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Short communication

Tetragenococcus koreensis is part of the microbiota in a traditional Italian raw fermented sausage

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ABSTRACT

This study reports the isolation of *Tetragenococcus koreensis*, a bacterial species currently represented only by the type strain isolated from kimchi, from a raw fermented and ripened Italian sausage, Ventricina Vastese, all over the ripening period of five months. Rep-PCR genotyping showed that different *T. koreensis* strains, identified by sequencing of the 16S rRNA gene, were present in the same production batch. Tests on representative isolates showed intra-species physiological variability and the possession of phenotypic traits relevant for the production of fermented sausages, i.e. ability to grow at high salt concentrations, to induce some changes in the peptide profile of the culture medium and inability to produce histamine and tyramine, confirmed by the absence of the respective decarboxylase genes. Therefore the opportunity to further investigate the suitability of *T. koreensis* as a starter for fermented meat products was suggested.

1. Introduction

Traditional food products, defined by the Italian law as those that have been manufactured by a well established process in a given geographical area for at least 25 years (DM 350/1999), achieve distinctive compositional, sensorial and safety characteristics by action of the natural microbiota, so that the development of starter cultures composed by autochthonous microorganisms permits the safeguard of both quality and hygiene.

Ventricina Vastese is a low-acid fermented sausage (final pH values over 5.3) typical of the Abruzzi and Molise regions that undergoes a spontaneous fermentation at low temperatures and is made from pork meat hand cut into pieces with sides of 2–7 cm, spiced with sweet and hot chilli pepper, stuffed in pig intestine, stomach and bladders or bovine intestine and ripened for 5–12 months. Microbial species found to predominate in Ventricina Vastese are *Lactobacillus sakei*, *Staphylococcus xylosus* and *Staphylococcus equorum* (Amadoro et al., 2013). In addition, in previous investigations, also Gram-positive, catalase negative cocci, identified by sequencing of the 16S rRNA gene as *Tetragenococcus koreensis*, were isolated during the whole ripening period (Colavita, unpublished). This species was not found in fermented meat products before and is currently represented only by the type strain isolated from kimchi (Lee et al., 2005).

Among tetragenococci, only *Tetragenococcus halophilus* was previously isolated from salted meat products (La Pietra et al., 1999; Holzapfel et al., 2006) and was detected by a culture independent analysis in two PDO (Protected Designation of Origin) meat products from Northern Italy (Busconi et al., 2014).

This study was aimed at increasing knowledge on the physiology of *T. koreensis* and at elucidating if further studies on its role in the manufacture of fermented meat products are opportune. New isolates were genetically typed by Rep-PCR and isolates representative of different genotypic clusters were identified by sequencing of the 16S rRNA gene. Moreover, technologically relevant traits, such as nitrate reduction, growth at different combinations of temperature/NaCl concentration, biogenic amine production and proteolytic capacity, were analysed.

2. Materials and methods

2.1. Bacterial strains and culture conditions

Two batches of Ventricina Vastese sausage were manufactured in separate production plants according to the traditional method (Piccirilli and Colavita, 2008) and without the addition of starter cultures. The mixture was constituted by pork meat added of 2.5%

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w/w salt, and 1.5% w/w of a blend of sweet and hot chili pepper powder. Ripening was allowed to occur in plug-in showcase cabinets at controlled temperature and humidity for 5 months. At each sampling time, two samples per batch were brought to the laboratory in sterile bags and in refrigerated conditions. About 10 g of sample were homogenized in 9 volumes of sterile saline (0.9% NaCl) and serially diluted. The order of magnitude of *T. koreensis* number was estimated after the genetic characterization and identification of pure cultures of bacteria isolated from agarized LM17 medium (M17 medium, Biolife, Milan, Italy, supplemented with 0.5% w/v lactose) after 5 days incubation at 28 °C in anaerobiosis created with the Anaerocult system (VWR International, Milan, Italy). These cultures were purified by double streaking on LM17 medium of two-three colonies of characteristic shape isolated for each production batch at each sampling time that were Gram-stained and tested for catalase activity.

2.2. Physiological tests

In all the physiological tests a 1% (v/v) inoculum of cells, harvested by centrifugation at 8000 rpm for 10 min from a fresh culture, washed with an equal volume of sterile phosphate buffered saline (1.1 g/l K_2HPO_4, 0.32 g/l KH_2PO_4, 8.5 g/l NaCl, pH 7.2) and resuspended in an equal volume of this buffer, was used and incubation was protracted for 15 days. The ability to grow in different conditions was determined by measuring the OD_{600} at the stationary phase or at the end of incubation. Growth capacity was examined also in an 8% (w/v) meat extract suspension (Lab Lemco, OXOID, Milan, Italy) at 28 °C. Gas production was tested with durham tubes in modified MRS broth without beef extract and trisodium citrate and containing 1% glucose. The type of fermenting metabolism was ascertained by striking the isolates on HHD (Biolife) agar plates. Proteolysis was tested in plate count agar (PCA) plus skim milk (Biolife) as described by Hantsis-Zacharov and Halpern (2007). Histamine and tyramine production was tested according to Joosten and Northolt (1989). Fermentation of carbohydrates was assayed in M17 medium without disodium-β-glycerophosphate, with 1% (w/v) of the sugar to be tested and pH indicator chlorophenol red.

2.3. Molecular methods

DNA extraction from pure cultures was carried out as described by Rossi et al. (2006).

Rep-PCR with the GTG_5 (5′GTGGTGGTGGTGGTG3′) primer was done according to Versalovic et al. (1994). Amplification of the intergenic ribosomal spacer 16S–23S region was done according to Jensen et al. (1993) with primer pair G1 (5′GAAGTCGTAACAAGG3′)/L1 (5′CAAGGCATCCACCGT3′). Amplification of a c.a. 1500 bp region of the 16S rRNA gene was done according to Bringel et al. (2005) with primers Lac16S-for (5′AATGAGAGTTTGATCCTGGCT3′)/Lac16S-rev (5′GAGGTGATCCAGCCGCAGGTT3′). Detection of genes encoding histidine and tyrosine decarboxylase was done according to De las Rivas et al. (2006), with primers HIS1-F (5′GGNATNGTNWSNTAYGAY MGNGCNGA3′) and HIS1-R (5′ATNGCDATNGCNSWCCANACN CCRTA3′) and Torriani et al. (2008) with primers DEC3 (5′CCGCCAGCAGAATATGGAAYRTANCCCAT3′) and DEC5 (5′CGTTGTT GGTGTTGTTGGCACNACNGARGARG3′), respectively, using as positive controls the histamine producer *Staphylococcus capitis* 26 and the tyramine producer *Enterococcus faecalis* 14 from the collection of the Department of Medicine and Health Sciences, University of Molise. The amplification products were separated on a 1.5% (w/v) agarose gel in 1× TAE buffer (80 mM Tris–acetate, 2 mM EDTA, pH 8.0) at 120 V. Gels were stained in a 0.5 μg/ml ethidium bromide solution.

Sequencing of the 16S rRNA gene was done by Eurofins Genomics (Ebersberg, Germany) in both directions with the same oligonucleotides used for the amplification. The species identification was carried out by BLAST alignment with the public database.

2.4. Genotypic profile comparison

Genotypic profiles were clustered according to distance values by the PyElph 1.4 software (Pavel and Vasile, 2012) with the Unweighted Pair Group using Arithmetic Averages (UPGMA) method.

2.5. RP-HPLC analysis

The peptide profile of 20 μl culture supernatants obtained by separating cells by centrifugation from cultures of 24 h in BHI broth and subsequent filtration through a sterile membrane filter with 0.22 μm pore size, was analysed by RP-HPLC using a Varian Prostar Solvent Delivery apparatus equipped with a C18 RP-HPLC column (Inertsil 150 × 4.6 mm ODS-3 column with 5 μm porosity, Altech Italia Srl., Milan, Italy) and a photodiode array detector (Agilent Technologies, Milan, Italy). Elution was performed at 1.0 mL/min flow rate using the following gradient: 100% eluent A (0.1% trifluoroacetic acid, TFA, in water) for 10 min, linear increase of eluent B (0.1% TFA and 60% acetonitrile in water) from 0% to 80% over 80 min, 100% eluent B for 10 min and finally 100% A for 20 min. Separated peptides were monitored at 280 nm.

3. Results and discussion

3.1. Genotypic and phenotypic features

The isolation of cocci with colony and cell morphology similar to bacteria previously identified as *Tetragenococcus koreensis* (Colavita, unpublished) was possible at all sampling times. The colonies developed after five days of incubation at 28 °C on LM17 plates and were round shaped and white, convex, smooth, slightly slimy, with regular margins and c.a. 2 mm diameter. The cells were perfectly circular and arranged in short chains of four or five.

The isolates were preliminarily assigned to the same taxonomic group on the basis of the amplification profiles of the ribosomal intergenic spacer 16S–23S region (Fig. 1), that were identical to those obtained for the first isolates from Ventricina Vastese sausage (Colavita, unpublished). Based on the highest dilution from which these microorganisms were isolated, the order of magnitude of their number in sausages was approximately as follows: 4 log CFU/g at time 0, 5 log CFU/g at day 10, 6 log CFU/g at day 20 and 8 log CFU/g at days 50 and 150.

Twenty six isolates, labelled according to the production batch (B followed by 1 or 2), the isolate number (I followed by a number)

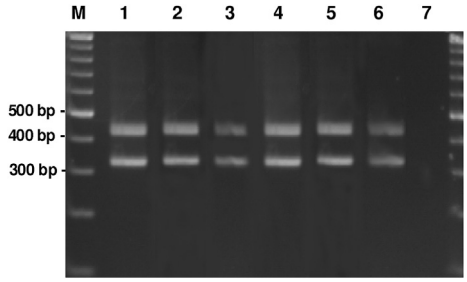

Fig. 1. Amplification profiles of the intergenic ribosomal spacer 16S–23S for isolates of *T. koreensis*. M: GeneRuler 100 bp Plus DNA ladder (Fermentas); lines 1–6, isolates B1-I1-D0, B2-I1-D0, B1-I2-D0, B2-I2-D0, B1-I1-D20, B1-I2-D20; line 7, negative control.

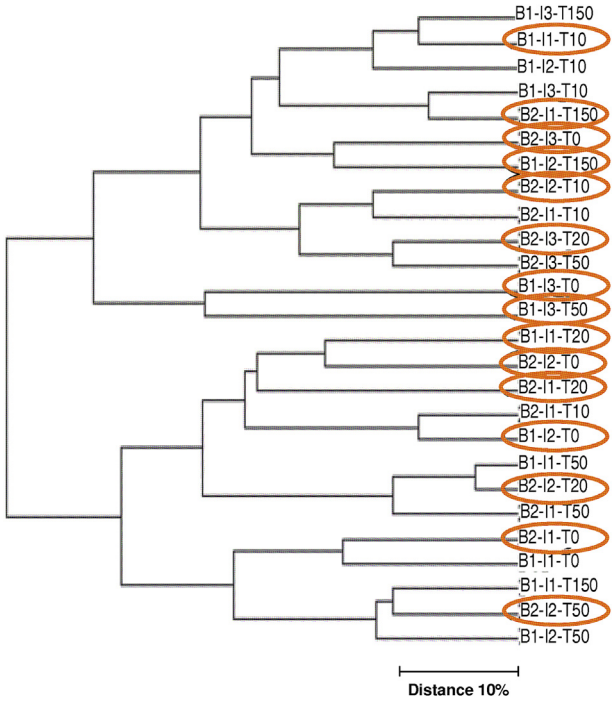

Fig. 2. Distance based clustering of genetic profiles obtained by Rep-PCR with the primer GTG_5 for *T. koreensis* isolates.

and the days of ripening (D followed by 0, 10, 20, 50 or 150), were used in Rep-PCR genotyping. The isolates circled in the dendrogram in Fig. 2 were representative of clusters separated at a cut off of 15% distance and were identified by sequencing of the 16S rRNA gene, all exhibiting 99% similarity with *Tetragenococcus koreensis*. Therefore, this is the first report on the presence of this species in meat products. Like for *T. halophilus* (Holzapfel et al., 2006), a possible source of *T. koreensis* might be the pig faeces, though also the spice mixture used in Ventricina Vastese sausage might be a source since this organism was first isolated from kimchi, a product made from vegetables and spices. This is the first study concerning the genotypic and physiological characterization of tetragenococci isolated from fermented meat products of Southern Italy and showed that, in some instances, strains from different batches grouped in the same cluster and therefore were closely related. Some strains isolated from the same batch at different days of ripening, until 150 days, grouped together, thus indicating the persistence of some biotypes and their contribution to the ripening process for its whole duration.

All the representative strains shared general features of the *Tetragenococcus* genus being homofermentative, Gram-positive, non-motile, non-pigmented, catalase-negative, halophilic and unable to reduce nitrate. All fermented D-glucose, D-fructose, D-mannose, did not ferment D-maltose, D-ribose, D-xylose, D-melibiose and xylitol and had phosphatase activity, being able to hydrolize β-naphthyl phosphate. Characters found to be variable are reported in Table 1. Differently from the *T. koreensis* strain isolated from kimchi (Lee et al., 2005), the isolates did not ferment D-maltose, D-xylose, xylitol and methyl-αD-glucopyranoside and showed variable capacity to ferment D-mannitol, D-saccharose, D-trehalose and N-acetyl-glucosamine. Just one strain was urease positive, so that this character cannot be considered as distinctive for the species like previously proposed. Physiological traits tested only in this study, i.e. the hydrolysis of arginine and fermentation of D-lactose and sodium pyruvate, resulted to be variable. The strains grew well in MRS broth, in brain heart infusion (BHI) broth and in meat extract suspension.

Table 1
Variable physiological characteristics of *T. koreensis* strains representative of distinct genotypic clusters.

	Strain													
	B1-I1-D10	B2-I1-D150	B2-I3-D0	B1-I2-D150	B2-I1-D10	B2-I3-D20	B1-I3-D0	B1-I3-D50	B1-I1-D20	B2-I2-D0	B2-I1-D20	B1-I2-D0	B2-I2-D20	B2-I1-D0	B2-I2-D50
D-lactose	+	+	–	–	–	–	–	+	–	+	+	–	+	+	–
D-trehalose	+	+	–	–	+	–	–	+	–	+	+	–	+	+	+
D-mannitol	+	–	–	–	+	–	–	–	–	+	+	–	+	+	–
Sodium pyruvate	–	–	–	–	–	–	–	–	–	+	+	–	–	–	–
D-saccharose	–	–	–	–	–	–	–	–	–	+	+	–	–	–	–
N-acetyl-glucosamine	+	–	–	–	+	–	–	–	–	+	+	–	+	+	–
L-arginine	+	–	–	–	–	–	–	+	–	+	+	–	–	+	–
Urea	–	–	–	–	–	–	–	–	–	–	+	–	–	–	–

+, Positive reaction.
–, Negative reaction.
W, Weakly positive reaction.

Table 2
Growth capacity of *T. koreensis* strains at different temperature/NaCl concentration combinations in MRS and LM17 broths.

Temperature	15 °C						28 °C						37 °C					
NaCl %	MRS			LM17			MRS			LM17			MRS			LM17		
	10	15	20	10	15	20	10	15	20	10	15	20	10	15	20	10	15	20
Strain																		
B1-I1-D10	–	–	–	w	–	–	w	vw	vw	+	w	w	–	–	–	+	–	–
B2-I1-D150	+	vw	–	+	+	–	+	+	vw	+	+	vw	–	–	–	+	+	vw
B2-I3-D0	w	w	vw	+	+	+	+	+	vw	+	+	vw	–	–	–	+	+	vw
B1-I2-D150	–	vw	–	w	–	–	w	vw	vw	+	+	w	–	–	–	+	+	–
B2-I2-D10	+	vw	–	+	+	–	+	+	vw	+	+	w	–	–	–	+	+	–
B2-I3-D20	–	vw	–	w	+	–	w	vw	vw	+	w	w	–	–	–	+	vw	vw
B1-I3-D0	+	vw	–	+	+	–	+	+	vw	+	+	vw	–	–	–	+	+	–
B1-I3-D50	vw	vw	–	w	w	–	w	w	vw	+	+	vw	–	–	–	+	+	–
B1-I1-D20	+	vw	vw	+	+	+	+	+	vw	+	+	w	–	–	–	+	+	vw
B2-I2-D0	+	vw	–	+	+	–	+	+	vw	+	+	vw	–	–	–	+	+	vw
B2-I1-D20	+	vw	–	+	+	–	+	+	vw	+	+	w	–	–	–	+	+	vw
B1-I2-D0	vw	vw	–	+	+	–	w	vw	vw	+	w	w	–	–	–	+	w	vw
B2-I2-D20	+	vw	–	+	+	vw	+	+	vw	+	+	w	–	–	–	+	+	–
B2-I1-D0	+	w	–	+	+	+	+	+	vw	+	+	w	–	–	–	+	+	vw
B2-I2-D50	+	vw	–	+	+	–	+	+	vw	+	+	vw	–	–	–	+	+	–

+, OD_{600} higher than 1.0.
w, Weak, OD_{600} between 0.3 and 0.5.
vw, Very weak, OD_{600} between 0.2 and 0.3.

3.2. Characteristics relevant for fermented meat production

All strains had a very low acidifying capacity after 24 h growth at 28 °C in a not buffered medium (LM17 broth without disodium-β-glycerophosphate), with the lowest pH value of 5.95 for strain B2-I3-D0. At 48 h pH values were all comprised between 6.00 and 6.14. These bacteria are therefore suitable for products like Ventricina Vastese sausage, where high acidity is undesired.

The ability to grow at different combinations of temperature/NaCl concentration is reported in Table 2. In addition, all strains grew very weakly or weakly at 5 °C also in presence of 5% (w/w) NaCl, while strains B1-I1-D10, B2-I2-D20 and B2-I3-D20, grew well in these conditions. The ability to grow at 5 °C, not shown by the fermented meat isolates of La Pietra et al., 1999, indicates that *T. koreensis* can carry out biochemical activities also during cold storage of products. The upper temperature limit for growth was 28 °C in MRS medium and 37 °C in LM17 medium for all strains. All strains grew well in presence of 7% (w/w) NaCl at 15 °C. Differently from the type strain (Lee et al., 2005), all tolerated concentrations of NaCl higher than 8% when grown in MRS medium at 28 °C.

A concern posed by tetragenococci in fermented food is histamine and tyramine production for the possible presence of amino acid decarboxylation gene clusters on plasmids or on the chromosome (Satomi et al., 2014). However, none of the strains tested produced histamine or tyramine in plate and none was positive for the consensus PCR tests for histidine and tyrosine decarboxylases, so that they do not pose the risk of biogenic amine production in fermented sausages.

In agreement with the observations of Lee et al., 2005, the *T. koreensis* strains did not exhibit strong proteolytic activity:

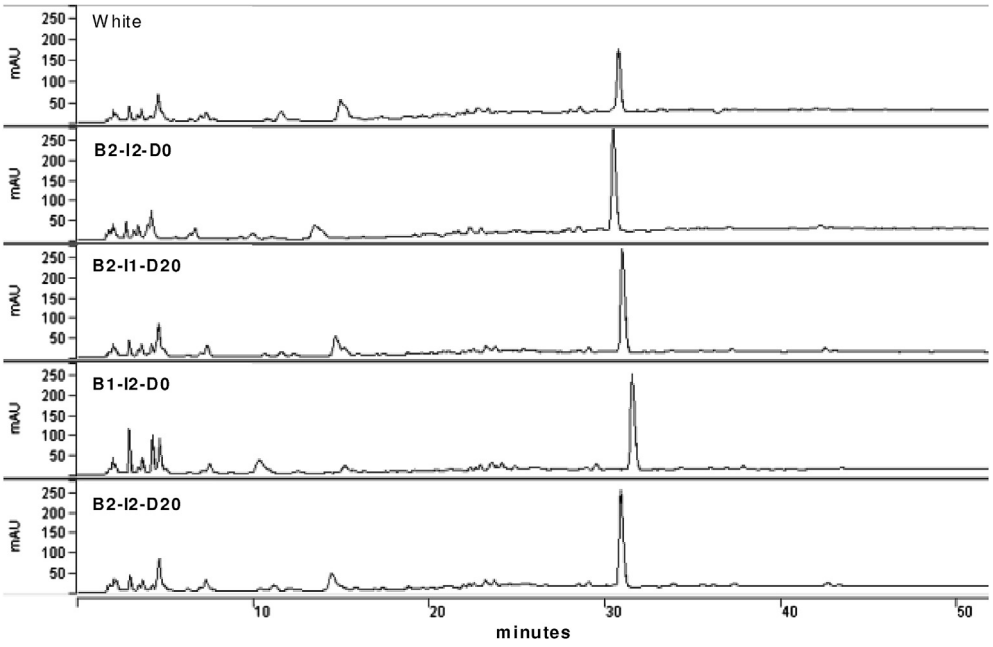

Fig. 3. RP-HPLC protein/peptide profiles of culture supernatants of *T. koreensis* strains grown in BHI medium for 24 h.

proteolysis was not observed in plate and RP-HPLC analysis highlighted a low capacity to modify the protein/peptide profile in BHI broth, as shown by the representative chromatograms of the culture supernatants (Fig. 3). All strains were able to increase the size of a main peak eluting at 31–33 min at a strain-dependent extent and strain B1-I2-D0 showed a chromatogram profile more different from the control compared to the other strains. Strains B1-I1-150 and B1-I3-D50 exhibited a profile similar to strain B1-I2-D0 (data not shown). Thus the opportunity to better characterize the peptide degrading activities was suggested, since information on this aspect is currently available only for *T. halophilus* (Udomsil et al., 2010).

In conclusion, this first report on the features of *Tetragenococcus koreensis* isolated from sausages demonstrated intra-species diversity in genotypic, phenotypic, and technologically relevant aspects. Safe use was indicated by the inability to form biogenic amines and therefore the evaluation of *T. koreensis* strains as autochthonous starter cultures in sausage production is opportune.

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