Evolution of lactic acid bacterial populations during lysine fortification of sourdough breads by addition of pistachio powder

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Short title: Characteristics of sourdough pistachio bread

ABSTRACT

Pistachio powder was added to flour or semolina to evaluate its contribution to increase the amount of lysine in bread. Bread production was carried out by sourdough technology using a selected 3-species (*Lactobacillus sanfranciscensis/Leuconostoc citreum/Weissella cibaria*) lactic acid bacterial (LAB) starter culture. All sourdoughs were subjected to a long-time fermentation (21 h) and showed levels of LAB around 10^9 CFU/g, indicating the suitability of pistachio powder for lactic fermentation. Yeasts were also detected, in particular in semolina trials. MySeq Illumina technology was applied to investigate the bacterial composition of sourdoughs evidencing a different distribution of LAB species among the trials with *Lactobacillus* as major LAB group in almost all sourdoughs. Physicochemical parameters were comparable among the trials. After baking, pistachio powder was found not to influence the height of the breads, but pistachio breads were more firm than control breads. Colour of the breads, void fraction and cell density, were influenced by pistachio powder. The amount of lysine increased consistently thanks to pistachio supplementation which also determined a higher presence of o-Xylene, p-Cymene and Limonene and the appearance of α-Pinene and 1-Octen-3-ol in breads. Sensory tests showed the best appreciation scores for the breads produced with flour and pistachio powder.

Keywords: Bread fortification; Lactic acid bacteria; Lysine; *Pistacia vera*; Sourdough; Volatile organic compounds

1. Introduction

In the last decades, consumers became more and more aware of the food components (Krystallis and Chrysochou, 2012) and started making their alimentary choices preferring healthy foods (Rayner et al., 2001). We are now living an age where most people acquired the belief that some foods promote healthfulness while others cause disease and death (Oakes and Slotterback, 2004). In particular, high-calorie foods are perceived as unhealthy, while low-calorie foods are considered

healthy (Carels et al., 2007). For this reason, especially the demand for vegetables is continuously on the increase (Settanni et al., 2012). In general, plant foods exert several positive effects on the human body (Lobo et al., 2010).

Cereal-based foods, especially those derived from rice, wheat and maize, represent the main energy sources for humans in the world (Spiertz and Ewert, 2009). However, processed cereals are deficient in essential amino acids (Torbatinejad et al., 2005), especially lysine (Young et al., 1994) and a cereal-based diet needs to be integrated with other sources of amino acids. Among plant-derived foods, nuts, as confirmed by epidemiological and/or clinical trials, play beneficial roles on the human health for the prevention of several diseases such as hypertension, diabetes and cancer. Pistachios are characterised by a lower fat and energy content than other nuts, but the contents of some vitamins and certain minerals are highest (Bulló et al., 2015). Furthermore, pistachios are rich in lysine (Soliman, 2012).

Food fortification represents the most important strategy applied in different productions. This practice has a long history of use in industrialized Countries for the successful control of deficiencies of vitamins A and D, several B vitamins (thiamine, riboflavin and niacin), iodine and iron. Conventional bread presents low content of protein, high carbohydrate, high glycemic index, low-resistant starch and low level of dietary fibers (Dhinda et al., 2011). From the early 1940s onwards, the fortification of cereal products with thiamine, riboflavin and niacin became common practice (Allen et al., 2006). Recently, the use of dietary fibers and ingredients or by-products rich in fibers (De Angelis et al., 2007, 2009; Rizzello et al., 2012), like mixture of soy proteins, oat bran and chickpea flour (Dhinda et al., 2011) and pea or chickpea flours (Dhinda et al., 2011; Kamaljit et al., 2010; Mohammed et al., 2012; Sadowska et al., 2003), as well as faba bean flour (Coda et al., 2017a) have been investigated to enhance the nutritional features of bread. These legumes are rich in lysine and deficient in sulfur containing amino acids, whereas cereal proteins are deficient in lysine but have adequate levels of sulfur amino acids (Eggum and Beame, 1983).

The main objective of this study was to evaluate the behavior of a mixed lactic acid bacterial starter culture in presence of pistachio powder in order to develop pistachio enriched baked goods and breads fortified in lysine. To this purpose flour and semolina were separately tested and the resulting productions characterized for the microbial populations, evaluated by culture methods as well as a next generation sequencing approach, and the physicochemical parameters during fermentation, and for lysine content, volatilome emission and acceptability by consumers after baking.

2. Materials and methods

2.1. Strains and growth conditions

The obligate heterofermentative strains *Lactobacillus sanfranciscensis* PON100336, *Leuconostoc citreum* PON10079 and *Weissella cibaria* PON10030 belonging to the culture collection of the Department of Agricultural, Food and Forest Sciences (University of Palermo, Italy) were used as mixed starter culture. These strains were all originated from wheat matrices (Alfonzo et al., 2013), applied for the production of tender wheat sourdough breads (Settanni et al., 2013) and also tested in form of liquid starters for the direct fermentation of durum wheat semolina (Alfonzo et al., 2016). The strains belonging to the species *Ln. citreum* and *W. cibaria* were chosen also because showed abilities to carry out sourdough fermentations at industrial level (Coda et al., 2018; Choi et al., 2012; Corona et al., 2016). *Lactobacillus sanfranciscensis* PON100336 was reactivated after overnight growth at 30 °C in Sour Dough Bacteria (SDB) broth (Kline and Sugihara, 1971), while the other two starters were propagated overnight at 30 °C in modified-de Man-Rogosa-Sharpe (mMRS) broth prepared as described by Corsetti et al. (2008).

2.2. Raw materials and dough production

The experimental breads were produced starting from pistachio powder packaged under vacuum (L'agricola di Cartillone, Bronte, Italy) in addition to the commercial tender wheat flour (Grandi Molini Italiani, Siracusa, Italy) and durum wheat semolina (Salvia Gaspare, Partinico, Italy). The experimental plan included two pistachio enriched sourdoughs, FPB [flour + 5% (w/w) pistachio powder and starter LAB] and SPB [semolina + 5% (w/w) pistachio powder and starter LAB] and the corresponding control sourdoughs, FB (flour and starter LAB) and SB (semolina and starter LAB).

Starter LAB were used in multi-strain combination. The bacterial mixture was prepared after the individual overnight growth of each strain as reported by Francesca et al. (2019) by centrifugation of the pellet at 5000 × g for 5 min and double washing in Ringer's solution (Sigma-Aldrich, Milan, Italy) and re-suspension to the optical density (evaluated spectrophotometrically at 600 nm) 1.00 which roughly corresponds to a cell density of about 10^9 CFU/ml for LAB. All trials were inoculated with the triple combination of LAB at approximately 10^6 CFU/g in dough.

Each dough of 300 g was produced with a dough yield (weight of the dough/weight of the flour × 100) of 160. Control doughs (FB and SB) were obtained adding 112.5 ml of tap water, containing the multi-strain LAB suspension, to 187.5 g of wheat flour or semolina. Experimental pistachio doughs were obtained with the same volume of inoculums to 178.1 g of wheat flour or semolina and 9.4 g (representing 5% of flour/semolina weight) of pistachio powder.

One-hundred grams of each dough were placed into the rectangular stainless steel baking pans (143 × 79 mm, top inside; 129 × 64 mm, bottom outside; depth inside 57 mm) indicated by the Method 10-10B of the American Association of Cereal Chemists (AACC, 2000) and covered with aluminium foils, while the rest of the doughs (200 g each) were placed into 500 ml-volume sterile glass beakers covered with parafilm. All doughs in pans as well as those in beakers were incubated at 30 °C for 21 h. Sourdough productions were carried out in duplicate and repeated twice after two weeks.

2.3. Physicochemical and microbiological analyses of sourdoughs

The acidification of the sourdoughs was monitored electrometrically using the portable pH meter Russell RL060P (Thermo Fisher Scientific, Beverly, MA), and measuring the total titratable acidity (TTA) with the official American Association of Cereal Chemistry method (AACC, 2003) (the results were expressed in terms of ml of NaOH/10 g of sourdough). The values of pH and TTA were determined in triplicate at t_0 and at 2 h intervals for the first 8 h and, subsequently, at 21 h.

The microbial loads were determined on raw materials, doughs soon after ingredient mixing and sourdoughs at 8 and 21 h of fermentation. Ten grams of each sample were first suspended in 90 ml Ringer's solution (Sigma-Aldrich, Milan, Italy), homogenized with a stomacher (BagMixer® 400, Interscience, Saint Nom, France) for 2 min at the highest speed, and then subjected to the decimal serial dilution. Cell suspensions were plated and incubated as follows: total mesophilic count (TMC) on plate count agar (PCA), incubated aerobically at 30 °C for 72 h; rod LAB on mMRS agar, incubated anaerobically at 30 °C for 48 h; sourdough LAB on Sour Dough Bacteria (SDB) incubated aerobically at 30 °C for 48 h; and total yeasts on yeast extract peptone dextrose (YPD) nutrient agar incubated at 28 °C for 48 h. To inhibit fungal growth, cycloheximide (10 mg/ml) was added to mMRS and SDB, while chloramphenicol (0.05 mg/ml) was added to YPD in order to inhibit the growth of bacteria. Microbiological counts were performed in triplicate.

2.4. Preparation of the MiSeq library and Illumina data analysis by QIIME2

The V3-V4 region of bacterial 16S rRNA gene was amplified using the primers 341F and 805R described by Baker et al. (2003). In order to conduct multiplexing of samples within a single sequencing run and to assign sequences to samples in bioinformatics analysis, a specific tag was added to both forward and reverse primers. PCR products were purified through Agencourt® AMPure® XP beads (Beckman Coulter, Indianapolis, USA) according to manufacturer's instructions and combined in equal molar concentration to generate a library pool. The 16S rRNA

gene library was sequenced at Genomic Platform – Fondazione Edmund Mach (San Michele a/Adige, Trento, Italy) using the Illumina MiSeq system (Illumina, USA).

After sequencing was completed, raw amplicon sequencing data were processed using Quantitative Insights Into Microbial Ecology (Qiime2, version 2018.2). First, raw reads were quality filtered, de-replicated and merged using DADA2 (Callahan et al., 2016); this tool was also used to detect and remove substitution and chimera errors. Taxonomic and compositional analyses were conducted by using plugins feature-classifier (https://github.com/qiime2/q2-feature-classifier). In order to generate taxonomy tables, paired-end sequence reads were classified by means of a pre-trained Naive Bayes classifier based on the Greengenes 13.8 97% Operational Taxonomic Units (OTUs) database (http://greengenes.secondgenome.com/), which had been previously trimmed to the V4 region of 16S rDNA, bound by the 341F/805R primer pair. Raw Illumina sequencing data are available at NCBI's Short Read Archive (SRA) under accession number PRJNA543801.

2.5. Baking process and bread analyses

At the end of fermentation, the doughs were baked in the oven Compact Combi (Electrolux, Pordenone, Italy) applying a 2-step program, including 5 min at 200 °C with the "Combi cooking" function, followed by 15 min at the same temperature with the "Convection heat" function.

Bread quality attributes were evaluated after cooling at ambient temperature. After weighed to determine the weight loss, breads were cut transversely in two halves and the central slice was measured with a caliper (Schober et al., 2005). The measurement of colorimetric parameters was performed on four points of the crust and three points of the crumb of the central slices with a Chroma Meter (CR-300; Minolta, Osaka, Japan). The Hunter's scale parameters were expressed as lightness (L*), redness (a*), and yellowness (b*), according to the International Commission on Illumination (CIE) L*a*b* system. The hardness of crumb was determined as reported by Corsetti et al. (2000) by means of the Instron-5564 (Instron Corp., Canton, MA). The two central slices of each bread were scanned (Epson Perfection 4180 Photo, Seiko Epson Corp., Japan) with 350 dpi of

resolution and the images were saved in TIFF format. The images were analysed with the ImageJ software (National Institutes Health, Bethesda, Md, USA). Each image was cropped to a square of 207 × 207 pixels (representing 15 × 15 mm of the slice area) and converted to grey-level image (8 bit). The Otsu's threshold algorithm was applied and void fraction (the fraction of the total area corresponding to the bread pores), cell density (number of cells/cm^2) and mean cell area (in mm^2) were calculated.

2.6. Determination of lysine content

2.6.1. Reagents

Lysine standards (99%) were supplied by Aldrich (Milwaukee, USA). Formic acid (98%) and acetic acid (glacial) at analytical grade were purchased from Merck (Darmstadt, Germany). Ultra-pure water (Milli-Q system, Millipore, Bedford, MA, USA) was used throughout the experiments.

2.6.2. Sample preparation

Stock solution of lysine (1000 µg/ml) was prepared by dissolving 25 mg of the compound in 25 ml distilled water and kept at 4 °C for a week for daily uses. Working standards were prepared daily by diluting the stock solution to different concentrations in the range 0.05–5.00 µg/ml with 0.2 mM acetic acid.

According to the method reported by Özcan and Şenyuva (2006) aliquot of 2 grams of pistachio powder, flour, semolina and bread samples were added with 0.2 mM acetic acid (10 ml) and homogenised with an Ultra-Turrax system (T 25 basic IKA labortechnik, Staufen Germany). After mixing by vortex for 2 min, each mixture was centrifuged at 5000 rpm for 10 min at −5 ◦C. The clear supernatant was quantitatively transferred into a vial avoiding the top oil layer if present and filtered through 0.45µm filter (Sartorius, Muggiò, Italy . prior to LC/MS analysis. An external calibration was obtained by analysing nine standard solutions at different concentrations. Analyses were performed in triplicate to ensure stability and reproducibility of the method.

2.6.3. Chromatographic procedure and measurement

The chromatographic system consisted of Agilent 6130 Series Quadrupole LC/MS Systems with a G1311A Quaternary Pump, G1329A High Performance Autosampler, G1316A Thermostated Column Compartment and G1315D Diode Array Detector (DAD).

The analytical separation was performed on a Zorbax Eclipse XDB C18 (75mm×4.6 mm, 5µm) supplied by Agilent Technologies (Wilmington, DE, USA). Identification and quantification of lysine were obtained using a G6120B Single Quadrupole LC/MS system equipped with an electrospray ionisation source (ESI). MS tune was optimised to the best experimental conditions. For target compound analysis, a flow injection analysis (FIA) was carried out to determine the fragmentor setting to improve the compound response. The potential chosen was 200 V. ESI work conditions were as follows: capillary voltage 5000 V, gas flow rate 13 L/m, gas temperature 300 °C and nebuliser pressure 60 Psi. To obtain the best sensitivity, the quadrupole was used in SIM mode. Optimum separation was achieved with a binary mobile phase gradient at 0.3 ml/m flow rate, the column temperature was kept at 30 °C and the injection volume was 20 µl. Solvents were (A) water/formic acid pH 3.1, and (B) acetonitrile. The gradient elution program was 0–15 min, 10–60% B, 15–20 min, 60–10% B.

The MS parameters subjected to optimisation were drying gas flow and temperature, nebuliser pressure and fragmentor voltage. The lysine compounds were identified and quantified by HPLC–ESI–MS. The identification was performed by comparing their mass spectra determined from standard solutions. In order to avoid or minimise interferences from background and side products, as well as to enhance the sensitivity, quantification was performed using HPLC-MS in SIM mode. The characteristic fragments of lysine amino acids and ions used in SIM mode for quantification are as follows: fragment ions m/z 147,130, 84; selected ion m/z 147; retention time 6.2. min. A peak corresponding to M+1 was found which was attributed to the formation of [M+H]+. [M+H]+ peak of the analyte was primarily exploited for quantitative purposes. Apart from [M+H]+, the remaining most abundant ion was used for confirmation purposes.

2.7. Volatile organic compounds emitted from breads

The analysis of the volatilomes from pistachio powder and bread samples was performed by gas chromatography (GC) technology. Each sample (5 g) was placed into 250 ml glass vials closed with a silicon septum and put into a dry heat block at 40.0±0.1 °C overnight to reach the equilibrium. The selected solid phase microextraction (SPME) fiber divinylbenzene/carboxen/polydimethylsiloxane (DVB/CAR/PDMS, 50 μm, Supelco), holder for manual sampling, was conditioned for 2 h at 250 °C in the inlet of the gas chromatograph according to the manufacturer's recommendations. The SPME fibre was manually inserted into the sample vial headspace for 60 min at 25 °C and, then, manually introduced into the GC×GC injection port at 250 °C kept for 5 min for desorption.

Chromatographic analyses were conducted on a GC-MS system comprising a GC instrument (Agilent 6890) and a mass selective detector (Agilent 5975 c). The column set used for GC×GC experiments comprised a fused silica capillary column Carbowax (30 m length, 0.25 mm internal diameter, and 0.25 μm film thickness; Supelco). GC–MS instrument was operated at 70 eV in the EI mode over the m/z range 30–550. Helium carrier gas at 1 ml/min. All analyses were performed using splitless injection mode at 250 °C. The temperature of the oven was programmed from 40 to 230 °C at 4 °C/min and then held isothermally for 40 min; the injector temperature and the transfer line were selected at 250 °C.

The identification of the compounds was achieved by comparing the fragmentation patterns of the experimental mass spectra with a commercial library (NIST05). The relative proportions of the individual components are expressed as percent peak areas normalisation, with all relative response factors being taken as one. Three replicates of each sample were analysed. Blanks, corresponding to analysis of the coated fibre not submitted to any extraction procedure, were run between sets of three analyses.

2.8. Sensory evaluation

A panel of 15 judges, including 6 women and 9 men (aged between 21 – 65 years old), were specifically trained for bread attribute evaluation. The sensory attributes of the final breads were analyzed following the guidelines of the ISO 6658. The judges were asked to evaluate 20 descriptors considering those suggested by Comendador et al. (2012), Rodrigues et al. (2014) and Martins et al. (2015), including color and thickness of crust, color, porosity, alveolation, and alveolation uniformity for crumb, intensity, bread and unpleasant odour, intensity, bread and unpleasant aroma, salty, acid, astringent, bitter, taste persistency, adhesiveness in mouth, crispness and the overall assessment. The scores were given using a line scale anchored on the left (visual analogue scale) with dislike/low quality and on the right with like/high quality. The hedonic scale results were converted as distance (cm) of mark from the left end of the line.

2.9. Statistical analyses

All data were tested for differences using the one-way analysis of variance (ANOVA; general linear model) followed by Tukey's multiple range test for $P \leq 0.05$ whereas Student's /-test was used to determine the significance and the difference between controls and pistachio breads using XLStat® add-in ver. 2014.5.03 (Addinsoft) for Microsoft Excel®.

3. Results and Discussion

3.1. Microbiological counts and acidification process

The microbial loads detected on the different samples collected during the experimentations are reported in Table 1. Regarding raw materials, flour showed the presence of detectable levels of TMC and LAB (but only on MRS), while semolina was characterised by all microbial groups investigated being below the detection limits. Previous investigation showed that the levels of LAB, as well as yeasts, are greatly variable (from undetectable to levels until 10^4 CFU/g) among flour and semolina samples (Alfonzo et al., 2013, 2016; Mamhoud et al., 2016; Nachi et al., 2019). Pistachio powder showed the presence of TMC at 3.25 CFU/g, but LAB and yeasts were undetectable. Al-

Moghazy et al. (2014) reported levels of about 10^5 CFU/g for under vacuum pistachio powder (indicated as pistachio flour in the reference work), but to our knowledge no published paper focussed on the presence of LAB on this matrix.

In doughs at t_0 the levels of LAB were slightly higher than those of TMC. These results confirm previous observations (Alfonzo et al., 2017; Corona et al., 2016) and are due to the nutritional requirements of LAB poorly satisfied by PCA. The levels of LAB as evaluated both on MRS and SDB were confirmed to be at levels of 10^6 CFU/g just after inoculation in doughs for all trials. After 8 h of fermentation, both control doughs reached levels (of about 1 log cycle) higher than those of the corresponding pistachio trials. However, these differences were much lower at the end of the experimentation (21 h), since the counts carried out on MRS as well as on SDB showed almost the same levels (around 10^9 CFU/g), indicating the suitability of pistachio powder for long fermentations carried out by LAB. Regarding the yeast component of sourdough microbiota, these eukaryotic organisms were undetectable until 8 h in both trials carried out with wheat flour, while they were detected at levels of 3.00 – 3.20 log CFU/g for both semolina trials at t_8. After 21 h of fermentation, all sourdoughs were characterised by the presence of yeasts, but the highest levels were displayed by SB and SBP trials.

The acidification process of pistachio sourdoughs is reported in Table 2. No significant differences were found among the four trials until 8 h of fermentation. All doughs started from a pH at around 6.6 and decreased at almost 4.3 at 8 h and below 4.0 after 21 h of fermentation. At that time, higher pHs were registered for the semolina trials in comparison to those carried out with flour and within the same raw materials, the trials with added pistachio showed higher pHs than the corresponding control trials. The decrease of pH values over time corresponded to the increase of TTA. Even though pH kinetics showed the lowest values for flour trials, the highest increase in TTA was showed by the trials carried out with semolina. Francesca et al. (2019) explained these data with the higher buffering capacity of semolina due to its higher protein content than flour. These results are comparable with those registered during fermentation of sourdoughs with legume addition (Coda et

al., 2017b; Diowksz et al., 2014; Mbata et al., 2009). Data on the acidification process of the four trials confirmed that all doughs could be considered sourdoughs.

3.2. Culture-independent analysis of sourdough bacteria

Figure 1 reports the distribution of the relative abundances (%) of bacterial genera identified by MySeq Illumina in raw materials (wheat flour, semolina and pistachio powder) and processed sourdoughs after 21 h of fermentation. A total of 18 bacterial groups were identified at different taxonomic levels. The sample with the highest percentage of unassigned OTUs was FBP, while almost all OTUs from unprocessed wheat flour were correctly identified. No LAB were detected in semolina and pistachio powder, while flour showed the presence of four genera (*Lactobacillus*, *Leuconostoc*, *Streptococcus* and *Weissella*) belonging to the LAB group. These data confirmed those obtained by plate counts that showed undetectable levels of LAB in pistachio powder and semolina while 1.85 log CFU/g of LAB were estimated in flour. Although pistachio powder showed the highest levels of TMC, MySeq Illumina revealed that to the viable counts of this raw material did not contribute LAB. Pistachio powder showed the highest percentage of *Enterobacteriaceae* among the samples analysed. Generally, pistachio or products processed from pistachio are reported as sources of bacteria included into *Enterobacteriaceae* family (Akbas and Ozdemir, 2006; Al-Moghazy et al., 2014; Iversen and Forsythe, 2004).

In general, fermented sourdoughs were characterised by the presence of *Delftia*, *Oxalobacteriaceae*, *Erwinia* and *Lactobacillus* as major bacterial groups. However, in terms of LAB presence, only *Lactobacillus* was found in both trials processed from semolina, FPB was characterised by *Weissella* and *Streptococcus* in addition to *Lactobacillus*, while, surprisingly, the control flour trial LAB community was only represented by *Weissella*. The suitability of *W. cibaria* to carry out lactic acid fermentation of sourdoughs is well documented (Alfonzo et al., 2016; Corona et al., 2016), but its dominance over *Lactobacillus* is not particularly common.

3.3. Bread attributes

The quality characteristics of the breads obtained with the addition of pistachio powder are reported in Table 3. The height of the breads registered on the central slices were not statistically different among the trials carried out with the same cereal material (with and without pistachio powder), but the breads obtained from both flour trials were significantly higher than those from semolina trials. The results obtained for control flour bread were comparable with those reported by Settanni et al. (2013) with the same three LAB inoculated singly and all producing breads with an average height higher than 30 mm. Also the values observed for control semolina breads were comparable with those registered by Alfonzo et al. (2016) who used different semolina genotypes.

The firmness of the breads was negatively correlated with the height, in fact flour breads were characterised by lower values than semolina trials. However, the addition of pistachio powder determined a higher firmness of breads than the corresponding control breads. The fortification might have different effects of bread firmness; Das et al. (2013) reported that fennel seed powder addition determined an increase of this parameter in final breads, while breads fortified with hulls and cotyledon fibres from legumes were characterised by the same firmness of control breads (Dalgetty and Baik, 2006).

Crumb colour was more influenced than crust colour by the presence of pistachio powder in the doughs. Regarding crust, yellowness resulted different between the two trials carried out with semolina, while no differences were registered between flour trials. In particular, the highest levels of yellowness were found for SB breads. The parameters a* and b* were particularly influenced by the type of wheat as well as pistachio addition. Also for crumb, the highest differences in L* were found between SB and SPB. All a* values were negative and the highest b* values were obtained with semolina breads. Generally, the addition of vegetable or mushroom powders might exert a strong effect on the colour of the final breads (Angioloni and Collar, 2012; Gaglio et al., 2019; Mohammed et al., 2012). The image analysis indicated that all breads were different especially for what concerns void fraction and cell density, whose values were at the highest levels in control

semolina breads. Higher values for void fraction of semolina breads in comparison with flour breads fermented with the same bacteria were also previously noticed (Alfonzo et al., 2016; Settanni et al., 2013).

3.4. Lysine content of breads

Lysine content was determined for raw materials and processed breads (Table 4). The amount of lysine detected in pistachio powder in our study was 2.15 mg/g that is much higher than that reported in the paper used as reference for lysine determination methodology (Özcan and Şenyuva, 2006). Although Özcan and Şenyuva (2006) detected 3.6 mg/100 g of fresh weight for entire pistachios, data are not comparable. Probably, the different results are due to the fact that pistachio subjected to shredding presents a higher levels of protein fragmentation during storage with generation of lysine.

The amount of lysine in pistachio powder was not particularly higher than that of flour (0.99 mg/g) and semolina (0.68 mg/g). Although soft wheat cultivars generate flours containing a lower protein percentage than hard wheat semolinas, from about 8 to 11% *versus* 10 to 14% protein, respectively (Delcour et al., 2012), the lysine content detected in this study for flour was higher than that displayed by semolina.

Control breads were characterized by amounts of this amino acid slightly lower than those detected in the corresponding unprocessed raw materials. These results are the consequence of the baking process that is reported to determine a loss of lysine of about 15% (Rosenberg and Rohdenburg, 1951). When pistachio powder was added, lysine content greatly increased and this effect was stronger for semolina rather than flour breads. Considering that barely 5% of pistachio powder was added, the higher contents of lysine estimated for FPB and SPB cannot be only due to the addition of pistachio, but it might be supposed that the action of fermenting microorganisms on pistachio proteins freed more lysine units than those obtained from wheat proteins.

3.5. Volatile profiles

The volatilome of the control breads (without pistachio powder supplementation) was characterised by 12 compounds when made from flour and 15 when produced with semolina (Table 5). In particular, the main volatile compound of all sourdough breads was 3-Methyl-1-butanol, typically reported as "fermentation" flavour of sourdoughs (Salim-ur-Rehman et al., 2006) which provides balsamic, alcoholic and malty notes (Pico et al., 2015).

Pistachio powder emitted 18 compounds, but only five impacted the volatilomes of the enriched breads; in particular, α-Pinene and 1-Octen-3-ol were present in FPB and SPB and absent in control breads, while o-Xylene, p-Cymene and Limonene were emitted at higher concentrations from the breads added with pistachio powder than from control breads. FPB and SPB were also characterised by the presence of Isoterpinolene, a terpenoid compound not present in control breads and not even in pistachio powder. This compound can be originated from Isolimonene (Heyen and Harder, 1998), but its presence in a sourdough has not been previously reported. However, terpens like L- and D-Limonene are generally associated to sourdough breads (Corona et al., 2016; Pétel et al., 2017). Indeed, pistachio powder showed 21.38% of Terpinolene and the presence of Isoterpinolene in pistachio powder supplemented breads might derive from this compound during baking; in fact, Comelli et al. (2005) reported that Terpinolene isomerization at 120 °C results also in the generation of Isoterpinolene. This compound is characterised by odor descriptors of herbal, woody and pine (Multari et al., 2018).

3.5. Sensory test

The spider plot reported in Fig. 2 shows the sensory evaluation of the final breads by the judges. The four breads were evaluated as different. For both wheat raw material trials (flour depicted in Fig. 2A and semolina depicted in Fig. 2B) the addition of pistachio powder impacted consistently crust and crumb color, odor and aroma intensity, astringency, sweet and taste persistency. Both breads supplemented with pistachio were characterized by a crispiness of the crust higher than that

of the control breads. Although porosity and regularity of alveolation were not different between breads produced with and without pistachio powder, alveolation was scored lower when pistachio was added. Control breads resulted more acidic with a higher crumb elasticity and characterized by higher levels of bread aroma and odor than pistachio breads. According to the judges, none of the breads showed strange odor and aroma. The addition of the pistachio powder determined a quite different sensory profile of the resulting breads and with regards to the overall assessment, intended as an overall rating of the breads expressed considering all parameters with their levels of evaluation, both pistachio breads were scored higher than the corresponding control bread, in particular FPB that was the most appreciated bread.

4. Conclusions

The present work was carried out to evaluate the behaviour of a mixed LAB starter culture in flour or semolina sourdoughs produced with the addition of pistachio powder. This supplementation was performed at 5% (w/w), following the common supplementation with legume flours, to increase the availability of lysine in cereal-based fermented products. The sourdoughs were employed to produce breads using AACC baking pans in order to evaluate quality parameters. The breads were produced without the addition of salt in order to develop a “basic” bread to taste the peculiar sensory characteristics due to pistachio powder for the development of future breads or sweet baked goods including this ingredient.

The results showed that all sourdoughs were dominated by *Lactobacillus* or *Weissella* strains. Thus, they clearly indicated that obligate heterofermentative species, necessary for the fermentation of cereal-based products, are able to grow at high levels in flour/semolina supplemented with pistachio powder. Pistachio powder affected firmness, crust and crumb color of resulting breads, which were characterised by an increased lysine content and were well appreciated, more than control breads, by judges. Sensory analysis indicated that pistachio enriched products should be preferably developed starting from flour rather than semolina.

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Table 1 Microbial loads (log CFU/g) of raw materials and sourdoughs[a].

Samples	PCA			MRS			SDB			YPD		
	T_0	T_8	T_{21}	T_0	T_8	T_{21}	T_0	T_8	T_{21}	T_0	T_8	T_{21}
Flour	2.10 ± 0.14	n.d.	n.d.	1.85 ± 0.04	n.d.	n.d.	<2	n.d.	n.d.	<2[a]	n.d.	n.d.
Semolina	<2	n.d.	n.d.	<1	n.d.	n.d.	<2	n.d.	n.d.	<2[a]	n.d.	n.d.
Pistachio powder	3.25 ± 0.21	n.d.	n.d.	<1	n.d.	n.d.	<2	n.d.	n.d.	<2[a]	n.d.	n.d.
FPB	5.85 ± 0.24[a]	6.62 ± 0.61[ab]	8.56 ± 0.27[a]	6.26 ± 0.19[a]	7.56 ± 0.72[a]	8.91 ± 0.38[a]	6.10 ± 0.29[a]	7.65 ± 0.18[a]	8.60 ± 0.37[a]	<2[a]	<2[b]	4.00 ± 0.49[b]
FB	5.70 ± 0.27[a]	7.48 ± 0.12[ab]	8.08 ± 0.39[a]	6.41 ± 0.11[a]	8.45 ± 0.81[a]	9.33 ± 0.67[a]	6.48 ± 0.22[a]	8.54 ± 0.41[a]	9.23 ± 0.63[a]	<2[a]	<2[b]	4.48 ± 0.34[ab]
SPB	5.83 ± 0.19[a]	6.32 ± 0.15[b]	8.43 ± 0.65[a]	6.58 ± 0.31[a]	7.70 ± 0.21[a]	8.89 ± 0.45[a]	6.22 ± 0.11[a]	7.40 ± 0.56[a]	8.95 ± 0.33[a]	<2[a]	3.20 ± 0.24[a]	5.18 ± 0.27[a]
SB	5.64 ± 0.30[a]	7.76 ± 0.87[a]	8.78 ± 0.61[a]	6.23 ± 0.19[a]	8.53 ± 0.63[a]	9.18 ± 0.32[a]	6.34 ± 0.02[a]	8.74 ± 0.74[a]	8.87 ± 1.11[a]	<2[a]	3.00 ± 0.18[a]	5.34 ± 0.36[a]
Statistical significance[b]	NS	*	NS	NS	NS	NS	NS	*	NS	NS	***	NS

[a] Units are log CFU/g. Results indicate mean values ± standard deviation (SD) of four plate counts (carried out in duplicate for two independent productions).
[b] Data within a line followed by the same letter are not significantly different according to Tukey's test. P value: *, P≤0.05; **, P≤0.01; ***, P≤0.001; NS, not significant.
Abbreviations: FPB, flour + 5% (w/w) pistachio powder and starter LAB; FB, flour and starter LAB; SPB, semolina + 5% (w/w) pistachio powder and starter LAB; SB, semolina and starter LAB; n.d. not determined.

Table 2 Acidification kinetics of pistachio sourdoughs.

Trials	t_0		t_2		t_4		t_6		t_8		t_{21}	
	pH	TTA	pH	TTA	pH	TTA	pH	TTA	pH	TTA	pH	TTA
FPB	6.60 ± 0.00[b]	1.00 ± 0.10[a]	5.59 ± 0.06[a]	2.50 ± 0.00[b]	5.48 ± 0.05[a]	2.80 ± 0.10[c]	5.31 ± 0.03[b]	4.20 ± 0.20[b]	4.28 ± 0.08[a]	5.60 ± 0.10[b]	3.70 ± 0.05[b]	10.00 ± 0.20[b]
FB	6.68 ± 0.04[a]	1.00 ± 0.10[a]	5.68 ± 0.07[a]	2.00 ± 0.00[c]	5.58 ± 0.05[a]	2.10 ± 0.10[d]	5.47 ± 0.02[a]	3.30 ± 0.20[c]	4.31 ± 0.09[a]	4.50 ± 0.10[c]	3.35 ± 0.06[c]	8.60 ± 0.20[c]
SPB	6.60 ± 0.03[b]	1.10 ± 0.00[a]	5.64 ± 0.11[a]	4.00 ± 0.20[a]	5.53 ± 0.04[a]	4.50 ± 0.10[a]	5.43 ± 0.08[ab]	5.40 ± 0.20[a]	4.26 ± 0.07[a]	6.10 ± 0.10[a]	4.05 ± 0.10[a]	11.60 ± 0.20[a]
SB	6.61 ± 0.02[ab]	1.10 ± 0.10[a]	5.64 ± 0.04[a]	2.50 ± 0.00[b]	5.59 ± 0.03[a]	3.50 ± 0.10[b]	5.49 ± 0.04[a]	3.90 ± 0.10[b]	4.30 ± 0.04[a]	4.50 ± 0.10[c]	3.90 ± 0.04[a]	10.10 ± 0.20[b]
Statistical significance[a]	**	NS	NS	***	*	***	*	***	NS	***	***	***

Results indicate mean values ± SD of four determinations (carried out in duplicate for two independent productions).

[a] Data within a column followed by the same letter are not significantly different according to Tukey's test. P value: *, $P < 0.05$ **, $P < 0.01$; ***, $P < 0.001$; NS, not significant.

Abbreviations: FPB, flour + 5% (w/w) pistachio powder and starter LAB; FB, flour and starter LAB; SPB, semolina + 5% (w/w) pistachio powder and starter LAB; SB, semolina and starter LAB; TTA, total titratable acidity.

Table 3 Bread attributes.

Trials	Height (mm)	Crust color			Crumb color			Firmness value (N)	Void fraction (%)	Cell density ($n.cm^{-2}$)	Mean cell area (mm^2)
		L^*	a^*	b^*	L^*	a^*	b^*				
FPB	32.00 ± 1.28[a]	59.58 ± 3.71[b]	2.18 ± 0.52[ab]	24.30 ± 2.02[c]	58.42 ± 2.63[a]	-0.48 ± 0.78[a]	13.24 ± 0.88[c]	29.29 ± 0.04[c]	3.48 ± 0.19[c]	1.41 ± 0.06[c]	0.06 ± 0.01[a]
FB	31.00 ± 1.09[a]	58.96 ± 4.57[b]	6.28 ± 3.32[a]	28.60 ± 2.53[bc]	63.06 ± 2.22[a]	-0.60 ± 0.26[a]	13.38 ± 0.60[c]	28.45 ± 0.01[d]	2.82 ± 0.03[d]	0.54 ± 0.11[c]	0.10 ± 0.03[a]
SPB	26.00 ± 1.55[b]	61.61 ± 1.94[b]	1.71 ± 0.27[b]	32.96 ± 1.99[ab]	50.93 ± 1.64[b]	-1.81 ± 0.32[b]	16.79 ± 1.13[b]	33.14 ± 0.08[a]	6.69 ± 0.14[b]	4.55 ± 0.56[b]	0.10 ± 0.06[a]
SB	23.00 ± 1.27[b]	70.29 ± 1.21[a]	-0.1 ± 0.54[b]	34.59 ± 0.63[a]	60.67 ± 0.87[a]	-2.80 ± 0.14[b]	22.12 ± 0.18[a]	32.19 ± 0.10[b]	12.88 ± 0.01[a]	18.07 ± 1.21[a]	0.09 ± 0.04[a]
Statistical significance[a]	***	***	**	***	***	***	***	***	***	***	NS

Results indicate mean values ± SD of four determinations (carried out in duplicate for two independent productions).

Data within a column followed by the same letter are not significantly different according to Tukey's test. P value: *, P≤0.05; **, P≤0.01; NS, not significant.

Abbreviations: FPB, flour + 5% (w/w) pistachio powder and starter LAB; FB, flour and starter LAB; SPB, semolina + 5% (w/w) pistachio powder and starter LAB; SB, semolina and starter LAB.

Table 4 Lysine content of breads.

Samples	Mean (mg/g)	SD[1]
Pistachio	2.15	0.19
Flour	0.99	0.02
Semolina	0.74	0.03
FPB	1.47	0.04
FB	0.80	0.08
SPB	1.82	0.03
SB	0.68	0.07

Results indicate mean values ± SD of three determinations.
Abbreviations: FPB, flour + 5% (w/w) pistachio powder and starter LAB; FB, flour and starter LAB; SPB, semolina + 5% (w/w) pistachio powder and starter LAB; SB, semolina and starter LAB; SD, standard deviation.

Table 5 Volatile organic compounds emitted from breads.

Chemical compounds[a]	Samples				
	Pistachio powder	**FPB**	**FB**	**SPB**	**SB**
Monoterpens					
α-Pinene	20.78	2.40	n.d.	2.22	n.d.
Camphene	1.53	n.d.	n.d.	n.d.	n.d.
Sabinene	1.90	n.d.	n.d.	n.d.	n.d.
Mircene	2.84	n.d.	n.d.	n.d.	n.d.
β-Pinene	4.42	n.d.	n.d.	n.d.	n.d.
Isosylvestrene	1.13	n.d.	n.d.	n.d.	n.d.
3-carene	6.96	n.d.	n.d.	n.d.	n.d.
p-Cymene	6.48	0.89	0.43	1.28	0.78
Limonene	16.40	4.63	4.06	5.50	4.36
β-Ocimene	0.77	n.d.	n.d.	n.d.	n.d.
γ-Terpinene	n.d.	0.36	0.29	0.46	0.40
β-Phellandrene	0.82	n.d.	n.d.	n.d.	n.d.
Isoterpinolene	n.d.	17.16	n.d.	10.8	n.d.
Terpinolene	21.38	n.d.	n.d.	n.d.	n.d.
1,3,8-p-Menthatriene	1.58	n.d.	n.d.	n.d.	n.d.
Monotherpens oxygenated					
Terpineol	5.85	n.d.	n.d.	n.d.	n.d.
Carvacrol	1.93	n.d.	n.d.	n.d.	n.d.
Carbonylic compounds					
Hexanal	n.d.	4.50	7.48	6.79	8.31
Benzaldehyde	n.d.	1.90	4.03	4.93	3.69
5hepten2-one.6methyl	n.d.	1.52	1.58	1.87	1.65
Acetophenone	n.d.	n.d.	0.45	0.78	0.56
3,5-Octadien-2one	n.d.	n.d.	n.d.	0.85	0.58
Nonanal	n.d.	3.25	2.13	5.39	4.20
n-Amyl isovalerate	n.d.	0.85	n.d.	3.19	2.18
Alcohols					
3-Methyl-1-butanol	n.d.	56.24	73.85	45.27	65.65
Undecanol	1.36	n.d.	n.d.	n.d.	n.d.
1-Octen-3-ol	0.83	0.88	n.d.	1.98	n.d.
2-Ethylhexanol	n.d.	0.74	0.75	1.59	1.36
Aromatic compounds					
o-Xylene	3.03	3.09	2.75	3.56	3.05
Anethole	n.d.	1.62	2.20	2.31	2.65
Coumarin	n.d.	n.d.	n.d.	1.24	0.58

[a] Results indicate mean percentage values of three measurements and are expressed as relative peak areas (peak area of each compound/total area of the significant peaks to all samples) x l00.

Legend to figures

Fig. 1. Relative abundances (%) of bacterial groups identified by MySeq Illumina in sourdough fermented for 21 h and raw materials. Only genera occurring at > 0.1% abundance in at least one sample were included. Abbreviation: FB, flour and starter LAB; FPB, flour + 5% (w/w) pistachio powder and starter LAB; SB, semolina and starter LAB; SPB, semolina + 5% (w/w) pistachio powder and starter LAB.

Fig. 2. Spider diagrams of descriptive sensory analysis of breads. (A) flour breads. (B) semolina breads. Abbreviation: FB, flour and starter LAB; FPB, flour + 5% (w/w) pistachio powder and starter LAB; SB, semolina and starter LAB; SPB, semolina + 5% (w/w) pistachio powder and starter LAB.

Fig. 1.

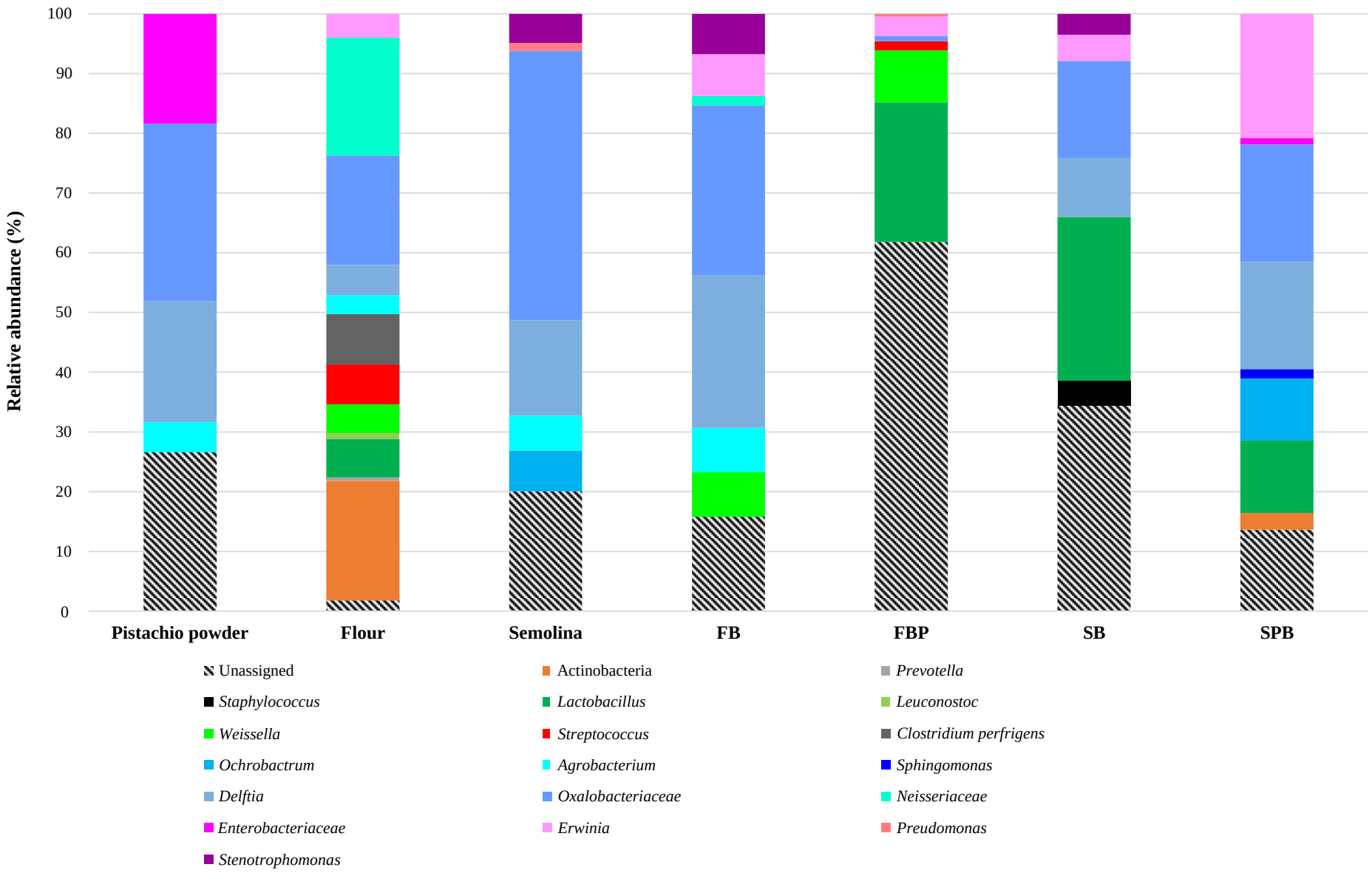
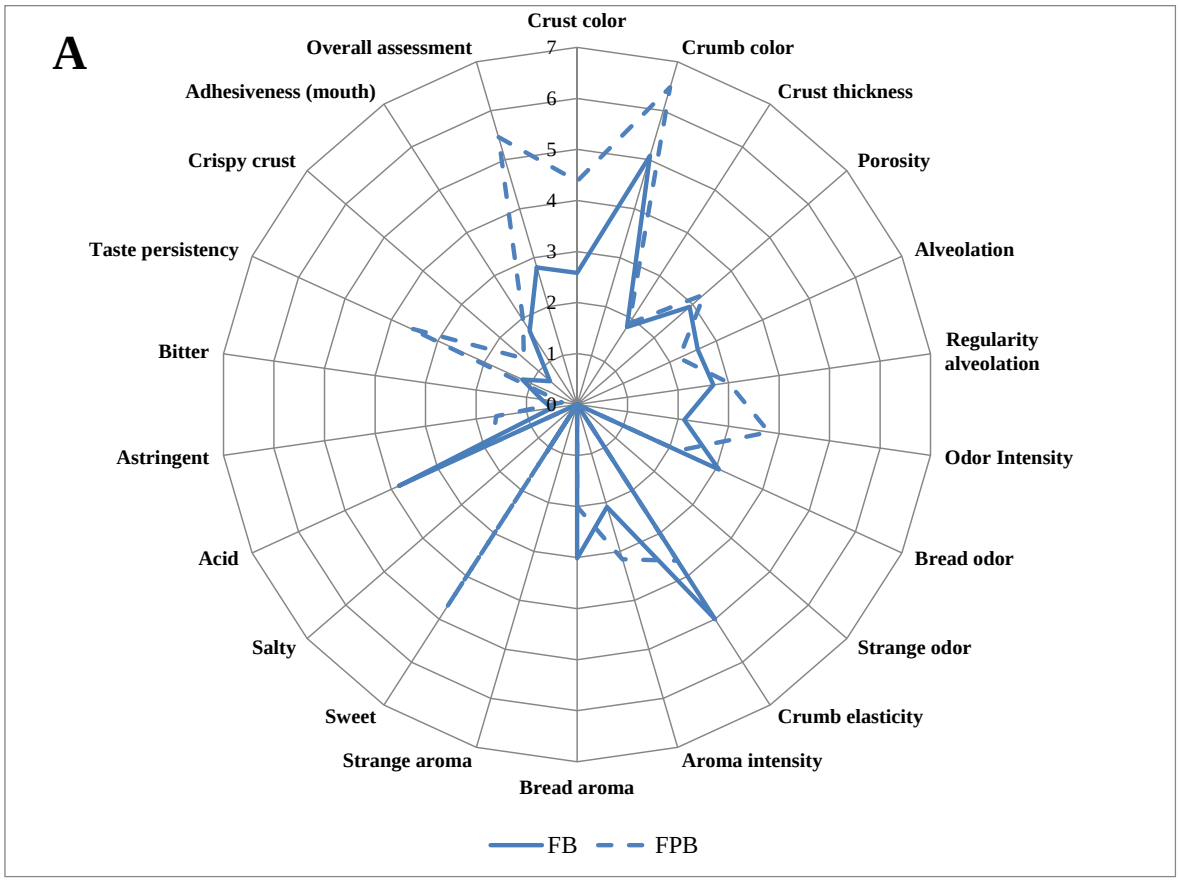
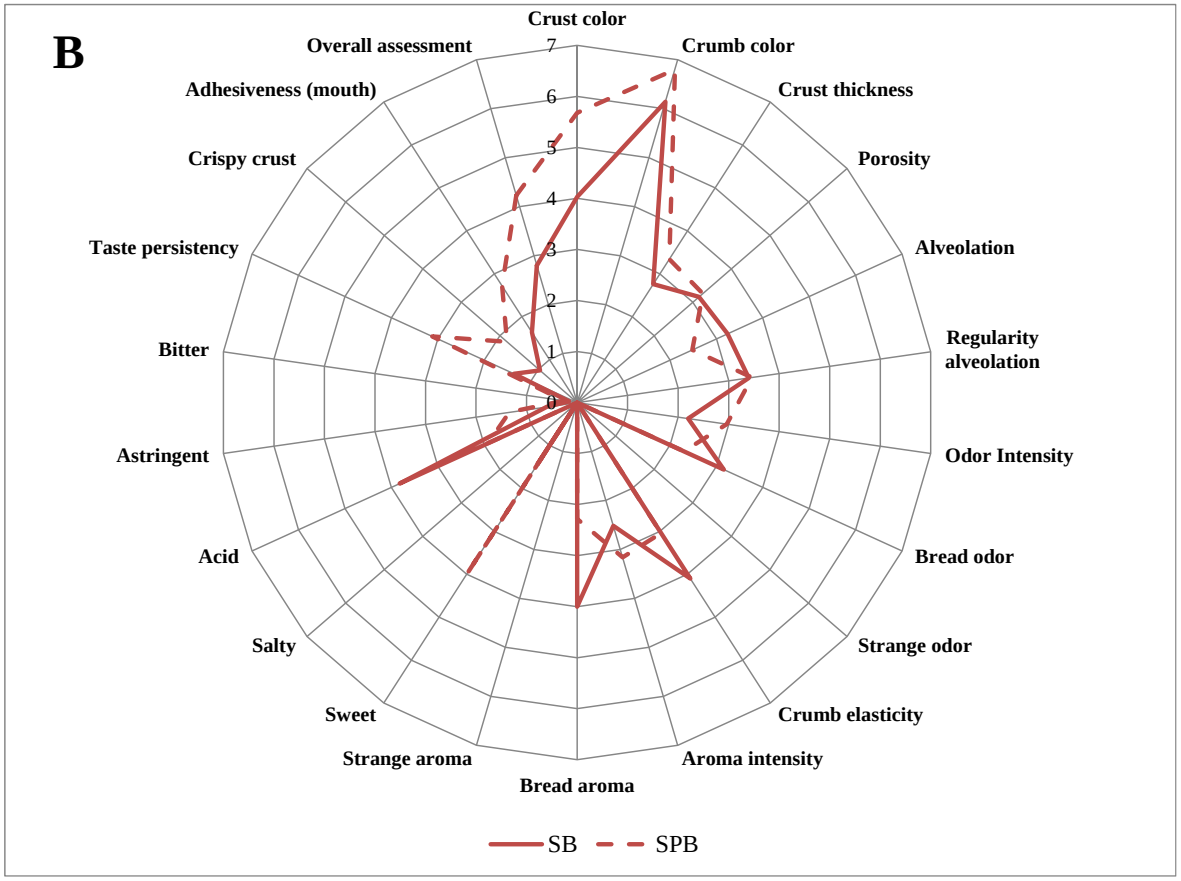

Relative abundance (%)
100
90
80
70
60
50
40
30
20
10
0
Pistachio powder
Flour
Semolina
FB
FBP
SB
SPB
Unassigned
Actinobacteria
Prevotella
Staphylococcus
Lactobacillus
Leuconostoc
Weissella
Streptococcus
Clostridium perfrigens
Ochrobactrum
Agrobacterium
Sphingomonas
Delftia
Oxalobacteriaceae
Neisseriaceae
Enterobacteriaceae
Erwinia
Preudomonas
Stenotrophomonas

Fig. 2.


A
Crust color
Crumb color
Crust thickness
Porosity
Alveolation
Regularity alveolation
Odor Intensity
Bread odor
Strange odor
Crumb elasticity
Aroma intensity
Bread aroma
Strange aroma
Sweet
Salty
Acid
Astringent
Bitter
Taste persistency
Crispy crust
Adhesiveness (mouth)
Overall assessment
0
1
2
3
4
5
6
7
FB
FPB


B
Crust color
Crumb color
Crust thickness
Porosity
Alveolation
Regularity alveolation
Odor Intensity
Bread odor
Strange odor
Crumb elasticity
Aroma intensity
Bread aroma
Strange aroma
Sweet
Salty
Acid
Astringent
Bitter
Taste persistency
Crispy crust
Adhesiveness (mouth)
Overall assessment
0
1
2
3
4
5
6
7
SB
SPB