Lactic Acid Bacteria in Durum Wheat Flour Are Endophytic Components of the Plant during Its Entire Life Cycle

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This study aimed at assessing the dynamics of lactic acid bacteria and other *Firmicutes* associated with durum wheat organs and processed products. 16S rRNA gene-based high-throughput sequencing showed that *Lactobacillus*, *Streptococcus*, *Enterococcus*, and *Lactococcus* were the main epiphytic and endophytic genera among lactic acid bacteria. *Bacillus*, *Exiguobacterium*, *Paenibacillus*, and *Staphylococcus* completed the picture of the core genus microbiome. The relative abundance of each lactic acid bacterium genus was affected by cultivars, phenological stages, other *Firmicutes* genera, environmental temperature, and water activity (a_w) of plant organs. Lactobacilli, showing the highest sensitivity to a_w, markedly decreased during milk development (Odisseo) and physiological maturity (Saragolla). At these stages, *Lactobacillus* was mainly replaced by *Streptococcus*, *Lactococcus*, and *Enterococcus*. However, a key sourdough species, *Lactobacillus plantarum*, was associated with plant organs during the life cycle of Odisseo and Saragolla wheat. The composition of the sourdough microbiota and the overall quality of leavened baked goods are also determined throughout the phenological stages of wheat cultivation, with variations depending on environmental and agronomic factors.

Lactic acid bacteria are ubiquitous microorganisms with potential beneficial effects on crop and livestock production (1). Together with lactic acid bacteria, other *Firmicutes* (e.g., *Bacillus* species) are ubiquitous, often found as soil inhabitants and easily transferred from soil to roots. Some strains of *Bacillus* produce cytokinins, thus acting as plant growth-promoting bacteria (2). Other strains inhibit the pathogenic fungus *Fusarium graminearum* in durum wheat (3). From the agronomic point of view, wheat is a well-characterized crop, with a worldwide economic relevance, whose associated bacteria could influence plant growth (4). Several plant-associated bacteria contaminate grains, and hence flour, affecting the overall quality of leavened baked goods (5, 6). It is well known that some spore-forming bacteria (e.g., *Bacillus* species) cause rope spoilage in bread (7).

Some *Bacillus* and other undesired microorganisms (e.g., molds belonging to *Aspergillus* and *Penicillium*) can be inhibited by the use of sourdough, a biological leavening agent containing high numbers of yeasts and, especially, lactic acid bacteria (8, 9). Other advantages that are consistent with the use of sourdough regard the sensory, shelf life, and nutritional features of leavened baked goods. Most of these advantages are attributed to the metabolisms of lactic acid bacteria (10). They are not deliberately added but are naturally present in the dough. Indeed, traditional sourdough originates from multiple steps (backslopping) of fermentation. Since they are not added, the bakery environment (the bakers, insects, and animals) and the flour are the two main routes for contaminating lactic acid bacteria. Although an abundance of the literature has dealt with the characterization of the mature sourdough microbiota (11–16), the origin of lactic acid bacteria has only in part been elucidated. Some lactic acid bacteria circulate throughout the environment of propagation. *Lactobacillus sanfranciscensis*, which was detected in the air of the storage and work rooms, as well as on benches and dough mixers, dominated in the related traditional sourdoughs (17, 18). *Lactobacillus plantarum* and *Lactobacillus spicheri* dominated in the bakery environment and in the sourdough produced in the same bakery (17). Evidence

has indicated that sourdough fermentations carried out in the laboratory with the flour as the only nonsterile ingredient were characterized by species and/or strains of lactic acid bacteria different from those in artisan sourdoughs (14, 19–22). This would suggest that the flour microbiota shares very few taxa with that of the corresponding mature sourdough. Evidence has suggested that the flour microbiota was affected by contamination from the bakery environment, especially from humans. For instance, sourdough isolates of *Lactobacillus reuteri* were human lineage strains (23). Furthermore, many species (e.g., *Lactobacillus rossiae*, *L. plantarum*, and *L. reuteri*) isolated from type II sourdoughs were also identified from rodent and/or swine feces (24–26). Apart from this contamination, the inherent presence of lactic acid bacteria in the flour also has to be considered. Nevertheless, lactic acid bacteria contaminating cereals in the outer layers of kernels are removed during milling and tend to stay in bran-rich fractions (27). Consequently, the lactic acid bacteria contaminating flour or semolina should theoretically be a part of the endophytic microbiota of cereals. Epiphytic lactic acid bacteria were isolated from forage crops (28, 29), and lactobacilli inoculated into *Lolium perenne* were able to colonize roots at the endophytic level (30). To

the best of our knowledge, no data are available for the microbiota of *Triticum aestivum* or *Triticum durum* cultivars during plant cultivation.

This study was aimed at assessing, through culture-dependent and -independent approaches, the composition of the wheat plant microbiota during the different phases of growth and to assess the extent of flour contamination by endophytic lactic acid bacteria and other *Firmicutes*, which from the field become relevant for sourdough fermentation.

MATERIALS AND METHODS

Plant growth and sample collection. The Odisseo and Saragolla varieties of durum wheat were cultivated in slimy loam soil (36% sand, 40% silt, and 24% clay) in Gravina di Puglia (Bari, Italy). For each cultivar, four comparable allotments (named A, B, C, and D), with individual sizes of 1.5×15 m, were established at approximately 500 m apart within the same field in November 2013. Plants were harvested from the following stages of crop growth: tillering, stem elongation, booting, flowering, milk development, and physiological maturity. At each sampling time, a group of 10 plants was carefully removed with intact roots, and the soil adhering to the roots was removed by shaking. Plants were divided by means of a sterile scalpel into hypogeous (roots) and epigeous (leaves or spikes, depending on the phenological stage) organs, which were inserted into Whirl-Pak sterile sampling bags (Nasco, Fort Atkinson, WI), kept at a cool temperature, and processed in the laboratory within 4 h of collection.

Confocal laser scanning microscopy (CLSM). Small pieces of roots and leaves were taken from wheat and put in isopentane cooled by liquid nitrogen at $-150°C$, in order to freeze the samples without formation of ice crystals. Twenty-micrometer-thick sections were achieved at $-27°C$ using a cryotome. After drying on a slide, sections were stained by using a LIVE/DEAD BacLight bacterial viability kit (Molecular Probes, Cambridge Bioscience, Cambridge, United Kingdom) according to the manufacturer's instructions. After 15 min, sections were rinsed using distilled water, and an upper lip was sealed on the section using nail polish. Microscopic observations were performed using a Leica confocal microscope (Leica Microsystems, Heidelberg GmbH, Wetzlar, Germany) in epi mode (31).

DNA extraction from plant organs and processed grain samples. Ten-gram quantities of washed plant organs (roots and leaves or spikes) were immersed in 200 ml of DNA-free 0.1 M potassium phosphate buffer (pH 7.0) in a 500-ml Erlenmeyer flask and treated (7 min at 30°C) in an ultrasonic bath (RK103H Sonorex Super, Ultra Center Europe, Zwolle, The Netherlands) (32). Plant organs were taken out of the flask for further treatment, whereas epiphytic microorganisms released into the buffer were pelleted by centrifugation ($20,000 \times g$ for 30 min at 4°C) and then resuspended in 5 ml of potassium phosphate buffer (33). Suspensions of epiphytic microorganisms were used as starting material for extraction of DNA. Plant organs were surface sterilized by soaking in 150 ml of a 15% (vol/vol) H_2O_2 solution. They were treated for 15 min with gentle rotary shaking, protected from light. Then plant organs were twice washed in sterile demineralized water and finally dried in a laminar flow hood for 1 h (34). Surfaces of plant organs were checked for sterility by blotting them tightly on 1/10-strength tryptic soy agar and incubating plates at 30°C for 48 h (35). No growth was observed. Surface-sterilized plant organs were homogenized (3 min) with 80 ml of 0.1 M potassium phosphate buffer (pH 7.0) in a BagMixer 400P (Interscience, Saint Nom, France) blender. The homogenate, free of plant debris, contained endophytic microorganisms and was used as starting material for extraction of DNA.

Processed grain samples were obtained as follows. Chaff was removed from grain by using a Wintersteiger AG (Ried, Austria) thresher (model Seedmech Classic) and stored for further analyses. The grain was further separated from residual chaff and tempered to a moisture content of 18%. An aliquot of tempered grain (100 g) was used for extraction of epiphytic and endophytic microorganisms. Epiphytic microorganisms were extracted according to the protocol described for plant organs. Endophytic

microorganisms were extracted upon grinding of chemically surface-sterilized grain, devoid of epiphytic microorganisms. Ten grams of ground grain was homogenized (3 min) with 90 ml of 0.1 M potassium phosphate buffer (pH 7.0) in a BagMixer 400P blender. The homogenate, free of grain debris, containing endophytic microorganisms, was used as starting material for extraction of DNA. Another aliquot of tempered grain (10 kg) was milled into flour with a Bühler-Miag MLI-204 grinder (Bühler S.p.A., Segrate, Milan, Italy). Chaff, bran, and flour were used for detection of lactic acid bacteria and for DNA extraction. Commercial grains and the related bran and flour were also analyzed as controls.

Genomic DNA was extracted from 0.5 ml of suspensions containing either epiphytic or endophytic microorganisms, using the FastDNA Pro Soil-Direct kit (MP Biomedicals, Santa Ana, CA) coupled to the FastPrep instrument (MP Biomedicals), according to the protocol described by Minervini et al. (36). The quality and concentration of DNA extracts were assayed by spectrophotometric measurements using a NanoDrop ND-1000 (Thermo Fisher Scientific Inc., Marietta, OH).

Illumina MiSeq analysis. DNA extracted from plant organs collected in each of the four allotments (A, B, C, and D) was pooled and used as the template for Illumina MiSeq diversity analyses, which were carried out at the Research and Testing Laboratory (RTL; Lubbock, TX). Primers Firm350F and Firm814R were used to amplify a fragment of the 16S rRNA gene for analysis of diversity inside the phylum of *Firmicutes* (37). PCR and sequencing analyses were carried out according to the protocol of the RTL.

The sequenced reads were processed through denoising and chimera detection. Denoising was performed through the following steps: (i) merging together the forward and reverse reads using the PEAR Illumina paired-end read merger (38); (ii) reading the runs through an internally developed quality trimming algorithm that truncates reads having an average quality higher than 25; (iii) grouping reads by using the USEARCH (39) algorithm (prefix dereplication) into clusters (4% dissimilarity among sequences of the same cluster), so that each sequence of shorter length to the centroid sequence must be a 100% match to the centroid sequence for the length of the sequence; and (iv) operational taxonomic unit (OTU) selection by using the UPARSE OTU selection algorithm (40) to classify the large number of clusters into OTUs. Following denoising, the selected OTUs were chimera checked using the UCHIME software executed in *de novo* mode (41). Each trimmed read was mapped to its corresponding nonchimeric cluster using the USEARCH global alignment algorithm (39). Each sequence in a cluster was then aligned to the consensus sequence. Each sequence was corrected base by base in order to remove noise. Analysis of microbial diversity was finally performed by running the centroid sequence from each cluster against the USEARCH algorithm, using a database of high-quality sequences derived from the NCBI database. Lastly, the output was analyzed using an internally developed python program that assigns taxonomic information to each sequence.

The percentage of each bacterial OTU was analyzed individually for each sample, providing relative abundance information among the samples based on the relative numbers of reads within each (42).

Enumeration and isolation of endophytic lactic acid bacteria from plant organs. Surface-sterilized plant organs and chemically surface-sterilized grain and flour were used. Ten-gram quantities of roots, leaves, and spikes were immersed in 90 ml of sterile 0.1 M potassium phosphate buffer (pH 7.0) and homogenized for 3 min in a BagMixer 400P. Endophytic microorganisms from 10 g of grain or flour were also analyzed. Lactic acid bacteria were enumerated and isolated from plant organs, grain, and flour by the use of two selective media: de Man, Rogosa, and Sharpe medium (MRS) with maltose (10 g liter^{-1}) and fresh yeast extract (50 ml liter^{-1}), pH 5.6 (modified MRS [mMRS]), and sourdough bacterial medium (SDB) (43). Cycloheximide (0.1 g liter^{-1}) was added to both media in order to prevent fungal growth. One milliliter of suspension containing endophytic microorganisms was serially diluted in sterile saline (NaCl 9 g liter^{-1}) solution and plated onto mMRS and SDB agar.

Plates were incubated at 30°C for 48 h. For each medium, a number of colonies equal to the square root of the total number recorded in plates coming from the highest dilution was randomly picked up. Gram-positive, catalase-negative, nonmotile rods and coccal isolates were cultivated in mMRS or SDB at 30°C for 24 h and restreaked onto the same agar medium. All isolates considered for further analyses were able to acidify the culture medium.

Phenotypic identification of endophytic lactic acid bacteria. Metabolic profiles of endophytic lactic acid bacteria isolated from plant organs, grain, and flour were determined using Biolog AN microplates (Rigel Life Sciences, Rome, Italy). The wells of the microplates were inoculated with 150 μl of bacterial suspensions adjusted to 0.6 ± 0.05 unit of absorbance (at a wavelength of 620 nm). Positive reactions were automatically recorded after incubation (for 24 h at 30°C under anaerobic conditions) using a microplate reader with a 590-nm wavelength filter.

Genotypic characterization of endophytic *L. plantarum* isolates by RAPD-PCR analysis. Genomic DNA of *L. plantarum* isolates was extracted using a DNeasy blood and tissue kit (Qiagen, SA, Courtaboeuf, France) according to the manufacturer's instructions (44). Three oligonucleotides, P4 (5′-CCGCAGCGTT-3′), P7 (5′-AGCAGCGTGG-3′), and M13 (5′-GAGGGTGGCGGTTCT-3′), with arbitrarily chosen sequences were used for biotyping isolates of *L. plantarum* from leaves, spikes, and processed grain samples of Odisseo and Saragolla wheat. The reaction mixture and PCR conditions for primers P4 and P7 were those described by De Angelis et al. (45). PCR conditions for primer M13 were as described by Siragusa et al. (46). Randomly amplified polymorphic DNA-PCR (RAPD-PCR) profiles were acquired by the MCE-202 MultiNA microchip electrophoresis system (Shimadzu Italia s.r.l., Milan, Italy), using the DNA-12000 reagent kit (100 to 12,000 bp) and the 2-log DNA ladder (0.1 to 10.0 kb) according to the manufacturer's instructions. The reproducibility of the RAPD profiles was assessed by comparing the PCR products obtained with primers P4, P7, and M13 and DNA prepared from three independent cultures of the same strain. Nine strains were studied, and the patterns for the same strain were 95% similar, indicating that the reproducibility of the technique under the conditions used was high.

Statistical analyses. Data were obtained at least in triplicate. Analysis of variance (ANOVA) was carried out on transformed data, followed by separation of means with Tukey's honestly significant difference (HSD) test, using the statistical software Statistica 7.0 for Windows (StatSoft, Vigonza, Italy). Weighted and unweighted UniFrac distance matrices and OTU tables were used to perform ADONIS and ANOSIM statistical tests through the compare_category.py script of QIIME to verify the microbial populations in the different plant organs. Permutation analysis was also performed for the Illumina MiSeq data. Multiple testing of corrected pairwise Spearman correlations was computed between OTU at the genus level (abundance of >0.1% in at least 5 samples) and environmental conditions. Only significant correlations (false discovery rate [FDR] < 0.050) were considered (47). Cell density of presumptive lactic acid bacteria, lactic acid bacterium OTU (genus level), environmental temperature, and water activity (a_w) of the wheat organs were analyzed by principal component analysis (PCA), using Statistica 7.0.

Nucleotide sequence accession number. The 16S rRNA gene sequences are available in the Sequence Read Archive of NCBI (accession number PRJNA268304).

RESULTS

Phenological stages of durum wheat samples and endophytic bacteria. Plants of Odisseo and Saragolla durum wheat were sampled at six phenological stages of crop growth (see Fig. S1 in the supplemental material). Compared to Odisseo, Saragolla is a premature cultivar accelerating the flowering stage of ca. 10 days (see Table S1 in the supplemental material). Consequently, flowering, milk development, and physiological maturity of the two cultivars occur under different climatic conditions (e.g., temperature and
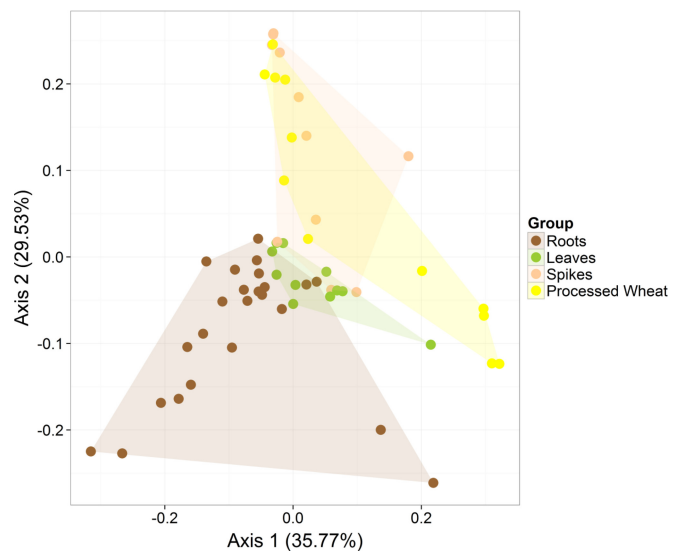

FIG 1 Principal coordinate analysis (PCoA) based on unweighted UniFrac analysis of all 16S rRNA gene sequences of epiphytic and endophytic *Firmicutes* found on hypogeous organs (roots), epigeous organs (leaves and spikes), and processed wheat (grain, chaff, bran, and flour) of Odisseo and Saragolla. Hypogeous and epigeous organs were analyzed at the tillering, stem elongation, booting, flowering, milk development, and physiological maturity stages.

rainfall). Preliminarily, the presence of bacteria in plant organs (roots and leaves or spikes) of durum wheat was analyzed by confocal laser scanning microscopy (CLSM). At the sampling times corresponding to tillering, stem elongation, and booting, leaves were analyzed. The samples of booting contained both leaves and spikes. Spikes, instead of leaves, were analyzed in correspondence to flowering, milk development, and physiological maturity. Bacterial cells were visible in all plant organs. An example is given in Fig. S2 in the supplemental material, related to roots and leaves at the stem elongation stage.

***Firmicutes* diversity through next-generation sequencing.** Total DNA from fractions of roots and leaves or spikes was amplified by *Firmicutes*-specific primers and subjected to next-generation sequencing. A distinction was made between the surface (epiphytic microbiota) and inside (endophytic microbiota) of plant organs. Total DNA from Odisseo and Saragolla grain (epiphytic and endophytic), chaff, bran, and flour was also analyzed. Commercial durum wheat grain, bran, and related flour were also analyzed. A total of 2,456,502 quality-trimmed sequences of 16S rRNA gene amplicons were obtained. The average number of sequences per sample was 40,942, with an average length of 447 bp. Good's estimated sample coverage (median value of ca. 98%; $P > 0.05$) indicated that a satisfactory coverage was reached for all the samples analyzed.

The community of *Firmicutes* was analyzed for number of operational taxonomic units (OTUs), richness estimator (ACE, Chao1), and diversity index (Shannon and Simpson) (see Table S2 in the supplemental material). The analysis revealed a significantly ($P < 0.050$) lower microbial diversity of leaves and spikes compared to that of root samples. As shown by principal coordinate analysis (PCoA), roots, leaves, and spikes of Odisseo and Saragolla wheat were clearly differentiated based on unweighted UniFrac distances (Fig. 1). In contrast, spikes and processed wheat samples were not differentiated. In addition, both ADONIS and ANOSIM
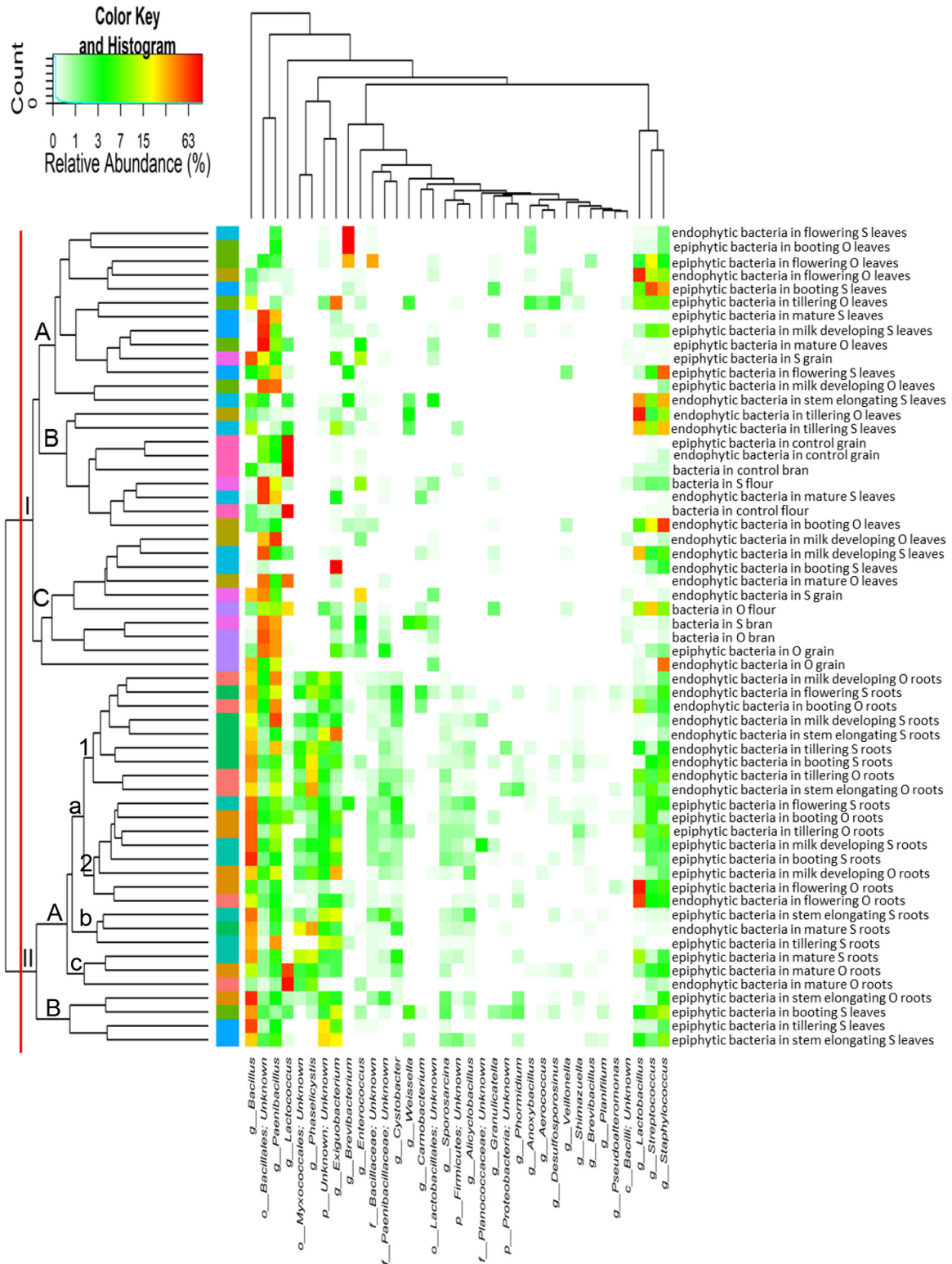
FIG 2 Heat map summarizing the relative abundances of the 35 most dominant genera in DNA samples directly extracted from epiphytic and endophytic bacteria found on hypogeous organs (roots), epigeous organs (leaves and spikes), and processed wheat (grain, chaff, bran, and flour) of the Odisseo (O) and Saragolla (S) varieties. Hypogeous and epigeous organs were analyzed at the tillering, stem elongation, booting, flowering, milk development, and physiological maturity stages. Commercial grain, bran, and flour were also analyzed as a control. The color key defines the percentages of OTUs in the samples.
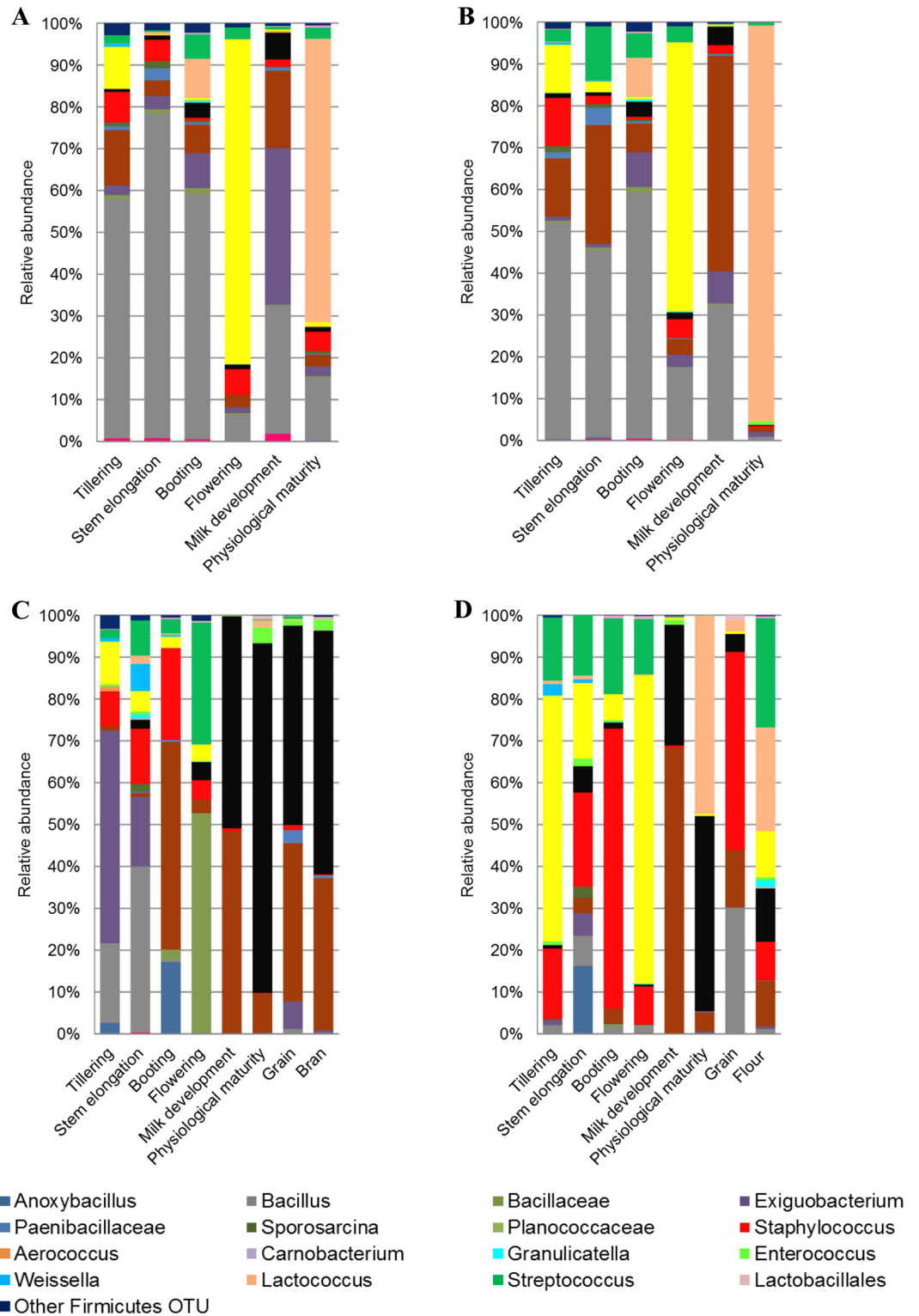
FIG 3 Relative abundance of epiphytic (A and C) and endophytic (B and D) *Firmicutes* genera found in hypogeous organs (roots) (A and B), epigeous organs (leaves and spikes), and processed wheat (grain, bran, and flour) (C and D) of Odisseo. Hypogeous and epigeous organs were analyzed at the tillering, stem elongation, booting, flowering, milk development, and physiological maturity stages.
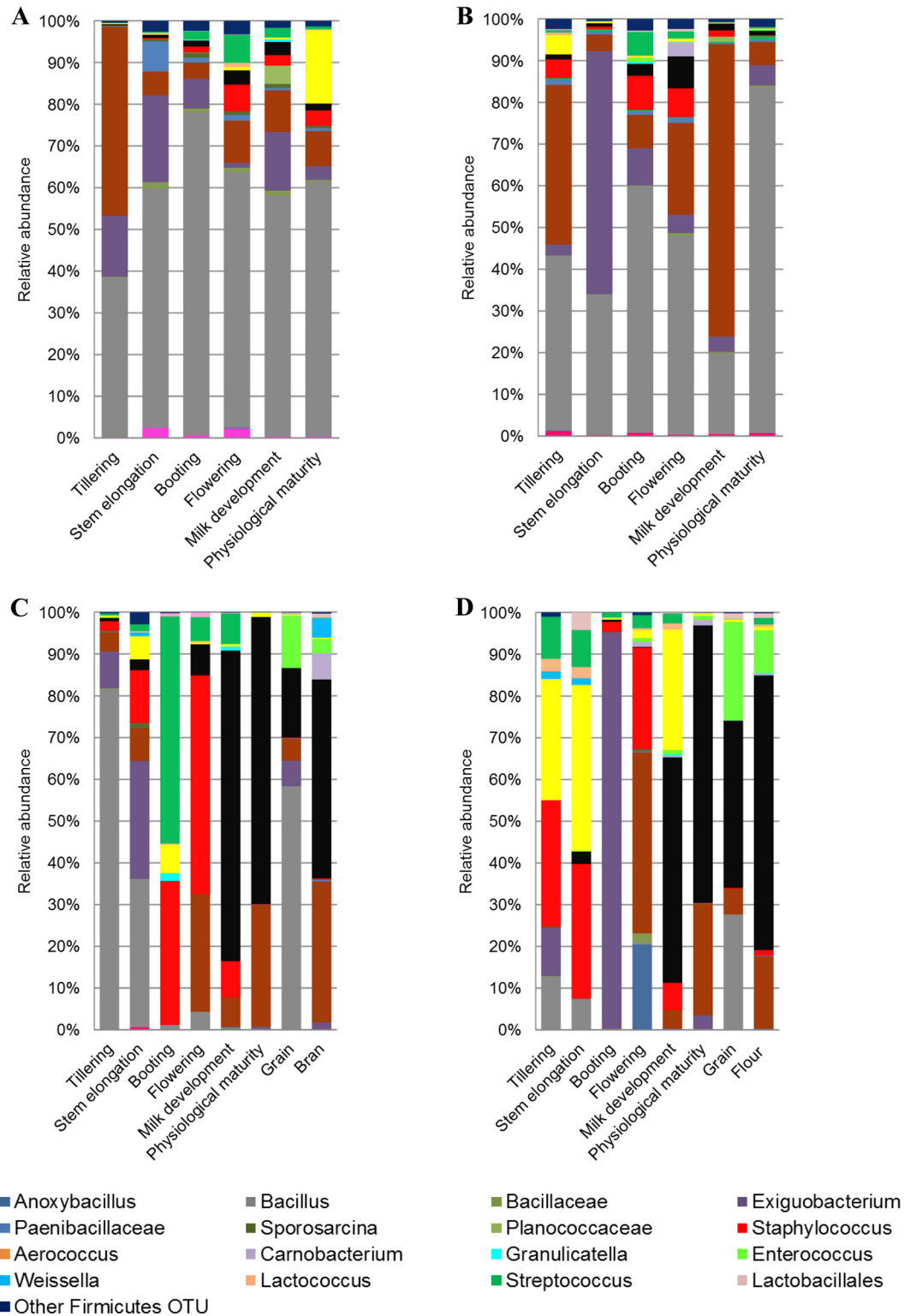
FIG 4 Relative abundance of epiphytic (A and C) and endophytic (B and D) *Firmicutes* genera found in hypogeous organs (roots) (A and B), epigeous organs (leaves and spikes), and processed wheat (grain, bran, and flour) (C and D) of Saragolla. Hypogeous and epigeous organs were analyzed at the tillering, stem elongation, booting, flowering, milk development and physiological maturity stages.
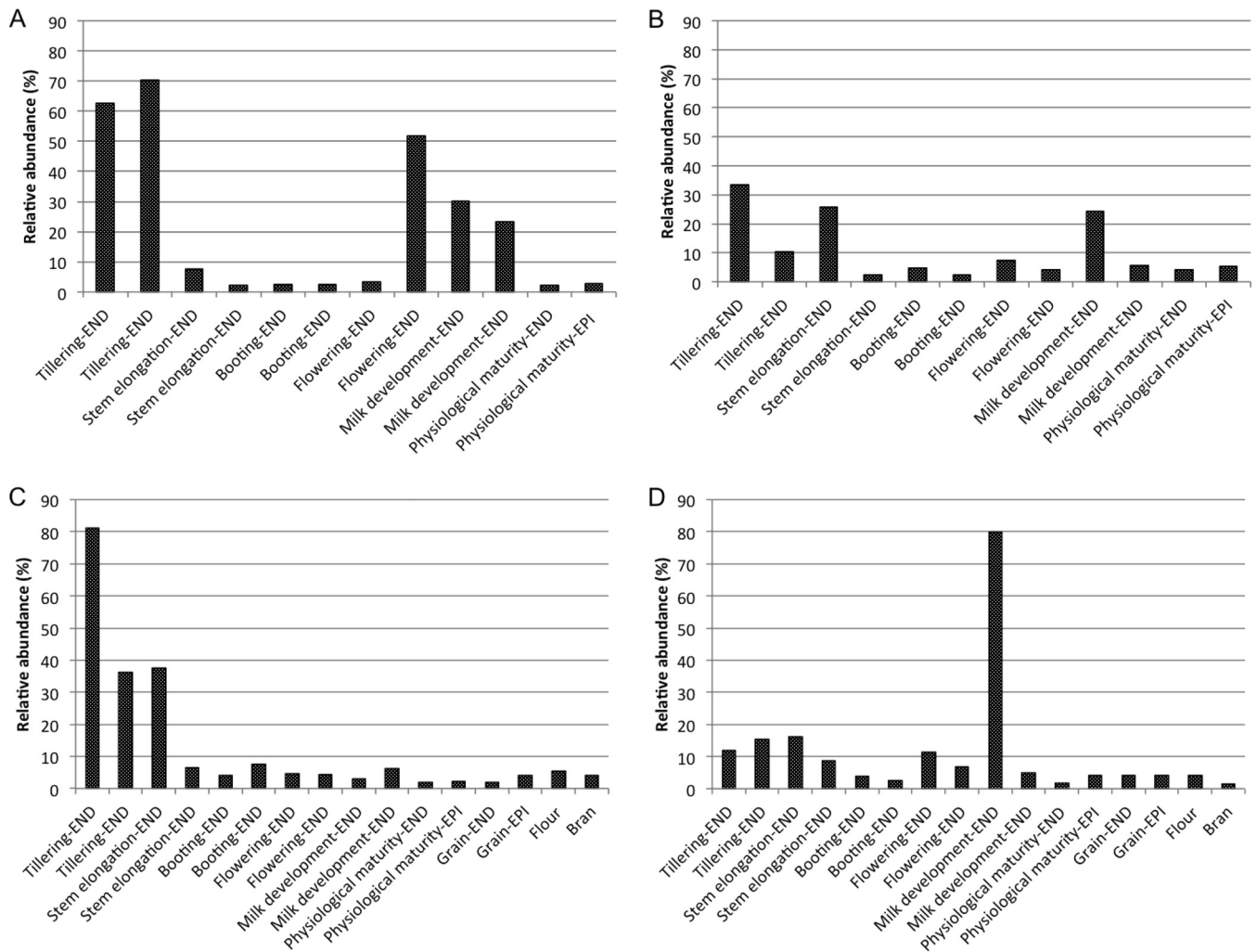
FIG 5 Relative abundance of endophytic (END) and epiphytic (EPI) *Lactobacillus plantarum* within the OTUs belonging to lactic acid bacteria found on hypogeous organs (roots) (A and B), epigeous organs (leaves and spikes), and processed wheat (grain, bran, and flour) (C and D) of Odisseo (A and C) and Saragolla (B and D). Hypogeous and epigeous organs were analyzed at the tillering, stem elongation, booting, flowering, milk development, and physiological maturity stages.

statistical tests indicated a significant influence of roots and leaves or spikes on the microbial diversity (FDR < 0.050). Leaves did not differ ($P > 0.050$) from spikes and processed wheat samples.

Core *Firmicutes* microbiome. According to alpha- and beta-diversity, and considering the 35 most dominant genera of all samples, roots and leaves or spikes were distributed in two clusters (Fig. 2). Exceptions were the epiphytic bacteria belonging to tillering, stem elongation, and booting of Saragolla wheat. With few exceptions, epiphytic and endophytic bacteria grouped in different subclusters (cluster IA versus clusters IB and IC; cluster IIAa1 versus cluster IIAa2). Samples corresponding to endophytic bacteria found in the last phenological stages (milk development and physiological maturity) clustered together with processed wheat samples of both cultivars (cluster IC). A core *Firmicutes* microbiome, defined at the genus level and occurring in >98% of the samples analyzed was constituted by *Bacillus, Exiguobacterium, Lactobacillus, Paenibacillus, Staphylococcus,* and *Streptococcus.* Besides these genera, OTUs belonging to genera *Enterococcus* and *Lactococcus* occurred in >85% of the samples.

Among *Firmicutes, Bacillus* was mainly found at the endophytic and, especially, epiphytic levels of roots throughout the wheat life cycle, whereas it was only occasionally detected in leaves and spikes (Fig. 3 and 4). *Paenibacillus* was found at the endophytic and epiphytic levels of roots regardless of phenological stage. However, the relative abundance of *Paenibacillus* was lower than that of *Bacillus* in more than 60% of root samples. In contrast, in samples of leaves and spikes, from flowering to physiological maturity, *Paenibacillus* was more frequently detected than *Bacillus. Exiguobacterium* was found in roots throughout the wheat life cycle, whereas it was rarely detected in leaves. However, it was the dominant *Firmicutes* genus at the epiphytic level of Odisseo leaves at tillering (Fig. 3C) and at the endophytic level of Saragolla leaves at the booting stage (Fig. 4D). *Bacillales* were among the dominant OTUs at milk development, at physiological maturity, and in processed wheat, especially for Saragolla.

At the endophytic level, *Lactobacillus* was the dominant *Firmicutes* genus at the flowering stage of Odisseo, both in roots and in spikes (Fig. 3B and D). *Lactobacillus* dominated also in roots at the
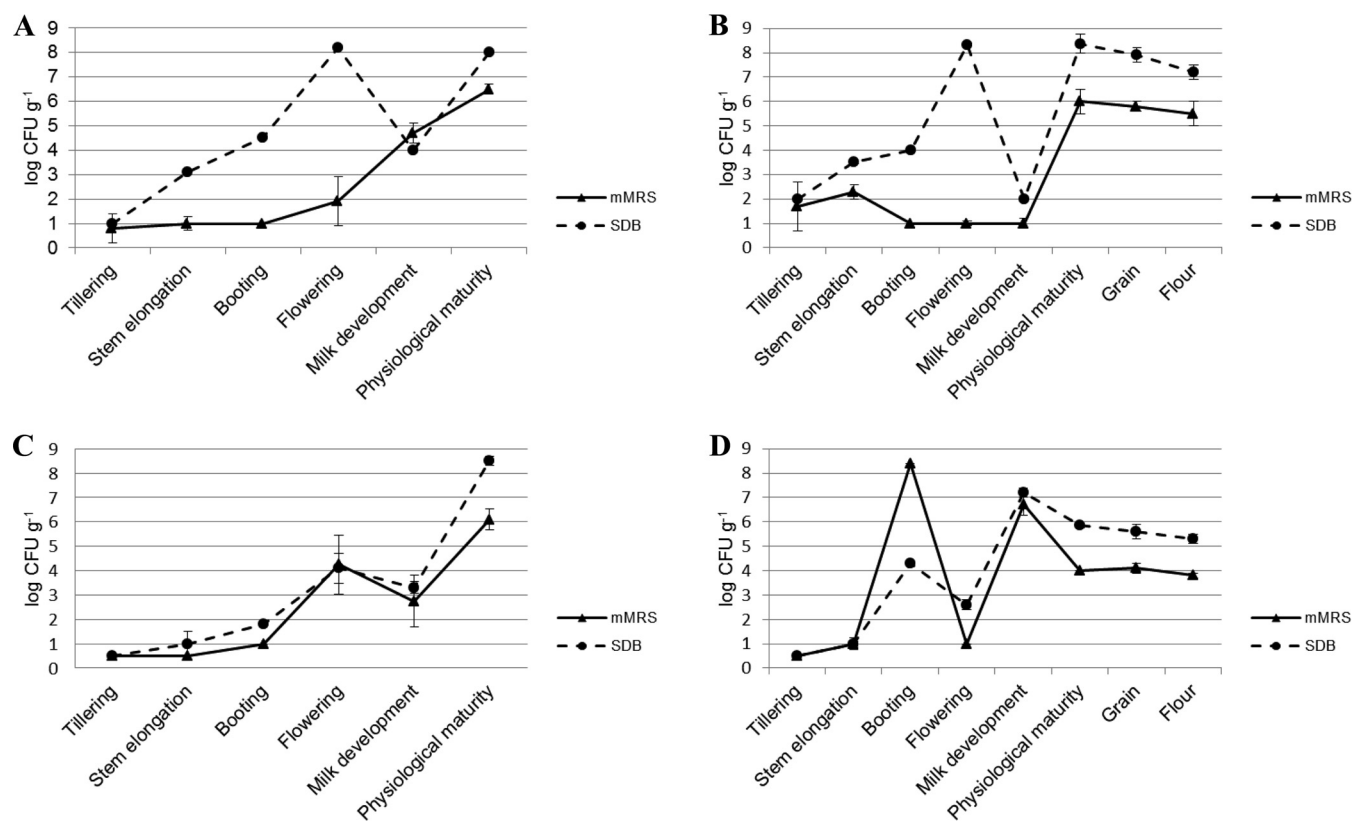
FIG 6 Cell numbers of endophytic presumptive lactic acid bacteria enumerated on mMRS and SDB in hypogeous (roots) and epigeous (leaves and spikes) organs of Odisseo (A and B, respectively) and Saragolla (C and D, respectively) durum wheat during the life cycle from tillering to flour.

epiphytic level during flowering (Fig. 3A). *Lactococcus* dominated in roots (endophytic and epiphytic levels) and spikes (endophytic level) at physiological maturity. *Lactococcus* was the lactic acid bacterium mostly present in grain at the endophytic level and dominated in flour together with *Streptococcus*. The genus *Lactobacillus* was found at a lower level in Saragolla than in Odisseo wheat (Fig. 4). At the flowering stage, *Streptococcus* was the dominant lactic acid bacterium in roots and spikes. *Lactobacillus* was the dominant lactic acid bacterial genus only at the endophytic level of spikes during milk development (Fig. 4D) and at the epiphytic level of roots during physiological maturity (Fig. 4A). *Enterococcus* was the dominant genus of lactic acid bacteria in grain at the endophytic level and dominated in flour. *Lactococcus* dominated in commercial grain and in the related bran and flour samples (see Table S3 in the supplemental material). At the species level, *L. plantarum* was the dominant species of *Lactobacillus* in almost all samples (Fig. 5; see also Table S3). Other lactic acid bacteria (*Aerococcus viridans*, *Carnobacterium maltaromaticum*, *Enterococcus faecalis*, *Lactobacillus brevis*, *Lactobacillus fermentum*, *Lactobacillus gasseri*, *Lactobacillus iners*, *Lactococcus garviae*, and *Lactococcus lactis*) were variously identified.

Cell density of endophytic presumptive lactic acid bacteria. Selective media (mMRS and SDB) were used to detect endophytic presumptive lactic acid bacteria (Fig. 6). Lactic acid bacteria were detected in all plant organs of Odisseo and Saragolla durum wheat at different phenological stages, with cell densities varying from ca. 0.5 to 8.5 log CFU g^{-1}. SDB was the medium showing the highest cell densities. Roots of Odisseo showed a higher cell den-

sity of presumptive lactic acid bacteria during tillering, stem elongation, and booting stages than did Saragolla. Roots showed the highest cell density of presumptive lactic acid bacteria during the flowering and physiological maturity stages of Odisseo and Saragolla durum wheat, respectively. Leaves and spikes showed the highest cell density during booting for Saragolla and flowering and physiological maturity for Odisseo.

Isolation and phenotypic identification of endophytic lactic acid bacteria. Presumptive lactic acid bacteria were isolated (10 colonies for roots or leaves/spikes in each phenological stage) and tested for the ability to acidify the culture medium. All Gram-positive, catalase-negative, acidifying rods or cocci were identified by Biolog AN microplates. *L. plantarum* was identified in all plant organs at all different phenological stages. Other lactic acid bacteria (*Lactobacillus helveticus*, *Lactobacillus coryniformis*, *Lactobacillus delbrueckii*, *L. sanfranciscensis*, *Weissella confusa*, *Weissella minor*, *Streptococcus anginosus*, *Leuconostoc citreum*, and *Pediococcus parvulus*) were variously identified (data not shown). Within lactobacilli, *L. plantarum* dominated in roots and samples of leaves and spikes together with *L. coryniformis* until the flowering stage. *L. plantarum*, *L. helveticus*, and *L. delbrueckii* dominated in both roots and spikes during the milk development and physiological maturity stages of Odisseo and Saragolla wheat (data not shown).

Genotypic characterization of endophytic *L. plantarum* strains. Since *L. plantarum* was the only lactic acid bacterial species identified at all phenological stages, RAPD-PCR analysis was applied to track the strains of this species. All isolates identified as *L. plantarum* were characterized by using single primer P4, P7, or
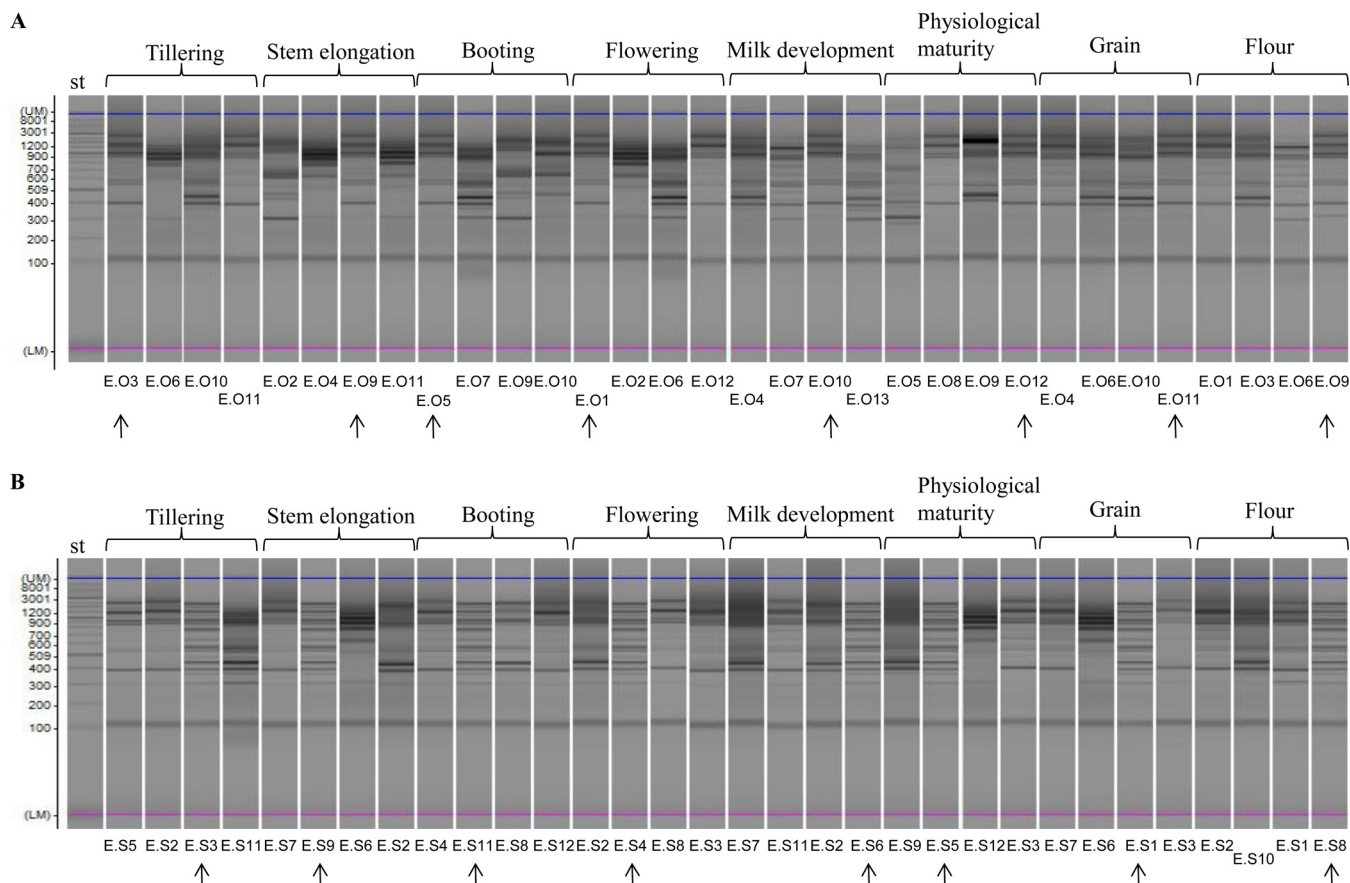
FIG 7 Representative randomly amplified polymorphic DNA-PCR (RAPD-PCR) profiles of *Lactobacillus plantarum* isolated at the endophytic level from leaves and spikes, grain, or flour of Odisseo (A) and Saragolla (B) durum wheat. Primer M13 was used for RAPD-PCR analysis. A 2-log DNA ladder (0.1 to 10.0 kb) was used as a molecular size standard (st). Capillary electrophoretic profiles were singly acquired by MultiNA. Strains isolated from different phenological stages and processed wheat and showing similar RAPD-PCR profiles are indicated by arrows.

M13. Several isolates of *L. plantarum* at different phenological stages of growth of Odisseo (Fig. 7A; see also Fig. S3A and S4A in the supplemental material) and Saragolla (Fig. 7B; see also Fig. S3B and S4B) wheat as well as in the processed wheat samples (grain and flour) (Fig. 7; see also Fig. S3 and S4) showed the same profile.

Correlation between lactic acid bacteria, environmental temperature, and wheat organ a$_w$. OTU correlation was investigated considering *Firmicutes* genus level (Fig. 8) taxonomic assignments and significant correlations at an FDR of <0.050. *Lactobacillus* showed positive correlation with *Anoxybacillus*, *Staphylococcus*, *Streptococcus*, and *Weissella*. *Lactobacillus* was negatively correlated mainly with *Bacillus* and *Paenibacillus*. *Weissella*, *Lactococcus*, and *Enterococcus* showed positive correlation with many genera, including other lactic acid bacteria (*Aerococcus*, *Granulicatella*, and *Carnobacterium*). Cell density of presumptive lactic acid bacteria, relative abundance of OTU belonging to lactic acid bacterial genera, environmental temperature, and wheat organ a$_w$ data were analyzed using PCA. The discrimination of samples between tillering, stem elongation, and booting and milk development, physiological maturity, grain, chaff, flour, and bran was evident (see Fig. S5 in the supplemental material). The cell density of presumptive lactic acid bacteria, *Lactococcus* and *Enterococcus*, was positively correlated (r > 0.5) with environmental

temperature. *Lactobacillus* showed a higher positive correlation (r = 0.72) with a$_w$ than did *Lactococcus* and *Enterococcus*.

DISCUSSION

This study used the 16S rRNA gene-based high-throughput sequencing approach targeting DNA to describe the ecology dynamics of *Firmicutes* and, especially, lactic acid bacteria during growth of durum wheat plants. Epiphytic and endophytic *Firmicutes* were analyzed to track the main source of contaminating lactic acid bacteria from plant to flour. Previously, lactic acid bacteria were found to be components of the epiphytic and/or endophytic microbiota of forage crops and roots of *Lolium perenne* (28–30). The biodiversity of *Firmicutes* was higher in roots than in leaves and spikes. At the contact zone with soil, plants host a distinctive root-associated bacterial microbiota, which is believed to have a positive role in plant nutrition and health (48). Diverse bacterial populations were described for the plant rhizosphere and phyllosphere. Beta-diversity indices showed that the composition of *Firmicutes* in durum wheat spikes was similar to that of processed grains. *Bacillus*, *Exiguobacterium*, *Paenibacillus*, *Staphylococcus*, *Lactobacillus*, *Streptococcus*, *Enterococcus*, and *Lactococcus* were the core genera of wheat plant and processed samples. All the above-mentioned *Firmicutes* were previously identified in wheat flour (16, 18, 49), strengthening the hypothesis that the wheat
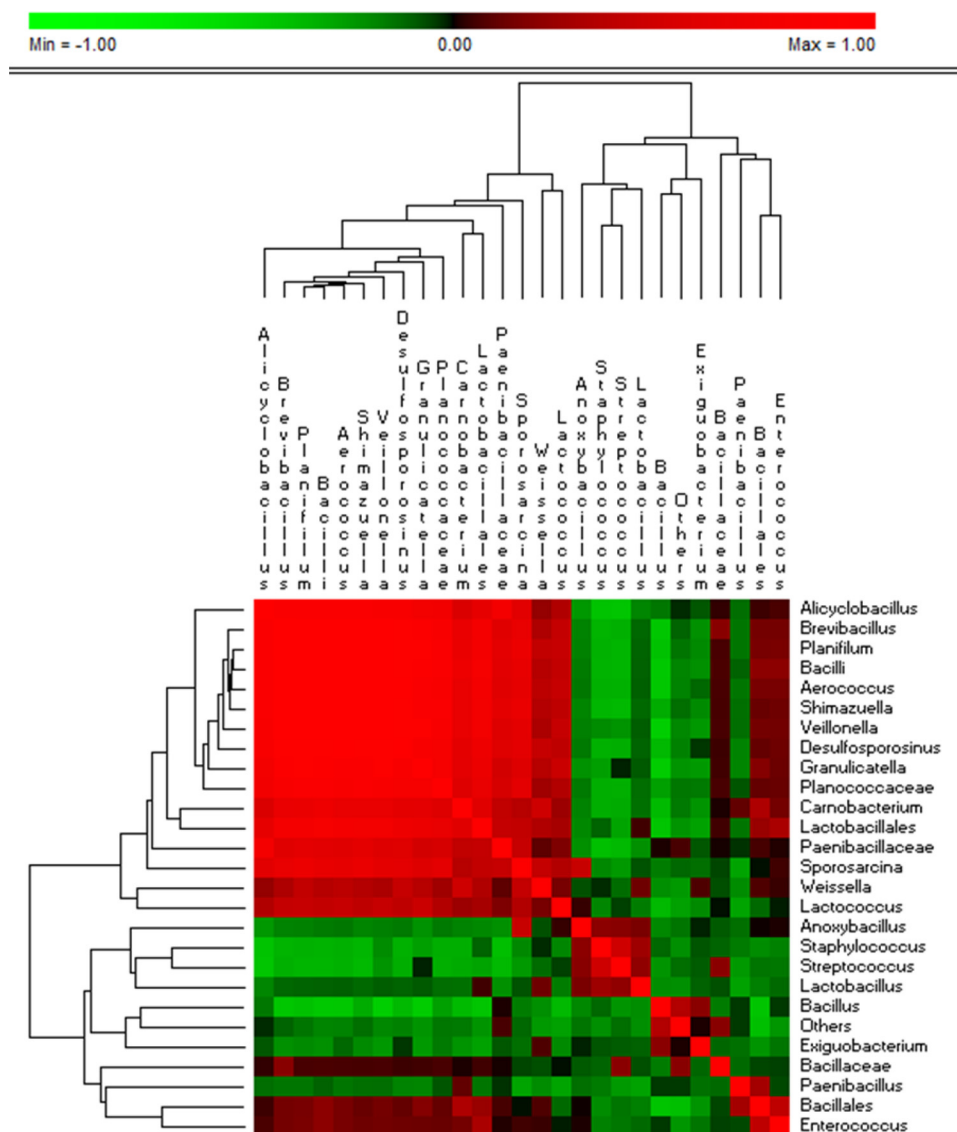
FIG 8 Significant correlations between lactic acid bacterial OTUs (genus level) found on hypogeous organs (roots), epigeous organs (leaves and spikes), and processed wheat (grain, chaff, bran, and flour) of Odisseo and Saragolla. Hypogeous and epigeous organs were analyzed at the tillering, stem elongation, booting, flowering, milk development, and physiological maturity stages. The colors of the scale bar denote the nature of the correlation, with 1 indicating a perfectly positive correlation (red) and −1 indicating a perfectly negative correlation (green) between two microbial orders, families, or genera. Only significant correlations (FDR < 0.05) are shown.

plant microbiota affected the microbial composition of the flour. Based on the results of this study, the spike or grain microbiota strongly contaminated the related flour. When flour is used as the base for producing sourdough, the most adapted microorganisms (mainly consisting of lactobacilli) are selected, leading to mature sourdough, whose microbiota greatly differs from that of flour (18). Dominant lactic acid bacteria and spore-forming bacteria in wheat flour could affect the quality of bread and other baked goods.

Within the core microbiota of wheat plant and processed samples, the relative abundance of each genus was affected by the plant organs, the cultivars, and the phenological stages. Previously, it was shown that microorganisms can randomly emerge from neighboring environmental ecosystems, but their survival is regulated by the plant (50). Overall, *Bacillus* was the most abundant

genus in roots, especially at the epiphytic level. In this study, *Bacillales* were found as dominant OTUs in the Saragolla flour. Besides *Bacillus*, other *Firmicutes* commonly found in the rhizosphere, such as *Paenibacillus* and *Exiguobacterium*, promote plant growth (51). *Exiguobacterium acetylicum*, inoculated on wheat seeds, positively affected plant growth during the first phenological stages (52). In this study, *Exiguobacterium* was found as the dominant genus at the endophytic level of roots and leaves, at the stem elongation and booting stages, respectively, of Saragolla wheat.

Recently, a relatively high number and diversity of lactic acid bacteria was demonstrated in agricultural soils (53). Culture-dependent, denaturing gradient gel electrophoresis (DGGE), and 16S rRNA gene sequencing analyses revealed the following main genera: *Lactococcus*, *Leuconostoc*, *Weissella*, *Lactobacillus*, and *Enterococcus* (53, 54). Saragolla showed a lower relative abundance of

OTUs belonging to *Lactobacillus* in roots, leaves, and spikes than did Odisseo durum wheat. Saragolla had the fastest increase of OTUs belonging to lactic acid bacteria (highest abundance for *Streptococcus*) in roots and, especially, at epiphytic levels (leaves and spikes) during booting. At the endophytic level (spikes), Saragolla showed the highest number of OTUs belonging to *Lactobacillus* during milk development. However, the relative abundance of *Lactobacillus* markedly decreased at physiological maturity. The influence of pesticides, farming practice, and atmosphere also on the composition of the microbial communities should not be excluded. Since the accelerated flowering stage of Saragolla, the environmental conditions differed between the phenological stages of the two cultivars. The lactic acid bacterial composition of spikes directly affected the bacterial community of processed wheat samples. As hypothesized, the OTUs found as endophytic bacteria of grains (*Lactobacillus*, *Lactococcus*, *Enterococcus*, and *Streptococcus*) were also found in the flour. *L. plantarum* was the only species identified in both cultivars at all the phenological stages. Commercial processed wheat grain (used as the control) harbored a high relative abundance of endophytic *Lactococcus*, which reflected on the related flour. Some strains of *Lactococcus lactis* showed significant biocontrol activity and/or direct plant growth promotion (55). Recently, it was shown that the inoculum of wheat roots with *L. plantarum* decreased the oxidative stress (56). Lactobacilli were shown to be effective against biotic stresses, due to their high antagonistic activity against plant pathogens (57). With the exception of *L. plantarum*, which is known to be a member of the sourdough microbiota, other lactic acid bacteria are present only as intermediate organisms in spontaneous sourdough until more characteristic microorganisms become predominant (16).

As shown by cultivation on mMRS and SDB, the cell density of presumptive lactic acid bacteria differed depending on the cultivar and/or the phenological stage. Saragolla durum wheat, which had an accelerated flowering stage, showed a faster increase of the cell density of presumptive lactic acid bacteria in leaves and spikes during booting than did Odisseo wheat. According to the 16S rRNA gene-based high-throughput sequencing, *L. plantarum* was the endophytic lactic acid bacterial species found in both cultivars at all the phenological stages, grain, and flour. This finding reinforced the concept that *L. plantarum* has a strong ecological or metabolic adaptability to different habitats (58, 59). Mesophilic lactobacilli (e.g., *L. coryniformis*) were mainly isolated in the first phenological stages. In contrast, thermophilic lactobacilli (*L. helveticus* and *L. delbrueckii*) were the highest in the milk development and physiological maturity stages.

Overall, the relative abundance of lactic acid bacterial genera was affected by other *Firmicutes* genera, environmental temperature, and a_w of plant organs. Lactobacilli showed the highest positive correlation with a_w. This finding could explain the strong decrease of OTU belonging to *Lactobacillus* during milk development (Odisseo) and physiological maturity (Saragolla). At these stages, *Lactobacillus* was mainly replaced by *Streptococcus*, *Lactococcus*, and *Enterococcus*. With the goal of decreasing chemical treatments (e.g., fertilizers, herbicides, and fungicides), microorganism-based commercial products were used to promote plant growth and to combat biotic disease (50, 60).

The results of this study highlight that the wheat microbiota differed from those of the grains and flour. Only a few microorganisms identified in wheat plants play a role in sourdough. Further experimental approaches, e.g., experiments involving competition of wheat isolates with sourdough isolates, are necessary to understand whether the *L. plantarum* strains from wheat grains dominate in mature sourdoughs.

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