of Ventricina del Vastese sausage highlighted particular aspects of this product, common to four producers, i.e. the predominance of the species *S. succinus* which comprises microorganisms of proven capacity to form aroma compounds; the low occurrence of species of dubious safety status, such as *S. epidermidis* and *S. saprophyticus*; and the absence of isolates harboring SEs. However, the occurrence of the *S. xylosus* strains with the tyrosine decarboxylase gene *tdc*A, isolates bearing the β-lactamase gene *bla*Z and the tetracycline efflux pumps *tet*K and *tet*A indicated that an improvement in the safety status of the product with respect to the occurrence of hazardous genetic traits is necessary.

Defining the specific CNS microbiota of Ventricina del Vastese sausage and the individual safety status of isolates is a first step toward the exploitation of autochthonous bacterial strains to supplant those with hazardous traits that naturally occur. The evaluation of single strains for their potential use as starter cultures, with the participation of producers, should follow. Indeed, further investigations are needed, including the full genetic characterization of the isolates using whole genome sequencing and the definition of their technological properties in production trials.

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