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Wholemeal wheat flours drive the microbiome and functional features of wheat sourdoughs

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ABSTRACT

This study aimed to evaluate the effect of soft (*Triticum aestivum*) and durum (*Triticum durum*) wheat flours at different extraction rate (type 00, 0, 1 and 2) and wholemeal flours on the microbial composition and functionality of type I sourdough. *Enterobacteriaceae* constituted the main component of the microbiome of refined soft and durum wheat flours. On the contrary, wholemeal durum wheat flour harboured mainly *Xanthomonadaceae*. Differences were also found between the soft and durum wheat flours. After 8 h of fermentation (1 day), a different behaviour of the microbiome components was observed. All the mature sourdoughs harboured a core microbiome constituted by 4 species (*Pediococcus pentosaceus*, *Lactobacillus brantae*, *Pediococcus argentinicus* and *Weissella cibaria*). Based on the type of flour, the relative abundance of each core species differed among sourdoughs. In addition, other dominant lactic acid bacteria species were variously detected in sourdoughs. Mature sourdoughs showed marked variations for the concentrations of glucose, fructose, maltose, lactic and acetic acids, ethanol and free amino acids (FAA). Specific correlations ($r > 0.7$; $FDR < 0.05$) were found between the microbiome and total phenols, fibres and metabolome of mature sourdoughs. Breads made by using wholemeal soft wheat or durum wheat doughs started by wholemeal wheat flour-based sourdoughs were characterized by the highest total amount of free cations (Ca^{2+}, Zn^{2+}, Fe^{2+}, Mg^{2+}), antioxidant activity and improved sensory traits.

1. Introduction

Sourdough, the oldest starter for leavened baked goods, consists in a mixture of water and flour from wheat or other cereals, which is fermented by lactic acid bacteria and yeasts (De Vuyst et al., 2016; Gobbetti et al., 2016). The microbiota composition of the sourdoughs affects the sensory, technological, safety, nutritional and functional properties of cereal based foods (De Vuyst et al., 2014; Gänzle, 2014), including different bread varieties (Thiele et al., 2002). The microbiota assembly in sourdough results from a complex interplay among the type of flour used, other ingredients and technological parameters (Gänzle and Ripari, 2016). Different types of flour such as those from soft wheat (*Triticum aestivum*), durum wheat (*Triticum durum*), C4-grains, non-cereals, and other grains are used to produce bread and other bakery products (Van Kerrebroeck et al., 2017). Overall, sourdoughs are produced after 5 to 10 days of back-slopping of the flour/water mixture followed by fermentation steps, in order to increase cell density of lactic acid bacteria and yeasts (Minervini et al., 2014). At the end of the production process, in the mature sourdoughs the developed microbial consortium often encompasses one to several lactic acid bacteria species and one or two yeast species (De Vuyst et al., 2014; Huys et al., 2013; Minervini et al., 2014). The microbial dynamics turning flours into mature rye or soft or durum wheat sourdoughs mainly consist in the initial dominance of flour and house microbiotas (*Acinetobacter*, *Pantoea*, *Pseudomonas*, *Comamonas*, *Enterobacter*, *Erwinia* and *Sphingomonas*) which are inhibited after 1 day of propagation, except for the *Enterobacteriaceae* (Ercolini et al., 2013; Minervini et al., 2015). Bacteria dominating the mature sourdoughs mainly belong to the genera *Lactobacillus*, *Pediococcus*, *Weissella* and *Leuconostoc* (Van Kerrebroeck et al., 2017). The comparison of microbial composition of mature sourdoughs made by using wheat and rye flours showed little differences, suggesting that the type of flour has a limited role on the microbiota assembly (De Vuyst et al., 2014; Van der Meulen et al., 2007; Vogel et al., 1999). On the contrary, the use of white flour or wholemeal flour or bran resulted in different buffering ability and endogenous enzymatic activities of the dough, modifying the assembly and fitness of

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the sourdough lactic acid bacteria (Meroth et al., 2003).

Flour microbiota reflects the niches contamination of the cereal grains which depend on many factors including climatic conditions (e.g., temperature and rainfall), insects or fungi contamination and agricultural practices used (e.g., use of insecticides, fungicides) (Corsetti et al., 2007). Microbial cells contaminating cereals are present at the highest density on the outer layers of kernel, remaining on the fractions rich in bran during milling. Consequently, white flour obtained from milling contains lower bacterial load than caryopses (Berghofer et al., 2003; Minervini et al., 2015). Microbial content of durum wheat grains, bran and flour and the related mature sourdoughs was analysed through culture-dependent approach highlighting the dominance of coccus-shaped (e.g. *Enterococcus* sp.) lactic acid bacteria (Corsetti et al., 2007). Except for *Lactobacillus curvatus*, lactic acid bacteria detected in durum wheat grain differed from those isolated from mature sourdoughs (Corsetti et al., 2001). Bread can be produced by using both high and low extraction rate flours. Extraction rate of 100% corresponds to wholemeal flour, which presents all of the caryopsis constituents. On the contrary, low extraction rate flour is refined, whiter flour, which, according to the Italian Legislation, is normally classified as type 00 or 0. These flours are progressively deprived of increasing amounts of bran and germ, and thus they are poor in B vitamins, fat, iron, and other minerals. This is a key point, since the more or less abundant presence of bran in flour may impact the resulting sourdough microbiota; indeed the relevant content of dietary fibre and bioactive compounds in bran may impact the microbial diversity (Katina et al., 2012; Liukkonen et al., 2003; Poutanen et al., 2009; Rizzello et al., 2010; Ross et al., 2004). A better knowledge of the contribution of the type of flour on the preparation of sourdough and the related composition of the microbiota may help to produce traditional sourdoughs with high biotechnological performances to ferment wholemeal, soft or durum, wheat flours.

This study aimed to evaluate the effect of extraction rate of *T. aestivum* and *T. durum* flour on the microbial composition and functionality of type I sourdough. The microbiota of *T. aestivum* and *T. durum* flours at different extraction rate, and its evolution during the preparation of the related mature sourdoughs were studied mainly through high throughput sequencing technologies. Besides, microbial counts with selective media and concentrations of main metabolites were used as complementary information to the results from metagenetic analysis.

2. Materials and methods

2.1. Dough ingredients and sourdough preparation

Codes used for flours/sourdoughs and breads used in this study are reported in Table S1. Commercial soft wheat flour from *Triticum aestivum* (SW) type 00 (SW_00); type 0 (SW_0); type 1 (SW_1), type 2 (SW_2), and wholemeal flour (WSW) and *Triticum durum* flour (DWF), semolina (DWS) and wholemeal semolina (WDW) were used. The gross compositions of the flour and semolina samples are reported in Table 1. Samples were purchased from two different industries located in the South of Italy. Three different batches from each producer were pooled and used to prepare the respective sourdoughs in triplicate.

All sourdoughs were produced according to traditional protocols (Minervini et al., 2012; Onno and Roussel, 1994) without using starter cultures or baker's yeast. Doughs were prepared as follows: tap water and SW_00, SW_0, SW_1, SW_2, WSF, DWF, DWS or WDW flour were kneaded for 5 min in a continuous high-speed mixer (Chopin&Co., Boulogne, Seine, France), to produce 200 g of doughs which were fermented (30 °C, 8 h). After a resting step (16 h at 4 °C), the fermented dough was used as inoculum of new dough (weighing 200 g), using the recipes reported in Supplementary Table 2 (Table S2). Dough was subjected to fermentation (30 °C, 6 h). After resting (ca. 18 h at 4 °C), the fermented dough was used again as inoculum and so on, until reaching constant final pH, which occurred after the tenth fermentation

Table 1
Chemical characteristics of the *Triticum aestivum* flours (SW) at different extraction rate (type 00, 0, 1 and 2) or wholemeal flour (WSW) and *Triticum durum* flour (DWF), semolina (DWS) or wholemeal semolina (WDW).[a]

Flours	Type	Moisture (%)	Protein (% of d.m.)	Ash (% of d.m.)	Starch (% of d.m.)	Maltose (mM)	Glucose (mM)	Fructose (mM)	Fat (% of d.m.)	FAA (mg/kg)	Fibre (% of d.m.)	Total phenols (mM)
SW_00	*Triticum aestivum*	13.19b	10f	0.81b	71.6a	56c	32d	15d	2b	403c	2d	4.01f
SW_0	*Triticum aestivum*	14.21a	11e	0.79b	69.6ab	45d	32d	19 cd	2b	510b	2d	4.15f
SW_1	*Triticum aestivum*	11.15d	12d	0.85ab	69.9a	65b	39 cd	19 cd	2.2b	528b	3.5bc	6.98c
SW_2	*Triticum aestivum*	12.22c	13c	0.78b	67.0b	48d	55b	37b	2.6b	554ab	4b	7.64c
WSW	*Triticum aestivum*	14.58a	11e	0.80b	59.8c	34e	66a	55a	3.8a	623a	7.5a	8.98b
DWF	*Triticum durum*	14.45a	14b	1.05a	60.6c	46d	34d	21c	2.5b	511b	4.4b	5.07e
DWS	*Triticum durum*	11.08d	13c	0.92a	69.9ab	90a	42c	15d	2b	527b	2.7 cd	6.31d
WDW	*Triticum durum*	14.21a	15a	0.99a	56.4d	43d	42c	23c	3.8a	620a	7.5a	10.02a

[a] Values in the same column followed by different letters significantly differed ($p < 0.05$).

(day 10). pH and volume of doughs were measured daily at the beginning and end of fermentation. At the end of the first (day 1) and tenth fermentation, microbiological and biochemical analyses were performed on the doughs. In parallel to microbiological and biochemical analyses, aliquots of each fermented dough were sampled and kept at −20 °C for analysis of bacterial microbiome.

2.2. *Enumeration of lactic acid bacteria and yeasts*

Cell densities of presumptive lactic acid bacteria were enumerated using MRS agar medium with cycloheximide (0.1 g/l) and SDB agar with cycloheximide (0.1 g/l). Yeasts were enumerated using Sabouraud Dextrose agar with chloramphenicol (0.1 g/l). All media were purchased from Oxoid Ltd., (Basingstoke, UK). Before inoculating the media, flours and sourdoughs were homogenized with 90 ml of sterile peptone water as previously described (Ercolini et al., 2013; Minervini et al., 2012). Colonies were counted after 48 h of incubation.

2.3. *Bacterial microbiome*

Before extracting total bacterial DNA from flour, the latter (22.7 g) was mixed with filtered demineralized water (27.3 g) to give 50 g of dough. Five grams of dough or sourdough were mixed with 45 ml of saline solution and subject to 3-min long homogenization in a BagMixer 400P (Interscience, Saint Nom, France). After two centrifugation steps (Cavallo et al., 2017), the microbial pellet was suspended in 2 ml of saline solution and added with 200 mg of glass beads (Sigma-Aldrich, USA) (Lattanzi et al., 2013). The microbial suspension was then moved into an ice-bath and sonicated using a Vibra-Cell sonicator (Sonic and Materials Inc., Danbury, CT) equipped with a micro-tip and set at 0.6 Amp for 15 min (3 cycles of 5 min each, with a 5-min-long pause between cycles). After sonication, 500 μl of microbial suspension was treated with the FastPrep Instrument (BIO 101) and DNA was extracted by using the FastDNA Spin Kit for Soil (MP Biomedicals, Illkrich, France), according to the manufacturer's instructions (Lattanzi et al., 2013). Genomix4Life (Salerno, Italy) performed 16S metagenetics using the Illumina MiSeq platform.

In order to analyse the bacterial diversity, the 16S rRNA gene (V1–V3 region, corresponding to *Escherichia coli* position 27–519) was amplified using the universal primers 28F and 519R (Suchodolski et al., 2012). Internal protocols of Genomix4Life were followed for PCR and sequencing analyses. The FLASH v1.2.11 software was used to merge forward and reverse reads. Merged reads that did not reach a minimum quality of 30 were discarded from further processing.

Clustering of Operational Taxonomic Units (OTUs) was carried out using Qiime v 1.9.1 (method: pick_closed_reference:otus.py). OTUs were allotted at the highest possible taxonomic level by using the BLAST search in the NCBI 16S ribosomal RNA sequences database (Altschul et al., 1990). Relative abundance of OTUs was calculated for comparing the different samples (Andreotti et al., 2011). Qiime was used to calculate the alpha diversity (observed species, Chao1 richness and Shannon diversity) indices (Chao and Bunge, 2002; Shannon and Weaver, 1949).

2.4. *Determination of carbohydrates, organic acids, ethanol, free amino acids and phenols*

Carbohydrates and fermentation metabolites were extracted from a homogeneous suspension of flour or sourdough (5 g) and H_2SO_4 10 mM (45 ml). Glucose, fructose, maltose, lactic acid, acetic acid and ethanol were quantified using the HPLC method described by Minervini et al. (2012). A second water-soluble extract was prepared for analysis of free amino acids (FAA). In detail, 5 g of sourdough were homogenized with 45 ml of Tris-HCl 50 mM pH 8.8, respectively. After extraction (25 °C, 30 min, under stirring) and centrifugation (12,857 ×*g*, 10 min, 4 °C), the supernatant (water-soluble extract) was filtered (0.20 μm) and analysed using the Biochrom 30 Amino Acid Analyser (Biochrom LTD, Cambridge, England), as previously described (De Angelis et al., 2007). Total phenols were estimated as described by Slinkard and Singleton (1997), and expressed as gallic acid equivalent.

2.5. *Bread-making*

Mature SW, WSW, DWS and WDW sourdoughs were used as starter for bread-making. To evaluate the different ability of the mature sourdoughs to ferment wholemeal *T. aestivum* and *T. durum* flours, six different breads were produced (Fig. S1): i) white soft wheat bread (B-SW_0) using SW_0 flour and the related sourdough as starter; ii) wholemeal wheat bread (WB-SW_0) using WSW flour and the SW_0 sourdough as starter; iii) wholemeal wheat bread (WB-WSW) using WSW flour and the related WSW sourdough as starter; iv) durum wheat bread (DB-DWS) using DWS and the related sourdough as starter; v) wholemeal durum wheat bread (WDB-DWS) using WDW and DWS sourdough as starter; and vi) wholemeal durum flour wheat bread (WDB-WDW) using WDW and the related sourdough as starter. Two additional wholemeal *T. aestivum* (WB) and *T. durum* (WDB) breads were made, without sourdough fermentation but using only baker's yeast (ca. 1.0%, w/w) as starter, and used as controls.

Bread-making was based on two-step fermentation, the first of which consisted of 6 h-sourdough fermentation. In detail, the six doughs were prepared using the same values of dough yield reported in Table S2 and without the addition of yeasts. The only modifications concerned the use of appropriate combination of flour and sourdoughs specific for each one of the six breads (Fig. S1). After fermentation, the second step consisted in the addition of the appropriate flour, tap water and baker's yeast (ca. 1.0%, w/w) to the previously fermented dough. After fermentation (30 °C, 1 h), the final doughs were baked (220 °C, 40 min) in a rotary oven (Combo 3, Zucchelli, Verona, Italy).

2.6. *Phytase activities of sourdoughs and phytate and total cations content in breads*

Phytase activity of final doughs before being baked was determined by monitoring the rate of hydrolysis of *p*-nitrophenyl phosphate (p-NPP) (Sigma-Aldrich). Water-soluble extracts (400 ml) of dough were mixed with 200 ml of 0.2 M sodium acetate, pH 5.2 solution containing p-NPP at final concentration of 1.5 mM. After incubating the mixture at 45 °C, 600 ml of 0.1 M NaOH were added to stop the reaction. The *p*-nitrophenol released during the reaction was determined by measuring the absorbance at 405 nm (De Angelis et al., 2003). The amount of enzyme necessary to release 1 mmol of *p*-nitrophenol in one minute was defined as the unit (U) of phytase activity.

Phytate content was determined on extracts of bread obtained upon homogenization of 2 g of bread with 100 ml of 500 mM HCl. The homogenates were treated at 90 °C for 5 min to denature the enzymes and centrifuged (4000 ×*g*, 10 min, 20 °C). The supernatant was dried and re-dissolved in 15 ml of a solution consisting of 1 M HCl and methanol in a 3:7 ratio (Caputo et al., 2015).

Free Ca^{2+}, Fe^{2+}, Zn^{2+}, and Mg^{2+} in the water-soluble extracts of breads were determined following an inductively coupled plasma method with atomic absorption spectrophotometric (AAS; IRIS Intrepid, Thermo Elemental, Thermo Fisher Scientific, Waltham, MA, USA) analysis and an air-acetylene flame.

2.7. *Antioxidant activity of breads*

Antioxidant activity was determined through measurement of the 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity of the methanolic extracts (ME) of the bread. The latter were obtained by mixing 3 g of previously ground bread with 30 ml of 80% methanol. The mixture was fluxed with nitrogen for 5 min and then centrifuged (4600 ×*g*, 20 min, 4 °C). The supernatant was collected and fluxed with nitrogen for 5 min. The MEs were stored at 4 °C until analysed. The free radical scavenging capacity was determined spectrophotometrically following the method described by Yu et al. (2003). The free radical scavenging activity was calculated as follows: DPPH scavenging activity (%) = [(blank absorbance − sample absorbance) / blank absorbance] × 100, wherein blank absorbance refers to the absorbance of DPPH alone. The positive control was represented by a solution (75 ppm) of butyl-hydroxy-toluene (BHT).

2.8. Sensory analyses

Breads were subject to a panel test after 24 h from baking. The panel was composed of eight volunteers (3 male and 5 female, mean age 30 years old, range 22–42 years old) from laboratory staff, which were previously trained about the meaning of the sensory attributes and scores. Slices (1.5 cm thick) of bread were cut into four pieces and served at 22 °C and under daylight illumination. Each panelist evaluated two pieces of bread per thesis, each bread being identified by a code letter and served in random order on a single tray. The attributes were: crust colour, odour, salt, sweet, acid taste, masticability, aroma and overall acceptability. The score for each sensory attribute ranged from 1 (lowest) to 5 (highest) (Haglund et al., 1998).

2.9. Statistical analysis

Data (at least three biological replicates) for maltose, glucose, fructose, FAA, pH, organic acids, ethanol, and cell density were subjected to one-way analysis of variance (ANOVA), and pair comparison of treatment means was achieved by Tukey's procedure at $p < 0.05$, using the statistical software Statistica v6.0 for Windows. Principal component analysis (PCA) using a correlation matrix was carried out to visualize the effects of type of flour on the sourdough microbiotas (Minervini et al., 2012; Scheirlinck et al., 2008). The chemical composition of the flours (concentrations of maltose, glucose, fructose, and total and individual FAA) and microbial community data were used as variables for PCA analyses. In addition, microbial community data were also analysed together with the biochemical characteristics (pH, organic acids, ethanol, and FAA) of sourdoughs. All data were standardized before PCA analysis using the Statistica software. OTU correlations were investigated considering family- and genus-level taxonomic assignments by using Statistica and PermutMatrix (Caraux and Pinloche, 2005).

3. Results

3.1. Lactic acid bacteria and yeasts, leavening and acidification during sourdoughs preparation

After 8 h of fermentation (1 day), cell density of presumptive lactic acid bacteria, as estimated on SDB, ranged from ca. 5.2 in wholemeal soft wheat dough (WSW) to ca. 6.8 log cfu/g ($p < 0.05$) in soft wheat flour type “0” (SW_0) and durum wheat semolina (DWS) doughs (Fig. 1). Except for WSW, all the other soft wheat flour doughs harboured a lower number ($p < 0.05$) of presumptive lactic acid bacteria on MRS medium compared to SDB. No statistical differences were found between presumptive lactic acid bacteria estimated on SDB and MRS media for durum wheat flour doughs (DWF, DWS, WDW) after 8 h of fermentation. After 5 days of back-slopping, cell number of presumptive lactic acid bacteria reached values ranging from ca. 8.6 (SW_2) to ca. 9.7 (WSW, DWF, DWS, WDW) log cfu/g ($p < 0.05$). After 5 days of back-slopping, no statistical differences ($p > 0.05$) were found between presumptive lactic acid bacteria estimated on SDB and MRS media.

At ten days of back-slopping, cell number of presumptive lactic acid bacteria reached values ranging from ca. 9.3 (SW_00) to ca. 9.5 (WSW) log cfu/g without statistical differences ($p > 0.05$). Yeasts increased in all doughs until 5 days of back-slopping reaching the highest value (3.8 log cfu/g) in SW_00 (Fig. 1). Yeast numbers decreased during back-slopping especially for DWF, DWS, and WDW sourdoughs. Nevertheless, the dough volume (ΔV) was higher in durum wheat sourdoughs (DWF, DWS, WDW) than in soft wheat sourdoughs (SW_00, SW_0, SW_1, SW_2, WSW) (Fig. 1). Before the first fermentation, the median value of the pH was higher ($p = 0.004$) in durum wheat doughs than in soft wheat doughs (6.25 and 5.96, respectively).

Within soft wheat samples, SW_1, SW_2 and, especially, WSW showed higher pH values than SW_00 and SW_0 (Fig. 1). Except for the first fermentation, the pH values decreased during back-slopping reaching the lowest value after 6 (SW_00 and SW_0, pH = 4.16), 7 (DWF, pH = 4.39), 9 (SW_1 and SW_2, pH = 4.15; DWS, pH = 4.20; and WDW, pH = 4.35) and 10 (WSW, pH = 4.15) days. The final pH was higher in durum wheat than in soft wheat sourdoughs (median value of 4.35 and 4.15, respectively).

3.2. Illumina MiSeq data analysis and alpha diversity

An average of 68,216 reads/sample and an average length of 465 bp, calculated after primer removal, were obtained. The highest number of OTUs (average value of 366) and Shannon index (average value of 0.55) were found for soft wheat flour type 0 (SW_0) and durum wheat semolina samples. The lowest values of OTUs (197 and 248) and Shannon index (0.369 and 0.375) were found for durum wheat flour and wholemeal wheat flour, respectively (Fig. S2). After the first day of fermentation, the total number of OTUs ranged from ca. 293 to 327 found in wholemeal wheat flour dough and durum wheat flour and semolina doughs, respectively. The Shannon index values ranged from ca. 1.252 to 2.415, found in whole durum wheat flour and soft wheat flour type 00 doughs, respectively (Fig. S2). After ten day of fermentation, mature sourdoughs showed a marked simplification of the microbial diversity. The lowest number of OTUs (243) and Shannon index (0.42) values were found in the mature sourdoughs made by using soft wheat flour type 00. Wholemeal wheat- and durum wheat flour-based sourdoughs showed the highest microbial diversity compared to other samples.

3.3. Structure of the sourdough microbiomes

The 16S rRNA sequences assigned to bacterial phyla and their relative abundances (%) varied depending on flour and number of propagations (Fig. S3). Proteobacteria dominated in all flour samples and doughs after 8 h of fermentation (1 day). The only exception was found for the WDW dough, which harboured 59% ± 0.85% of Firmicutes and 39% ± 0.91% of Proteobacteria. Firmicutes dominated in all sourdoughs after 10 day of back-slopping. Within mature sourdoughs, the relative abundance of Proteobacteria ranged from ca. 2.4% (SW_00) to 18.5% (WSW).

Refined soft and durum wheat flours harboured the highest percentage of *Enterobacteriaceae* (relative abundance of ca. 35% in SW_00, SW_0 and DWS) (Fig. 2). Wholemeal flours harboured the lowest abundance of *Enterobacteriaceae* (19 and 8% in WSW and WDW, respectively). After 8 h of fermentation (1 day), the relative abundance of *Enterobacteriaceae* increased, reaching the highest values in SW_00, SW_1, SW_2 and, especially, SW_0 doughs. Refined soft wheat flours, as well as their related doughs, also harboured the highest percentage of *Moraxellaceae*. *Pseudomonadaceae* was found at the highest level in DWS (7.8%). *Thiotrichaceae* family was lowest (ca. 2.2%) in refined soft wheat flour (SW_00 and SW_0) but increased in SW_1 (4.8%), SW_2 (5.7%) and, especially, in WSW (10.8%). *Xanthomonadaceae* dominated the microbiome of WDW (ca. 40%) and was subdominant (relative abundance < 3%) in all the other flours. WDW was also characterized by the highest value of *Staphylococcaceae* and *Leuconostocaceae*. After 8 h of fermentation (1 day), *Leuconostocaceae* strongly increased showing the highest values in DWF, DWS and, especially, in WDW.

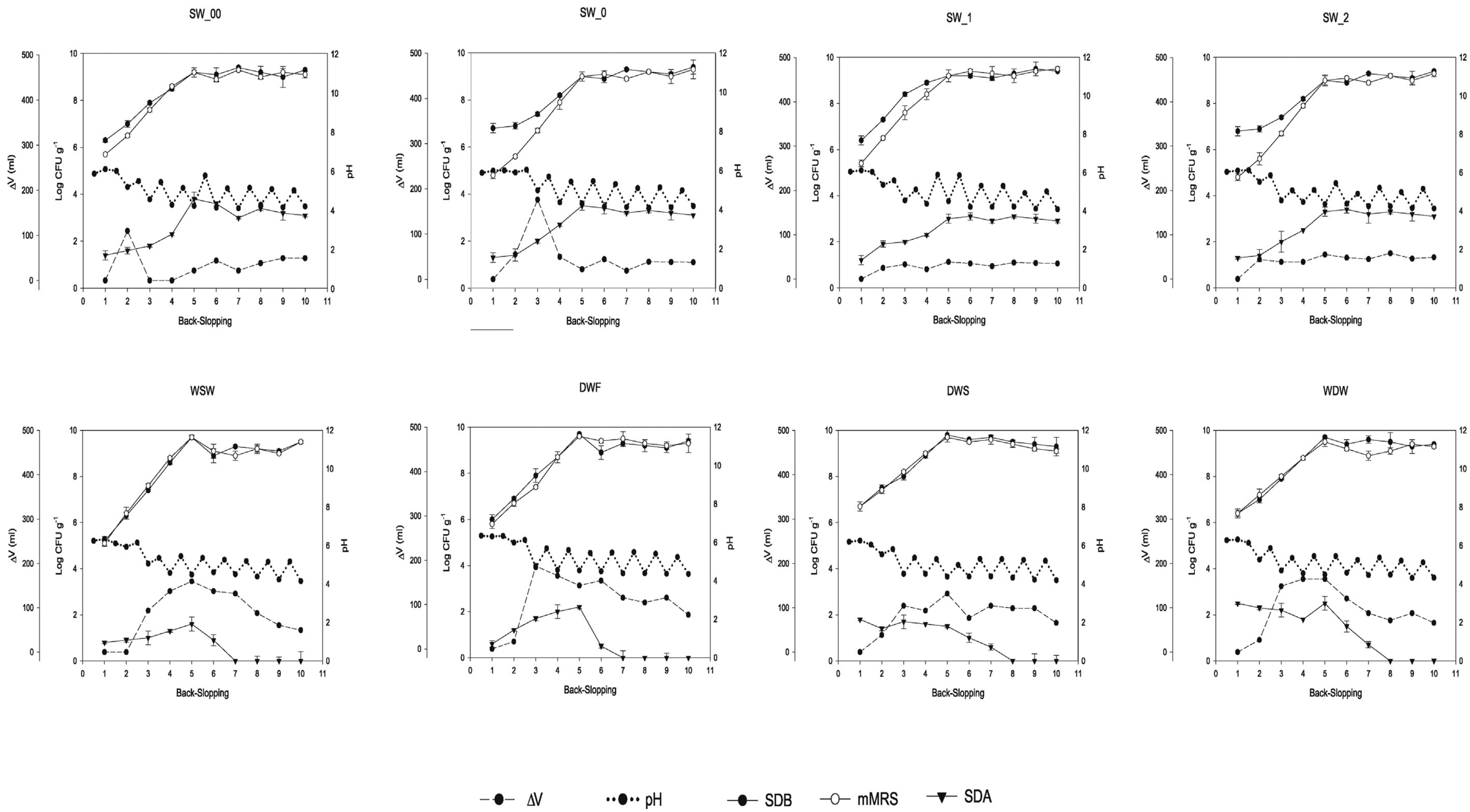

Fig. 1. Cell density (log cfu/g) of presumptive lactic acid bacteria (enumerated on both SDB and MRS), yeasts (enumerated on SDA), dough volume (ΔV) and kinetics of acidification (pH) of sourdoughs prepared with soft wheat flours (SW) at different extraction rate (type 00, SW_00; 0, SW_0; 1, SW_1; and 2, SW_2) or wholemeal flour (WSW) and durum wheat flour (DWF), semolina (DWS) or wholemeal semolina (WDW). Sourdoughs were daily propagated for 10 days; numbers on the x axis indicate days of propagation. Day 0 refers to the dough prior to fermentation and before becoming sourdough. Data are the means of two independent experiments analysed in triplicate (n = 6).

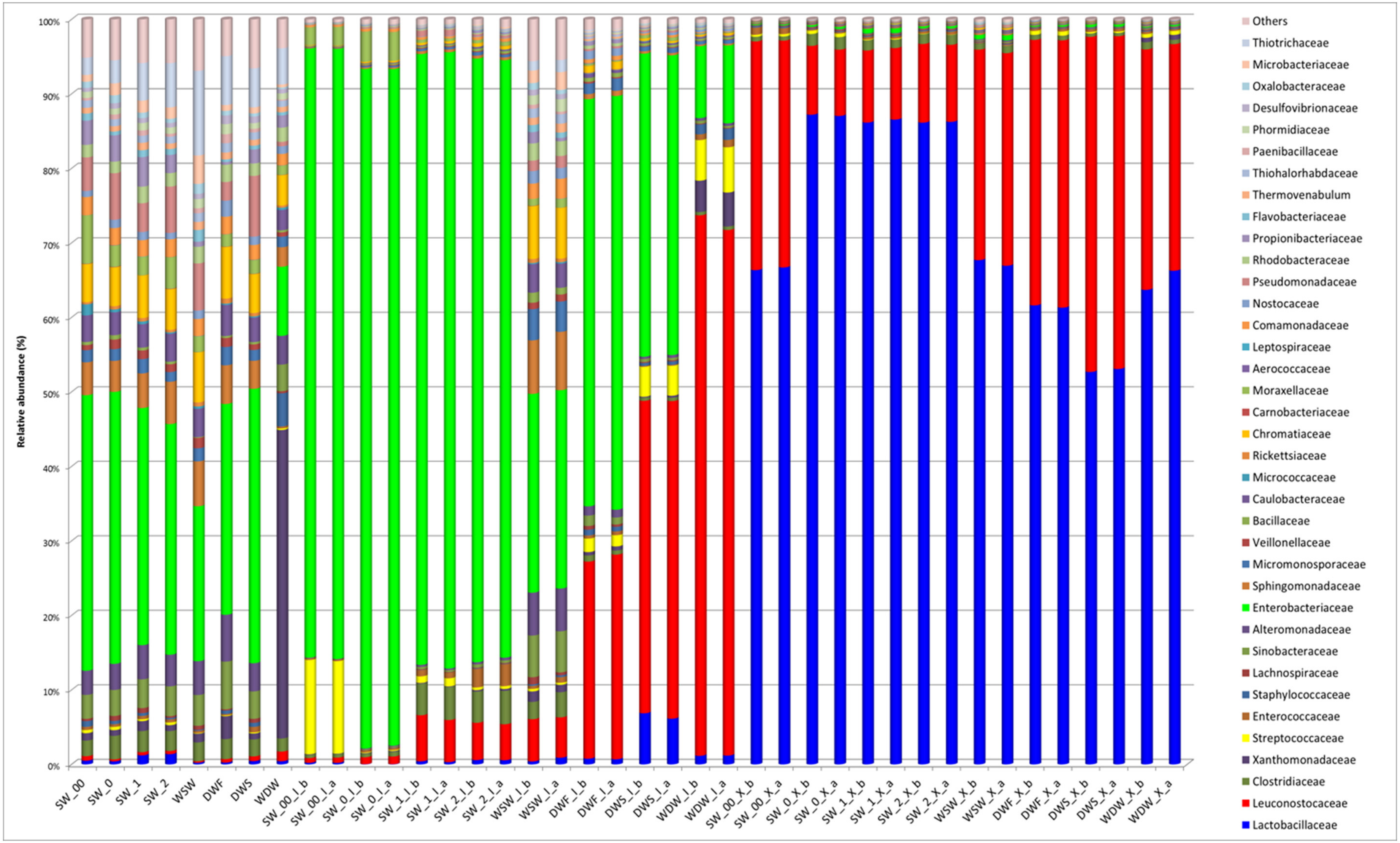

Fig. 2. Bacterial families distribution (%) found in soft wheat flours (SW) at different extraction rate (type 00, SW_00; 0, SW_0; 1, SW_1; and 2, SW_2) and wholemeal flour (WSW) and durum wheat flour (DWF), semolina (DWS) and wholemeal semolina (WDW) and related doughs after 8 h of fermentation (SW_00_I, SW_0_I, SW_1_I, SW_2_I, WSW_I, DWF_I, DWS_I and WDW_I); and mature sourdoughs (SW_00_X, SW_0_X, SW_1_X, SW_2_X, WSW_X, DWF_X, DWS_X and WDW_X). Data from flours refer to one pool of three different batches from two producers. Data from doughs and the sourdoughs refer to two independent experiments (a, b) performed in triplicate.

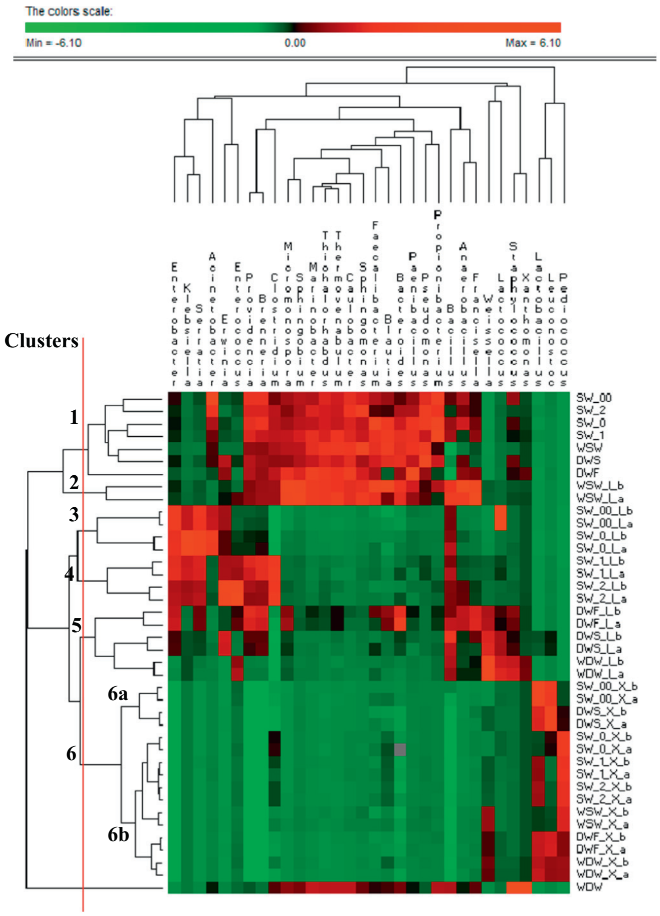

Fig. 3. Permutation analyses summarizing the relative abundance of the genera (A) and species (B) found in soft and durum wheat flours, dough after the first fermentation (8 h at 30 °C) and the related mature sourdoughs. Samples belonging to soft wheat flours (SW) at different extraction rate (type 00, SW_00; 0, SW_0; 1, SW_1; and 2, SW_2) and wholemeal flour (WSW) and durum wheat flour (DWF), semolina (DWS) and wholemeal semolina (WDW); related doughs after 8 h of fermentation (SW_00_I, SW_0_I, SW_1_I, SW_2_I, WSW_I, DWF_I, DWS_I and WDW_I); and mature sourdoughs (SW_00_X, SW_0_X, SW_1_X, SW_2_X, WSW_X, DWF_X, DWS_X and WDW_X). Data from flours refer to one pool of three different batches from two producers. Data from doughs and the sourdoughs refer to two independent experiments (a, b) performed in triplicate. Euclidean distance and McQuitty's criterion were used for clustering. Colours correspond to normalized mean data levels from low (green) to high (red). The colour scale, in terms of units of standard deviation, is also shown. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

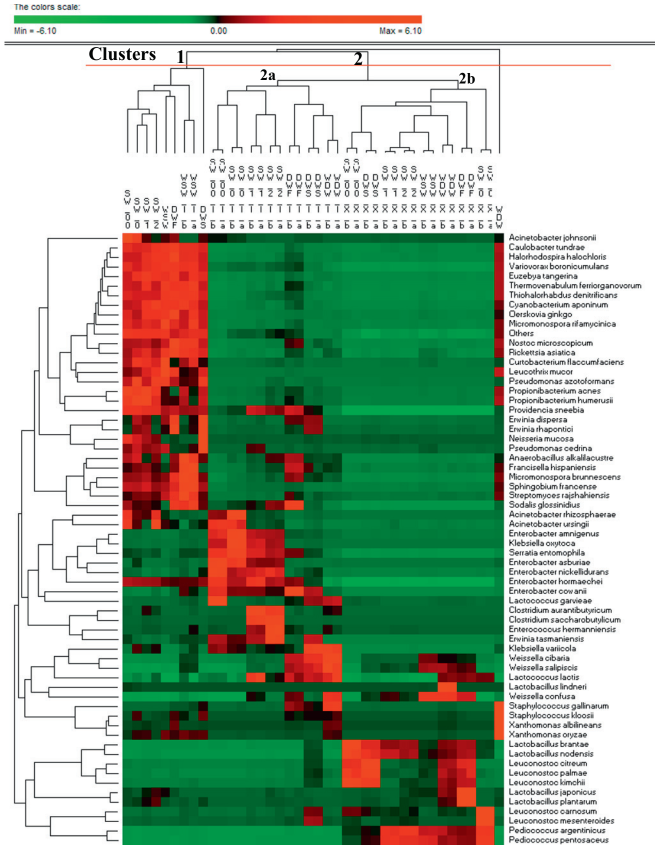

Fig. 3. (*continued*)

Streptococcaceae were harboured mainly in SW_00 and SW_0 flours and markedly increased after 8 h of fermentation especially in SW_00 dough. *Lactobacillaceae* was found at the highest level in DWS doughs. At 10 days, *Lactobacillaceae* dominated in all the sourdoughs, followed by *Leuconostocaceae*.

Multivariate statistical analyses were used to cluster sourdough microbial community composition, at genus (Fig. 3A) and specie levels (Figs. 3B and S4), onto time of propagation and type of flour. The separation of OTUs and samples depended on the time of propagation and type of flours. Permutation analysis, based on OTUs classified at genus level, grouped flour samples in the cluster 1. The only exception was WDW flour, which differed from the others mainly for the highest presence of *Staphylococcus* (*Staph. gallinarum* and *Staph. kloosii*) and, especially, *Xanthomonas* (*X. albilineans* and *X. oryzae*) (Fig. 3A; Fig. 3B, cluster 2). WSW dough fermented for 8 h (1 day) showed the highest similarity with the microbial profiles of flour. Except for *Acinetobacter*, *Providencia*, *Brenneria*, and *Clostridium* species, all the other dominant OTUs of flours decreased after 8 h of dough fermentation (1 day) (Fig. 3A, clusters 2–5; Fig. 3B, clusters 1-2a). Compared to flour, SW_00, SW_0, SW_1 and SW_2 doughs were mainly characterized by a strong increase of *Enterobacter* (e.g., *E. amnigenus*, *E. asburiae*, *E. hormaechei*), *Klebsiella* (mainly *K. oxytoca*) and *Serratia* (*S. entomophila*) species. SW_1 and SW_2 doughs were also characterized by the highest abundance of *Erwinia* (*E. tasmaniensis*), *Clostridium* (*C. aurantibutyricum* and *C. saccharobutylicum*) and *Enterococcus* (*Ent. hermanniensis*). DWF, DWS and WDW doughs fermented for 8 h (day 1) were mainly characterized by the highest abundance of *Weissella* (*W. cibaria*, *W. confusa* and *W. salipiscis*), *Lactococcus* (*Lc. garvieae*) and *Staphylococcus* (*Staph. gallinarum*, detected mainly in WDW). DWF dough also harboured *Bacteroides*, *Anaerobacillus* and *Francisella* species. With few exceptions, all these genera and species almost disappeared in the mature sourdoughs (Figs. 3, S4 and Table S3).

After 10 days of back-slopping, SW_00 and DWS mature sourdoughs showed the highest similarity, being characterized by high relative abundance of *Lactobacillus* (mainly *L. brantae* and *L. nodensis*, ca. 30 and 1%, respectively) and *Leuconostoc* (mainly *Leuc. kimchii*). In detail, *L. brantae* was among the dominant bacterial species in all the mature sourdoughs with relative abundance ranging from ca. 7 (WSW) to 31% (SW_00 and DWS). *Leuconostoc carnosum* and *Leuconostoc mesenteroides* were mainly associated to SW_0 sourdough. *Pediococcus argentinicus* and *Pediococcus pentosaceus* were harboured at the highest levels in SW_0, SW_1 and SW_2 sourdoughs. In detail, *P. pentosaceus* was a dominant species in all the mature sourdoughs with relative abundances ranging from ca. 19–35% in durum wheat sourdoughs to 42–72% in soft wheat sourdoughs. At the specie level, WSW showed the highest similarity with WDW and, especially with SW_1 and SW_2 mature sourdoughs (Fig. 3B, cluster 2B). Compared to other mature sourdoughs, WSW showed the highest relative amount of *W. cibaria* (15%). *W. confusa* (7%) and *W. salipiscis* (3%) did not differ between WSW and WDW mature sourdoughs. Compared to other sourdoughs, WDW showed the highest percentage of *Lactobacillus lindneri* (5%). On the contrary, the DWF mature sourdough harboured the highest relative abundance of *Lactobacillus plantarum*.

3.4. Biochemical characteristics of sourdoughs

Mature sourdoughs showed marked variations in terms of glucose, fructose and maltose concentrations (Fig. S5; Table S4). The highest amounts of glucose and fructose were found for WDW (ca. 42 and 14 mM/kg, respectively) and, especially, WSW (71 and 33 mM/kg, respectively) sourdoughs. Wholemeal soft wheat flour showed the highest levels of glucose and fructose (Table 1). Except for maltose in SW_00, SW_0 and DWF sourdoughs showed the lowest amount of residual fermentable carbohydrates. SW_00 and DWF showed the lowest level of glucose. Compared to other sourdoughs, SW_2, WSW and WDW were characterized by the highest levels of fibre (determined only in flours, Table 1), total free amino acids (FAA), and phenols (Table S4). Lactic acid ranged from 51 mM (WSW) to 69 mM (DWF). Acetic acid was found at the highest levels in WSW and WDW sourdoughs. On the contrary, ethanol was found at the highest levels in SW_00, DWS and WDW.

3.5. Correlations between microbiota and biochemical characteristics of flour and sourdoughs

Glucose, fructose and FAA increased during sourdough fermentation and they were positively correlated ($r > 0.70$; FDR < 0.05) with the level of fibre in flours. Fibre, phenols, FAA, glucose and fructose in flours and/or sourdoughs were positively correlated with the relative amount of *Weissella* (*W. salipiscis*, *W. confusa* and *W. cibara*) and *Pediococcus* (*P. argentinicus* and *P. pentosaceus*) found in the mature sourdoughs (Fig. 4). Fibre, fructose, FAA and *Weissella* species in sourdough were positively correlated with acetic acid. Maltose was positively correlated with *L. brantae*, *L. nodensis*, *Leuc. citreum*, *Leuc. palmae* and *Leuc. kimchii*. No statistical (FDR > 0.05) positive correlations were found for *Leuconostoc* species (*Leuc. carnosum* and *Leuc. mesenteroides*).

3.6. Phytase activity of sourdoughs and phytate and total cations content in breads

Based on the different microbiotas and biochemical characteristics, SW, WSW, DWS and WDW sourdoughs were selected and used to ferment white and wholemeal doughs for bread production. Controls were represented by doughs based on either wholemeal soft wheat flour or wholemeal durum wheat semolina, both started with baker's yeast alone. Phytase activity was estimated on final doughs before being baked (Table 2). The highest values of phytase activity were found for both wholemeal soft and durum wheat doughs started with WSW and WDW sourdoughs (ca. 5.7 ± 0.15 and 5.86 ± 0.13 U respectively, $p = 0.657$). Compared to controls, the doughs fermented by SW and DWS sourdoughs showed higher ($p < 0.05$) phytase activity, ranging from ca. 0.80 (controls) to 2.5 (wholemeal soft wheat flour dough fermented by SW sourdough) U. Compared to controls, soft and durum wheat wholemeal doughs started with WSW and WDW sourdoughs showed the lowest concentrations of phytate (Table 2). Compared to controls, the sourdough fermentation increased the total amount of free cations (Ca^{2+}, Zn^{2+}, Fe^{2+}, Mg^{2+}). Compared to SW_0 and DWS, wholemeal soft and durum wheat breads obtained by using WSW and WDW sourdoughs increased by ca. 2-fold the total amount of free cations.

3.7. Antioxidant activity of breads

The antioxidant properties of breads were determined based on the scavenging activity towards the DPPH radical (Fig. 5). The lowest antioxidant activity was found in control wholemeal breads produced by baker's yeast alone (WDB) and, especially, WB. Compared to control breads, the B-SW_0, WB-SW_0, DB-DWS and WDB-DWS showed higher ($p < 0.05$) antioxidant activity. Wholemeal durum wheat bread obtained by using WDW sourdough (WDB-WDW) showed the highest antioxidant activity. Compared to SW_0, wholemeal soft wheat bread obtained by using WSW sourdough (WB-WSW) increased by ca. 2-fold the antioxidant activity.

3.8. Sensory analyses of whole meal flour breads made by using selected sourdoughs

The sensory characteristics of the sourdough breads were determined 24 h after baking through a panel test (Fig. S6). Overall, breads based on wholemeal flours and started with wholemeal flour-based sourdoughs (WB-WSW and WDB-WDW) received higher scores than the corresponding breads started with refined flour-based sourdoughs. In detail, WB-WSW was characterized by higher ($p < 0.05$) values of odour, aroma, masticability and overall acceptability, compared to WB-SW_0 started with soft wheat flour-based sourdough. WDB-WDW received higher scores ($p < 0.05$) for the same above mentioned attributes than WDB-DWS started with durum wheat semolina-based sourdough.

4. Discussion

The consumption of wholemeal breads is strongly recommended for the positive effects on human health compared to white wheat breads (SACN, 2015). In Italy, *T. aestivum* and *T. durum* flours at different extraction rate (type 00, 0, 1 and 2 and wholemeal flours) are used to produce commercial and traditional breads (Minervini et al., 2012). Consequently, there is strong interest by bakers and industrial bakeries to control and supplement their sourdoughs, in order to improve bread organoleptic properties (Clément et al., 2018; Taccari et al., 2016). First, the phylogenetic composition and metabolic activities of the bacterial communities during sourdough preparation using soft wheat flours at different extraction rate and wholemeal soft and durum wheat flours were described through integrate approaches. Although there were slight variations between flours, complementary evidences suggested that sourdoughs achieved maturity during 6 to 10 days of propagation. Overall, sourdoughs made by soft wheat flours types 1 and 2 and especially wholemeal soft wheat flour required longer time to reach maturity compared to highly refined flours (type 00 and 0) (Taccari et al., 2016). This could be attributed to higher pH buffering capacity of wholemeal compared to refined flours. Indeed, pH, resulting from a balance between acidifying activity of lactic acid bacteria and flour buffering capacity, is one of the main drivers contributing to achieve sourdough maturity. If pH keeps above certain levels (e.g., 4.5–5.0), environmental pressure is low and microorganisms more adapted to low pH will outgrow other microorganisms in longer time. Previously, other studies (Meroth et al., 2003; Ercolini et al., 2013; Van der Meulen et al., 2007) showed that sourdoughs became mature within 1 week, with slight variations that depended on environmental and technological determinants. All the mature sourdoughs harboured presumptive lactic acid bacteria above 9 log cfu/g. According to previous findings (Taccari et al., 2016), yeasts were not detected (durum wheat flour-based sourdoughs) or found at very low levels (< 4 log cfu/g in soft wheat flour-based sourdoughs) in sourdoughs prepared under sterile laboratory conditions. This may occur especially for spontaneous fermentations performed at laboratory, where yeast contamination is much lower than at a bakery using baker's yeast. Under our conditions, we used flours with very low level of yeast contamination. Maybe if we had used flours highly contaminated by yeasts, we would have found higher yeast cell density in our sourdoughs.

The alpha diversity analysis showed that microbial diversity was

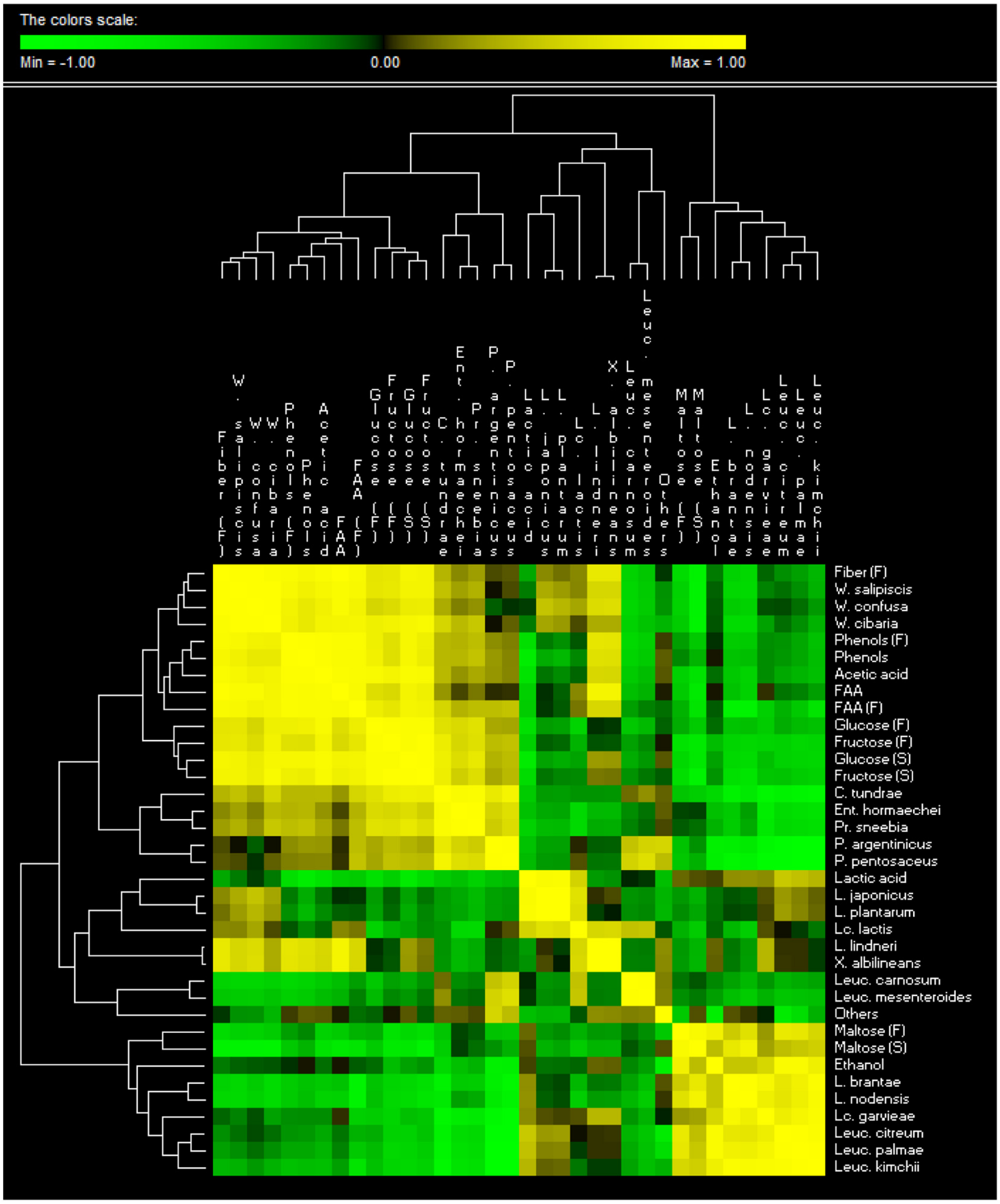

Fig. 4. Correlations between dominant (relative abundance > 1%) bacterial OTUs found in sourdoughs and concentrations of glucose, fructose, maltose, total free amino acids (FAA), total phenols and fibre in flours (F) and sourdoughs (S) and lactic acid, acetic acid, ethanol, in sourdoughs. Only significant correlations (FDR < 0.05) are shown.

markedly simplified in highly refined soft wheat flours (type 00, 0, and 1). This could be due to the low microbial contamination (epiphytic microbes are supposed to be absent or lower in wheat flours type 00, 0, and 1) and amount of fibres in refined flours. Flours were strongly contaminated by Proteobacteria (> 90% of the total microbiota). Additional phyla found in flour samples were Firmicutes, Actinobacteria and Bacteroides. Although with some quantitative variations, these phyla formed the common microbiome of wheat flours (Ercolini et al., 2013; Celano et al., 2016). This study highlighted strong differences among flour samples at family level. *Enterobacteriaceae* constituted the main component of the microbiome of refined soft and durum wheat flours. Proteobacteria represents one of the most commonly found plant endophytic bacteria (Liu et al., 2017). On the contrary, wholemeal durum wheat flour mainly harboured *Xanthomonadaceae*. Differences were also found between soft and durum wheat flours. Bacteria contaminating flour may originate from milling, external layers of wheat plant organs (epiphytic) or may represent a part of the endophytic microbial community of wheat. Previously, it was shown that after 1 day of propagation (8 h at 25 °C), flour bacterial population was almost completely inhibited (Ercolini et al., 2013). In the present study, after 8 h of fermentation (1 day), a different behaviour of the microbiome components was observed. Several OTUs found in flours disappeared, whereas other OTUs (*Enterobacteriaceae*, *Leuconostocaceae*, *Streptococcaceae*) increased depending on the extraction rate of flours. Wholemeal soft wheat flour (WSW) permitted the growth of the highest number of bacterial species during the first fermentation. Such phenomenon could be related to the higher buffer capacity of the system that did not allow rapid decrease of dough pH and the different chemical composition (fibres, phenols, maltose, etc) of the flours. After 8 h of fermentation (1 day), doughs were characterized by some

Table 2
Phytase activity of doughs before baking and concentration (mg/g) of phytate and total free cations (Ca^{2+}, Zn^{2+}, Fe^{2+}, Mg^{2+}) in wholemeal soft and durum wheat flour-based sourdough and control breads[a].

Sample	Phytase activity (U)	Phytate[b]	Total free cations
Wholemeal soft bread	0.85c	7.7a	0.76c
Wholemeal durum bread	0.80c	8.1a	0.68c
Bread based on wholemeal soft wheat flour and started with SW_0 sourdough	2.5b	5.21b	1.56b
Bread based on wholemeal soft wheat flour and started with WSW sourdough	5.7a	1.09c	3.38a
Bread based on wholemeal durum wheat flour and started with DWS sourdough	2.24b	5.17b	1.45b
Bread based on wholemeal durum wheat flour and started with WDW sourdough	5.86a	0.98c	3.52a

[a] Controls are represented by breads based on wholemeal soft wheat flour or wholemeal durum wheat semolina, both started with baker's yeast alone. Sourdough breads were based on: wholemeal soft wheat flour and started with soft wheat 0-based sourdough (SW_0); wholemeal soft wheat flour and started with wholemeal soft wheat flour-based sourdough (WSW); wholemeal durum wheat semolina and started with durum wheat semolina-based sourdough (DWS), or wholemeal durum wheat semolina and started with wholemeal durum wheat semolina-based sourdough (WDW).

[b] Values in the same column followed by different letters significantly differed ($p < 0.05$). The concentration of the phytate was the average of three replicates twice analysed.

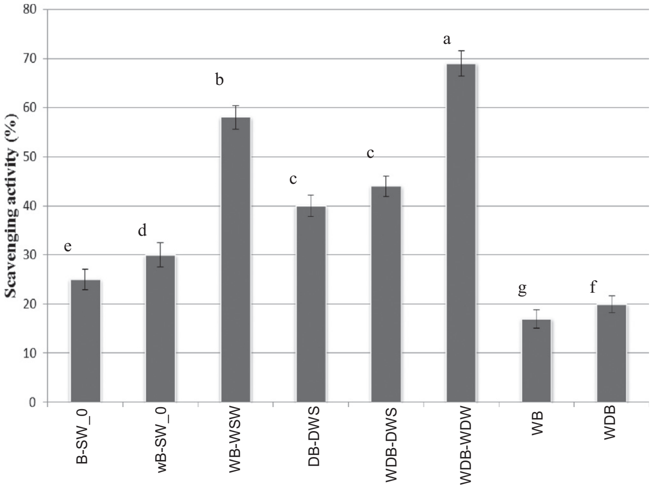

Fig. 5. Antioxidant activity of white soft wheat bread (B-SW_0) using SW_0 flour and the related sourdough; wholemeal soft wheat bread (WB-SW_0) using WSW flour and the SW_0 sourdough; wholemeal soft wheat bread (WB-WSW) using WSW flour and the related sourdough; durum wheat bread (DB-DWS) using DWS and the related sourdough; wholemeal durum wheat bread (WDB-DWS) using WDW semolina and DWS sourdough; and wholemeal durum wheat bread (WDB-WDW) using WDW and the related sourdough. Wholemeal soft wheat flour (WB) and durum wheat semolina (WDB) breads were made without sourdough fermentation but using only baker's yeast. Antioxidant activity, calculated as scavenging activity on DPPH radical, was expressed as follows: DPPH scavenging activity (%) = [(blank absorbance − sample absorbance) / blank absorbance] × 100. Data are the means of two independent experiments analysed in triplicate ($n = 6$). Different letters indicate that data are significantly different ($p < 0.05$).

microbiome traits such as: (i) *X. albilineans* and *X. oryzae* in wholemeal durum wheat flour-based dough; (ii) *Caulobacter tundrae*, *Micromonospora brunnescens*, and *Sphingobium francense* in wholemeal soft wheat flour-based dough; (iii) *Enterobacter*, *Klebsiella* and *Serratia* species in refined soft wheat flour doughs; (iv) *Erwinia*, *Clostridium* and *Enterococcus* species in low refined soft wheat flour-based doughs; (v) *Bacteroides*, *Anaerobacillus* and *Francisella* species and *Enterobacter cowanii* in highly refined durum wheat flour-based dough; and (vi) *W. cibaria*, *W. confusa*, *W. salipiscis* and *Staph. gallinarum* in durum wheat flour-based doughs.

The differences found among the microbiome of flours and the related doughs after the first fermentation could lead to different microbial assembly in the final mature sourdoughs (Celano et al., 2016). Indeed, all mature sourdoughs harboured a core microbiome constituted by 4 species (*P. pentosaceus*, *L. brantae*, *P. argentinicus* and *W. cibaria*). Previously, *P. pentosaceus* and *W. cibaria* were found at high numbers in many soft and durum wheat flour-based sourdoughs (Minervini et al., 2012; Scheirlinck et al., 2008; Taccari et al., 2016; Van Kerrebroeck et al., 2017). *L. brantae*, isolated from the faeces of Canada geese (*Branta canadensis*) in 2010 is phylogenetically very close to the *Lactobacillus casei* group (Volokhov et al., 2012). *L. brantae* has also been isolated from other food matrices (Bozoudi et al., 2016). However, further studies will be necessary to highlight the role of *L. brantae* in sourdoughs. Based on the type of flour, the relative abundance of each core species differed among sourdoughs. In addition, other dominant lactic acid bacteria species were variously detected in the mature sourdoughs. As showed by multivariate statistical analyses, specific lactic acid bacteria microbiome profiles were found depending on the extraction rate of flours. Overall, sourdoughs based on highly refined wheat flour harboured the highest levels of *L. brantae* (soft wheat flour) and *Leuc. citreum* (durum wheat semolina). Sourdoughs based on wholemeal wheat flours also harboured *W. cibaria*, *W. confusa*, and *W. salipiscis*. *W. cibaria* is a nomadic species with high adaptability to various environmental niches, including cereals and pseudocereals (De Vuyst et al., 2014; Minervini et al., 2014; Van Kerrebroeck et al., 2017).

The type of flour by-product (bran, shorts and germ) affected performances of a 36-year old type I sourdough, modifying the concentration of lactic and acetic acids and the specific volume (Clément et al., 2018). This study showed marked variation in terms of biochemical characteristics of mature sourdoughs. Specific correlations were found between the content of total phenols, fibre, glucose, fructose, FAA and maltose with both the relative abundance of some lactic acid bacteria species and the synthesis of lactic and acetic acids and ethanol. These findings could have technological/practical implications for the production of sourdough breads made by using wheat flours at different extraction rate (e.g., types 1 and 2) and wholemeal flours.

Compared to refined wheat flours, wholemeal flours contain high amount of phenolic acids and flavonoids in the free, soluble conjugated and insoluble bound forms (Vaher et al., 2010). Most of phenols are bioactive molecules, having mainly antioxidant activity, but processing conditions (e.g., milling, malting/germination, fermentation) markedly influence their levels and bioavailability (Hassani et al., 2016). These compounds have shown antioxidant, antimicrobial and anticancer effects *in vitro* and could be drivers of the microbiome assembly of sourdoughs (Koistinen et al., 2018). However, most of the studies created sourdough using starter strains to ferment refined wheat flour enriched with by-products and pseudo-cereals or wholemeal dough, which did not allow the evaluation of the impact of flour change on the formation of a stable traditional bakery sourdough (Clément et al., 2018; Coda et al., 2015; Rizzello et al., 2012). Compared to refined wheat flours, wholemeal flours contain high amount of phytates (Buddrick et al., 2014), which sequester and thereby prevent the intestinal absorption of some essential minerals, including Ca^{2+}, Zn^{2+},

Fe^{2+}, and Mg^{2+} (Kumar et al., 2010). Selected lactic acid bacteria strains and sourdough fermentation are undoubtedly efficient in reducing to less than one-half the phytate content of wholemeal wheat bread. This is mainly due to the reduction of the pH value, which provides favourable conditions for cereal endogenous phytase activities (Gobbetti et al., 2014). The hypothesis of this study is that the composition of mature sourdoughs is affected by the type of flour used. Consequently, traditional white flour-based sourdoughs could lack biochemical properties necessary to exploit the potential of the different type of flours, especially wholemeal flours (Gänzle, 2014). According to this hypothesis, this study showed that mature sourdoughs made by wholemeal soft or durum wheat flours had better performances when used to ferment wholemeal wheat flour, compared to the sourdoughs made by using refined flours. In detail, breads based on wholemeal soft or durum wheat flour and started by wholemeal wheat flour-based sourdoughs were characterized by the highest total amount of free cations (Ca^{2+}, Zn^{2+}, Fe^{2+}, Mg^{2+}), antioxidant activity and some improvement of sensory traits. The sourdough fermentation of whole meal flours has shown the most promising nutritional and functional results, using a biotechnological strategy that includes the use of wholemeal flours for sourdough preparation.

5. Conclusion

The present study evaluated the effect of extraction rate of *T. aestivum* and *T. durum* flours on the microbial composition and functionality of type I sourdough. First, this study showed that the use of flour type 00, 0, 1 and 2 and, especially, wholemeal flours affected the microbiome assembly of mature sourdoughs. Compared to refined flour based-sourdoughs, the use of wholemeal flours based-sourdoughs appears to be a more suitable biotechnological tool to produce wholemeal breads with improved mineral content, antioxidant activities and sensory features. These findings create a foundation for future research on the use of wholemeal flour-based sourdoughs in the bakery industry.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijfoodmicro.2018.08.009.

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