Temporal diversity and resistance of lactic acid bacterial community on durum wheat kernels during aging

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ABSTRACT

In the present work the dynamics of lactic acid bacteria (LAB) on wheat kernels were evaluated over time. The main aim of this research was to study the resistance of this bacterial group associated to unprocessed cereals used for bread making during long term conservation. To this purpose four *Triticum durum* Desf. genotypes including two modern varieties (Claudio and Simeto) and two Sicilian wheat landraces (Russello and Timilia) were analysed by a combined culture-independent and -dependent microbiological approach after one, two or three years from cultivation and threshing. DNA based Illumina technology was applied to reveal the entire bacterial composition of all semolina samples. The samples showed a different distribution of bacterial taxa per variety and time of storage. The groups mostly represented were *Stenotrophomonas*, *Pseudomonas*, *Erwinia*, *Delftia* and *Sphingomonas* genera, *Enterobacteriaceae* and *Oxalobacteriaceae* families, and Actinobacteria phylum. Among LAB, only *Enterococcus* genus was detected barely in a single sample (Simeto stored for one year) by the next generation sequencing, indicating that LAB remained unassigned or their abundances were below 0.1% or their DNAs were rendered inaccessible. Plate counts showed consistent differences in relation to genotypes and duration of storage, with the highest levels found for total mesophilic microorganisms detected until 6.81 Log CFU/g. Colonies of presumptive sourdough LAB were detected only in a few samples. Cocci constituted the major group of LAB in almost all samples. Following the enrichment procedure, almost all samples were characterised by the presence of acidifying microorganisms. All isolates collected before and after enrichment represented 28 different strains belonging to 10 species of *Enterococcus*, *Lactobacillus* and *Pediococcus* genera. The most resistant species during aging were *Enterococcus faecium*, *Enterococcus durans*, *Lactobacillus brevis*, *Lactobacillus pentosus* and *Lactobacillus paracasei* demonstrating that lactobacilli and enterococci are able to overcome the stressing conditions represented by cereal storage better than other LAB genera commonly found associated to cereals after harvest.

Key words: Bacterial persistence; Lactic acid bacteria; MiSeq Illumina; Sourdough; Durum wheat; Wheat kernels

1. Introduction

Wheat is strictly related to the history of mankind since the Stone Age (Gifford and Baer-Sinnot, 2007). Durum and bread wheat (*Triticum durum* Desf. and *Triticum aestivum* L., respectively), whose milling products are namely semolina and flour, respectively (Alfonzo *et al.*, 2016), are consumed almost worldwide, mainly for the production of pasta or leavened products (Alfonzo *et al.*, 2017). Durum wheat semolina is commonly used for pasta production, but in southern Italy it is also used for the production of bread (Corona *et al.*, 2016; Pasqualone *et al.*, 2004; Rizzello *et al.*, 2015). Furthermore, in the last years there has been a rediscovery of the ancient durum wheat genotypes not only to diversify the agricultural productions and to expand the portfolio of pasta and baked products (Alfonzo *et al.*, 2017; Coda *et al.*, 2014), but also for dietary purposes (Shewry *et al.*, 2010). Regarding breads, historically their making was performed thanks to the sourdough technology (Corsetti and Settanni, 2007), a practise still applied today to produce traditional products, especially breads (Sakandar *et al.*, 2019).

Sourdough is a very complex environment originated from the fermentation of flour/semolina with water where the indigenous lactic acid bacteria (LAB) and yeasts constitute the main populations (Corsetti and Settanni, 2007; De Vuyst and Vancanneyt, 2007; Vogel *et al.*, 1999). Although mature sourdoughs are characterised by an almost stable LAB/yeasts community (Minervini *et al.*, 2014) the ratio between species and strains may change over time due to the continuous addition of flour/semolina. As a matter of fact, raw materials (flours and/or semolina) used in bread making cannot be thermally treated before addition and their living microorganisms are transferred to the sourdough during refreshments (Alfonzo *et al.*, 2013,2017) and, together with those present on the milling equipment (Berghofer *et al.*, 2003), might modify its microbiological composition.

Although the microbial composition of raw materials used in sourdough bread making has not been well characterised as that of mature sourdoughs of the different typologies (Type I, II and III sourdough), in the last years some information have been acquired, especially on LAB populations (Alfonzo *et al.*, 2013, 2017; Mamhoud *et al.*, 2016; Minervini *et al.*, 2015; Pontonio *et al.*, 2015). All these works evidenced a quite constant presence of several LAB species of marginal technological interest in sourdough propagation, while the typical species with relevance during a successful fermentation, such as *Lb. sanfranciscensis* or other obligate heterofermentative lactobacilli were found at low frequencies. A few works focussed on the characterization of LAB from wheat kernels in order to retrieve the primary source of those hosted in flour/semolina (Alfonzo *et al.*, 2017; Corsetti *et al.*, 2007a; Minervini *et al.*, 2015). To this purpose, Minervini *et al.* (2015) demonstrated that the cultivation (soil) environment represents a source for certain species, such as *L. plantarum*, even though some species and strains of LAB can be transferred by the seeds (Alfonzo *et al.*, 2017).

The practise of aging flour is applied to cause changes that improve its suitability to produce baked goods such as breads and cakes (Seguchi, 1993). The chemical composition and the quality status of the flour determine the microbial community dynamics during the sourdough fermentation processes (Harth, 2016) and the variations registered using different flour batches, in terms of dominating LAB and yeasts, might reflect the different age of the flours (Harth *et al.*, 2018). Due to the lack of information on the microbiological composition of aged raw materials used to produce bread, in this work the kernels belonging to four different durum wheat varieties, including old and modern genotypes, aged for one, two and three years were aseptically milled and the resulting semolina characterized for the levels, composition and distribution of LAB by a multiphasic combined culture-independent and –dependent approach.

2. Materials and methods

2.1. Wheat and milling

Wheat kernels object of this study (Table 1) belonged to two modern (Claudio and Simeto) and two Sicilian landraces (Russello and Timilia) *T. durum* Desf. genotypes. All varieties were cultivated in adjacent experimental fields (6 m × 50 m) in a single farm (Pietranera) located at Santo Stefano Quisquina (Agrigento province, Italy) during three consecutive production years. A total of 12 kernel masses (three production years × four varieties) were sampled. After threshing, each mass of wheat kernels was put into 5 kg plastic bags and stored in a warehouse at room temperature for 12 months. After that, wheat kernels were stored under refrigeration at 4°C and 90/95% relative humidity. Following this storage protocol, the kernels from the three years old production (aged for three years, Ag3Y) were kept refrigerated for two years, those from the two years old production (aged for two years, Ag2Y) only for one year, while the kernels from the one year old production (aged for one year, Ag1Y) were not subjected to any refrigeration step. All kernel samples (2 kg for each genotype and production year) were transferred into sterile plastic bags and transported under refrigeration by means of insulated boxes containing reusable ice packs to the Agricultural Laboratory of University of Palermo. Milling was performed aseptically as reported by Alfonzo *et al.* (2017) under a laminar flow hood and sanitizing the grid (1 mm) and the rotor of the Retsch centrifugal Mill ZM1 (Haan, Germany) through a step in 96% (v/v) ethanol for 10 min followed by 30 min UV treatment. The sanitizing procedure was applied for each storage year/genotype sample (400 g of kernels).

2.2. Preparation of the MiSeq library

A 464-nucleotide sequence of the bacterial V3-V4 region (Baker *et al.*, 2003) of the 16S rRNA gene (*Escherichia coli* positions 341 to 805) was amplified. Unique barcodes were attached before the forward primers to facilitate the pooling and subsequent differentiation of samples. To prevent preferential sequencing of smallest amplicons, the amplicons were cleaned using the Agencourt AMPure kit (Beckman coulter) according to manufacturer's instructions. The DNA concentration of amplicons was determined using the Quant-iT PicoGreen dsDNA kit (Invitrogen) following the

manufacturer's instructions. In order to ensure the absence of primer dimers and to assay the purity, the generated amplicon libraries quality was evaluated by a Bioanalyzer 2100 (Agilent, Palo Alto, CA, USA) using the High Sensitivity DNA Kit (Agilent). Following quantitation, the cleaned amplicons were mixed and combined in equimolar ratios. Library building and pair-end sequencing were carried out at the Genomic Platform – Fondazione Edmund Mach (San Michele a/Adige, Trento, Italy) using the Illumina MiSeq system (Illumina, USA).

2.3. Illumina data analysis and sequences identification by QIIME2

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). Sequences were quality filtered, trimmed, de-noised, and merged using DADA2 (Callahan *et al.*, 2016). Chimeric sequences were identified and removed via the consensus method in DADA2. Representative sequences were aligned with MAFFT and used for phylogenetic reconstruction in FastTree using plugins alignment and phylogeny (Katoh and Standley, 2013; Price *et al.*, 2009). 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 97% Operational Taxonomic Units (OTUs) database (http://greengenes.secondgenome.com/), which had been previously trimmed to the V4 region of 16S rDNA, bound by the 341F/805R primer pair, was applied to paired-end sequence reads to generate taxonomy tables.

The data generated by Illumina sequencing were deposited in the NCBI Sequence Read Archive (SRA) and are available under Ac. PRJNA548083

2.4. Classical microbiological analyses

All whole-meal semolina samples (15 g) were homogenized by the stomacher BagMixer® 400 (Interscience, Saint Nom, France) at the maximum speed for 2 min in Ringer's (Sigma-Aldrich,

143 Milan, Italy) solution (135 ml) and then subjected to the serial decimal dilution. Basically, the
144 microbial groups investigated belonged to the sourdough technological relevant populations and to
145 the spoilage groups. Plate count agar (PCA), incubated at 30°C for 72 h, was used for the total
146 mesophilic microorganisms (TMM). de Man-Rogosa-Sharpe (MRS) agar and M17 agar, both
147 incubated at 30°C for 48 h in anaerobiosis using the AnaeroGen AN25 (Oxoid, Milan, Italy) in jars
148 closed hermetically, for generic food LAB rods and cocci, respectively. Sourdough LAB were
149 specifically investigated on Sour Dough Bacteria (SDB) (Kline and Sugihara, 1971) agar and San
150 Francisco Medium (SFM) (Vogel *et al.*, 1994) agar incubated aerobically at 30°C for 48 h. Yeasts
151 were grown on yeast peptone dextrose (YPD) agar incubated at 28°C for 48 h. Moulds were
152 detected on malt agar (MA) after incubation at 25°C for 7 days. Members of the
153 *Enterobacteriaceae* family were plated on violet red bile glucose agar (VRBGA) incubated
154 aerobically for 24 h at 37 °C. MRS, M17, SDB and SFM were supplemented with tetracycline
155 hydrochloride (50 μg/mL) to inhibit bacterial growth. All media and supplements were purchased
156 from Oxoid (Milan, Italy). All plate counts were carried out in duplicate.
157 All samples were also subjected to the enrichment procedure carried out in MRS, SDB and SFM.
158 Ten grams of semolina for each year/variety were inoculated in 50 ml of broth medium and
159 incubated at 30°C for 96 h. After that, the pH was measured with the laboratory pH-meter BASIC
160 20+ (Crison Instrument S.A., Barcelona, Spain).
161
162 *2.5. Isolation, phenotypic characterization and genetic identification of semolina LAB*
163 Presumptive LAB, as being Gram-positive (Gregersen KOH method) and catalase-negative (unable
164 to catalyse 5% H_2O_2 to H_2O), were randomly picked up from the highest plated dilutions of
165 semolina suspensions on MRS, M17, SDB and SFM agar considering all different colony types
166 (colour, morphology, edge, surface and elevation). The isolates were purified by successive sub-
167 culturing by streaking on the same media used for plate counts, transferred to the corresponding
168 broth media containing 20% glycerol (v/v) and stored at −80 °C until further characterization.

169 The isolates were phenotypically investigated by observing their cell morphology through an optical
170 microscope, by determining their growth at 15 and 45 °C, and their metabolic characteristics such
171 as CO_2 production from glucose, carried out in Durham's tubes with the optimal growth media that
172 did not contain citrate, acid production from different sources (arabinose, ribose, xylose, fructose,
173 galactose, lactose, sucrose and glycerol), NH_3 production from arginine (Abd-el-Malek and Gibson,
174 1948), and aesculine hydrolysis (Qadri *et al.*, 1980). LAB cocci were also evaluated for their ability
175 to grow in presence of 0.65 % (w/v) NaCl and at pH 9.2 to directly identify enterococci, showing
176 growth in both conditions.
177 Genomic DNA from whole-meal semolina LAB was extracted after overnight growth in the optimal
178 media using the Instagene Matrix kit (Bio-Rad, Hercules, CA) following manufacturer's
179 instructions and used for differentiation of the isolates at strain level as well as for the genetic
180 identification.
181 Strain typing was approached by randomly amplified polymorphic DNA (RAPD)-PCR analysis as
182 described by Gaglio *et al.* (2017) using singly the primers AB111, AB106 and M13 (Stenlid *et al.*,
183 1994; van den Braak *et al.*, 2000). Electrophoresis on 2% (w/v) agarose gels (Gibco BRL, Cergy
184 Pontoise, France) was performed to separate DNA amplicons which were visualised, after staining
185 with the SYBR® safe DNA gel stain (Molecular probes, Eugene, OR, USA), by an UV trans-
186 illuminator. RAPD profiles were analysed through Gelcompare II software version 6.5 (Applied-
187 Maths, Sint-Marten-Latem, Belgium) and the isolates showing different patterns were considered to
188 represent different strains.
189 All different LAB strains were identified genetically by sequencing of the 16S rRNA gene and
190 comparison of the sequences in public databases (GenBank and EZ-taxon) by BLAST search. PCR
191 reactions were carried out following the protocol described by Weisburg *et al.* (1991) with the
192 primer pair fD1 (5′-AGAGTTTGATCCTGGCTCAG-3′)/rD1 (5′-AAGGAGGTGATCCAGCC-3′).
193 After confirming the molecular size of the amplicons (about 1600 bp) on agarose gels, the PCR
194 products were purified using the QIAquick purification kit (Quiagen S.p.a., Milan, Italy) and

sequenced using the same primers used for PCR amplification at AGRIVET (University of Palermo, Italy).

2.6. Statistical analyses

Statistical analyses of microbiological counts were conducted using STATISTICA software (StatSoft Inc., Tulsa, OK, USA). Microbial data were converted to the Log scale before statistical elaborations. Differences between means were determined by the post-hoc Tukey's multiple-range test. A $P < 0.05$ was deemed significant.

3. Results

3.1. Characteristics of the Illumina data and taxonomic analysis of the bacterial community

Illumina technology was applied to reveal the entire bacterial composition of all semolina samples from the aged grains, including also the dormant and/or viable but not cultivable bacterial community. The total DNA extracted from the 12 samples was always successful in amplification of the bacterial V3-V4 16S rRNA gene region. After splitting quality trimming of raw data, 588,192 reads were further analysed.

The samples showed a different distribution of bacterial taxa per genotype (Fig. 1). The Sicilian wheat landraces were characterised by 10 and nine groups for Russello and Timilia old genotype, respectively. The semolinas from these two durum wheat genotypes shared the presence of *Stenotrophomonas*, *Pseudomonas*, *Erwinia*, *Delftia* and *Sphingomonas* genera, *Enterobacteriaceae* and *Oxalobacteriaceae* families, and members of *Actinobacteria* phylum; in addition to these groups, Timilia genotype semolinas also showed the presence of *Serratia* genus, while *Paenibacillus* and *Chryseobacterium* genera were detected in Russello genotype semolinas. The two modern varieties showed a quite different distribution of bacterial taxa, because only seven groups were identified for Claudio variety and 12 for Simeto variety semolinas. These two varieties shared the presence of *Pseudomonas*, *Erwinia*, *Delftia*, *Sphingomonas* and members of

221 *Oxalobacteriaceae* family and *Actinobacteria* phylum. Claudio variety semolinas showed in
222 addition to the previous groups also *Lysinobacillus*, while Simeto variety semolinas *Serratia*,
223 *Paenibacillus*, *Chryseobacterium*, *Enterobacteriaceae* and *Enterococcus* which is the unique genus
224 ascribable to the groups of LAB detected by the culture independent approach.
225 A different temporal distribution of bacterial taxa among semolina samples was also found for the
226 three aging periods analysed. *Erwinia* percentage diminished with time in Russello genotype, but
227 increased for Timilia genotype and showed the highest proportion at the second year of storage
228 (Ag2Y) for both modern varieties. *Delftia*, and members of *Oxalobacteriaceae* and
229 *Enterobacteriaceae* increased constantly for Russello semolinas, but this trend was confirmed only
230 for *Delftia* in Timilia and Claudio genotypes. *Serratia* constituted 33.48% of total bacterial
231 community of Simeto Ag1Y, but was not found in the samples subjected to further aging. Simeto
232 Ag1Y was the only sample to present OTUs allotted into a genus (*Enterococcus*) belonging to the
233 group of LAB and its percentage was quite low (5.65%). Regarding LAB species detected after the
234 isolation procedure, but not revealed by Illumina, they were probably part of the unassigned OTUs
235 ranging between 0.60 (Russello Ag3Y) and 17.22% (Claudio Ag1Y) of total bacterial community
236 or they were below 0.1% of abundance or their DNAs were rendered inaccessible by nucleases.
237
238 *3.2. Microbiological counts*
239 The samples of durum wheat semolinas showed consistent differences in terms of cell densities of
240 all microbial groups object of investigation (Table 2) in relation to the varieties and the aging
241 duration. For all samples, the highest cell counts were displayed by TMM ranging between 3.95 and
242 6.81 Log CFU/g. Except AT, for the other varieties the samples characterized by the lowest TMM
243 numbers were those of Ag2Y. When aging occurred for two years, all samples were characterized
244 by the complete absence of sourdough LAB (below the detection limit on SDB and SFM) as well as
245 LAB rods (on MRS). Regarding the last group, it was undetectable also for all Ag3Y semolina
246 samples. Bacteria able to develop colonies on the media generally used for the growth of the typical

247 sourdough LAB were mainly detected in Ag3Y (only on SDB) and Ag1Y (only on SFM) samples.
248 The modern variety Claudio was characterised by undetectable levels of these bacteria. Regarding
249 LAB cocci (on M17) all samples showed consistent levels, except the old genotype Timilia for the
250 sample Ag2Y. Yeasts and moulds were detected in all samples at levels of 10^3 – 10^4 CFU/g.
251 Members of the *Enterobacteriaceae* family were below the detection limit in Claudio samples,
252 present at almost constant levels in Simeto samples, while their levels decreased with aging in both
253 ancient varieties.
254 The results of plate counts clearly indicated that rod LAB and those presumptively of technological
255 relevance for sourdough production did not represent the dominating populations of aged durum
256 wheat kernels. From the technological application perspective, LAB to be used in sourdough
257 propagation have to be rapid acidifiers (Ravyts and De Vuyst, 2011). For this reason, all semolina
258 samples were subjected to the enrichment procedure to check for the acidification of the broth
259 media (Table 2). Consistent levels of acidification were found on all three media used to this scope
260 (MRS, SDB and SFM) only for the samples Ag2Y and Ag1Y of the variety Simeto and for both the
261 old genotypes at one year of aging. In general, almost all samples were characterized by a value of
262 pH lower than that registered at the moment of inoculation, indicating the presence of acidifying
263 microorganisms in semolinas. Only one sample (Simeto Ag3Y) determined the increase of the pH
264 of the enrichment broth (SFM) from 5.40 to 5.96. No correlation was found between LAB count
265 levels and pH of enrichment broths, since almost all samples were able to acidify the broths
266 independently on the cell densities detected on MRS, SDB and SFM.
267
268 *3.3. Phenotyping grouping and genetic identification of LAB*
269 All colonies showing different appearance on the media used for LAB (MRS, M17, SDB and SFM)
270 counts were isolated from the plates inoculated with the most diluted cell suspensions of semolinas
271 and purified to cell homogeneity. The isolation occurred also from the enriched broths after
272 streaking on the corresponding agar media.

Gram type and catalase test indicated that only 139 isolates from semolinas before enrichment and barely 28 from the enriched broths could be still considered as presumptive LAB. After microscopic inspection, coccus was the main cell morphology found and, regarding the spatial disposition of the cells, the following groups were recognized: cocci in pairs or short chains; and tetrads. All rods were organized into single cells or short chains. The preliminary characterization based on the evaluation of the physiological and biochemical characteristics grouped all cultures into five groups, three for rods and two for cocci (Table 3).

All presumptive LAB were processed by RAPD-PCR analysis to differentiate them at strain level. Two or more bacterial isolates were considered to represent the same strain when the RAPD patterns were identical (Charlton *et al.*, 1999). The comparison of the RAPD profiles obtained with three different primers allowed to identify a total of 28 strains (Fig. 2) from the total of 167 LAB collected which were subsequently subjected to 16S rRNA gene sequencing. The dendrogram clearly showed that all strains grouped per species, even though strains of the same genus were quite different from one another. For example *E. casseliflavus* grouped with pediococci and *L. graminis* rather than other enterococci, while *L. brevis*, *L. paracasei* and *L. pentosus* were quite distant from *L. graminis*. Furthermore, *P. pentosaceus* were found into two subgroups.

The identification process showed that the LAB isolated from aged durum wheat kernels was composed of 10 species allotted into *Enterococcus* (*E. casseliflavus* Ac. No. MN166293 – MN166296, *E. durans* Ac. No. MN166297 – MN166299, *E. faecalis* Ac. No. MN166300 and *faecium* Ac. No. MN166301 – MN166305), *Lactobacillus* (*L. brevis* Ac. No. MN166306, *L. graminis* Ac. No. MN166307 – MN166309, *L. paracasei* Ac. No. MN166310 and *L. pentosus* Ac. No. MN166311), and *Pediococcus* (*P. acidilactici* Ac. No. MN166312 and *P. pentosaceus* Ac. No. MN166313 – MN166320) genera.

3.4. LAB distribution

298 Figure 2 also allows to retrieve the distribution of the LAB strains identified among the kernel (in
299 form of semolina) samples analysed. Basically, only members of *Enterococcus*, *Pediococcus* and
300 *Lactobacillus* were found associated to the aged kernels. In particular, the most aged kernels
301 (Ag3Y) were found to host *L. brevis* (modern variety Claudio), *L. pentosus* (modern variety
302 Claudio), *L. paracasei* (old genotype Russello), *E. durans* (both old genotypes and modern variety
303 Claudio) and *E. faecium* (both old genotypes and modern variety Simeto). *E. casseliflavus* was
304 detected until two years of aging (old genotype Russello and modern variety Claudio), while *E.*
305 *faecalis* only after one year (modern variety Claudio). Pediococci were only isolated from old
306 genotypes and mostly after one year of aging even though *P. pentosaceus* and *P. acidilactici* were
307 collected after two years from Russello and Timilia genotype kernels, respectively. Three different
308 strains of *L. graminis* were isolated from a single variety (modern variety Simeto) after one and two
309 years of aging.
310 The majority of the strains were collected after the enrichment procedure, showing their ability to
311 overcome the growth of other LAB, when and if they were present. In fact, *E. casseliflavus* strains
312 S50, S52 and S76 were isolated from semolinas (AR Ag1Y and Ag2Y and AT Ag1Y) prior
313 enrichment, but they were overcome by *P. pentosaceus* S163, S164, S165 and S141 and *E. faecium*
314 S156 and S157. Furthermore, it is worth noting that the same strains, specifically *L. brevis* S154, *P.*
315 *pentosaceus* S165 and *E. casseliflavus* S52 were found associated to a given genotype (S154 in
316 modern variety Claudio and S165 and S52 in old genotype Russello) over time and that some
317 strains, such as *E. durans* S161 and *E. casseliflavus* S76 were found associated to different
318 genotypes.
319
320 **4. Discussion**
321 The factors affecting the stability of a microbial community in durum wheat semolina include plant
322 growth conditions (Minervini *et al.*, 2015), genotypes, geographical area of production as well as
323 the microbial contamination of planted seeds (Alfonzo *et al.*, 2017). In general, wheat kernels and

semolina/flour microbial community composition might play a key role in the dynamics of sourdough ecosystem processes (Corsetti *et al.*, 2007a,b), although several other factors such as bakery environment (Minervini *et al.*, 2012), propagation conditions (Gänzle and Ripari, 2016) and even the water used during dough mixing (Minervini *et al.*, 2019) might exert defining influences on the final microbial community of mature sourdoughs.

The need to produce cereal-based products all year round means that grains such as wheat can be stored for some time before processing (Arumugam, 2012). During prolonged storage, there is a progressive reduction in the rate of germination and seedling growth, before seed death takes place (Bernal-Lugo *et al.*, 1999). The aging process causes changes that improves the characteristics of the wheat kernels and, hence, enhances their utilization; age- related changes have great influence on viscosity of the batter and the water-binding ability of the wheat flour (Arumugam, 2012). Also, the starch granule surface protein is found to be increased up to three to four times after various aging treatments compared to the control wheat flour (Shelke *et al.*, 1992). The practice of aging flour is applied to cause changes that improve its suitability to produce baked goods such as breads and cakes (Seguchi, 1993). Thus, due to the relevance of the indigenous microbial community of wheat kernels and, consequently, of the resulting flour/semolina for producing baked goods and to the lack of knowledge on the microbiological composition of aged grains, this work was aimed to evaluate the resistance of the LAB of modern and old genotypes of durum wheat during a prolonged storage (until three years) and to evaluate their dynamics during aging. Recently, LAB composition of kernels and semolinas from different durum wheat genotypes has been studied and unrevealed at the end of the cultivation period (Alfonzo *et al.*, 2017; Minervini *et al.*, 2015), but, so far, no work has been focused on their resistance over time, where resistance is intended as the degree to which microbial composition remains unchanged in the face of a disturbance (Allison *et al.*, 2008).

In this work, the four genotypes investigated for their microbial content and composition belonged to two old genotypes (Russello and Timilia) and two modern varieties (Claudio and Simeto). They were chosen because are commonly cultivated in Sicily and also because they are different for plant

growth habit, efficient use of resources, yield and quality grain (Giambalvo et al., 2010; Ruisi et al., 2015; Vita et al., 2016), so that they represented different sources for the evaluation of LAB resistance over time. The approach followed in this study was the same described by Alfonzo *et al.* (2017) who applied a combined culture-dependent and –independent strategy to retrieve the origin of LAB in sourdoughs produced from durum semolinas. However, contrarily to that work, the next generation sequencing (NGS) methodology, performed by Illumina technology, was applied prior to the cultural tools.

NGS tools reveal the presence of microorganisms also when they are in a dormant and/or viable but not cultivable state, but identifies even the dead cells as long as the DNAs remain accessible. Analysing the total bacterial diversity through amplification of the V3-V4 16S rRNA gene region, the samples showed a different distribution of bacterial taxa per variety and aging duration. Considering that the plants were grown in adjacent fields of the same area, the differences could be attributed to a variety-specific interaction of certain bacterial groups. In particular, the groups ubiquitous among the samples were *Stenotrophomonas*, *Pseudomonas*, *Erwinia*, *Delftia* and *Sphingomonas* genera, *Enterobacteriaceae* and *Oxalobacteriaceae* families, and members of *Actinobacteria* phylum, while *Serratia* genus was associated to Timilia and Simeto, and *Paenibacillus* to Russello and Simeto semolinas. The lowest biodiversity was displayed by the modern variety Claudio. This variety was only characterised by *Erwinia* among the members of *Enterobacteriaceae* family. The group of *Enterobacteriaceae* that includes besides *Erwinia* also *Serratia* among the taxa identified at genus level constituted the major bacterial group in almost all samples analysed. *Enterobacteriaceae* were also detected after pyrosequencing by Ercolini *et al.* (2013) in rye and tender wheat flour and durum semolina. Alfonzo *et al.* (2017), applying MySeq Illumina, detected high percentages of *Enterobacter* and *Erwinia* in doughs prepared from semolinas of the modern genotypes Duilio, Iride, Saragolla and Simeto milled ca. three months after harvesting and mixed individually with sterile water, stating that before acidification takes place *Enterobacteriaceae* are not inhibited.

In the present study, a different distribution of bacterial taxa among semolina samples was also found for the three aging years analysed and, from the application point of view, LAB were only detected in Simeto semolinas obtained from kernels aged for one year only that showed the presence of *Enterococcus* at 5.65%. These results indicated that LAB species could be absent in the other samples, or their OTUs remained unassigned, or simply that their presence was below 0.1% of abundance. Looking at the results of Alfonzo *et al.* (2017), semolinas from kernels not subjected to long storage were characterised by abundances above 0.1% of the genera *Enterococcus*, *Lactococcus* and members of the *Leuconostocaceae* family, while lactobacilli were detected at extremely low percentages. The work of Ercolini *et al.* (2013) reported that the LAB community of durum wheat doughs, at the first fermentation step, ascertained exclusively by a culture-independent approach, was composed of *Lactococcus*, *Lactobacillus*, *Leuconostoc* and *Weissella*, but no information on the storage of the samples analysed was provided. Thus, the storage determined a strong reduction of LAB biodiversity and this group was barely represented by *Enterococcus* only in one sample stored for one year.

However, even though DNA based culture-independent approaches are being used alone to study the microbial, especially bacterial, diversity of food matrices and raw materials used in fermented food processing (Marino *et al.*, 2019; Stellato *et al.*, 2015) they do not provide any information about the viability of the different taxa identified. Thus, plate counts were performed to evaluate the potential of growth of aged wheat kernel LAB by developing colonies on agar media. To this purpose, PCA was used to estimate the levels of all microorganisms present, while the viable LAB were cultivated on MRS and M17 that are generally used for LAB (Liu *et al.*, 2005) and SDB and SFM that are employed to isolate sourdough LAB (Vera *et al.*, 2009). In addition, members of *Enterobacteriaceae* family were also investigated, since they are commonly associated to flours and found during the first stages of durum wheat sourdough propagation (Ercolini *et al.*, 2013), even though they are not generally found to persist during the propagation of mature sourdoughs (Siepmann *et al.*, 2018).

The highest levels of cell densities were found for TMM, followed by LAB cocci, yeasts and moulds and members of *Enterobacteriaceae* family. For the majority of samples, LAB rods and sourdough LAB were below the detection limit. However, after enrichment in MRS, SDB and SFM broths, several samples showed a clear acidification, indicating the possible presence of LAB. For this reason, the colonies obtained after streaking were subjected to investigation at species and strain level resulting in the identification of *E. faecium*, *E. durans*, *L. brevis*, *L. pentosus* and *L. paracasei* in the most aged kernel samples (Ag3Y) of all four wheat genotypes (at least one species per variety), indicating a high resistance of these species over time.

From a deepen look at the distribution of the strains, it was found out that some strains persisted on a given wheat genotype over time showing a strict variety relation. On the contrary, some other strains were found on different wheat varieties. These results might be due to the fact that the four wheat genotypes were cultivated in adjacent fields, thus, the geographical area is quite restricted. Similar conclusions were draft by Alfonzo *et al.* (2017).

In conclusion, an approach combining culture-dependent and –independent methods is important to deeply analyse the entire bacterial biodiversity of raw materials subjected to stressing conditions. Briefly, this work did not evidence the presence of LAB in 11 samples out of 12 by NGS technology and this group was also undetectable on several media when plate count method was applied while only the enrichment procedure revealed their presence. These results clearly showed that the numbers of LAB on kernels decrease over time, but some members of *Lactobacillus* and *Enterococcus* showed a certain resistance in the conditions applied during storage. In comparison to previous works carried out on semolinas from kernels not subjected to long storage and analysed within three months from harvest, the biodiversity of aged kernels was strongly reduced, since no strain belonging to *Lactococcus* and *Leuconostoc* was isolated. However, also in the work of Alfonzo *et al.* (2017) lactobacilli were below 0.1% abundance after Illumina analysis but present in a viable state on durum wheat kernels, since after enrichment *Lactobacillus coryniformis* and *L.*

brevis were isolated. These findings undoubtedly demonstrate that even at very low levels, *Lactobacillus* together with *Enterococcus* show the highest degree of resistance over time.

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561 **Table 1.** Wheat genotypes used for the study of lactic acid bacteria evolution over time.

562

Genotypes		Year of release	Group	Plant stature	Heading time	Pedigree
1	Russello	before 1915	Old	Tall	Late	Indigenous landrace from Sicily
2	Timilia	before 1915	Old	Tall	Early	Indigenous landrace from Sicily
3	Simeto	1988	Modern	Short	Early	Capeiti8/Valnova
4	Claudio	1998	Modern	Short-mid	Early-mid	CIMMYT'sselection35/Durango//IS1938/Grazia

Table 2. Characteristics of whole-meal semolinas.

T. durum genotypes	Aging (years)	Microbial loads (Log CFU/g)								pH after enrichment		
		PCA	MRS	M17	SDB	SFM	YPD	MA	VRBGA	MRS	SDB	SFM
Claudio	3	5.7 ± 0.3^{ab}	<1^{a}	4.4 ± 0.3^{b}	<2^{a}	<2^{a}	3.5 ± 0.3^{b}	3.7 ± 0.2^{a}	<1^{a}	4.8 ± 0.1^{b}	4.4 ± 0.2^{b}	4.1 ± 0.1^{a}
	2	5.4 ± 0.2^{b}	<1^{a}	5.0 ± 0.2^{ab}	<2^{a}	<2^{a}	4.2 ± 0.2^{ab}	4.1 ± 0.2^{a}	<1^{a}	5.0 ± 0.2^{b}	5.3 ± 0.1^{a}	4.9 ± 0.4^{a}
	1	6.3 ± 0.2^{a}	<1^{a}	5.3 ± 0.2^{a}	<2^{a}	<2^{a}	4.9 ± 0.5^{a}	4.2 ± 0.3^{a}	<1^{a}	5.7 ± 0.1^{a}	4.7 ± 0.4^{ab}	4.2 ± 0.2^{a}
Statistical significance[a]		*	NS	*	NS	NS	**	NS	NS	**	*	NS
Simeto	3	6.4 ± 0.4^{ab}	<1^{b}	5.6 ± 0.3^{ab}	4.8 ± 0.2^{a}	<2^{b}	4.2 ± 0.1^{a}	4.4 ± 0.1^{a}	2.7 ± 0.3^{a}	4.9 ± 0.4^{a}	4.8 ± 0.3^{a}	6.0 ± 0.3^{a}
	2	5.8 ± 0.1^{b}	<1^{b}	4.8 ± 0.2^{b}	<2^{b}	<2^{b}	4.3 ± 0.1^{a}	4.0 ± 0.4^{a}	2.6 ± 0.2^{a}	4.1 ± 0.3^{a}	3.9 ± 0.2^{b}	4.1 ± 0.2^{b}
	1	6.8 ± 0.3^{a}	1.1 ± 0.1^{a}	6.2 ± 0.3^{a}	<2^{b}	2.0 ± 0.1^{a}	4.8 ± 0.4^{a}	4.5 ± 0.3^{a}	2.6 ± 0.1^{a}	4.4 ± 0.1^{a}	3.9 ± 0.3^{b}	4.1 ± 0.2^{b}
Statistical significance[a]		*	***	**	***	***	NS	NS	NS	NS	*	**
Russello	3	5.8 ± 0.5^{a}	<1^{b}	5.5 ± 0.5^{a}	4.8 ± 0.3^{a}	<2^{b}	3.9 ± 0.4^{a}	3.7 ± 0.2^{b}	3.9 ± 0.1^{a}	4.8 ± 0.1^{a}	4.4 ± 0.5^{a}	4.3 ± 0.2^{ab}
	2	4.9 ± 0.2^{a}	<1^{b}	4.0 ± 0.5^{a}	<2^{b}	<2^{b}	3.9 ± 0.3^{a}	3.3 ± 0.3^{b}	1.5 ± 0.1^{b}	5.1 ± 0.2^{a}	4.0 ± 0.2^{a}	5.0 ± 0.3^{a}
	1	5.1 ± 0.1^{a}	2.0 ± 0.3^{a}	3.9 ± 0.4^{a}	<2^{b}	2.0 ± 0.1^{a}	4.1 ± 0.2^{a}	4.5 ± 0.2^{a}	1.3 ± 0.2^{b}	4.1 ± 0.1^{b}	3.8 ± 0.3^{a}	4.0 ± 0.2^{b}
Statistical significance[a]		NS	***	NS	***	***	NS	**	***	**	NS	*
Timilia	3	5.0 ± 0.4^{a}	<1^{a}	5.1 ± 0.2^{a}	3.5 ± 0.5^{a}	3.0 ± 0.2^{a}	3.9 ± 0.3^{ab}	4.2 ± 0.4^{a}	3.5 ± 0.1^{a}	4.7 ± 0.3^{a}	4.9 ± 0.1^{a}	4.8 ± 0.2^{a}
	2	4.3 ± 0.3^{a}	<1^{a}	2.3 ± 0.3^{c}	<2^{b}	<2^{b}	3.3 ± 0.5^{b}	3.0 ± 0.2^{a}	2.4 ± 0.2^{b}	4.4 ± 0.4^{a}	4.2 ± 0.4^{ab}	4.6 ± 0.4^{a}
	1	3.9 ± 0.4^{a}	<1^{a}	3.8 ± 0.3^{b}	<2^{b}	3.0 ± 0.1^{a}	3.9 ± 0.5^{a}	3.9 ± 0.3^{a}	2.0 ± 0.1^{b}	3.9 ± 0.2^{a}	3.8 ± 0.1^{b}	4.1 ± 0.2^{a}
Statistical significance[a]		NS	NS	***	***	***	*	NS	***	NS	*	NS

Abbreviations: PCA, plate count agar for total mesophilic count; MRS, de Man-Rogosa-Sharpe agar for mesophilic rod LAB; M17, medium 17 agar for mesophilic coccus LAB; SDB, sourdough bacteria agar for sourdough LAB; SFM, San Francisco medium for sourdough LAB; YPD, yeast peptone dextrose agar for yeast; MA, malt agar for molds.
P value: *, P < 0.05; **, P < 0.01; ***, P < 0.001; N.S., not significant.
For a given wheat variety and type of sample, data within a column followed by the same letter are not significantly different according to Tukey's test.
Results indicate mean values ± SD of two determinations.

Table 3. Phenotypic grouping of LAB isolated aged kernels.

Characters	Clusters				
	I (n=5)	II (n=4)	III (n=20)	IV (n=34)	V (n=76)
Cell morphology	Rod	Rod	Rod	Coccus (tetrads)	Coccus (short chain)
Growth:					
15°C	+	+	+	+	+
45°C	–	+	–	+	+
pH 9.2	n.d.	n.d.	n.d.	+	+
6.5% NaCl	n.d.	n.d.	n.d.	+	+
CO_2 from glucose	+	–	–	–	–
Pentose fermentation	n.d.	+	+	n.d.	n.d.

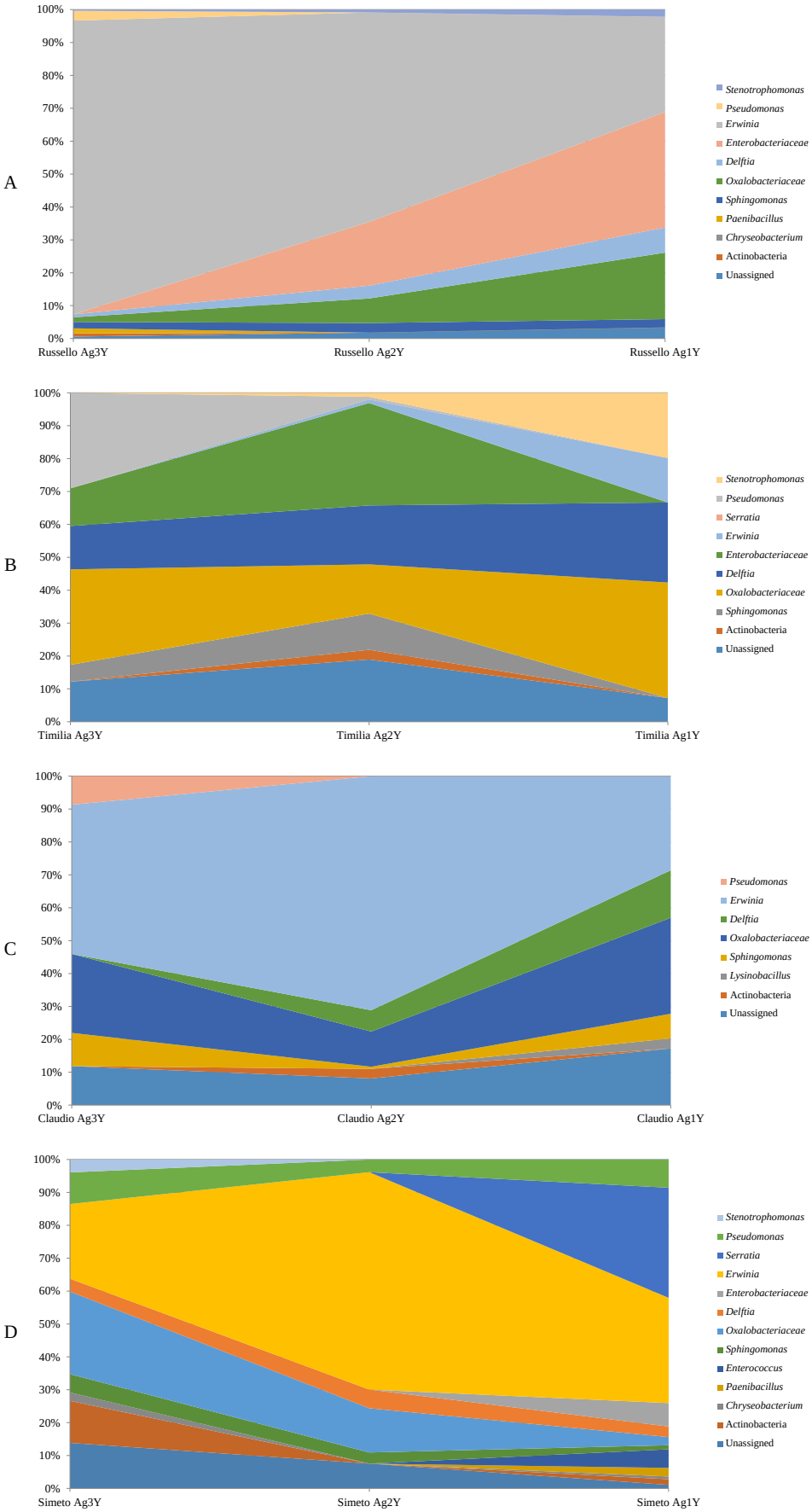
n.d. not determined.

Legend to figures.

Fig. 1. Relative abundances (%) of bacterial genera identified by MySeq Illumina on durum wheat semolinas obtained from aged kernels. A, Russello genotype; B, Timilia genotype; C, Claudio genoype; D, Simeto genotpe. Only genera occurring at > 0.1% abundance in at least one sample were included. Abbreviations: Ag1Y, aged for one year; Ag2Y, aged for two years; Ag3Y, aged for three years.

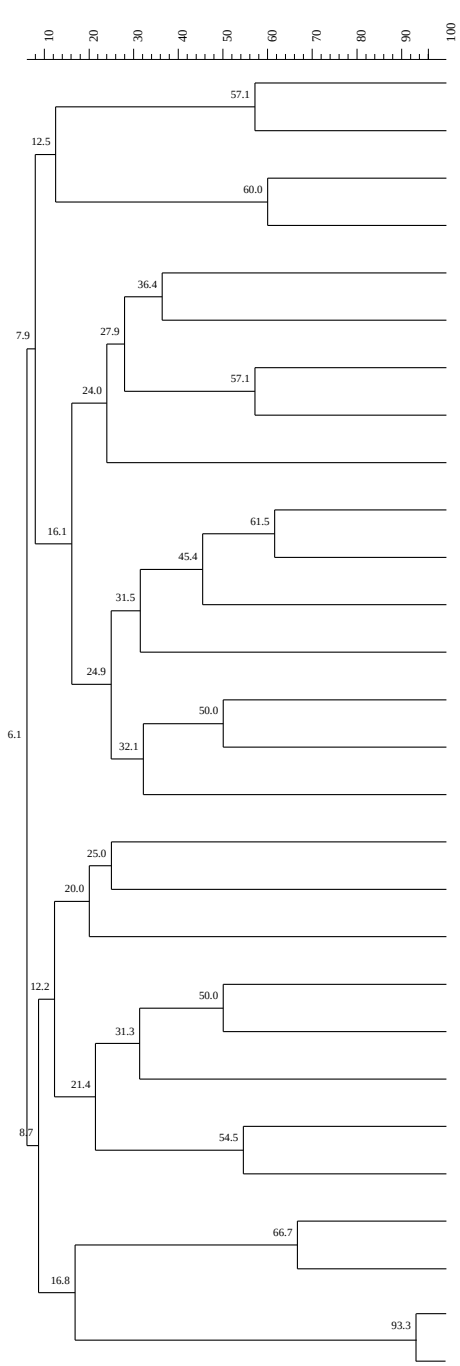
Fig. 2. Dendrogram obtained with combined RAPD-PCR patterns generated with three primers for LAB strains of different durum wheat semolinas obtained from aged kernels. The line at the top indicates percentages of similarity. Abbreviations: *E.*, *Enterococcus*; *L.*, *Lactobacillus*; *P.*, *Pediococcus*.

607 **Fig.1.**


A
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
Russello Ag3Y
Russello Ag2Y
Russello Ag1Y
Stenotrophomonas
Pseudomonas
Erwinia
Enterobacteriaceae
Delftia
Oxalobacteriaceae
Sphingomonas
Paenibacillus
Chryseobacterium
Actinobacteria
Unassigned
B
Timilia Ag3Y
Timilia Ag2Y
Timilia Ag1Y
Stenotrophomonas
Pseudomonas
Serratia
Erwinia
Enterobacteriaceae
Delftia
Oxalobacteriaceae
Sphingomonas
Actinobacteria
Unassigned
C
Claudio Ag3Y
Claudio Ag2Y
Claudio Ag1Y
Pseudomonas
Erwinia
Delftia
Oxalobacteriaceae
Sphingomonas
Lysinobacillus
Actinobacteria
Unassigned
D
Simeto Ag3Y
Simeto Ag2Y
Simeto Ag1Y
Stenotrophomonas
Pseudomonas
Serratia
Erwinia
Enterobacteriaceae
Delftia
Oxalobacteriaceae
Sphingomonas
Enterococcus
Paenibacillus
Chryseobacterium
Actinobacteria
Unassigned

608

609 **Fig. 2.**



STRAIN	ISOLATION SOURCE	ENRICHMENT	PHENOTYPIC GROUP	SPECIES
S50	AR Ag1Y		V	*E. casseliflavus*
S76	AT Ag1Y – AR Ag2Y		V	*E. casseliflavus*
S52	AR Ag1Y – AR Ag2Y		V	*E. casseliflavus*
S168	MC Ag2Y	*	V	*E. casseliflavus*
S163	AR Ag1Y	*	IV	*P. pentosaceus*
S164	AR Ag1Y	*	IV	*P. pentosaceus*
S162	MS Ag2Y	*	III	*L. graminis*
S145	MS Ag1Y	*	III	*L. graminis*
S143	MS Ag1Y	*	III	*L. graminis*
S122	AR Ag1Y		IV	*P. pentosaceus*
S165	AR Ag1Y - AR Ag2Y	*	IV	*P. pentosaceus*
S125	AR Ag1Y		IV	*P. pentosaceus*
S132	AR Ag1Y		IV	*P. pentosaceus*
S147	AT Ag2Y	*	IV	*P. acidilactici*
S140	AT Ag1Y	*	IV	*P. pentosaceus*
S141	AT Ag1Y	*	IV	*P. pentosaceus*
S154	MC Ag1Y - MC ag3Y	*	I	*L. brevis*
S159	AR Ag3Y	*	II	*L. paracasei*
S151	MC Ag3Y	*	III	*L. pentosus*
S149	MC Ag3	*	V	*E. durans*
S161	AR Ag3Y – AT Ag3Y - MC Ag2Y	*	V	*E. durans*
S47	AR Ag 3Y	*	V	*E. durans*
S170	MS Ag3Y	*	V	*E. faecium*
S161	MS Ag2Y	*	V	*E. faecium*
S157	AT Ag3Y - AR Ag3Y - AR Ag2Y	*	V	*E. faecium*
S153	MC Ag1Y	*	V	*E. faecalis*
S146	AT Ag2Y	*	V	*E. faecium*
S176	AT Ag3Y	*	V	*E. faecium*

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