Monitoring of wheat lactic acid bacteria from the field until the first step of dough fermentation

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A R T I C L E   I N F O
Article history:
Received 22 March 2016
Received in revised form 3 October 2016
Accepted 7 October 2016
Available online 11 October 2016

Keywords:
Ears
Kernels
Lactic acid bacteria
MiSeq Illumina
Semolina
Sourdough
Wheat

A B S T R A C T
The present work was carried out to retrieve the origin of lactic acid bacteria (LAB) in sourdough. To this purpose, wheat LAB were monitored from ear harvest until the first step of fermentation for sourdough development. The influence of the geographical area and variety on LAB species/strain composition was also determined. The ears of four Triticum durum varieties (Duilio, Iride, Saragolla and Simeto) were collected from several fields located within the Palermo province (Sicily, Italy) and microbiologically investigated. In order to trace the transfer of LAB during the consecutive steps of manipulation, ears were transformed aseptically and, after threshing, milling and fermentation, samples of kernels, semolinas and doughs, respectively, were analysed. LAB were not found to dominate the microbial communities of the raw materials. In general, kernels harboured lower levels of microorganisms than ears and ears than semolinas. Several samples showing no development of LAB colonies acidified the enrichment broth suggesting the presence of LAB below the detection limit. After fermentation, LAB loads increased consistently for all doughs, reaching levels of 7.0–7.5 Log CFU/g on M17. The values of pH (5.0) and TTA (5.6 mL NaOH/10 g of dough) indicated the occurrence of the acidification process for several doughs. LAB were phenotypically and genotypically differentiated by randomly amplified polymorphic DNA (RAPD)-PCR into eight groups including 51 strains belonging to the species Lactobacillus brevis, Lactobacillus coryniformis, Lactobacillus plantarum, Lactococcus lactis, Lactococcus garvieae, Enterococcus casseli flavus, Enterococcus faecalis, Enterococcus faecium, Leuconostoc citreum, and Pediococcus pentosaceus. Lactobacilli constituted a minority the LAB community, while lactococci represented more than 50% of strains. Lower LAB complexity was found on kernels, while a richer biodiversity was observed in semolinas and fermented doughs. For broader microbiota characterisation in doughs before fermentation, the 16S rRNA gene profiling was conducted on the unfermented doughs using MiSeq Illumina. LAB group was represented by Enterococcus and members of Leuconostocaceae family whose relative abundances differed according to both geographical area and variety of wheat. The culture-independent approach confirmed that pediococci and lactobacilli constituted low abundance members of the semolina LAB microbiota and that although some strains may pass from wheat ear to fermented doughs, most are likely to come from other sources.

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1. Introduction
Foods derived from cereals are popular worldwide (http://www.fao.org/worldfoodsituation/csdб/en/) and differ depending on the local culture and traditions (De Vuyst and Neyesens, 2005; Jenson, 1998; Spicher, 1999). Wheat is the crop plant that best represents the history of mankind and has been a staple for the populations that inhabited today’s Europe since the Stone Age (Gifford and Baer-Sinnott, 2007). Wheat has been also a major agent of landscape
change (Head and Atchison, 2016). Within Triticum genus, the two species predominantly cultivated are Triticum aestivum and Triticum durum (Toderi, 1989), commonly known as tender wheat and durum wheat, respectively. Durum wheat is a key component of diet, economy, and culture in Italy (Flagella, 2006) where it is used mainly to produce pasta, even though in some Southern regions (especially Apulia and Sicily) it is also used for the production of several bread types (Corsetti et al., 2001; Quaglia, 1988). The raw materials, the microbiota developing during the fermentation process and the technological parameters affect the characteristics of the final breads (De Vuyst et al., 2014).

Bread production is within the most ancient of human biotechnological activities (Pomeranz, 1987) and, probably, represents one of the first examples of a food obtained from raw materials transformed through fermentation. This process has been carried out with sourdough for millennia (Manetta, 2016). The introduction of baker’s yeast accelerated the leavening process to the detriment of the organoleptic and nutritional characteristics of the final products (Gobbetti and Corsetti, 2010). However, thanks to its numerous advantages over the baker’s yeast, the sourdough technology has not been abandoned (Venturi et al., 2012), especially for traditional and typical breads (Corsetti and Settanni, 2007).

Basically, sourdough originates from a mixture of flour and water that undergoes a fermentation carried out by indigenous lactic acid bacteria (LAB) and yeasts present in flour (De Vuyst and Vancanneyt, 2007; Vogel et al., 1999). LAB are responsible for the acidification of dough, but partly contribute to volume increase of dough (Gobbetti et al., 1995). The leavening action is exerted only by the obligate heterofermentative species and is basically due to the CO₂ produced through 6-phosphogluconate/phosphoketolase pathway (Eno and Dicks, 2014). Since raw materials are not subjected to thermal treatments before fermentation, they are contaminated by microorganisms, which is one of the causes for eventual change of sourdough microbial community (Alfonzo et al., in press; Harth et al., 2016).

Wheat flours (from T. aestivum) and semolinas (from T. durum) are naturally contaminated by LAB (Alfonzo et al., 2013). These bacteria have at least a double origin: LAB might be endophytic components of wheat plants (Minervini et al., 2015) or derive from the outer layers of kernels (Berghofer et al., 2003; Gobbetti et al., in press). In the latter case, LAB should derive from the environment. Thus, the LAB present on the awns, palea, lemma, glumes and rachis might be transferred to the kernels during threshing and from the kernels to the flour/semolina during milling. Wheat kernels have been found to host several LAB species that do not typically dominate the mature sourdoughs (Corsetti et al., 2007a), but are present in these ecosystems at subdominant levels (Corsetti et al., 2007b). Kernel LAB were found to play a defining role during the first step of sourdough production, mainly inhibiting the indigenous undesired microorganisms by lowering the pH, thus preparing the environment for the establishment of the typical sourdough (e.g. Lactobacillus) species (Corsetti et al., 2007b). The strong competitiveness of kernel LAB was directly related to their bacteriocin production (Corsetti et al., 2008). Several LAB are applied in the process of bakery products also for their antifungal activities (Valerio et al., 2016).

Based on these considerations, the aim of the present study was to follow the route of LAB from the field, where wheat is cultivated, to bakery, where the resulting semolina is processed, in order to clarify the origin of the LAB populations found in sourdough. The specific objectives of our study were (i) to characterise the LAB populations on ears and kernels, and in semolinas and fermented doughs by classical culture-dependent methods, (ii) to assess the microbial diversity of unfermented doughs by culture-independent 16S rRNA gene sequencing, and (iii) to evaluate the influence of the geographical area and T. durum variety on the bacterial populations before the first fermentation step of traditional sourdough production.

2. Materials and methods

2.1. Wheat fields, threshing, milling and collection of samples

In this study, four T. durum varieties (Duilio, Iride, Saragolla, and Simeto) cultivated in adjacent experimental fields (20 m × 500 m) of five farms located in four geographical areas within the Palermo province (Sicily, Italy) were microbiologically investigated. The sites for the wheat cultivation were as follows: Campofiorito (37°76’N, 13°25’E, elevation 483 m), Cininna (37°86’N, 13°52’E, elevation 329 m), Contessa Entellina (two sites: A, 37°75’N, 13°15’E, elevation 378 m; B, 37°76’N, 13°15’E, elevation 320 m), and Valledolmo (37°71’N, 13°79’E, elevation 619 m). Once the plants reached the physiological maturity stage, the ears with approximately 10 cm of culm were manually randomly collected with disposable gloves and dissecting scissors, transferred into paper bags (two aliquots of 100 and 300 ears) and immediately transported at ambient temperature to the laboratory of Agricultural Microbiology – University of Palermo, where they were kept in a refrigerated (7 °C) chamber. The smaller aliquots of ears were used for the microbiological analyses, while the larger ones for threshing.

Threshing was carried out aseptically under a laminar flow hood. The external segments were removed manually. To exclude any contamination of the kernels with the microorganisms hosted in the inner layers of wheat, all damaged kernels were also removed. The kernels were then mixed and 100 g were transferred into sterile plastic bags for the microbiological analyses, while the rest, put in sterile bags, was used for milling without conditioning. Both aliquots were kept refrigerated before processing.

Milling was performed at ambient temperature with a Retsch centrifugal Mill ZM1 (Haan, Germany) equipped with a 1 mm grid. In order to perform the process in aseptic conditions, the mill was put under the laminar flow hood, the grid and the rotor were cleaned with ethanol and subjected to 30 min UV treatment before milling each sample of kernels. The resulting whole-meat semolinas were put in sterile plastic bags and kept refrigerated.

2.2. Dough production, monitoring of acidification and sampling

The doughs were prepared by mixing manually 125 g of each semolina with 75 mL of sterile tap water to a dough yield (weight of dough/weight of flour × 100) of 160. Dough production (in duplicate for each semolina) was performed aseptically under a laminar flow hood in sterile glass beakers using a stainless steel spoon for mixing. The doughs were fermented for 21 h at 30 °C. The acidification process was followed every 2 h for the first 8 h and at 21 h by pH, determined electrometrically with the pH meter BASIC 20+ (Crison Instrument S.A., Barcelona, Spain), and total titratable acidity (TTA), determined by titration (expressed as mL of NaOH/10 g of dough) (Lonnert et al., 1986). All determinations were carried out in duplicate.

2.3. Microbiological analyses

The ears were analysed after removal of culms by dissecting scissors. Samples of ears (five ears, approximately 15–20 g) and kernels (15 g) were separately transferred into 500 mL sterile glass flasks and added with Ringer’s solution (Sigma-Aldrich, Milan, Italy) till reaching a ratio 1:10 (sample:diluent) (Hartnett et al., 2016).
The flasks were subjected to agitation by an orbital shaker at 240 rpm for 1 h at room temperature. Semolina and fermented dough samples (15 g) were added with 135 mL of Ringer’s solution and homogenised by a stomacher (BagMixer® 400, Interscience, Saint Nom, France) for 2 min at the highest speed. The microbial suspensions obtained from ears and kernels were used for the analysis of the microbial cells contaminating the outer layers of wheat. Aliquots of 1 mL from the supernatants of ears and kernels and 1 mL from the semolinas and doughs homogenised were subjected to the decimal serial dilution.

The following microbial groups were investigated for all samples: total mesophilic microorganisms (TMM) spread-plateed on plate count agar (PCA, Oxoid, Milan, Italy); LAB rods pour-plated on MRS (Oxoid); LAB cocci pour-plated on M17 (Oxoid); sourdough LAB pour-plated on Sour Dough Bacteria (SDB) (Kline and Sugihara, 1971) and San Francisco Medium (SFM) (Vogel et al., 1994). All media were incubated at 30°C and incubation lasted 72 h for PCA and 48 h for the other media. Microbiological counts were carried out in duplicate.

When no colony developed on any of the four media used for LAB growth (MRS, M17, SDB and SFM), the enrichment procedure was applied. These matrices (10 g of each sample) were inoculated in 50 mL of modified-MRS (mMRS) broth (maltose, lactose and fresh yeast extract were added to the final concentrations of 1.1 and 10%, respectively, and the final pH was adjusted to 5.6 with 5 M lactic acid) and, after 96 h of incubation at 30°C, the pH was measured.

2.4. Isolation and grouping of lactic acid bacteria

At least four colonies of presumptive LAB sharing color, shape, edge, surface and, when developed onto the surface of the agar media, elevation, originated from the highest plated dilutions of the microbial cells were collected for all different morphologies. The isolates were subjected to Gram determination (Gregersen KOH method) and catalase test (determined by transferring fresh colonies from the agar media to glass slides and adding 5% H2O2) and only the Gram-positive/catalase-negative cultures were purified by successive sub-culturing in the optimal media and stored in glycerol (20% v/v) stocks at −80°C.

The phenotypic grouping of the cultures was based on the cell morphology, determined microscopically, growth at 15 and 45°C and metabolism type, testing the ability to produce CO2 from glucose. The last test was carried out in the optimal broth media containing glucose as the only carbohydrate source and without citrate. Washed cells of pure cultures, prepared as reported by Ventimiglia et al. (2015), were inoculated (1% v/v) into test tubes which were then sealed with H2O agar (2% w/v). After 48 h of incubation, the test indicated an obligate heterofermentative metabolism if a rising of the agar cap, due to CO2 production, was detected. LAB scored negative for CO2 generation were cultivated in presence of pentose carbohydrates as described by Settanni et al. (2012). The growth in these conditions indicates a facultative heterofermentative metabolism, whereas the cultures unable to grow belong to obligate homofermentative species. Coccus isolates were further tested for their growth at pH 9.2 and in the presence of 6.5% (w/v) NaCl.

2.5. Genetic differentiation and identification of lactic acid bacteria

DNAs from LAB cultures was extracted using the InstaGene Matrix kit (Bio-Rad, Hercules, CA, USA) following the manufacturer’s instructions. Cells were harvested after overnight growth of the isolates in the optimal broth media at 30°C, washed as reported by Ventimiglia et al. (2015) using sterile distilled H2O in place of Ringer’s solution. Crude cell extracts were used as templates for PCRs.

The random amplification of polymorphic DNA (RAPD)-PCR technique was applied for the differentiation of the isolates at strain level. The amplification was performed with the primers M13, AB111, and AB106, used singly, following the methodology described by Settanni et al. (2012). The amplified products were separated by electrophoresis on a 2% (w/v) agarose gel (Gibco BRL, Cergy Pontoise, France) and, after staining with SYBR® Safe DNA gel stain (Molecular Probes, Eugene, OR, USA), visualised by UV transillumination and acquired. The analysis of the RAPD patterns was performed with the Gelcompar II software, version 6.5 (Applied-Maths, Sint-Martens-Latem, Belgium).

The identification of the LAB strains was obtained through 16S rRNA gene sequencing applying the method reported by Weisburg et al. (1991). The amplicons of approximately 1600 bp were purified using 10 U of Exonuclease I and 1 U of Shrimp Alkaline Phosphatase (Fermentas). DNA sequencing reaction was carried out using BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) with 5 μM of the same primers used in the PCR reaction. Cycle sequencing reaction was performed according to the manufacturer's instruction following Ethanol/EDTA/Sodium Acetate precipitation. Sequencing analyses were performed in an ABI PRISM 3130xl Genetic Analyzer (Applied Biosystems).

The sequences were compared with those available in two databases: EzTaxon-e (http://eztaxon-e.ezbiocloud.net/) (Chun et al., 2007) that includes only the sequences of the type strains; GenBank/EMBL/DDBJ (http://www.ncbi.nlm.nih.gov) (Altschul et al., 1997). The members of Lactobacillus plantarum group were unequivocally identified by the recA gene based multiplex PCR described by Torriani et al. (2001) that distinguishes among Lb. plantarum, Lactobacillus paraplanarum and Lactobacillus plantarum. The identity of Lactobacillus brevis was further verified by the multiplex PCR strategy targeting the rrn operon reported by Settanni et al. (2005). Enterococci were also subjected to the sodA gene-based multiplex PCR developed by Jackson et al. (2004).

2.6. DNA amplification and Illumina sequencing

Total DNA extraction from the unfermented doughs was carried out using the NucleoSpin Food Kit (Macherey-Nagel GmbH & Co. KG, Düren, Germany) according to the manufacturer's instructions. For each sample, a 464-nucleotide sequence of the V3-V4 region (Baker et al., 2003; Claesson et al., 2010), of the 16S rRNA gene (Escherichia coli positions 341 to 805) was amplified starting from the extracted genomic DNA. Unique barcodes were attached before the forward primers to facilitate the pooling and subsequent differentiation of samples.

To prevent preferential sequencing of the smaller (shorter than 100 bp) amplicons, the amplicons were cleaned using the Agencourt Ampure kit (Beckman Coulter) according to the manufacturer’s instructions; subsequently, DNA concentrations of the amplicons were 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 quality of the generated amplicon libraries was evaluated by a Bioanalyzer 2100 (Agilent, Palo Alto, CA, USA) using the High Sensitivity DNA Kit (Agilent). Following the quantitation, cleaned amplicons were mixed and combined in equimolar ratios. Pair-end sequencing using the Illumina MiSeq system (Illumina, USA) was carried out at CIBIO (Center of Integrative Biology) – University of Trento (Trento, Italy).
2.7. Illumina data analysis and sequence identification by QIIME

Sequences obtained from Illumina Sequencing were processed using Quantitative Insights Into Microbial Ecology (QIIME) software package version 1.9 (Caporaso et al., 2010). Briefly, reads were assigned to each sample according to the unique barcode; pairs of reads from the original DNA fragments were firstly merged using a script implemented in QIIME; quality trimming of dataset removed sequences with a mean quality score less than 20, the primer sequences were excluded before alignment. The remaining sequences were assigned into operational taxonomic units (OTUs) at a threshold of 97% pair-wise sequence identity by uclust as implemented in QIIME (Edgar, 2010). OTU representative sequences were then classified taxonomically using Ribosomal Database Project (RDP) classifier 2.0.1 (Maidak et al., 2001). The OTUs belonging to the LAB genera that could not be identified at species level using the databases implemented in QIIME were manually checked and recovered through a BLAST search against the NCBI database (http://www.ncbi.nlm.nih.gov). The OTUs were aligned using PyNAST software/tool with a minimum alignment length of 150 bp and a minimum identity at 80%. Rarefaction plots were constructed and diversity indices were estimated as implemented in QIIME (Caporaso et al., 2010). The species richness within each sample was estimated by QIIME pipeline using Chao1 metric that is a non-parametric abundance-based estimator of species richness (Chao and Bunge, 2002). The data generated by Illumina sequencing were deposited in the NCBI Sequence Read Archive (SRA) and are available under Ac. No. SRP071270.

2.8. Statistical analyses

Microbiological data, pH and TTA were subjected to one-way analysis of variance (ANOVA). Pair comparison of treatment means was achieved by Tukey's procedure at P < 0.05. The distribution of LAB species among T. durum varieties and geographical locations was determined by correspondence analysis. All data were analysed by the statistical software XLSTAT for Windows (XLSTAT version 2014.5.03, Addinsoft, New York).

3. Results

3.1. Microbiological characteristics of ears, kernels and semolinas

The enumeration of TMM and LAB on four media (MRS, M17, SDB and SFM), reported in Table 1, showed consistent differences in loads among samples. The levels of TMM were not directly correlated to the levels of LAB in ears, kernels and semolinas. As an example, despite a load of 5.3 Log CFU/g of TMM in Iride ears from Contessa Entellina B, presumptive LAB were 2 Log cycles lower on M17 and below the detection limit on the other agar media. In general, kernels (<2–3.0 Log CFU/g) harboured lower levels of TMM than the corresponding ears (2.6–5.3 Log CFU/g). Among LAB, the highest levels were displayed by M17. For Simeto kernels, all microbial groups investigated were below the detection limits. Compared to kernels, an increasing trend of the levels of LAB was observed in semolinas, especially for Saragolla and Simeto varieties. These observations indicated that LAB are not the dominating microbial groups investigated were below the detection levels.

Furthermore, all samples showing no development of presumptive LAB colonies were subjected to the enrichment in mMRS broth. After 4-d incubation, the pH of the fermented substrates were measured (Table 1). Several broth cultures showed a lower value, ranging between 4.6 and 5.0, than 5.6 characteristic of mMRS, confirming the presence of acidifying microorganisms in the raw materials. On the contrary, some samples such as Iride kernels and semolina from Contessa Entellina B, Saragolla kernels from Campofilorio, Ciminna and Valledolmo, Saragolla semolina from Campofilorio and Simeto kernels and semolinas from Ciminna, Contessa Entellina B and Valledolmo determined an increase of the pH of enrichment broth up to 6.8.

3.2. Characteristics of fermented doughs

Viable counts determined for fermented dough samples (Table 1) ranged between 6.7 and 8.7 for TMM. LAB loads were particularly different among the four media used, with the highest counts, up to 7.5 Log CFU/g, registered on M17 medium. The numbers of LAB estimated on MRS were higher than those counted on SDB and SFM. In particular, the latter medium determined the lowest LAB counts. An almost direct correlation was registered for TMM and LAB in some fermented doughs (Duilio from Contessa Entellina A, Iride from Campofilorio, Ciminna, Contessa Entellina A and Valledolmo), for which the loads of LAB, especially those registered on M17 (7.0–7.5 Log CFU/g), were comparable or even superimposable to those of TMM (6.7–7.7 Log CFU/g).

The fermented doughs were also analysed for the acidification capacity of the indigenous microorganisms by measuring pH and TTA (Table 1). The pH of the uncompromised doughs (results not shown in Table 1) was between 6.3 and 6.7. After 21 h of incubation, the pH remained almost unvaried for the samples Duilio from Campofilorio (6.4), Iride from Contessa Entellina B (6.4), Saragolla and Simeto from Ciminna (6.3 and 6.4, respectively), whereas values below 6.0, ranging from 5.0 to 5.8, were registered for the other fermented doughs. The majority of these, actually, showed pHs within the range 5.4 and 5.8. TTA measured at T0 (results not shown in Table 1) was 1.8–2.1 mL of NaOH/10 g of dough. This parameter was inversely correlated with pH, since low levels of TTA were registered when pHs were high and, viceversa, high levels of TTA were registered for fermented doughs characterised by low pHs. In particular, TTA was 2.1, 2.3, 2.6 and 2.0 for the samples Duilio from Campofilorio, Iride from Contessa Entellina B, Saragolla and Simeto from Ciminna, respectively, confirming that for these fermented doughs the acidification process did not occur or it was extremely limited. The values of TTA for the other fermented doughs was between 4.0 and 5.6 showing the production of organic acids.

All these results indicated that LAB present in the semolina/water mixture increased at consistent levels during fermentation for 16 out of the 20 samples object of investigation.

3.3. Phenotyping grouping of LAB

The colonies representative of the different morphologies of bacteria developed on MRS, M17, SDB and SFM were picked from the plates inoculated with the most diluted samples of ears, kernels, semolinas and fermented doughs. A total of 2368 bacteria were collected, purified and tested for the Gram type and presence of catalase. Only 705 cultures belonged presumptively to the LAB groups, as being Gram positive and catalase negative. The microscopic investigation recognised a rod shape for 89 cultures, while the majority of the presumptive LAB (616 cultures) were characterised by a coccus cell morphology. LAB cocci appeared mostly in pairs or short chains, but some isolates showed cells in tetrads. Based on the combination of the other characters evaluated during the phenotypic investigation, LAB cultures were separated into eight groups, three for rods and five for cocci (Table 2). Two groups, one for rods (I) and one for cocci (V) included bacteria with an obligate heterofermentative metabolism. The other two groups of rods were both facultative heterofermentative. Within cocci, three groups were able to grow at pH 9.2 and in presence of 6.5% NaCl.
### Table 1
Characteristics of ears, kernels, semolinas and fermented doughs.

<table>
<thead>
<tr>
<th>T. durum variety</th>
<th>Samples</th>
<th>Microbial loads (Log CFU/g)</th>
<th>pH after enrichment</th>
<th>Acidification of fermented doughs</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>PCA</td>
<td>MRS</td>
<td>M17</td>
</tr>
</tbody>
</table>

#### Durum

Ears:
- **Fermented doughs:**
  - Campoforito:
    - 3.5 ± 0.4ab
    - 2.0 ± 0.3bc
    - 2.6 ± 0.3ab
    - 1.9 ± 0.3bc
  - Cimanna:
    - 2.2 ± 0.4bc
    - 1.2 ± 0.3bc
    - 1.7 ± 0.3ab
    - 0.9 ± 0.2bc
  - Contessa Entellina A:
    - 2.2 ± 0.4bc
    - 1.2 ± 0.3bc
    - 1.6 ± 0.3ab
    - 0.8 ± 0.2bc
  - Contessa Entellina B:
    - 2.1 ± 0.3bc
    - 1.1 ± 0.2bc
    - 1.5 ± 0.2bc
    - 0.7 ± 0.2bc
  - Valledolmo:
    - 2.6 ± 0.4bc
    - 1.8 ± 0.4bc
    - 2.6 ± 0.4bc
    - 1.2 ± 0.3bc

Kernels:
- **Fermented doughs:**
  - Campoforito:
    - <2b
    - 1b
    - 1b
    - 1b
  - Cimanna:
    - 2.3 ± 0.2b
    - 2.2 ± 0.5a
    - 2.2 ± 0.5a
    - 2.3 ± 0.2a
  - Contessa Entellina A:
    - 2.0 ± 0.1b
    - 2.4 ± 0.3b
    - 2.3 ± 0.3a
    - 2.0 ± 0.3a
  - Contessa Entellina B:
    - 2.3 ± 0.2b
    - 1b
    - 1b
    - 1b
  - Valledolmo:
    - <2b
    - 1b
    - 1b
    - 1b

#### Iride

Ears:
- **Fermented doughs:**
  - Campoforito:
    - 3.2 ± 0.4bc
    - 1.1 ± 0.2bc
    - 1.1 ± 0.2bc
    - 1.1 ± 0.2bc
  - Cimanna:
    - 2.8 ± 0.3bc
    - 2.9 ± 0.4bc
    - 2.9 ± 0.4bc
    - 2.5 ± 0.3ab
  - Contessa Entellina A:
    - 3.9 ± 0.3b
    - 2.1 ± 0.1b
    - 2.9 ± 0.6b
    - 1.6 ± 0.4b
  - Contessa Entellina B:
    - 5.3 ± 0.5b
    - 5.3 ± 0.5b
    - 2.9 ± 0.6b
    - 1.5 ± 0.3b
  - Valledolmo:
    - 3.9 ± 0.3b
    - 3.0 ± 0.2b
    - 2.3 ± 0.2b
    - 1.5 ± 0.3b

Kernels:
- **Fermented doughs:**
  - Campoforito:
    - 3.0 ± 0.4a
    - 2.2 ± 0.3ab
    - 2.5 ± 0.2a
    - 2.0 ± 0.3a
  - Cimanna:
    - 2.2 ± 0.3a
    - 1.8 ± 0.4a
    - 2.5 ± 0.6a
    - 1.7 ± 0.3b
  - Contessa Entellina A:
    - 2.5 ± 0.3b
    - 1.3 ± 0.3b
    - 1.3 ± 0.3b
    - 1.4 ± 0.3a
  - Contessa Entellina B:
    - <2b
    - 1b
    - 1b
    - 1b
  - Valledolmo:
    - <2b
    - 1b
    - 1b
    - 1b

#### Semolinas:**

Ears:
- **Fermented doughs:**
  - Campoforito:
    - 3.6 ± 0.7a
    - 3.3 ± 0.6a
    - 2.4 ± 0.4a
    - 2.2 ± 0.6a
  - Cimanna:
    - 2.4 ± 0.7b
    - 1c
    - 1b
    - 1b
  - Contessa Entellina A:
    - 2.7 ± 0.3ab
    - 2.2 ± 0.4ab
    - 2.6 ± 0.7ab
    - 1.8 ± 0.3ab
  - Contessa Entellina B:
    - 2.7 ± 0.3ab
    - 1b
    - 1b
    - 1b
  - Valledolmo:
    - 3.0 ± 0.5ab
    - 2.0 ± 0.7ab
    - 3.0 ± 0.4ab
    - 2.1 ± 0.3b

Kernels:
- **Fermented doughs:**
  - Campoforito:
    - 7.4 ± 0.4ab
    - 4.7 ± 0.3ab
    - 7.0 ± 0.3a
    - 5.9 ± 0.8a
  - Cimanna:
    - 6.8 ± 0.3ab
    - 5.7 ± 0.2a
    - 7.1 ± 0.4b
    - 2.3 ± 0.7b
  - Contessa Entellina A:
    - 6.7 ± 0.3ab
    - 4.2 ± 0.6b
    - 7.3 ± 0.8b
    - 2.5 ± 0.8b
  - Contessa Entellina B:
    - 8.5 ± 0.3a
    - 4.2 ± 0.5a
    - 6.5 ± 0.9a
    - 2.9 ± 0.5b
  - Valledolmo:
    - 7.7 ± 0.5ab
    - 6.0 ± 0.6a
    - 7.5 ± 0.8a
    - 6.0 ± 1.0a

#### Saragolla

Ears:
- **Fermented doughs:**
  - Campoforito:
    - 4.1 ± 0.6a
    - 3.0 ± 0.2a
    - 3.4 ± 0.2a
    - 3.1 ± 0.7a
  - Cimanna:
    - 3.1 ± 0.2a
    - 2.4 ± 0.3a
    - 2.6 ± 0.4a
    - 2.4 ± 0.6a
  - Contessa Entellina A:
    - 3.6 ± 0.6ab
    - 1b
    - 2.0 ± 0.6b
    - 1c
  - Contessa Entellina B:
    - 4.2 ± 0.3a
    - 3.0 ± 0.6a
    - 3.3 ± 0.1
    - 3.3 ± 0.4a
  - Valledolmo:
    - 2.6 ± 0.4a
    - 1a
    - 1a
    - 1a

Kernels:
- **Fermented doughs:**
  - Campoforito:
    - <2b
    - 1b
    - 1b
    - 1b
  - Cimanna:
    - 2.6 ± 0.4a
    - 2.4 ± 0.4a
    - 2.7 ± 0.4a
    - 1.7 ± 0.3a
  - Contessa Entellina A:
    - 2.3 ± 0.2b
    - 1b
    - 1b
    - 1b
  - Contessa Entellina B:
    - 2.5 ± 0.4a
    - 1b
    - 1b
    - 1b

#### Semolinas:**

Ears:
- **Fermented doughs:**
  - Campoforito:
    - 2.6 ± 0.3a
    - 1b
    - 1b
    - 1d
  - Cimanna:
    - 2.7 ± 0.5b
    - 2.0 ± 0.6b
    - 2.3 ± 0.4b
    - 1.3 ± 0.1b
  - Contessa Entellina A:
    - 2.2 ± 0.3a
    - 2.2 ± 0.2a
    - 3.0 ± 0.6b
    - 2.0 ± 0.3a
  - Contessa Entellina B:
    - 2.7 ± 0.3a
    - 1b
    - 1b
    - 1b
  - Valledolmo:
    - 2.8 ± 0.5ab
    - 2.7 ± 0.4a
    - 2.9 ± 0.5b
    - 2.9 ± 0.6b

Kernels:
- **Fermented doughs:**
  - Campoforito:
    - 8.0 ± 0.3ab
    - 5.9 ± 0.7a
    - 6.0 ± 0.6a
    - 2.0 ± 0.9b
  - Cimanna:
    - 7.3 ± 0.5b
    - 4.3 ± 0.8b
    - 4.5 ± 0.5b
    - 2.2 ± 0.5b
  - Contessa Entellina A:
    - 8.5 ± 0.4a
    - 4.7 ± 0.4b
    - 6.5 ± 0.5b
    - 2.2 ± 0.7b
  - Contessa Entellina B:
    - 3.0 ± 0.4bc
    - 5.0 ± 0.8a
    - 6.6 ± 0.4a
    - 3.0 ± 0.4bc
  - Valledolmo:
    - 7.0 ± 0.6b
    - 4.0 ± 0.3b
    - 6.3 ± 0.6b
    - 2.5 ± 0.7b
Table 1 (continued)

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<th>Microbial loads (Log CFU/g)</th>
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<th>Acidification of fermented doughs</th>
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<td>Valledolmo</td>
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<td>&lt;1^b</td>
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<tr>
<td>Kernels:</td>
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<td>&lt;1^a</td>
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<td>2.6 ± 0.6^a</td>
<td>2.2 ± 0.5^a</td>
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<td>1.6 ± 0.4^a</td>
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<td>3.1 ± 0.2^a</td>
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<td>1.9 ± 0.5^a</td>
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<td>&lt;1^b</td>
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<td>Fermented doughs:</td>
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<tr>
<td>Campofiorito</td>
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<td>Cimmina</td>
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<td>Contessa Entellina A</td>
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<td>6.5 ± 0.6^a</td>
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<tr>
<td>Valledolmo</td>
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<td>6.5 ± 0.7^a</td>
<td>6.5 ± 0.6^a</td>
<td>5.7 ± 0.8^a</td>
</tr>
</tbody>
</table>

Abbreviations: n.n., not necessary; 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; P value: *, P < 0.05; **, P < 0.01; ***, P < 0.001; N.S., not significant. Results indicate mean values ± SD of two determinations. 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.

^1 mL of NaOH/10 g of dough.

The largest group (VI), composed of 312 isolates, included more than the half of cocci.

3.4. Strain typing and genetic identification of LAB

The representative isolates of the different ears, kernels, semolina and fermented doughs, for a total of 273 cultures, were selected from each phenotypic group and subjected to RAPD-PCR analysis. The combination of the three RAPD patterns for each isolate indicated that the cultivable presumptive LAB community isolated from raw materials and fermented doughs was composed of barely 51 different strains. When a given strain was found in different samples, it was reported in the dendrograms (Figs. 1 and 2) for all sources of isolation; e.g. the isolates 200587, 200682 and 200679 (Fig. 1) were the same Lb. plantarum strain, but they were isolated from dough of Irilde from Valledolmo and dough of Saragolla from Contessa Entellina A, respectively. Thus, Figs. 1 and 2 report the 118 isolates (corresponding to 51 strains) that cover all sources of isolation (ears, kernels, semolina and doughs) of the four varieties from the five areas. Due to the high numbers of isolates included into group VI, the resulting strains were reported into a separate dendrogram (Fig. 2). Several strains belonging to the same phenotypic groups clustered quite far apart from one another after analysis of the polymorphic profiles.

To verify whether bacteria sharing the same RAPD profile and isolated in different areas represented the same strain, all 118 isolates were identified by sequencing of the 16S rRNA gene and were confirmed to belong to the group of LAB (Figs. 1 and 2), since they were allotted into species of the genera Enterococcus, Lactobacillus, Lactococcus, Leuconostoc and Pediococcus. To unequivocally identify the species, some strains were further processed by multiplex PCRs.

Table 2

<table>
<thead>
<tr>
<th>Characters</th>
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<td>I (n = 24)</td>
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<td>6.5% NaCl</td>
<td>n.d.</td>
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</table>

n.d. not determined.
Fig. 1. Dendrogram obtained with combined RAPD-PCR patterns generated with three primers for LAB strains of different durum wheat raw material origin and fermented doughs belonging to different phenotypic groups. The line at the top indicates percentages of similarity. Abbreviations: E, Enterococcus; Lb, Lactobacillus; Lc, Lactococcus; Ln, Leuconostoc; P, Pediococcus; E, ears; K, kernels; S, semolina; D, dough; Du, Duilio; I, Iride; Sa, Saragolla; Si, Simeto; Ca, Campofiorito; C, Ciminna; C.E.A, Contessa Entellina A; C.E.B, Contessa Entellina B; V, Valledolmo.
Fig. 2. Dendrogram obtained with combined RAPD-PCR patterns generated with three primers for LAB strains of different durum wheat raw material origin and fermented doughs belonging to the phenotypic group VI. The line at the top indicates percentages of similarity. Abbreviations: Lc., Lactococcus; E, ears; K, kernels; S, semolina; D, dough; Du, Dulio; I, Iride; Sa, Saragolla; Si, Simeto; Ca, Campofiorito; C, Ciminna; C.E.A, Contessa Entellina A; C.E.B, Contessa Entellina B; V, Valledolmo.
The biodiversity in terms of species was quite limited due to the recognition of barely nine species: *Lb. brevis* (Ac. No. KU898963–KU898966), *Lactobacillus coryniformis* (Ac. No. KU898967–KU898971), *Lb. plantarum* (Ac. No. KU898972–KU898977), *Lactococcus lactis* (Ac. No. KU898987–KU8990386), *Lactococcus garvieae* (Ac. No. KU898978–KU898986), *Lactococcus casei* (Ac. No. KU898938–KU898942), *Enterococcus faecium* (Ac. No. KU898943–KU898962), *Leuconostoc citreum* (Ac. No. KU899039–KU899046), and *Pediococcus pentosaceus* (Ac. No. KU899046 e KU899055). Interestingly, the majority of strains were grouped per species, forming one major RAPD-PCR cluster for a given species. However, two strains of *P. pentosaceus* (200102 and 200292) were included into the cluster of *Ln. citreum* (Fig. 1).

### 3.5. Species distribution

Fig. 3 shows the distribution of the species among samples in relation to geographic area and *T. durum* variety. Lactobacilli constituted only the 13% of the LAB community, while lactococci represented the largest population reaching more than 50% of the total strains. LAB species isolated from ears were *P. pentosaceus*, *Ln. citreum*, *Lc. lactis*, *Lc. garvieae* and *Lb. coryniformis*. Not all samples resulted positive for the presence of LAB, even when plate counts showed consistent levels. For the majority of samples of ears from which presumptive LAB were counted only on M17 (Table 1), LAB showed consistent levels. For the majority of samples of ears from which presumptive LAB were counted only on M17 (Table 1), LAB were not identified at the highest dilutions. A lower complexity of the LAB community (*E. casseliflavus* and *Lc. garvieae*) was found on kernels, while a richer biodiversity was displayed by the semolinas which harboured *E. faecium*, *E. casseliflavus*, *Lc. lactis*, *P. pentosaceus*, *Ln. citreum*, *Lb. coryniformis* and *Lb. brevis*. Also for kernels and semolinas high counts on M17 did not always correspond to the development of LAB. After fermentation of the doughs obtained by mixing semolina with sterile water, the species identified at the highest levels (ranging between 10^5 and 10^7 CFU/g) among the LAB populations were *E. faecium*, *Lc. garvieae*, *Lc. lactis*, *Lb. coryniformis* and *Lb. brevis*. Also for kernels and semolinas high counts on M17 did not always correspond to the development of LAB. After fermentation of the doughs obtained by mixing semolina with sterile water, the species identified at the highest levels (ranging between 10^5 and 10^7 CFU/g) among the LAB populations were *E. faecium*, *Lc. garvieae*, *Lc. lactis*, *Lb. coryniformis* and *Lb. brevis*. Although different strains of lactococci were found at higher levels than lactobacilli, the latter group was more represented in the fermented matrices rather than the unprocessed raw materials. The fermented doughs (Duilio, Iride and Simeto, all from Valledolmo) showing the lowest pHs (5.0–5.2) and the highest levels of TTA (5.3–5.6 mL NaOH/10 g) showed the presence of *Lb. brevis* and/or *Lb. plantarum*.

The distribution of the LAB species was quite different among *T. durum* varieties. The highest biodiversity of LAB associated to ears and kernels was found for Iride, while the lowest was found for Simeto which harboured only *Ln. citreum* on ears, but did not show the presence of cultivable LAB on kernels. In semolinas, Duilio showed the highest richness of species (*E. casseliflavus*, *Lb. brevis*, *Lc. lactis* and *P. pentosaceus*). Semolinas from Duilio and Simeto showed a high diversity of pediococci. Enterococci were not found to dominate LAB populations after fermentation of doughs processed from Simeto variety hosting only one strain of *E. faecium*. Lactococci were present at high levels in all doughs independently on the variety. *Lb. plantarum* was found in doughs from all varieties, while *Lb. brevis* were associated only with the dough of Duilio and Iride semolina.

Regarding the geographic areas, the species distribution was strongly diverse. In particular, Valledolmo showed the lowest diversity of LAB, both in terms of species and strains, on ears and kernels. On the contrary, this area was characterised by the highest richness of *E. faecium* strains isolated from semolinas. The number of *Lc. lactis* strains isolated from doughs was almost equally distributed among the five areas. Lactobacilli were almost area specific with *Lb. brevis* found only in doughs from Valledolmo and *Lb. coryniformis* in those of Campofiorito and Valledolmo. The doughs from Contessa Entellina B did not show any *Lactobacillus*. The maximum number of species and strains was found for doughs from Valledolmo made with Duilio semolina, while the doughs from Campofiorito and Contessa Entellina A made with Duilio and Iride semolina, respectively, did not contain LAB at dominating levels.

To gauge the distribution of the 51 strains (118 isolates from different samples) their presence was mapped (Fig. 4). This map shows the LAB biodiversity associated to the different sources of isolation and allows finding quickly the strains shared by different samples. The species that dominated the first fermentation step was undoubtedly *Lc. lactis*. The simplified bipartite graph clearly shows that 14 strains of this species were found in more than one sample, including semolinas of different varieties cultivated either in the same site or in different geographical areas. It is worth noting that one *Lc. lactis* strain (indicated with the isolate reference numbers 200583, 200592, 200627, 200647, 200684, 200695 and 200703) was identified in seven doughs produced with semolinas different for variety (Iride, Duilio and Simeto) and geographical area (all five cultivation sites). The strains of *Lb. coryniformis*, *Lb. pentosaceus*, *Ln. citreum* and enterococci were mainly represented on the external parts of wheat or in semolinas, those of *Lc. garvieae* were distributed equally on ears, kernels and doughs, while those

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**Fig. 3.** Species distribution and strain proportion of LAB isolated from ears, kernels, semolinas and fermented doughs. Abbreviations: *E*. *Enterococcus*; *Lb*. *Lactobacillus*; *Lc*. *Lactococcus*; *Ln*. *Leuconostoc*; *P*. *Pediococcus*; *Du*. Duilio; *I*. Iride; *Sa*. Saragolla; *Si*. Simeto; *Ca*. Campofiorito; *C*. Ciminina; CEA, Contessa Entellina A; CEB, Contessa Entellina B; V, Valledolmo.
Fig. 4. Distribution of the LAB strains among samples. Colours for rectangles: grey, LAB species; black, Dullio; pink, Iride; orange, Saragolla; white, Simeto. The length of grey rectangles is in linear proportion to the number of isolates from different samples. Black lines indicate the presence of a strain in single samples. Lines with color different from black indicate the presence of a strain in more than one sample. Abbreviations: Ca, Campo Fiorito; Ci, Ciminna; CEA, Contessa Entellina A; CEB, Contessa Entellina B; V, Valledolmo. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
of *Lb. brevis* and *Lb. plantarum* were associated to the doughs.

The distribution of the species among wheat varieties, geographical areas and samples was also statistically confirmed by the correspondence analysis, as shown in Fig. 5. Considering the factors F1 and F2, the inertia explained was 87.58% for the *T. durum* varieties (Fig. 5A), 78.46% for the sites of cultivation (Fig. 5B) and 85.29% for the source of isolation (ears, kernels, semolinas and fermented doughs) (Fig. 5C). In all cases the greatest diversity was explained by F1.

3.6. Illumina data analysis of the microbial population in dough samples

In order to better investigate the microbial populations present soon after mixing of semolina with water, the doughs at T0 were analysed by MiSeq Illumina. The DNA extracted from the 20 unfermented dough samples was always successful in amplification of the bacterial V3-V4 16S rRNA gene region. After splitting quality trimming of raw data, 115,118 reads remained for subsequent analysis. By performing alignment, OTUs were clustered at a 3% distance and the doubles and singletons (OTUs counting only two reads) were discarded by a filter script implemented in QIIME. Chao1 estimator predicted an average of 106 ± 32 OTUs in dough samples and the average of observed count was 67 ± 15 OTUs suggesting that we were able to capture about 63% of the OTUs estimated as present in the dough bacterial populations.

3.7. Characterisation of unfermented dough microbiota by Illumina analysis

In all the samples, some OTUs were identified as not belonging to bacteria but to chloroplasts. These OTUs were not very abundant and accounted for never more than 6.4% of the total reads. The relative abundances (%) of the bacterial genera identified in unfermented doughs are reported for semolina variety (Fig. 6A) and area (Fig. 6B). Only taxonomic groups with an incidence of 0.1% in at least one sample were considered. Basically, seven bacterial genera were detected. The LAB group was represented by three genera (*Enterococcus, Lactococcus* and *Lactobacillus*). However, the major part of OTUs belonged to *Erwinia, Enterobacter* and *Bacillus*, while LAB did not exceed 2.7%, considering all doughs of Ciminna (Fig. 4A). To better analyse the distribution of the OTUs, their relative abundances are also shown per bacterial genus (Fig. 7). Among LAB, enterococci are mainly associated to Duilio doughs, followed by Simeto doughs, while lactococci are at the highest percentages (in similar proportions) in Duiilio and Saragolla doughs (Fig. 7A). *Leuconostocaceae* were only detected in Iride doughs. Regarding the geographical distribution, LAB were detected as follows: *leuconostocs* exclusively in doughs of Campofiorito, *Lactococcus* in doughs of Valedolmo and Ciminna, and *Enterococcus* mainly in doughs of Ciminna and Campofiorito, and, at a lesser extent, in those of Contessa Entellina A (Fig. 7B). OTUs corresponding to *Enterococcus* and *Lactococcus* were also found for two doughs of Contessa Entellina B, but their relative abundances were below 0.1% (0.01% for *Enterococcus of Saragolla* doughs and 0.05% for *Lactococcus of Simeto* doughs).

In order to retrieve information at species level, the OTUs belonging to *Lactococcus, Enterococcus* and *Leuconostocaceae* were manually blasted against the NCBI database. All the *Lactococcus* OTUs were identified as belonging to *Lactococcus lactis* species confirming the isolation data. The *Enterococcus* OTUs were identified as mainly belonging to *Enterococcus faecium* (2 OTUs representing the 84% of the reads classified as enterococci by QIIME). The minority of enterococci (only 1 OTU representing the 14% of all reads) was identified as belonging to *Enterococcus casseliflavus*. The only one OTU recovered for *Leuconostocaceae* family was identified as belonging to *Weissella cibaria*.

4. Discussion

Sourdough fermenting LAB are well known. Several works have been carried out to investigate their diversity, dynamics, interactions and properties during the fermentation of numerous sourdough types produced with different raw materials, ingredients and technologies. Recently, De Vuyst et al. (2014) and Minervini et al. (2014) reviewed these works. So far, a few papers reported the LAB composition of the raw materials, mainly flours/semolinas (Alfonzo et al., 2013; Ercolini et al., 2013), which could transfer their indigenous strains to the doughs. To our knowledge, only Corsetti et al. (2007a) tried to trace the source of LAB found in mature sourdoughs, analysing the LAB populations of several samples of durum wheat kernels, but the influence of field environment and wheat variety on the species/strain composition has not been investigated. Recently, Salvucci et al. (2016) isolated LAB from cereals with the only aim of investigating their technological traits useful to improve the nutritional properties and safety of food products.

In this work, the LAB populations of interest in sourdough production were followed from field to the first fermentation, which represents the initial step of the sourdough process. For this
reason, ears were collected and manipulated in controlled sterile conditions to follow the fate of LAB naturally occurring on these matrices after threshing, milling and fermentation of the semolina/water mixture. To explore the influence of the geographical areas and T. durum varieties on the LAB communities, wheat ears of four genotypes widely cultivated in Sicily and different for plant growth habit, grain yield potential and year of release (Rusini et al., 2015) were collected from five sites of production within the Palermo province, transformed and analysed at each phase of manipulation (ears, kernels, semolinas and fermented doughs). 

Due to the low levels of microorganisms generally detected on kernels (Corsetti et al., 2007a,b; Salvucci et al., 2016), in this study PCA was also used to ascertain the presence and estimate the levels of the total microbiota of the samples analysed. Cultivable LAB were investigated on different media, two generally used for food LAB (MRS and M17) and two specifically applied for sourdough LAB (SDB and SFM). The last two media were included to retrieve the primary source of LAB with potential to dominate the mature sourdough communities. 

Ears hosted up to 5.3 Log CFU/g of TMM, but the levels of presumptive LAB on the other media were consistently lower. In general, in the unfermented samples (ears, kernels and semolinas) the numbers of bacteria estimated on M17 were higher than those counted on MRS and, especially, those observed on SDB and SFM. 

The careful removal of broken kernels excluded the contamination of the outer layers with the microorganisms present in the inner layers. Kernels were generally characterised by lower counts than ears. For almost the half of samples analysed, the levels of LAB were below the detection limit. The highest cell densities (up to 2.7 Log CFU/g) were registered of M17. These data are almost in accordance with those reported by Corsetti et al. (2007a). In that study, Duilio kernels were characterised by undetectable levels of LAB and iride harbouring up to 2.1 Log CFU/g.

After milling, whole-meal semolinas were obtained from kernels. Thus, the LAB found in semolinas include those hosted on the external parts of kernels. An increasing trend of the levels of LAB was observed in semolinas. Berghofer et al. (2003) stated that, although the microorganisms that contaminate cereals are more concentrated in the outer layers of kernels and the flour should theoretically contain a lower bacterial load than caryopses, the conditioning phase before milling can increase flour’s microbial content. Furthermore, Minervini et al. (2015) demonstrated that LAB are also endophytic components of wheat plants. Thus, when they are found in semolina, they probably originate from inner layers of kernels. The levels of LAB in semolinas estimated in this work, are lower, on average, of about 1–1.5 Log cycles than those reported for the semolinas analysed by Alfonzo et al. (2013) which were collected in bakeries from semolina stocks kept at ambient temperature for longer periods. Furthermore, in our study milling was carried out in laboratory (controlled) conditions, whereas commercial milling would contribute to microbial contamination of flour.

After almost 1 day of incubation, the fermented doughs generally increased their loads on all media used for LAB. The results displayed by SDB and SFM confirmed that LAB of relevance in sourdoughs constitute the minor part of total LAB that were counted at higher levels on M17 and MRS. 

The main objective of this study was the evaluation of the transfer of LAB from ears to kernels, from kernels to semolinas and, finally, from semolinas to doughs. Thus, the main hypothesis of the work was to demonstrate that LAB from ears and kernels influence the dynamics of sourdough LAB. To pursue this objective, LAB were isolated not only to identify the metabolically active species during each step of contamination among samples (from ears to doughs), but also to characterise their polymorphic profiles in order to follow and monitor their evolution and to trace their effective transfer.

Ears were contaminated by P. pentosaceus, Ln. citreum, Ln. garvieae and Lb. coryniformis. After threshing, only E. casseliflavus and Ln. garvieae were found on kernels, but the semolinas harboured E. faecium, E. casseliflavus, Lc. lactis, P. pentosaceus, Ln. citreum and Lb. brevis. Since ears, kernels and semolinas are three unfermented matrices, a different composition among them due to the competition for nutrients has to be excluded. These findings could be due to the fact that the LAB present on the external parts of the ears were mostly removed, but remained on kernels, even though at low levels. During milling, microorganisms from the surface of kernels and from inner layers of kernels contaminated semolinas. This would explain why P. pentosaceus and Ln. citreum are found on ears and semolinas, but not on kernels. Furthermore, Lb. coryniformis was not even detected in semolinas, but it was recognised in the fermented doughs. The confirmation of these assumptions was obtained by comparison of the RAPD-PCR profiles of the isolates after each step of wheat manipulation which showed that, in some cases, they represented the same strain (Figs. 1 and 2 report only

**Fig. 6.** Relative abundances (%) of bacterial genera identified by MySeq Illumina in unfermented doughs represented per T. durum variety (A) and geographical site (B). Only genera occurring at > 0.1% abundance in at least one sample were included.

**Fig. 7.** Distribution of the relative abundances (%) of bacterial genera identified by MySeq Illumina in unfermented doughs among T. durum varieties (A) and geographical sites (B). Only genera occurring at > 0.1% abundance in at least one sample were included.
the source of first isolation). Enterococci were not detected on ears, but they were found on kernels and then in semolinas. Corsetti et al. (2007a) reported that these bacteria were the most frequently LAB isolated from durum wheat kernels. Several Enterococcus strains were also found after enrichment of the kernels from different wheat varieties cultivated in Argentina (Salvucci et al., 2016). Their presence at high numbers was not previously observed for the semolinas collected from Sicilian bakeries (Alfonso et al., 2013), probably because the maintenance at room temperatures determined a different equilibrium among LAB species. Corsetti et al. (2007a) reported also that strains of Lc. garvieae were isolated at consistent percentages from wheat kernels. In this work, Lc. garvieae was not found in the whole-meal semolinas, even though it was detected on kernels. This result could be explained by the very low levels of kernel Lc. garvieae strains that constitute only a small percentage of semolina LAB and, for this reason, not detected after growth on agar media.

After fermentation, the LAB found to dominate the microbiota of the doughs belonged to the following species: E. faecium, Lc. garvieae, Lc. lactis, Lb. coryniformis, Lb. brevis and Lb. plantarum. The work of Ercolini et al. (2013) reported that the LAB community of durum wheat dough, at the first fermentation step, ascertained exclusively by a culture-independent approach, was composed of Lactococcus, Lactobacillus, Leuconostoc and Weissella. In our work, the comparison of the polymorphic profiles showed that, in some cases, the strains (especially belonging to Ln. citreum and Lc. garvieae) found at the highest level originated from ear or kernel. Regarding lactobacilli, except for Lb. coryniformis, those present in fermented doughs were not found to originate from the external parts of wheat. Only one Lb. brevis strain was isolated from semolina and then found in doughs, but the other strains of this species and all Lb. plantarum were only detected after fermentation. Minervini et al. (2015) demonstrated that several LAB, including Lb. plantarum, Lb. brevis and lactococci were associated with plant organs during the life cycle of the durum wheat (Odisseo and Saragolla varieties) and found in semolina. Thus, it is possible that lactobacilli found in fermented doughs originate from inner layers of plant organs rather than the external parts of ears and kernels. Both routes if contamination occurred by Enterococcus.

The absence of colonies of Lb. plantarum developed from semolina samples might be due to the presence of this bacterium in a dormant state (Corsetti et al., 2007a). The presence of Lb. brevis in the fermented dough determined a consistent decrease of dough pH. Due to its performances during sourdough production, Lb. brevis is available as commercial starter (Meignen et al., 2001). Strains of Lb. plantarum have been recently reported to codominate with obligate heterofermentative LAB, especially Lb. brevis, in several sourdough samples made with semolina collected in western Sicily (Ventimiglia et al., 2015). Thus, this study support the assumption that LAB populations present on raw materials can influence the fermenting LAB dominating during sourdough fermentation, although the species and strain distribution of LAB clearly suggested that the microbiota contaminating ears and the outer part of the kernels is scarcely transferred to semolina and dough.

The culture-dependent approach allowed the monitoring of the strains among samples. The finding of a given strain from ears to fermented doughs clearly indicated the influence of the environment of cultivation on the dough LAB. Some strains were found on different varieties in the same geographical area. These findings are not surprising, because the different varieties were cultivated in adjacent fields within each site. Furthermore, some strains were found in different sites. This observation might be explained by the fact that the seeds planted in the different areas came from the same batches. Thus, seeds may act as vectors for LAB dissemination.

The culture-dependent approach alone does not allow to detect all the bacteria present in the unprocessed matrices, such as semolinas. For this reason, and especially to evaluate the influence of the geographical area and the variety on the LAB populations of semolinas, the doughs were processed by MiSeq Illumina soon after mixing of semolina with water, prior the fermentation took place. Basically, the LAB group of the unfermented doughs included, at abundances above 0.1%, only the genera Enterococcus, Lactococcus and members of the Leuconostocaceae family, indicating that pediococci and lactobacillii constituted only an extremely low percentage of semolina LAB.

In general, the plant-associated habitat is a dynamic environment in which many factors may affect the structure and species composition of bacterial communities (Araújo et al., 2002). Correspondence analysis evidenced a statistical significant influence of the geographical area in LAB species distribution, even though the cultivations were carried out at no more than 20–30 km from one another. Furthermore, wheat variety affected LAB. However, only for Lc. factsis the distribution was reflected along the soil/plant-dough axis. The lack of correspondence between the presence of Lb. plantarum and Lb. brevis in the fermented doughs and their absence on ears and kernels and in semolinas could be explained by the levels of these two species below the detection limit in the unprocessed raw materials. It is known that the type of flour, process technology, and other factors strongly influence the composition of sourdough microbiota (De Vuyst et al., 2002; Meroth et al., 2003; Scheirlinck et al., 2007).

With this study, we have provided evidence that the LAB composition of semolinas differ according to wheat variety and geographical area of production, but the findings that some strains are ubiquitous necessitates further studies to clarify the role of seeds in the dissemination of these bacteria.

Acknowledgements

This work was financed by the project “Innovation for Cereal-iculture in Sicily, I.C.S.” funded by “Assessorato alle Attivita Produttive” of Regione Siciliana with OP-ERDF 2007–2013 funds and the project for industrial research and training, PON01_00249 (“Application of Molecular Biotechnologies and Proteological Microorganisms for the Characterization and Valorisation of Dairy and Bakery Chains of Typical Products”), of the Italian Ministry of Education, University and Research (CUP:B11C1100430005).

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