Tracking the transfer of antimicrobial resistance genes from raw materials to sourdough breads

Valeria Gargano [a], Delia Gambino [a], Enrico Viola [b], Elena Franciosi [c], Antonio Alfonzo [b], Luca Cicero [a], Giovanni Cassata [a], Luca Settanni [b,*], Raimondo Gaglio [b]

[a] *Istituto Zooprofilattico Sperimentale della Sicilia "A. Mirri", 90129 Palermo, Italy*
[b] *Dipartimento Scienze Agrarie, Alimentari e Forestali, Università di Palermo, Viale delle Scienze 4, 90128 Palermo, Italy*
[c] *Research and Innovation Centre, Fondazione Edmund Mach (FEM), Via E. Mach 1, 38098 San Michele all'Adige, Italy*

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ABSTRACT

The present study hypothesizes that raw materials used in bread making can transfer antibiotic resistance genes (ARGs) to processed breads. Four types of flour and four types of semolina were purchased from supermarkets and inoculated with a commercial dried sourdough starter to make breads. The microbiological characteristics of all raw materials and fermented doughs were investigated. The levels of yeasts and lactic acid bacteria (LAB) increased up to 10^7 CFU/g. The values of pH decreased to 4.54–4.86 while total titratable acidity increased inversely. All unprocessed and processed samples, including breads, were analyzed by a molecular approach to detect bacterial and fungal DNAs and 17 antibiotic resistance genes for penicillins, macrolides, tetracyclines, and chloramphenicol. Illumina technology showed that the operational taxonomy units (OTUs) identified from unprocessed wheat milling products, fermented doughs, and baked products mainly belonged to Acetobacteraceae. Enterococci were present in all doughs. After baking, the relative abundance (RA)% of *Enterococcus* and Acetobacteraceae decreased. The DNA analyzed for fungal composition showed that *Kazachstania humilis* dominated dried sourdough starter and doughs, and its OTUs were also detected at high RA% in baked products. The search for ARGs revealed that all samples analyzed did not show resistance to penicillins, chloramphenicol, and macrolides. However, three of the semolinas included in this study (S1, S3 and S4) and the corresponding doughs (SD1, SD3 and SD4) were positive for *tet*(A) and *tet*(B) resistance genes. This work indicated that breads have a limited role in the dissemination of ARGs.

1. Introduction

Antimicrobial resistance is a natural adaptive mechanism through which microorganisms acquire the ability to survive or grow in the presence of antimicrobial agents that would otherwise inhibit or kill them (Arzanlou, Chai, & Venter, 2017). The continuous and excessive use of antimicrobials in humans, animals, and plants leads to the emergence of antimicrobial resistance. According to One Health approach, this phenomenon has a significant impact on the health of humans, animals, and the environment (Hernando-Amado, Coque, Baquero, & Martínez, 2019; McEwen & Collignon, 2018). Controlling the spread of antimicrobial resistance is challenging because the responsible genes can be transferred to different types of bacteria (Prestinaci, Pezzotti, & Pantosti, 2015). Antimicrobial resistance genes (ARGs) are carried on mobile genetic elements that allow their transfer between different bacterial genera (Flores-Orozco et al., 2023). Antimicrobial resistant bacteria can be carried by humans, animals, animal products, and the environment (Gardner et al., 2023). Among food-associated microorganisms, those carrying ARGs include micrococci, kokurias, coagulase-negative staphylococci (Gardini, Tofalo, & Suzzi, 2003), enterococci (Chajęcka-Wierzchowska, Zarzecka, & Zadernowska, 2021), non-enterococcal lactic acid bacteria (LAB), *Bifidobacterium* (Ammor, Flórez, & Mayo, 2007), several foodborne bacteria (Caniça, Manageiro, Abriouel, Moran-Gilad, & Franz, 2019), and some yeasts (Wolfe, 2023).

According to Zarzecka, Zadernowska, and Chajęcka-Wierzchowska (2020), starter strains are responsible for the dissemination of ARGs for antibiotics used to treat human diseases. Therefore, the European Food Safety Authority (EFSA) strongly recommends screening starter cultures for ARGs before commercialization (EFSA, 2018). Besides starter

cultures, raw materials are also involved in the dissemination of ARGs into fermented food production systems, especially in case these are of animal origin, because milk and meat may transfer antimicrobial resistant microorganisms (Frétin et al., 2018; Pisacane, Callegari, Puglisi, Dallolio, & Rebecchi, 2015). In fact, animals might be subjected to antibiotic treatments during their life, and the microorganisms associated with animal-derived raw materials may acquire ARGs (Catry, Laevens, Devriese, Opsomer, & de Kruif, 2003). Unlike animals, plants are not typically exposed to antibiotics on a large scale. The spreading of ARGs in crop systems has been addressed by analyzing soil, water, and manure samples (Heuer, Schmitt, & Smalla, 2011; Hölzel, Tetens, & Schwaiger, 2018). However, information on raw materials used in bread production is limited. Wolfe (2023) reported that the relative risk of antibiotic resistance in sourdough breads is low because the fermenting microbiota is no longer viable at consumption due to baking exposure.

The aim of this work was to monitor the transfer of ARGs through sourdough bread making and to evaluate the potential transfer of ARGs from residual DNA after baking. The microbiological characteristics of raw materials used in bread making, fermented doughs, and final breads produced from tender flour and durum semolina were investigated. All samples were also tested for the presence of ARGs toward the classes of antibiotics most commonly used in animal husbandry and agriculture: tetracyclines, penicillins, chloramphenicol, and macrolides. A metagenomic (culture-independent) approach was applied to search for ARGs in the raw materials (semolinas, flours, salt, water, dried sourdough starter), doughs, and finished baked products from the total extracted DNA of individual samples.

2. Materials and methods

2.1. Raw materials, bread production and sample collection

In this study, sourdoughs were processed from commercial durum wheat semolinas and tender wheat flours (Table 1). A commercial dried sourdough (Molino Rossetto S. p.A., Pontelongo, Italy) was used as starter. Kitchen salt (Sosalt S. p.A., Trapani, Italy) and tap water were added according to the recipe provided by the sourdough starter producer: semolina/flour (250 g); commercial dried sourdough starter (17.5 g); salt (5 g); tap water (150 mL).

The doughs were mixed using a planetary mixer model XBM10S (Electrolux Professional, SpA, Pordenone, Italy) equipped with a paddle at speed 1 for 5 min followed by 2 min at speed 2. Three aliquots of 100 g per dough were transferred into trapezoidal stainless steel baking pans of the dimension reported by the American Association of Cereal Chemists – Method 10-10 B of AACC (2000) and kept at 28 °C for 2 h (fermentation duration was indicated by the sourdough starter producer's instructions). After fermentation, all dough replicates were baked in a Compact Combi (Electrolux, Pordenone, Italy) semi-industrial oven at 200 °C for 5 min under hot air/steam followed by 200 °C for 15 min under hot air only. The process of bread production is illustrated in Fig. 1.

Samples of commercial flours (F1 – F4) and semolinas (S1 – S4), tap water (TW), kitchen salt (KS), dried sourdough starter (DSS), doughs (FD1 – FD4; SD1 – SD4) just after ingredient mixing (T0) and at the end of fermentation (T2), and final breads (FB1 – FB4; SB1 – SB4) were collected for analyses.

2.2. Fermentation process

The acidification of the doughs was monitored by measuring pH, total titratable acidity (TTA) and the levels of different microbial groups. The pH values were determined electrometrically using a pH meter (XS Instruments, Carpi, Italy) by directly inserting the probe. TTA was determined by titration with 0.1 N NaOH on 10 g of dough and expressed in terms of mL of NaOH.

To investigate the presence of microorganisms, plate counts were performed on 10 g of each dough. The dough was suspended into 90 mL of Ringer's solution (Sigma-Aldrich, Milan, Italy), homogenized through stomacher (BagMixer® 400, Interscience, Saint Nom, France) at the highest speed for 2 min, and serially diluted. The following microorganisms were studied: total mesophilic microorganisms (TMM) on plate count agar (PCA) incubated aerobically at 30 °C for 72 h; LAB rods on modified de Man, Rogosa, and Sharpe (mMRS) agar prepared as described by Corsetti, Settanni, Braga, de Fatima Silva Lopes, and Suzzi (2008), incubated anaerobically at 30 °C for 48 h; LAB cocci on Media 17 (M17) incubated anaerobically at 30 °C for 48 h; and total yeasts on yeast extract peptone dextrose (YPD) agar incubated at 28 °C for 48 h. To inhibit fungal growth, cycloheximide (10 mg/mL) was added to mMRS and M17, while chloramphenicol (0.1 mg/mL) was added to YPD to prevent bacterial growth. All media were purchased from Oxoid (Basingstoke, UK). Plate counts were performed in triplicate.

2.3. Culture-independent analysis of total microbial community

2.3.1. DNA extraction

Total genomic DNA was extracted from each sample (10 mg) using the QIAamp® DNA Investigator Kit (QIAGEN, Hilden, Germany) following the manufacturer's instructions. The quality and concentration of DNA were determined using the NanoDrop™ 8000 Microvolume UV–Vis spectrophotometer (ThermoFisher Scientific, Inc.,Wilmington, DE, USA).

2.3.2. MiSeq library preparation and illumina sequencing

At the Sequencing Platform, Fondazione Edmund Mach (FEM, San Michele a/Adige, Italy), the Illumina MiSeq system (Illumina, USA) was used to perform amplicon library preparation, quality and quantification of pooled libraries, and pair-end sequencing. Briefly, bacterial V3–V4 region (Baker, Smith, & Cowan, 2003; Claesson et al., 2010) of the 16 S rRNA gene (*Escherichia coli* positions 341 to 805), and ITS1F/ITS4 specific for the ITS1-5.8 S fungi region (Gardes & Bruns, 1993) were amplified for bacteria and fungi, respectively, from samples of flours, semolinas, DSS, TW, KS, doughs at T2, and breads.

To facilitate the pooling and subsequent differentiation of samples, unique barcodes were attached before the forward primers. The amplicons were cleaned using the Agencourt AMPure kit (Beckman coulter) according to the manufacturer's instructions to prevent preferential sequencing of the smaller amplicons. Subsequently, DNA concentrations of the amplicons were determined using the Quant-iT PicoGreen dsDNA kit (Invitrogen) following the manufacturer's instructions. To ensure the absence of primer dimers and to assay the purity, the quality of the generated amplicon libraries was evaluated by a Bioanalyzer 2100 (Agilent, Palo Alto, CA, USA) using the High Sensitivity DNA Kit

Table 1
Samples of wheat flours used to produce traditional sourdough breads in Sicily.

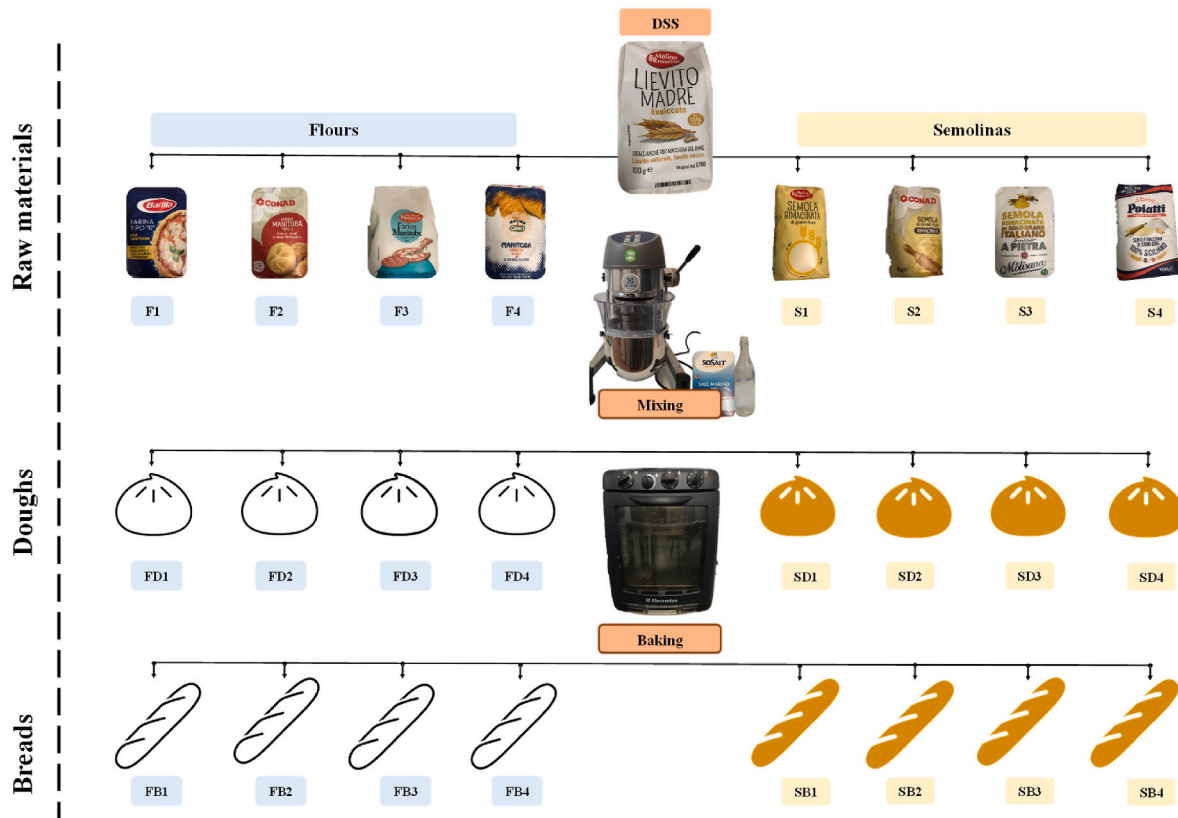| Samples	Type	Commercial name	Company
F1	Soft wheat flour	Manitoba Type "0"	Barilla S.p.A., Casalgrasso (CN)
F2	Soft wheat flour	Manitoba Type "0"	Molino Spadoni S.p.A., Coccolia (RA)
F3	Soft wheat flour	Manitoba Type "0"	Molino Rossetto S.p.A., Pontelongo (PD)
F4	Soft wheat flour	Manitoba Type "0"	Molino Oddo Vito, Valderice (TP)
S1	Durum wheat semolina	Semola rimacinata	Molino Rossetto S.p.A., Pontelongo (PD)
S2	Durum wheat semolina	Semola rimacinata	Molino Casillo, Ortona (CH)
S3	Durum wheat semolina	Semola rimacinata	La Molisana S.p.A., Ripalimosani (CB)
S4	Durum wheat semolina	Semola rimacinata	Poiatti S.p.A., Mazara del Vallo (TP)

Fig. 1. Graphical representation of the experimental plan followed to track the transfer of antimicrobial resistance genes from raw materials to sourdough breads. Abbreviations: DSS, dried sourdough starter; F1–F4, commercial flours; S1–S4, commercial semolinas; FD1–FD4, flour doughs; SD1−SD4, semolina doughs; FB1–FB4, flour breads. SB1–SB4, semolina breads.

(Agilent). After quantitation, cleaned amplicons were mixed and combined in equimolar ratios.

2.3.3. Illumina data analysis and sequences identification by QIIME2

The raw paired-end FASTQ files were demultiplexed using idemp (https://github.com/yhwu/idemp/blob/master/idemp.cpp) and imported into Quantitative Insights Into Microbial Ecology (Qiime2, version 2018.2). The sequences were quality filtered, trimmed, denoised, and merged using DADA2 (Callahan et al., 2016). Chimeric sequences were identified and removed via the consensus method in DADA2. Representative bacterial sequences were aligned with MAFFT and used for phylogenetic reconstruction in FastTree using plugins alignment and phylogeny (Katoh & Standley, 2013; Price, Dehal, & Arkin, 2009). For bacteria, taxonomic and compositional analyses were conducted by using plugins feature-classifier (https://github.com/qiime2/q2-feature-classifier). A pre-trained Naive Bayes classifier based on the Greengenes 13_8 99% Operational Taxonomic Units (OTUs) database, which had been previously trimmed to the V4 region of 16 S rDNA bound by the 341F/805 R primer pair, was applied to paired-end sequence reads to generate taxonomy tables. Fungal sequences were classified to the species-level using a 97 or 99% threshold using UNITE dynamic classifier version 8.0 released for Qiime2 (UNITE QIIME release for Fungi. Version November 18, 2018. UNITE Community. https://doi.org/10.15156/BIO/786334). The data generated by MiSeq Illumina sequencing were deposited in the NCBI Sequence Read Archive (SRA) and are available under Ac. No. PRJNA987557.

2.4. Detection of antimicrobial resistance genes

The search for 17 ARGs (Table 2) that may contribute to resistance toward penicillins, macrolides, tetracyclines, and chloramphenicol

(Campedelli et al., 2019; Lee, Heo, Jeong, & Jeong, 2019) was conducted on raw materials (semolinas, flours, water, salt, and dry commercial yeast), sourdoughs, and breads obtained by experimental baking. Briefly, 10 ng of sample DNA and 0.4 μM of forward and reverse primers listed in Table 2 were used in a total volume of 25 μL of Advanced Universal EVA Green Supermix 1 × (Bio-Rad Laboratories, Hercules, CA, USA). A portion of the 16 S rDNA of about 200 bp was used as a positive control (Lu, Perng, Lee, & Wan, 2000). The reaction conditions for DNA amplification included initial denaturation for 5 min at 94 °C, 30 cycles each of denaturation (94 °C for 1 min), annealing (refer to Table 2), and extension (72 °C for 2 min), followed by final extension at 72 °C for 10 min. Fluorescence during extension was recorded to generate the amplification curves. For real-time PCR, melting curve and peak analysis were performed at a melting rate of 0.2 °C/min from 65 to 95 °C. Moreover, all samples that tested positive by real-time PCR were verified by electrophoresis on E-Gel™ Go! Agarose gel, 2% (Thermo Fisher Scientific, Waltham, MA, USA). To confirm identity, the amplicons genes detected by PCR were then sequenced. DNA sequences were determined using the dideoxy chain termination method with the commercial DNA sequencing kit BigDye™ Terminator v3.1 Cycle Sequencing Kit (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's instructions. The obtained sequences were analyzed for nucleotide sequence identity by comparing them with reference strains in the GenBank database, using the Basic Local Alignment Search Tool (BLAST) and The Comprehensive Antimicrobial Resistance Database (https://card.mcmaster.ca, accessed on date 2 June 2023).

2.5. Statistical analyses

The data on pH, TTA, and plate count were statistically analyzed

Table 2

Primer pairs and PCR conditions used for detection of selected ARGs.

Antibiotic	Target gene	Primer sequence (5'→3')	Amplicon size (bp)	Annealing temperature (°C)	References
Chloramphenicol	*cat*	TTAGGTTATTGGGATAAGTTA GCATGRTAACCATCACAWAC	300	48	Guo et al. (2017)
	catA	GGATATGAAATTTATCCCTC CAATCATCTACCCTATGAAT	486	50	
Macrolides	*erm(A)*	CCCGAAAAATACGCAAAATTTCAT CCCTGTTTACCCATTTATAAACG	590	60	Duche et al. (2023)
	erm(B)	TGGTATTCCAAATGCGTAATG CTGTGGTATGGCGGGTAAGT	745	60	
	erm(C)	AATCGTCAATTCCTGCATGT TAATCGTGGAATACGGGTTTG	299	60	
Penicillin	*blaZ*	ACTTCAACACCTGCTGCTTTC TAGGTTCAGATTGGCCCTTAG	240	58	Guo et al. (2017)
	bla	CATARTTCCGATAATASMGCC CGTSTTTAACTAAGTATSGY	297	50	
	mecA	GGGATCATAGCGTCATTATTC AGTTCTGCAGTACCGGATTTGC	1429	58	
	blaTEM	ATCAGCAATAAACCAGC CCCCGAAGAACGTTTTC	516	55	Anisimova, Gorokhova, Karimullina, and Yarullina (2022)
Tetracycline	*tet(A)*	GCTACATCCTGCTTGCCTTC CATAGATCGCCGTGAAGAGG	210	60	Schneider, Müller, Miess, and Gross (2014)
	tet(B)	TTGGTTAGGGGCAAGTTTTG GTAATGGGCCAATAACACCG	659	60	
	tet(C)	CTTGAGAGCCTTCAACCCAG ATGGTCGTCATCTACCTGCC	418	62	
	tet(D)	AAACCATTACGGCATTCTGC GACCGGATACACCATCCATC	787	62	
	tet(M)	GGTGAACATCATAGACACGC CTTGTTCGAGTTCCAATGC	401	58	Anisimova et al. (2022)
	tet(K)	TTATGGTGGTTGTAGCTAGAAA AAAGGGTTAGAAACTCTTGAAA	348	55	Thumu and Halami (2012)
	tet(L)	GTMGTTGCGCGCTATATTCC GTGAAMGRWAGCCCACCTAA	696	55	
	tet(O)	AATGAAGATTCCGACAATTT CTCATGCGTTGTAGTATTCCA	781	55	

using one-way variance analysis (ANOVA). The Tukey's test was applied to determine the difference between means when p < 0.05. The detection of ARGs in all samples was graphically represented as a heat map generated using ascendant hierarchical clustering. The ARGs distribution was graphically represented by two colours: yellow (no ARGs detection) and red (detection of ARGs). Statistical processing of chemical and microbiological data and the graphic constructions of ARGs detection were performed with the XLStat software version 2020.3.1 for Microsoft Excel (Addinsoft, New York, NY, USA).

3. Results and discussion

3.1. Sourdough acidification

The evolution of pH and TTA during sourdough production are reported in Table 3. The kinetics of these two parameters was followed for only 2 h, which is the leavening duration indicated by the sourdough starter producer. Short sourdough fermentations are possible in the presence of baker's yeasts (Xu et al., 2019). All initial pH values were in the range 5.58–5.70 and decreased to 4.54–4.86 at the end of fermentation. Sourdoughs propagated from flour displayed slightly lower pHs than those propagated from semolina. This difference is due to the different particle size of tender and durum wheat milling products (Stoddard, 1999), as the texture endosperm of the cultivars of *Triticum aestivum* and *Triticum turgidum* L. ssp. *durum* wheat are different (Pauly, Pareyt, Fierens, & Delcour, 2013), with the result that flour is finer than semolina (Posner, 2000). The smaller the particle size, the greater the contact surface area for bacteria and yeasts, which leads to higher consumption of fermentable carbohydrates and lower pH levels registered in doughs at the end of fermentation (Ruisi et al., 2021).

The values of TTA at the beginning of the process were around 3.00 mL of 0.1 N NaOH and more than doubled after 2 h. The levels of total

Table 3

Acidification of flour and semolina doughs during fermentation.

Samples	pH		TTA	
	T_0	T_2	T_0	T_2
FD1	5.61 bc	4.68 bcd	2.95 bc	6.80 ab
FD2	5.64 abc	4.62 cde	3.10 abc	6.90 ab
FD3	5.58 c	4.54 e	3.40 a	7.10 a
FD4	5.68 ab	4.60 de	2.90 bc	7.20 a
SD1	5.63 abc	4.71 bc	3.00 abc	6.80 ab
SD2	5.70 a	4.86 a	2.80 c	6.60 b
SD3	5.58 c	4.75 b	3.30 ab	6.80 ab
SD4	5.66 abc	4.77 ab	3.00 abc	6.60 b
SEM	0.01	0.02	0.04	0.04
p value	0.001	< 0.0001	0.004	0.005

Results indicate mean values of four determinations (performed in duplicate for two independent experiments). Data within a column followed by the same letter are not significantly different according to Tukey's test. Abbreviations: TTA, total titratable acidity (mL of 0.1 N NaOH/10 g); T_0, doughs just after ingredient mixing; T_2, doughs at the end of fermentation; FD1–FD4, flour doughs; SEM, standard error of the mean; SD1−SD4, semolina doughs.

acidity of flour sourdoughs were, on average, higher (7.00 mL of 0.1 N NaOH) than those registered in semolina sourdoughs (6.70 mL of 0.1 N NaOH). These findings were expected, because a decrease in pH is directly and linearly related to an increase in TTA value (Alfonzo et al., 2016).

The values of both acidification parameters (pH and TTA) registered in this study are different from those generally recorded for mature semolina sourdoughs used in bread making in southern Italy (Alfonzo et al., 2016; Rizzello et al., 2015; Ventimiglia et al., 2015); in particular, pH is higher, while TTA is lower. This behaviour is undoubtedly due to the short fermentation time applied in this work. It is well known that

fermentation time is a process parameter affecting pH kinetics in sourdough (De Vuyst, Vrancken, Ravyts, Rimaux, & Weckx, 2009; Üçok & Sert, 2020).

3.2. Cell counts

Table 4 reports the levels of viable microorganisms hosted on all raw materials and sourdoughs. Plate counts specifically focused on TMM, yeasts, and LAB (both cocci and rods). All these groups were below the detection levels in tap water and kitchen salt, while the commercial sourdough starter was characterized by cell densities around 10^8 CFU/g for TMM and yeasts, while LAB were at almost two orders of magnitude lower. Indeed, yeast levels are comparable with those detected in other dried sourdough starters purchased in Italy (8.8–9.0 Log CFU/g) (Principato, Garrido, Massari, Dordoni, & Spigno, 2019). Principato et al. (2019) reported very low levels of LAB (2.5–4.7 Log CFU/g) for the same commercial starters and even discovered that one of the commercial dried sourdough starters analyzed lacked vital LAB. In this work, LAB rods and cocci detected in the dried sourdough starter were 7.30 and 7.44 Log CFU/g, respectively. A consistent reduction of viability of LAB in commercial sourdough starter is a common phenomenon observed after drying (Reale et al., 2019; Tafti, Peighambardoust, Hesari, Bahrami, & Bonab, 2013) and the reduction can account for four Log cycles when spray-drying is applied (Denkova, Georgieva, & Denkova, 2014).

Both flour and semolina samples showed comparable cell densities (around 10^3 CFU/g) for all four microbial groups investigated. Previous works on flours and semolinas showed that the levels of indigenous LAB and yeasts in these unprocessed raw materials can reach up to 10^4 CFU/g (Alfonzo et al., 2013, 2016; Mamhoud et al., 2016; Pontonio et al., 2015).

Once raw materials were mixed with the commercial dried starter,

the doughs were characterized by levels of TMM in the range 6.06–6.32 Log CFU/g in presence of flour and 5.95–6.19 Log CFU/g when semolina was used. As expected, LAB (both groups, cocci and rods) and yeasts were inoculated at levels of almost two orders of magnitude lower than those registered in dried sourdough starter (10^5 and 10^6 CFU/g, respectively). All microbial groups monitored increased in 2 h of fermentation, but the increase was more consistent for LAB populations (ca. 2.0 Log CFU/g more than inoculums) rather than yeasts. Except sourdough FD4, yeast densities increased of at least one Log cycle. However, all microbial groups investigated reached the same level (around 10^7 CFU/g) at the end of fermentation. Flour sourdoughs displayed the highest cell densities of LAB rods and yeasts. As per pH and TTA, the smaller particle size of flour determined a higher contact surface area for bacteria and yeasts than semolina (Ruisi et al., 2021). This explains their higher access to fermentable sugars and thus their higher viable counts in flour doughs rather than semolina doughs.

3.3. Composition of bacterial and fungal communities

All 27 samples' DNA was successfully amplified. This approach also involved baked products to deeply analyse the fungal and bacterial DNA involved in the possible transfer of ARGs during bread consumption. After merging and quality trimming of raw data, 767,749 reads for bacteria and 263,888 reads for fungi remained for subsequent analysis. After alignment, the remaining OTUs were clustered at a 3% of distance.

A total of 148 OTUs were identified from all samples. They were distributed among Acidobacteria, Actinobacteria, Bacteroidetes, Chloroflexi, Cyanobacteria, Firmicutes, Fusobacteria, Planctomycetes, Proteobacteria, Spirochaetes, Tenericutes and Verrucomicrobia phyla. Only the OTUs with a relative abundance (RA) above 0.1% (n = 16) are reported in Fig. 2 as this is the threshold for abundant communities

Table 4
Microbial loads of raw materials and doughs.

Samples	TMM		LAB rods		LAB cocci		Yeasts[a]	
	T₀	T₂	T₀	T₂	T₀	T₂	T₀	T₂
TW	0.00	n.a.	0.00	n.a.	0.00	n.a.	0.00	n.a.
KS	<2	n.a.	<1	n.a.	<1	n.a.	<2	n.a.
DSS	8.11	n.a.	7.30	n.a.	7.44	n.a.	7.97	n.a.
F1	2.82	n.a.	3.23	n.a.	3.11	n.a.	3.20	n.a.
F2	3.10	n.a.	3.44	n.a.	3.49	n.a.	3.00	n.a.
F3	2.84	n.a.	3.37	n.a.	3.22	n.a.	2.88	n.a.
F4	2.91	n.a.	3.27	n.a.	3.34	n.a.	3.12	n.a.
SEM	0.05	n.e.	0.04	n.e.	0.05	n.e.	0.04	n.e.
p value	0.546	n.e.	0.630	n.e.	0.270	n.e.	0.216	n.e.
FD1	6.22	7.56	5.25	7.70	5.41	7.61	6.04	7.21
FD2	6.18	7.45	5.44	7.85	5.54	7.53	6.13	7.36
FD3	6.32	7.30	5.40	7.68	5.30	7.27	5.99	7.27
FD4	6.06	7.23	5.34	7.43	5.18	7.58	6.21	7.03
SEM	0.05	0.06	0.04	0.06	0.06	0.06	0.06	0.05
p value	0.753	0.619	0.746	0.376	0.698	0.642	0.837	0.506
S1	3.09	n.a.	3.32	n.a.	3.56	n.a.	2.74	n.a.
S2	2.88	n.a.	3.30	n.a.	3.64	n.a.	2.99	n.a.
S3	3.11	n.a.	3.42	n.a.	3.69	n.a.	2.63	n.a.
S4	2.79	n.a.	3.12	n.a.	3.49	n.a.	2.81	n.a.
SEM	0.06	n.e.	0.05	n.e.	0.04	n.e.	0.05	n.e.
p value	0.487	n.e.	0.632	n.e.	0.741	n.e.	0.696	n.e.
SD1	6.11	7.15	5.27	7.24	5.33	7.40	5.80	6.94
SD2	6.01	6.91	5.15	7.19	5.42	7.29	5.94	7.15
SD3	6.19	7.03	5.33	7.30	5.59	7.68	5.75	6.88
SD4	5.95	7.10	5.18	7.14	5.35	7.37	5.90	7.09
SEM	0.05	0.04	0.05	0.03	0.07	0.05	0.04	0.05
p value	0.665	0.634	0.793	0.750	0.833	0.242	0.742	0.586

Results are expressed as Log CFU/g and indicate mean values of four plate counts (carried out in duplicate for two independent productions). Abbreviations: TMM, total mesophilic microorganisms counted on plate count agar (PCA); LAB, lactic acid bacteria counted on modified de Man, Rogosa, and Sharpe (mMRS) agar (rods) and Medium 17 (M17) agar (cocci); T₀, doughs just after ingredient mixing; T₂, doughs at the end of fermentation; TW, tap water; KS, kitchen salt; DSS, dried sourdough starter; F1–F4, commercial flours; FD1–FD4, flour doughs; S1–S4, commercial semolinas; SD1−SD4, semolina doughs; SEM, standard error of the mean; n. a., not analyzed; n.e., not evaluated.

[a] Yeasts were counted on yeast peptone dextrose (YPD) agar.
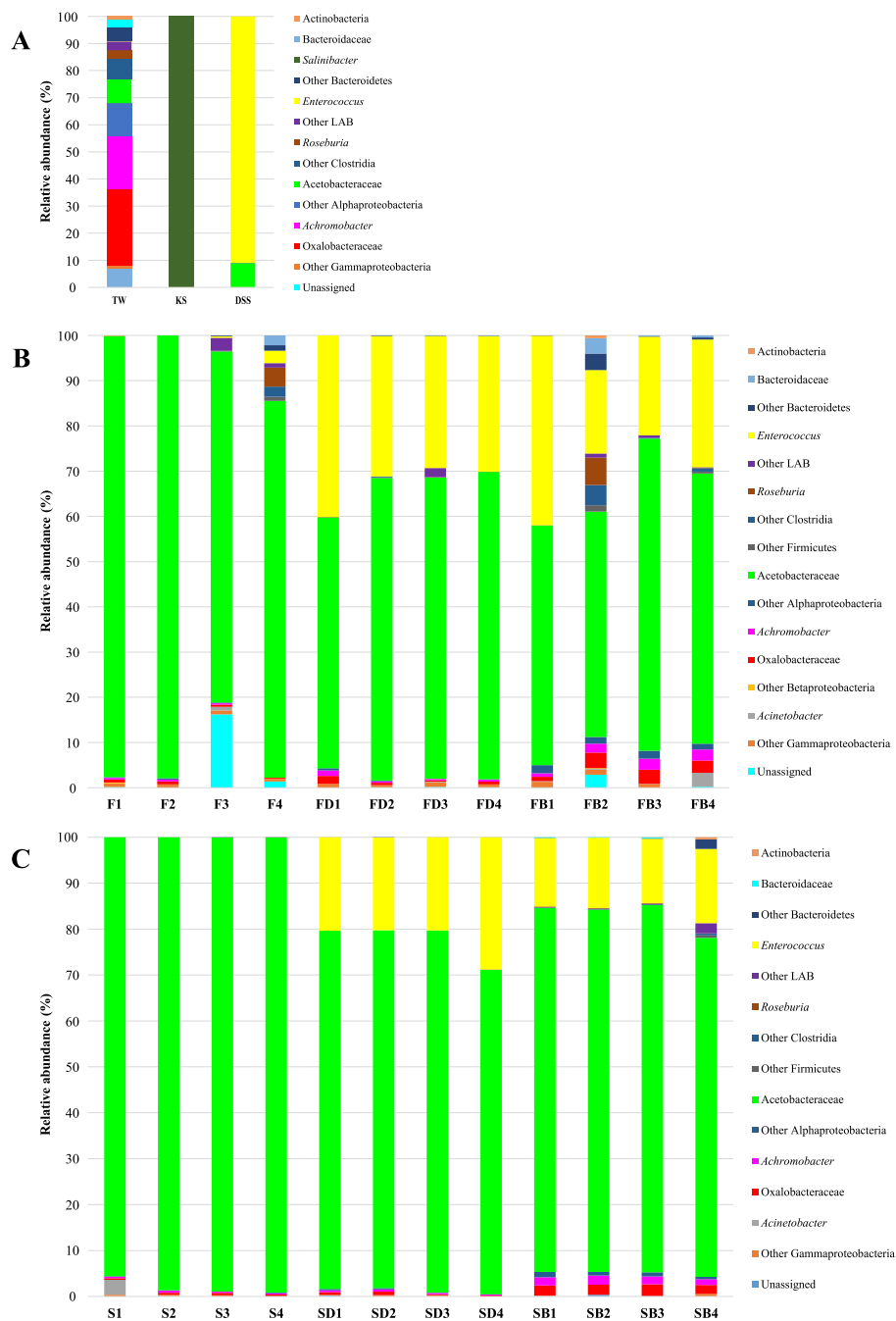
Fig. 2. Relative abundances% of the bacterial operational taxonomy units (OTUs) identified by MiSeq Illumina. A: TW, tap water; KS, kitchen salt; DSS, dried sourdough starter. B: F1–F4, commercial flours; FD1–FD4, flour doughs; FB1–FB4, flour breads. C: S1–S4, commercial semolinas; SD1−SD4, semolina doughs; SB1–SB4, semolina breads.

(Logares et al., 2014). The same threshold level was applied to yeast OTUs, and only 17 of the 61 OTUs are reported in Fig. 3.

The bacterial community composition of raw materials different from flours/semolinas (Fig. 2A) was very simple in case of kitchen salt and dried sourdough starter, except tap water. Kitchen salt only hosted *Salinibacter*, which are extremely halophilic bacteria (Oren, 2013). Surprisingly, dried sourdough starter preparation was characterized by 90.94% RA of *Enterococcus*, 8.96% RA of Acetobacteraceae, and barely 0.10% RA of other LAB. Although enterococci are part of the LAB populations particularly important during the first phases of sourdough fermentation (Corsetti, Settanni, Valmorri, Mastrangelo, & Suzzi, 2007), their dominance in mature sourdoughs is quite unusual. *Enterococcus* play a positive role in gluten protein degradation (Wieser, Vermeulen,

Gaertner, & Vogel, 2008), especially gliadins (M'hir et al., 2008), but they are considered sourdough-atypical LAB (De Vuyst, González-Alonso, Wardhana, & Pradal, 2023) and their cell densities are strongly reduced when sourdough pH decreases significantly (Oshiro, Zendo, & Nakayama, 2021; Zotta, Parente, & Ricciardi, 2009). Tap water OTUs were mainly identified as Oxalobacteraceae, Achromo- bacter, Alphaproteobacteria (especially Acetobacteraceae) and Clos- tridia, but Bacteroidetes, Actinobacteria, and among Firmicutes *Roseburia*, *Enterococcus* and other unspeciated LAB were also identified.

The OTUs identified from flours, fermented doughs, and baked products (Fig. 2B) mainly belong to Acetobacteraceae. This groups is generally associated with the fermentation of cereal-based foods (Kayitesi, Onojakpor, & Moyo, 2023; Shangpliang & Tamang, 2023),
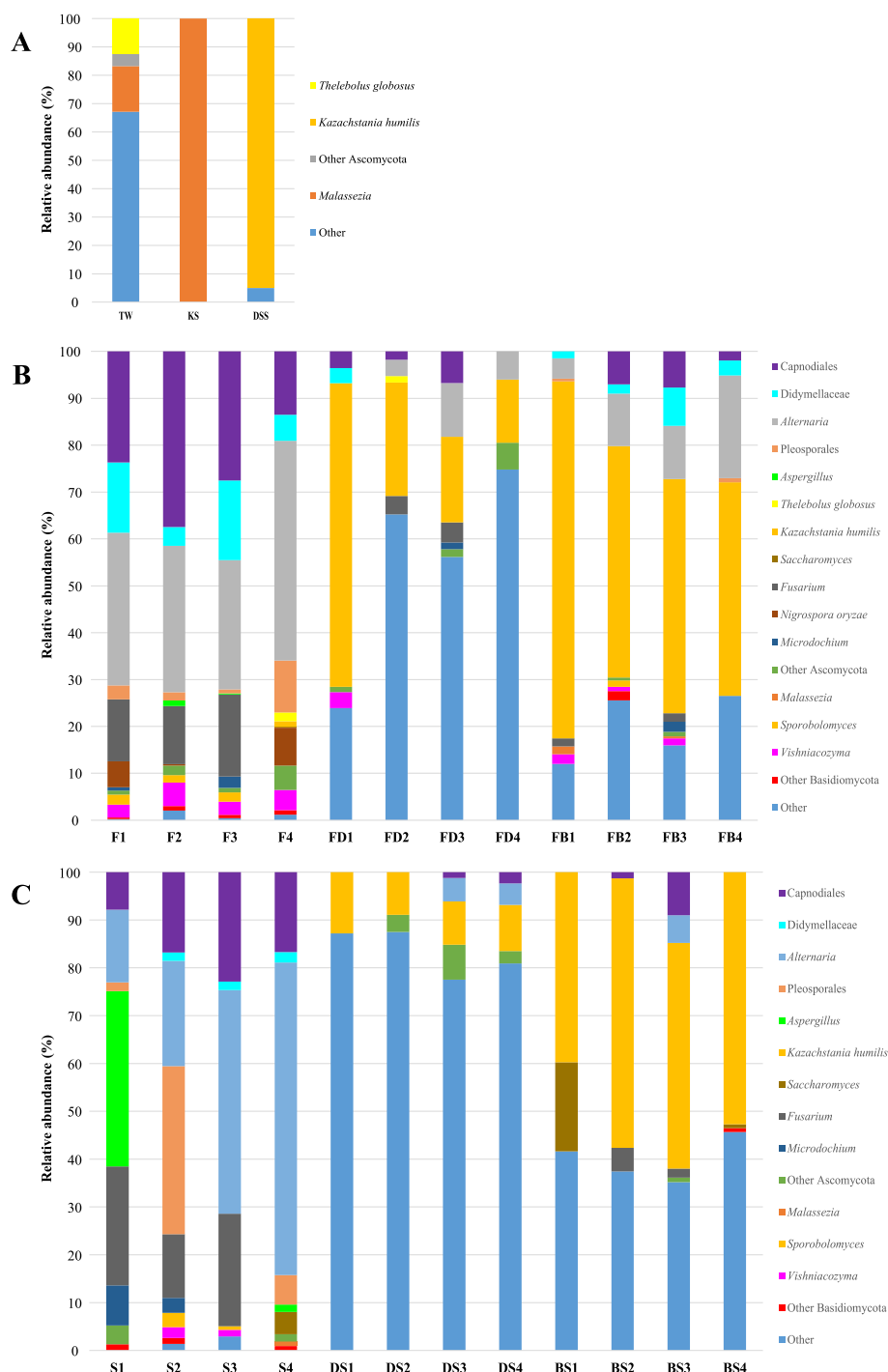
Fig. 3. Relative abundances% of the fungal operational taxonomy units (OTUs) identified by MiSeq Illumina. A: TW, tap water; KS, kitchen salt; DSS, dried sourdough starter. B: F1–F4, commercial flours; FD1–FD4, flour doughs; FB1–FB4, flour breads. C: S1–S4, commercial semolinas; SD1−SD4, semolina doughs; SB1–SB4, semolina breads.

and accounted for 77.62–97.88% in flours before processing. After 2 h of fermentation, RA% of Acetobacteraceae decreased to 55.46–67.96%, while *Enterococcus* were detected at 29.19–40.13%. Enterococci were the sole LAB present in doughs FD1, FD2 and FD4. Only FD3 showed the presence of unspeciated LAB other than *Enterococcus*, but at very low levels (2.02 RA%). The analysis of DNA extracted from breads showed that bacterial OTUs distribution remained almost unchanged after baking. However, consistent differences were detected for the production carried out with flour sample F2, because RA% of the OTUs allotted into Bacteroidetes, *Roseburia*, Clostridia, Oxalobacteraceae and

Achromobacter increased from dough (FD2) to bread (FB2), while that of *Enterococcus* and Acetobacteraceae decreased. An almost similar trend was registered when breads were produced from semolina in place of flour (Fig. 2C). Acetobacteraceae dominated the unprocessed durum wheat milling products (S1 − S4) and were found at higher RA% than *Enterococcus* in doughs and breads. The analysis of the bacterial composition of breads showed that bacterial DNA was still accessible after baking.

The DNA from raw materials and processed products was also analyzed for fungal composition (Fig. 3). Like for bacteria, kitchen salt

was characterized by 100% of a unique fungal genus *Malassezia* (Fig. 3A), which are lipid-dependent microorganisms inhabiting the skin and mucosa of humans and other warm-blooded animals (Theelen et al., 2018). This genus, together with *Thelebolus globosus* and other unidentified fungi, constituted the diversity of tap water, while dried sourdough starter was dominated by *Kazachstania humilis*. The last species, previously named *Candida humilis*, is a yeast commonly associated with sourdoughs (Palla et al., 2020) because it is typical of this ecological niche (De Vuyst, Harth, Van Kerrebroeck, & Leroy, 2016).

The fungal diversity of flours and semolinas (Fig. 3B and C) was higher than that revealed by bacteria. Except for Capnodiales, *Alternaria*, and *Fusarium* found in all unprocessed samples, the fungal composition of flours and semolinas differed for Didymellaceae, mainly associated with flours, Pleosporales found at high RA% (35.14) only in sample S2, and *Aspergillus*, accounting for 36.65% in sample S1. *Alternaria*, *Aspergillus*, and *Fusarium* are toxigenic moulds because of their mycotoxin generation (Medina et al., 2006) and are generally associated with cereals (Jedidi et al., 2018). Fermented doughs processed with dried sourdough starters from both flours and semolinas showed *K. humilis* as the dominating yeast. *Kazachstania humilis* DNA was highly accessible after baking; all breads were characterized by a RA% of this species in the range 45.55–76.17 in FB samples and 39.77–56.34 in SB samples.

Illumina technology was helpful in better interpreting data from plate counts in light of dominant bacterial and fungal groups. Although sourdough LAB and yeasts show different carbohydrate uptake kinetics (Gobbetti, 1998), the interaction between LAB and certain yeasts, such as *Kazachstania humilis*, is supposed to be commensal rather than competitive, unlike in the presence of *Saccharomyces cerevisiae* (Rogalski, Ehrmann, & Vogel, 2021). This might explain the co-evolution of LAB and yeasts in the doughs of the present study.

3.4. ARGs detection and amplicons sequencing

The search for ARGs was conducted on all samples representative of the different stages of sourdough bread production (Fig. 4). PCRs conducted to search for resistance genes to penicillins, chloramphenicol, and macrolides did not detect amplification products matching the chosen targets. However, the detection of amplicons corresponding to some of the analyzed targets for tetracyclines gave positive results. Indeed, the analysis of the sequences of the amplicons confirmed the presence of two *tet* genes [*tet*(A) and *tet*(B)] among the eight genes targeted. Specifically, the *tet*(A) gene was detected in semolina samples S3 and S4 and the resulting processed doughs. After baking, *tet*(A) gene was detected only in bread SB3. Regarding *tet*(B) gene, it was detected in semolina samples S1 and S4 and, after fermentation, in SD1 and SD4, but not after baking.

The genes *tet*(A) and *tet*(B) encode for efflux pumps that eject the antibiotic from the bacterial cell and are among the most prevalent ARGs (Gargano et al., 2021). Tetracyclines have been extensively used in the livestock sector for growth promotion and infection control in farms. Since antibiotics are not totally digested and processed in the intestines of animals, up to 90% of them are then excreted in the animal's urine and/or feces, which are often used as fertilizers, thereby contaminating soils and groundwater (Qing, Qigen, Jian, Hongjun, & Jingdu, 2022). Several studies have shown that animal organic fertilizers are a major vector of antibiotics, heavy metals, antibiotic resistance genes (ARGs), antibiotic-resistant bacteria (ARBs), and mobile genetic elements (MGEs) (Nõlvak et al., 2016). Therefore, the use of organic fertilizers of animal origin can significantly increase the level of antibiotic resistance in soil, as well as change its properties and bacterial community composition (Qing et al., 2022).

4. Conclusions

In this study, a metagenomic approach was applied to investigate the microbial communities involved in semolina or flour sourdough bread production and the transfer of ARGs from raw materials and baked products. The presence of ARGs toward macrolides, penicillins, chloramphenicol and tetracyclines was screened and the results showed that only two of the 17 target genes screened for, namely the *tet*(A) and *tet*(B) genes, were found in the analyzed samples. Although the agricultural sector does not undergo the same level of antibiotics pressure as the veterinary and animal production sector, soils can be considered a reservoir of ARGs when amended with organic fertilizers of animal origin. Wheat plants can acquire these genes through soil microbial communities. Even though only one bread was positive for barely one *tet* gene, this study demonstrated that raw materials used in bread making (flour and semolina) are able to transfer their ARGs to the final breads. Future works will be carried out to specifically investigate the presence of *tet*(A) and *tet*(B) resistance genes in bacteria and fungi isolated from
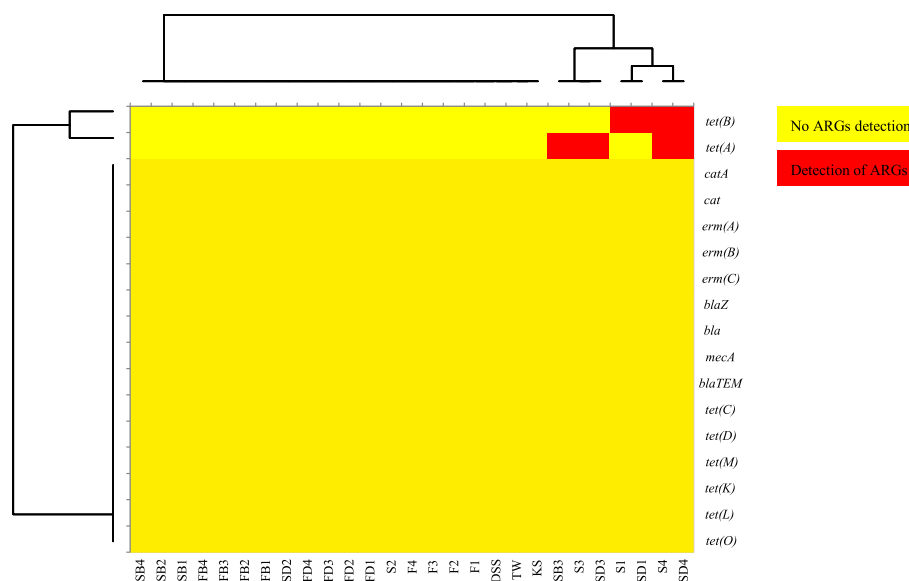

Fig. 4. Distribution of antibiotics resistance genes (ARGs) among samples. The heat map plot depicts the presence or absence of each ARGs. Abbreviations: TW, tap water; KS, kitchen salt; DSS, dried sourdough starter; F1–F4, commercial flours; FD1–FD4, flour doughs; FB1–FB4, flour breads; S1–S4, commercial semolinas; SD1−SD4, semolina doughs; SB1−SB4, semolina breads.

semolina samples S1, S3, and S4, whose DNAs were still accessible after the baking of the corresponding doughs (SD1, SD3, and SD4).

CRediT authorship contribution statement

Valeria Gargano: Methodology, Data curation. **Delia Gambino:** Methodology, Data curation. **Enrico Viola:** Methodology, Data curation. **Elena Franciosi:** Methodology, Formal analysis. **Antonio Alfonzo:** Software, Investigation. **Luca Cicero:** Funding acquisition. **Giovanni Cassata:** Supervision. **Luca Settanni:** Writing – review & editing. **Raimondo Gaglio:** Writing – original draft, Validation.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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