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Novel solid-state fermentation of bee-collected pollen emulating the natural fermentation process of bee bread

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ABSTRACT

Structure of lactic acid bacteria biota in ivy flowers, fresh bee-collected pollen (BCP), hive-stored bee bread, and honeybee gastrointestinal tract was investigated. Although a large microbial diversity characterized flowers and fresh BCP, most of lactic acid bacteria species disappeared throughout the bee bread maturation, giving way to *Lactobacillus kunkeei* and *Fructobacillus fructosus* to dominate long stored bee bread and honeybee crop. Adaptation of lactic acid bacteria was mainly related to species-specific, and, more in deep, to strain-specific features. Bee bread preservation seemed related to bacteria metabolites, produced especially by some *L. kunkeei* strains, which likely gave to lactic acid bacteria the capacity to outcompete other microbial groups. A protocol to ferment BCP was successfully set up, which included the mixed inoculum of selected *L. kunkeei* strains and *Hanseniaspora uvarum* AN8Y27B, almost emulating the spontaneous fermentation of bee bread. The strict relationship between lactic acid bacteria and yeasts during bee bread maturation was highlighted. The use of the selected starters increased the digestibility and bioavailability of nutrients and bioactive compounds naturally occurring in BCP. Our biotechnological protocol ensured a product microbiologically stable and safe. Conversely, raw BCP was more exposed to the uncontrolled growth of yeasts, moulds, and other bacterial groups.

1. Introduction

Bee bread (BB) and bee-collected pollen (BCP) are honeybee-derived products, which represent excellent sources of high nutritional and functional compounds for human (Markiewicz-Żukowska et al., 2013; Denisow and Denisow–Pietrzyk, 2016; Sobral et al., 2017; Kieliszek et al., 2018). BB is hive-stored pollen undergoing a maturation process, likely driven by bee's glandular secretions and bee-associated microbial communities. According to this hypothesis, the hive-stored pollen is the outcome of a microbial succession involving mould, yeasts, and bacteria. Among bacteria, lactic acid bacteria have a key role (Herbert and Shimanuki, 1978; Gilliam, 1979a, 1979b; 1997; Loper et al., 1980; Gilliam et al., 1989; Vásquez and Olofsson, 2009; Vásquez et al., 2009; Anderson et al., 2011; Mattila et al., 2012; De Grandi-Hoffman et al., 2013; Lee et al., 2015). The hive-stored pollen matures after few weeks, resulting in a stable and more nutritious food for honeybees than fresh pollen (Human and Nicolson, 2006; Ellis and Hayes, 2009; Podrižnik and Božič, 2015). Compared to the fresh pollen, the hive-stored pollen undergoes a decrease of complex polysaccharides, a shift in amino acids, proteins and lipids profiles, and an increase of simple carbohydrates and titratable acidity (Herbert and Shimanuki, 1978; Loper et al., 1980; Human and Nicolson, 2006; Anđelković et al., 2012; Lee et al., 2015). The BB results into a stable food, due to the high concentration of simple sugars (35–61% dry weight), low pH (3.8–4.3), and the presence of antimicrobial compounds (Vásquez et al., 2009; Anderson et al., 2014; Podrižnik and Božič, 2015). Differently, BCP is collected by beekeepers through pollen traps, before undergoing any maturation process (Campos et al., 2010).

Nutritional and functional value of BB and BCP within the human diet had been widely reported (Nagai et al., 2004; Maruyama et al., 2010; Markiewicz-Żukowska et al., 2013; Sobral et al., 2017; Urcan et al., 2017). Bee pollen has been applied for centuries in traditional medicine and during the last decades experienced a favorable market as functional food owing to the trend toward a natural diet supplementation (Campos et al., 2008; Kieliszek et al., 2018). Bee pollen is recognized as human food, and given its nutritionally balanced composition; it is also considered a "perfect food" (Yang et al., 2013; Denisow and Denisow–Pietrzyk, 2016; Kaplan et al., 2016; Sobral et al., 2017). Several studies revealed a wide range of bioactivities of the bee pollen, such as antimicrobial, antioxidant, antiradical, anticancer, anti-inflammatory, hepatoprotective, anti-atherosclerotic, and immunomodulatory activities (Denisow and Denisow–Pietrzyk, 2016).

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Marketing of BB for human consumption is rather stunted, since its collection from the hive is very labor intensive and detrimental to the hive. Conversely, pollen removal through the traps is convenient for beekeepers and harmless to the hive. Nevertheless, the resulting product is not shelf stable. Due to the high values of humidity of freshly trapped pollen (6.7–20.6%) and corresponding values of water activity (0.3–0.7), BCP is compatible with growth of toxigenic moulds (Medina et al., 2004; González et al., 2005; Nardoni et al., 2016). The use of storage treatments is mandatory to prevent the spoilage and to preserve the nutritional and functional quality of BCP. Nevertheless, the technologies traditionally implemented present several drawbacks due to the detrimental effect on nutritional value or to the high operating costs (Collin et al., 1995; Campos et al., 2008, 2010; Soares de Arruda et al., 2013; Dias et al., 2016). To overcome these limitations, alternatives for BCP processing were considered to preserve the nutritional and functional quality of BCP throughout storage. One of the most attractive opportunities is the simulation of the natural fermentation occurring within the hive leading to the bee bread (Zuluaga et al., 2014). Solid-state fermentation might be applied for a scaled production. First, the use of autochthonous acid resistant and osmotolerant fructophilic lactic acid bacteria like *Lactobacillus kunkeei* to ferment BCP was investigated (McFrederick et al., 2012; Anderson et al., 2013; Corby-Harris et al., 2014). This study was also aimed at setting up a novel protocol of solid-state fermentation of BCP by mixed selected starters simulating the microbial consortia involved in bee bread fermentation. We may not fail to report that some authors questioned the role in bee pollen conversion by bacterial communities associated with honeybee, and reported the lack of a bacterial community co-evolved to predigest pollen (Anderson et al., 2014; Carroll et al., 2017). As the literature is limited and conflicting concerning the prominent role of microbes and the nutritional changes occurring during bee bread maturation, the experimental condition of this study could provide a scientific basis for the improved nutritional value of the bee bread.

2. Materials and methods

2.1. Flowers, bee-collected pollen, and bee bread sampling

Flowers of ivy (*Hedera helix* L.), bee-collected ivy pollen (BCP), 15 and 30 days hive-stored bee bread (BB) were collected during September–October 2016 from organic fields of the Apulia region (Italy). Samples were collected from the same hives of four different fields.

2.2. Isolation, typing and identification of lactic acid bacteria

Ten grams of flowers, fresh BCP, or BB samples were suspended into sterile 0.1% (w/v) peptone-water solution and homogenized with a Classic Blender 400 (PBI International) for 2 min at room temperature. Serial dilutions were plated on MRS agar (Oxoid Ltd, Basingstoke, Hampshire, England) and fructose-yeast extract-polypeptone (FYP) agar (10 g D-fructose, 10 g yeast extract, 5 g polypeptone, 2 g sodium acetate, 0.5 g Tween 80, 0.2 g $MgSO_4 \cdot 7H_2O$, 0.01 g $MnSO_4 \cdot 4H_2O$, 0.01 g $FeSO_4 \cdot 7H_2O$, 0.01 g NaCl, 0.05 g cycloheximide, and 0.05 g sodium azide per liter of distilled water [pH 6.8]) supplemented with 0.5% $CaCO_3$ (w/v), and incubated aerobically at 30 °C for 24–48 h (Filannino et al., 2016). MRS and FYP broth (Oxoid) were also used as enrichment media. At least 10 colonies were isolated from the highest dilutions of the MRS and FYP plates, based on the morphology and size of the clearance zone surrounding the colonies, and streaked on the same agar medium. The clearance zone surrounding the colonies indirectly indicates the hydrolysis of $CaCO_3$ reacting with organic acids synthesized by bacteria. Gram-positive and catalase-negative isolates were cultivated in FYP broth at 30 °C for 24 h and restreaked on FYP agar. Genomic DNA was extracted from 2 ml of FYP culture broth of each isolate using the DNeasy blood and tissue kit (Qiagen, Valencia, CA), according to the manufacturer's instructions. Taxonomic strain identification was performed by partial sequencing of the 16S rRNA gene. Two primer pairs, LacbF/LacbR and LpCoF/LpCoR (Sigma Chemical Co., Milan, Italy), were used to amplify 16S rRNA gene fragment of lactic acid bacteria De Angelis et al. (2006). Differentiation between *Lactobacillus plantarum*, *Lactobacillus pentosus* and *Lactobacillus paraplantarum*, was carried out by partial sequencing of the *recA* gene (Torriani et al., 2001). *Lactobacillus curvatus/Lactobacillus fuchuensis/Lactobacillus sakei* isolates were differentiated by partial sequencing of the *rpoA* gene. PCR products were separated by electrophoresis. The expected amplicons were eluted from the gel, purified by the Nucleospin gel and PCR cleanup kit (Macherey-Nagel, Düren, Germany), and subjected to Sanger sequencing (Sanger et al., 1977). The identification was performed by comparing the sequences of each isolate with those reported in the Basic BLAST database (Altschul et al., 1997). Strains showing homology of at least 97% were considered to belong to the same species (Goebel and Stackebrandt, 1994). Gene sequences have been deposited in GenBank under the accession numbers MH666040-MH666046.

Biotyping of isolates through randomly amplified polymorphic DNA-PCR (RAPD-PCR) analysis was performed as described by De Angelis et al. (2001) and Siragusa et al. (2009). Cultures were maintained as stocks in 15% (v/v) glycerol at −80 °C.

2.3. Lactic acid bacteria isolated from the *Apis mellifera* L. bee-gut

Sixteen strains of fructophilic lactic acid bacteria, belonging to the Culture Collection of the Department of Soil, Plant and Food Science, University of Bari Aldo Moro, Bari, Italy, were also screened. All strains were previously isolated from the *Apis mellifera* L. bee-gut and identified as *Lactobacillus kunkeei* (BIII60, BIII59, B17, B7, B23I, B4I, BV61, BV20, BVI14, BVI17, BVI52) and *Fructobacillus fructosus* (MBIII2, MBIII5, B5, B4, B1) (Filannino et al., 2016). Strains were identified by partial sequencing of the 16S rRNA gene. Cultures were maintained as stocks in 15% (v/v) glycerol at −80 °C and routinely propagated at 30 °C. Gene sequences have been deposited in GenBank under the accession numbers KX833121-KX833136.

2.4. Growth and acidification capacities of lactic acid bacteria strains in pollen extract

Pollen extract (PE) was chosen as growth model system to carry out the kinetics of growth and acidification of lactic acid bacteria strains. PE was obtained from BCP through a multi-step sequential chemical extraction. One-hundred grams of BCP were added of glass beads, mixed with 1 L of distillated water, and shaken at 500 rpm for 2 h, using a MaxQ™ 4000 Benchtop Orbital Shakers (Thermo Fisher Scientific Inc., Asheville, NC). The mixture was centrifuged at 10,000 × *g* for 20 min. The supernatant was collected, and the residues were sequentially extracted with 1 L of acidified methanol (0.1% HCl, v/v), hexane/acetone/ethanol 50:25:25 (v/v/v), and boiling water, respectively, as described above. All extracts were dried under vacuum using a Büchi Rotavapor RE21 (Büchi, Flawil, Switzerland) or a freeze-drier. Solids from each extraction were dissolved in 10 ml of dimethyl sulfoxide (DMSO), mixed together, and further diluted in distillated water to a final volume of 1 L. The PE was sterilized by filtration on 0.22 μm membrane filters (Millipore Corporation, Bedford, MA, USA). The total phenolic content of PE was quantified according to Folin-Ciocalteu method (Singleton and Rossi, 1965).

Strains of lactic acid bacteria were propagated in FYP broth at 30 °C under stirring conditions (200 rpm), until the late exponential growth phase was reached (ca. 12 h). Cells were harvested by centrifugation (10,000 × *g*, 10 min at 4 °C), washed twice in 50 mM sterile potassium phosphate buffer (pH 7.0), and re-suspended into the PE to a final cell density corresponding to ca. 7.0 log cfu ml^{-1}. PE was incubated at 30 °C for 24 h. Growth was monitored by measuring the optical density

Table 1
Microorganisms and growth conditions used in this study as indicators to assay the antimicrobial activity of lactic acid bacteria during growth on pollen extract.

Indicator microorganism	Culture collection	Growth conditions
Staphylococcus aureus DSM 20231	DSMZ[a]	Trypticase soy yeast extract medium at 37 °C
Listeria monocytogenes ATCC 19115	ATCC[b]	Brain heart infusion medium at 37 °C
Escherichia coli DSM 30083	DSMZ	Luria-Bertani broth at 37 °C
Bacillus megaterium F6	DISSPA[c]	Luria-Bertani broth at 30 °C
Pantoea agglomerans DTB8	DISSPA (Celano et al., 2016)	Nutrient broth at 30 °C
Escherichia hermannii PS2	DISSPA (Celano et al., 2016)	Nutrient broth at 30 °C
Serratia marcescens DR8	DISSPA	Nutrient broth at 30 °C
Serratia marcescens DR10	DISSPA	Nutrient broth at 30 °C
Aspergillus versicolor CBS 117286	CCCS[d]	Potato Dextrose Agar at 25 °C
Aspergillus niger DPPMAF3	DISSPA	Potato Dextrose Agar at 25 °C
Penicillium roqueforti DPPMA1	DISSPA	Potato Dextrose Agar at 25 °C
Penicillium polonicum CBS 112490	CCCS	Potato Dextrose Agar at 25 °C
Penicillium albocoremium CBS 109582	CCCS	Potato Dextrose Agar at 25 °C
Aspergillus parasiticus CBS 971.97	CCCS	Potato Dextrose Agar at 25 °C
Penicillium paneum CBS 101032	CCCS	Potato Dextrose Agar at 25 °C
Penicillium bialowiezense CBS 110102	CCCS	Potato Dextrose Agar at 25 °C

[a] DSMZ, Leibniz Institute German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany).
[b] American Type Culture Collection (Manassas, VA, USA).
[c] DISSPA, Department of Soil, Plant and Food Sciences (University of Bari, Bari, Italy).
[d] Culture Collection of Centraalbureau voor Schimmelcultures (Utrecht, Holland).

at 620 nm or plating on FYP agar. The pH was measured by a Crison pH-meter (model 507; Crison).

2.5. Antimicrobial activity of lactic acid bacteria strains

Lactic acid bacteria strains showing the greatest growth and acidification capacities were screened for their antimicrobial activity. Lactic acid bacteria were grown in PE at 30 °C for 24 h as described above. The cell-free supernatants (CFS) was recovered by centrifugation (10,000 × *g*, 10 min at 4 °C) of fermented PE, sterilized by filtration through 0.22 μm membrane filters (Millipore Corporation) and used to assay the antibacterial and antifungal activities of lactic acid bacteria during growth on pollen. The antimicrobial activity of fermented PE was compared to the activity of unfermented PE. The antibacterial activity was assayed through the well diffusion assay (Schillinger and Lücke, 1989). Analyses were carried out on 15 ml of water-agar (agar 2%, w/v) overlaid with 5 ml of different soft agar media, which contained ca. 4.0 log CFU ml^{-1} of an overnight culture of the indicator strains (Table 1). Wells (5 mm in diameter) were cut into agar plates, and 100 μl of sterile CFS was added. Plates were stored for 1 h at 4 °C to permit the radial diffusion of CFS, and incubation was allowed at 30 or 37 °C for 24 h. The antifungal activity was assayed based on the hyphal radial growth rates of indicator fungi (Table 1) (Quiroga et al., 2001). The sterile CFS was added (30%, v/v final concentration) to PDA, and aliquots of 15 ml were poured into petri plates (90 mm diameter). Control plates contained PDA alone. The assay was carried out by placing 3 mm-diameter plugs of growing mycelia onto the center of petri dishes containing the culture medium. Plates were incubated aerobically at 25 °C. The radial growth of mycelia (colony diameter, in mm) on all plates was measured 8 days after inoculation. Each datum point is the mean for at least four measurements of a growing colony. The percentage of hyphal radial growth inhibition was calculated from the mean values as follows: percent inhibition = [(mycelial growth under control conditions - mycelial growth in the presence of cell free PE)/mycelial growth under control conditions] x 100.

The antibacterial and antifungal activities of cell-free supernatants recovered after growth (30 °C for 24 h) of lactic acid bacteria in synthetic medium (FYP broth) was also tested following the protocol described above.

2.6. Set up of the protocol to ferment bee-collected pollen

Moisture content, level of inoculum, fermentation time and temperature were the process parameters used to set-up the protocol for lactic fermentation of fresh BCP. Fifty milliliters sealed tubes were used. Lactic acid bacteria cells were cultivated until the late exponential growth phase was reached (ca. 12 h), washed as previously described, and used to inoculate the BCP. Pectinolytic enzymes Endozym ICS 10 Rouge (AEB group, CA, USA) or the mixed fermentation with the yeast *Hanseniaspora uvarum* AN8Y27B (from the Culture Collection of the Department of Soil, Plant and Food Science, University of Bari Aldo Moro, Bari, Italy) were evaluated. *H. uvarum* had been reported as pectinolytic yeast and pollen inhabitant (Masoud and Jespersen, 2006; De Melo et al., 2015). Endozym ICS 10 Rouge was used according the manufacturer instructions (AEB group). *H. uvarum* AN8Y27B was routinely propagated at 30 °C in Sabouraud dextrose broth (Oxoid), and cells at the late exponential growth phase were washed twice in 50 mM phosphate buffer, pH 7.0, and used to inoculate the BCP as described by Xiao (2006).

The final fermentation protocol we set up included: (i) mixed inoculum of *Lactobacillus kunkeei* PF12, PL13, and PF15 strains, and *H. uvarum* AN8Y27 B at the final density in fresh BCP of ca. 8 Log CFU g^{-1}; (ii) addition of sterile water to reach the final water content of 40% (w/w); (iii) and fermentation of BCP into sealed tubes at 30 °C for 216 h. BCP fermented according this protocol by the selected mixed starter (started-BCP) was characterized as described below. BCP treated under the same conditions, except for the use of microbial starters, was used as control (unstarted-BCP). Features of started-BCP and unstarted-BCP were also compared with that of fresh BCP subjected to no treatment (Raw-BCP).

2.7. Physical-chemical analysis of BCP

Moisture content of BCP was determined through a Moisture Analyzer MA35 (Sartorius Stedim Biotech GmbH, Germany). Total titratable acidity (TTA) was measured on 10 g of BCP homogenized with 90 ml of distilled water through a Classic Blender and expressed as the amount (ml) of 0.1 M NaOH needed to achieve a pH of 8.3. The pH was measured by a Crison pH-meter (model 507; Crison).

2.8. Determination of carbohydrates, polyols and organic acids

One gram of BCP was mixed with an equivalent volume of glass beads and 5 ml of perchloric acid (5%, v/v). The mixture was shaken for 30 min at 4 °C (500 rpm) using a MaxQ™ 4000 Benchtop Orbital Shakers (Thermo Fisher Scientific Inc.). Then, the mixture was subjected to sonication (amplitude 60) using a macroprobe (Vibra-Cell sonicator, Sonic and Materials Inc., Danbury, CT) for 1 min (2 cycles, 30 s/cycle, 5 min interval between cycles) in an ice-bath. The suspension was centrifuged (10,000 × g, 10 min), and filtered through 0.22 µm membrane filters (Millipore Corporation). The supernatant was analyzed through HPLC, using an ÄKTA Purifier system (GE Healthcare, Uppsala, Sweden) equipped with an Aminex HPX-87H column (ion exclusion, Biorad) and Perkin Elmer 200a refractive index detector. Organic acids, polyols, and sugars used as the standards were purchased from Sigma-Aldrich (Steinheim, Germany).

2.9. Determination of peptides and free amino acids (FAA) in bee-collected pollen

One gram of BCP was mixed with an equivalent volume of glass beads and 20 ml of ethanol (80%, v/v). The mixture was shaken and subjected to sonication as described above. The suspension was centrifuged (10,000 × g, 10 min) and the supernatant was collected. The extraction was repeated, and the ethanolic extract solution was dried using a vacuum centrifuge (SpeedVac Concentrator SPD121P, Thermo Fisher Scientific Inc., Asheville, NC). Solids were dissolved in a volumetric flask with ultrapure water to 10 ml and analyzed. Total and individual FAA were analyzed by a Biochrom 30 series Amino Acid Analyzer (Biochrom Ltd., Cambridge Science Park, England), with a Na-cation exchange column (20 by 0.46 cm inner diameter) as described by Rizzello et al. (2010). The peptides concentration was determined by the o-phtaldialdehyde (OPA) method (Church et al., 1983). Peptides profiles were obtained by gel-filtration chromatography using a Superose 12 10/300 GL column and the ÄKTA FPLC equipment, with an UV detector operating at 214 nm (GE Healthcare Bio-Sciences AB). Trifluoroacetic acid was added (0.05%, v/v final concentration) to the ethanolic extract. The supernatant was collected by centrifugation (10,000 × g for 10 min), filtered through 0.22 µm membrane filters (Millipore Corporation), and loaded onto the column. Elution was performed at room temperature in 50 mM phosphate-buffered saline (pH 7.0) containing 0.15 M NaCl at 0.5 ml min-1. β-amylase (200 kDa), alcohol dehydrogenase (150 kDa), and carbonic anhydrase (29 kDa) were used as molecular weight markers.

2.10. Determination of total free phenolic compounds in bee-collected pollen

Water and methanol soluble free phenolics were obtained from BCP through a multi-step sequential chemical extraction. One hundred grams of BCP were mixed with 1 L of distillated water. The mixture was purged with nitrogen stream for 30 min, under stirring condition, and centrifuged at 10,000 × g for 20 min. The supernatant was collected, and the residues were further extracted with 1 L of acidified methanol (0.1% HCl, v/v) following the same procedure. Total phenolics in water and methanol extracts was quantified according to Folin-Ciocalteu method and expressed as g gallic acid equ kg^{-1} dry weight of BCP (Singleton and Rossi, 1965).

2.11. In vitro digestibility of bee-collected pollen

The *in vitro* digestibility of BCP was determined by the method of Akeson and Stahmann (1964). The concentration of protein of the supernatant was determined by the Bradford method (Bradford, 1976). The precipitate was subjected to protein extraction, according to Vanderplanck et al. (2014), and the concentration of protein was determined. The *in vitro* protein digestibility was expressed as the percentage of the total protein, which was solubilized after enzyme hydrolysis.

2.12. Microbial stability of bee-collected pollen

Raw-, started-, and unstarted-BCP were stored at room temperature (25 °C) for 50 days in order to study their shelf-life. Bacteria, yeasts, and moulds were enumerated in BCP before and after fermentation, as well throughout the storage. Ten grams of BCP were suspended into sterile 0.1% (w/v) peptone-water solution and homogenized with a Classic Blender 400 for 2 min at room temperature. Serial dilutions were plated on the following selective media: FYP agar plus cycloheximide 0.05 $g\,l^{-1}$ and sodium azide 0.05 $g\,l^{-1}$ for lactic acid bacteria (48 h at 30 °C); Malt Extract Agar plus chloramphenicol 0.1 $g\,l^{-1}$ for yeasts (48 h at 30 °C); Plate Count Agar plus cycloheximide 0.1 $g\,l^{-1}$ for total mesophilic microorganisms (48 h at 30 °C); ChromoCult Coliform Agar for total coliforms (24 h at 37 °C); MacConkey Agar No2 for enterobacteria (48 h at 37 °C); GSP Agar plus penicillin-G 60 $g\,l^{-1}$ for *Pseudomonas* spp. and *Aeromonas* spp. (48 h at 25 °C); Baird Parker Agar for *Staphylococcus* spp. (48 h at 25 °C); Slanetz and Bartley Medium for enterococci (48 h at 37 °C); Wort Agar for moulds (5 days at 25 °C). ChromoCult Coliform Agar and GSP Agar were purchased by Merck KGaA (Darmstadt, Germany). Except for FYP agar, all the other media were purchased by Oxoid Ltd (Hampshire, England).

To further investigate the microbiological stability of BCP, the conidial germination assay was performed on BCP as described by Coda et al. (2008). After growth on PDA plates, conidia of *Aspergillus parasiticus* CBS 971.97 were harvested in sterile water, containing 0.05% (v/v) Tween 80. The count of the conidia in the suspension was carried out using the Petroff-Houser Counting chamber. Twenty grams of raw, started-, or unstarted- BCP were poured into petri plates (90 mm diameter) and inoculated by nebulization with a suspension of 10^2 conidia/ml of *A. parasiticus* CBS 971.97. *A. parasiticus* was used as indicator since it occurs frequently in bee pollen, and it had been reported as producer of mycotoxins, such as aflatoxin B_1 (Kostić et al., 2017). Petri plates were packed in polyethylene bags to maintain constant moisture and incubated at 25 °C for 10 days. The contamination was detected by visual observation of the BCP surface and approximately quantified as the percentage of the surface covered by the visible mycelia of the indicator mould.

2.13. Statistical analysis

Analyses were carried out in triplicate with three biological replicates for each condition. Analysis was performed using Student's *t*-test for single comparison, or analysis of variance (ANOVA) test for multiple comparisons (one-way ANOVA followed by Tukey's procedure at $P < 0.05$), using the statistical software, Statistica 7.0 (Statsoft). Data of growth and acidification in PE were subjected to permutation analysis using PermutMatrix.

3. Results

3.1. Isolation, typing and identification of lactic acid bacteria

Cell densities of presumptive lactic acid bacteria in ivy (*Hedera helix* L.) flowers, bee-collected ivy pollen (BCP), 15 and 30 days hive-stored bee bread were enumerated on FYP and MRS agar. Lactic acid bacteria were not detectable in 10 g of ivy flowers, and were isolated only after enrichment in FYP and MRS broths. Cell number of lactic acid bacteria ranged from 5.21 ± 0.43 to 5.83 ± 0.52 Log CFU $mL^{-1}\ g^{-1}$ in BCP. Samples of 15 and 30 days hive-stored bee bread showed a cell number of lactic acid bacteria ranging from 5.30 to 5.52 and from 3.18 to 3.54 Log CFU $mL^{-1}\ g^{-1}$, respectively. Gram-positive, catalase-negative, non-motile cocci and rods, able to acidify the culture medium were identified by partial sequencing of the 16S rRNA, and biotyped through

Table 2
Species of lactic acid bacteria identified from ivy (*Hedera helix* L.) flowers, bee-collected ivy pollen (BCP), 15 days hive-stored bee bread, and 30 days hive-stored bee bread. The table shows also the representative strains for each cluster recognized by combined RAPD patterns[a].

Source	Species	Representative strains of clusters
Ivy flowers	*Lactococcus lactis*	EF70, EF67
Bee-collected ivy pollen	*Fructobacillus fructosus*	PL10, PL22, PL21, PL25, PL17
	Lactobacillus kunkeei	PF5, PL3, PL27, PL31, PL24, PL13, PF18, PL15, PL33, PL34, PF15, PF7, PF16, PF6, PF29, PL9, PF12, PL28, PF10, PL12, PL20, PF1
	Lactobacillus curvatus	PL32
15 days hive-stored bee bread	*Lactobacillus plantarum*	PLB1, PLB15, PLB16
	L. kunkeei	PFB13, PLB20, PLB29, PLB30, PLB18, PLB34, PLB12, PFB7, PFB3, PLB17
	F. fructosus	PFB26, PLB6, PFB29, PFB34
	Lc. lactis	PFB15,PFB12
	Leuconostoc citreum	PFB11
	L. curvatus	PFB30, PFB8, PLB7, PFB10, PFB19
30 days hive-stored bee bread	*L. kunkeei*	PFA35, PFA7, PLA21, PFA5, PLA9, PLA8, PLA24, PLA6, PFA15, PFA2, PFA4, PFA9, PLA14, PFA12, PLA16, PLA13, PFA3
	F. fructosus	PFA18, PFA23, PLA1, PFA25, PFA34,

[a] The combined RAPD profiles were subjected to cluster analysis by unweighted pair-group method using arithmetic averages (UPGMA), aiming to exclude clonal relatedness.

RAPD-PCR analysis. All 45 isolates of lactic acid bacteria from ivy flowers were identified as *Lactococcus lactis*, which were grouped into 2 clusters at the similarity level of 88% based on RAPD patterns (Table 2). The 38 isolates from BCP were identified as *Fructobacillus fructosus*, *Lactobacillus kunkeei*, and *Lactobacillus curvatus*, and were grouped into 28 clusters (similarity level of 84%) (Table 2). *Lactobacillus plantarum*, *L. kunkeei*, *F. fructosus*, *Lc. lactis*, *Leuconostoc citreum*, and *L. curvatus* were identified from 15 days hive-stored bee bread, and the 64 isolates were grouped into 25 clusters (similarity level of 81%) (Table 2). Only *F. fructosus* and *L. kunkeei* species were identified in 30 days hive-stored bee bread, and the 67 isolates were grouped into 22 clusters (similarity level of 82%) (Table 2). One representative strain for each cluster, recognized by combined RAPD patterns, was selected and used for further characterizations.

3.2. Growth and acidification capacities of lactic acid bacteria strains in pollen extract

The 77 representative strains of lactic acid bacteria isolated from ivy flowers, BCP, and bee bread (Table 2), and 16 strains of fructophilic lactic acid bacteria previously isolated from the *Apis mellifera* L. bee-gut were screened based on the growth and acidification capacity in pollen extract (PE). PE reproduced the same selective pressure of BCP for the growth of lactic acid bacteria, due to its high acidity (pH 4.10 ± 0.04) and its high content of antimicrobial compounds, such as phenolics (56 ± 5 mg gallic acid equ L^{-1}). Based on the cell density increase (ΔOD_{620}) and pH decrease (ΔpH) after 24 h of incubation at 30 °C, strains were grouped into 4 clusters (A-D) (Fig. 1). Cluster A grouped strains with the highest ($P < 0.05$) values of ΔOD_{620} (1.73–2.10) and ΔpH (0.66–1.05), followed by clusters B (ΔOD_{620}, 1.11–1.68 and ΔpH, 0.55–0.88), C ($\Delta OD_{620,}$ 0.42–0.92 and ΔpH, 0.40–0.63) and D (ΔOD_{620}, 0.04–0.69 and ΔpH, 0.02–0.34). Only L. *kunkeei* strains were included in cluster A, whereas cluster B and C included also *L. plantarum* and *F. fructosus* strains. Cluster D, which grouped the less growth and acidification performing strains, included *L. curvatus*, *Lc. lactis*, and *Leuc. citreum*, together with *L. kunkeei* and *F. fructosus* species (Fig. 1).

3.3. Antimicrobial activity of lactic acid bacteria strains in pollen extract

Forty strains of lactic acid bacteria belonging to A, B, and C clusters (36 *L. kunkeei*, 1 *F. fructosus*, and 3 *L. plantarum* strains) (Fig. 1), which showed the greatest ($P < 0.05$) growth and acidification capacities, were also screened for their antimicrobial activity. Lactic acid bacteria were grown in PE at 30 °C for 24 h and the cell-free supernatants was recovered and used to assay the antibacterial and antifungal activities. No inhibition ($P > 0.05$) was found when unfermented PE was used. Significant ($P < 0.05$) antibacterial activity was observed towards all the indicator bacteria, although different inhibition spectra were observed depending on the lactic acid bacteria strains (Table 3). A significant ($P < 0.05$) antifungal activity was also found with 12 *L. kunkeei* strains and *F. fructosus* B4 towards most of all indicator moulds. The widest ($P < 0.05$) inhibition spectra were found with *L. kunkeei* PF12, PL13, and PF15 (Table 4). No inhibition was observed testing the cell free extract obtained after growing in synthetic medium.

3.4. Fermentation of bee-collected pollen

Based on growth and acidification capacities and antimicrobial activity, *L. kunkeei* PF12, PL13, and PF15 were chosen as the best performing strains and used as mixed starter for BCP fermentation. A protocol was set up, which included mixed inoculum of *Lactobacillus kunkeei* PF12, PL13, and PF15 strains, and *H. uvarum* AN8Y27 B at the final density of ca. 8 Log CFU g^{-1}, and fermentation at 30 °C for 216 h with water content of 40% (w/w). These conditions were selected based on preliminary trials (data not shown). BCP treated under the same conditions, except for the use of microbial starters, which was used as control (unstarted-BCP), underwent a spontaneous fermentation. With the addition of the selected mixed starter, the cell density of the lactic bacteria in the pollen reached ca. 9 Log CFU g^{-1} after 96 h, remained almost stable until 144 h, then decreased to 7.03 ± 0.14 Log CFU g^{-1} throughout the incubation time (Fig. 2). The co-inoculums of *H. uvarum* AN8Y27B was an essential condition to allow a rapid growth and acidification by lactic acid bacteria. When the selected lactic acid bacteria were added without the co-inoculums of *H. uvarum* AN8Y27B, their growth and acidification performances were lacking and comparable to those observed during spontaneous fermentation (data not shown). During the spontaneous fermentation of the unstarted-BCP, the lactic bacteria reached the density of ca. 9 Log CFU g^{-1} only after 120 h, and rapidly decreased until 3.95 ± 0.41 Log CFU g^{-1} during the remaining incubation time (Fig. 2). Decreasing trend of yeasts was found throughout 216 h of incubation both in started- and unstarted-BCP (Fig. 2). The kinetics of acidification during fermentation were consistent with the growth of lactic bacteria. Compared to the started BCP, the TTA of unstarted-BCP followed a trend slower and less intense ($P < 0.05$) (Fig. 2). The pH reflected the TTA values. The initial values of pH of raw-BCP was 4.19 ± 0.04, which decreased in started-BCP to

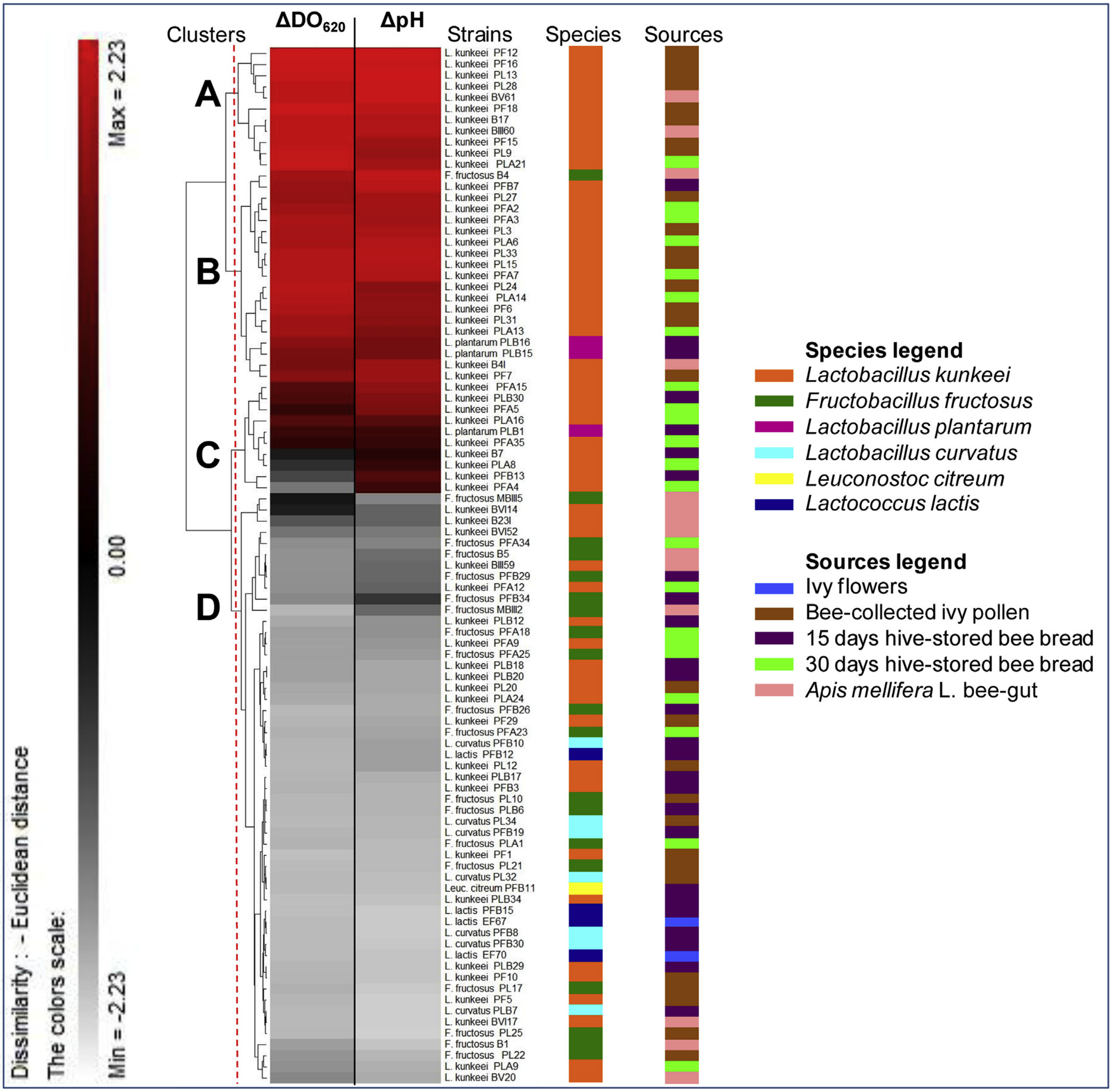

Fig. 1. Pseudo-heat map showing the cell density increase (ΔOD_{620}) and pH decrease (ΔpH) values in pollen extract (PE) inoculated with 77 strains of lactic acid bacteria after 24 h of incubation at 30 °C. The color scale shows differences, with red and light gray indicating the highest and lowest values of the standardized data for ΔOD_{620} and ΔpH. Lactic acid bacteria species and source of isolation were highlighted with different colors. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

3.95 ± 0.04 after 48 h and to 3.60 ± 0.03 after 216 h. The unstarted-BCP reached a similar final pH value (3.62 ± 0.12), but acidification was significantly ($P < 0.05$) slower than in started-BCP.

Glucose and fructose were the dominant carbohydrates of raw-BCP (223 ± 11 and 161 ± 9 $g\,kg^{-1}$ dry weight, respectively). During fermentation, started-BCP showed a substantial ($P < 0.05$) decrease of glucose and fructose (ca. 39 and 80% w/w, respectively). Main microbial metabolites were lactic (87 ± 4 $g\,kg^{-1}$ dry weight) and acetic (51 ± 3 $g\,kg^{-1}$ dry weight) acids, and mannitol (151 ± 8 $g\,kg^{-1}$ dry weight). Similarly, in unstarted-BCP the decrease ($P < 0.05$) in glucose and fructose (ca. 31 and 71% w/w, respectively) was followed by the production of lactic (70 ± 14 $g\,kg^{-1}$ dry weight) and acetic (42 ± 9 $g\,kg^{-1}$ dry weight) acids, and mannitol (112 ± 15 $g\,kg^{-1}$ dry weight). Ethanol was not found in any samples.

3.5. Nutritional characterization of fermented bee-collected pollen

Raw-BCP, BCP fermented by the selected mixed starter (started-BCP), and spontaneously fermented unstarted-BCP were characterized for the peptides, FAA, and free phenolics content and for *in vitro* digestibility of proteins. Concentration of peptides of raw-BCP was 14.12 ± 2.40 $mg\,kg^{-1}$ dry weight, which increased to 50.78 ± 1.86 $mg\,kg^{-1}$ dry weight after the fermentation with the mixed starter. An increase was also found in unstarted-BCP

Table 3
Antibacterial activity[a] of selected lactic acid bacteria strains grown in pollen extract (PE) at 30 °C for 24 h. Cell-free supernatants was recovered, and used to assay the inhibitory spectrum of PE. Unfermented PE was used as the control.

	Staphylococcus aureus DSM 20231	*Listeria monocytogenes* ATCC 19115	*Escherichia coli* DSM 30083	*Bacillus megaterium* F6	*Pantoea agglomerans* DTB8	*Escherichia hermannii* PS2	*Serratia marcescens* DR8	*Serratia marcescens* DR10
Lactobacillus kunkeei PF12	+	+ + +	+ + +	+ + + +	+ +	+ + +	+ +	+ + +
L. kunkeei PF16	+	+ +	+ +	+ + +	+ + + +	+ + +	+ +	+ +
L. kunkeei PL13	+	+ +	+ +	+ + + + +	+ + +	+ + +	+ +	+ +
L. kunkeei PL28	–	+ +	+ +	+ + + +	+ + + +	+ + +	+ +	–
L. kunkeei BV61	–	+ +	+ +	+ + + +	+ +	–	+ +	–
L. kunkeei PF18	–	+	+ +	+ + + +	–	–	+ +	+ + +
L. kunkeei B17	+	+ +	+ +	+ + + +	+ + + +	+	+ +	–
L. kunkeei BIII60	–	+	+ +	+ + + +	+	+	+ +	–
L. kunkeei PF15	+	+ +	+ + +	+ + + + +	+ + + +	+	+ + +	+ + +
L. kunkeei PL9	–	+	+ +	+ + + +	–	–	+ +	+ + +
L. kunkeei PLA21	–	+	+ + +	+ + + +	+ + +	–	+ +	+ + +
Fructobacillus fructosus B4	–	+	+ +	+ + + +	+ +	+ + +	+ +	+ + +
L. kunkeei PFB7	+	+ +	+ +	+ + + +	+ + +	+ + +	+ +	–
L. kunkeei PL27	–	+	+	+ + + +	+ +	+	+	+ +
L. kunkeei PFA2	–	+ +	+ +	+ + + + +	+ +	–	+	+ +
L. kunkeei PFA3	–	+ + +	+ +	+ + +	+ + + +	–	+ +	+ + +
L. kunkeei PL3	–	+	+	+ + + +	+ +	+	+	+ +
L. kunkeei PLA6	+	+ +	+ + +	+ + + + +	+	–	+ +	+ + +
L. kunkeei PL33	–	+	+	+ + + + +	+ + +	–	+ + +	–
L. kunkeei PL15	–	+	+	+ + + +	+ +	+	+	+ +
L. kunkeei PFA7	–	+ + +	+ +	+ + + +	+ + +	–	+	+ +
L. kunkeei PL24	–	+	+ +	+ + + +	+	+ +	+ +	+ +
L. kunkeei PLA14	+	+ + +	+	+ + + + +	+ +	+ +	+	–
L. kunkeei PF6	–	+	+ +	+ + +	+	+	+	–
L. kunkeei PL31	+	+ + +	+	+ + + + +	+ +	+ +	+	–
L. kunkeei PLA13	–	+ +	+ +	+ + + +	+ +	+ +	+ +	–
Lactobacillus plantarum PLB16	–	+	+ +	+ + + +	+	+	–	–
L. plantarum PLB15	–	+	+ +	+ + +	+	+	–	–
L. kunkeei B4I	+	+	+	+ + + +	+	–	+	–
L. kunkeei PF7	–	+ +	+ + +	+ + + +	+ + + +	+ + +	+ +	+ +
L. kunkeei PFA15	–	+	+ +	+ + + +	+	+	–	–
L. kunkeei PLB30	+	+ + +	+	+ + + + +	+ +	+ +	+	–
L. kunkeei PFA5	–	+ +	+ +	+ + + + +	+ +	+ +	+ +	+ +
L. kunkeei PLA16	+	+	+	+ + + +	+	–	+	–
L. plantarum PLB1	–	+	+	+ + + +	+	+	–	–
L. kunkeei PFA35	+	+ + +	+ + +	+ + + + +	+ +	+	+ +	–
L. kunkeei B7	–	+ +	+ +	+ + + + +	+ + + +	+ +	+ +	+ +
L. kunkeei PLA8	–	+	+ +	+ + + + +	+	+ + + +	+ +	–
L. kunkeei PFB13	–	+ +	+ +	+ + + + +	+ + + +	+ +	+ +	+ +
L. kunkeei PFA4	–	+ +	+ +	+ + +	+ + +	+ +	+ +	+ +

[a] Inhibitory activity was scored as follows: −, no inhibition; +, halo of inhibition radius of < 1 mm; + +, halo of inhibition radius of 1–2.5 mm; + + +, halo of inhibition radius of 2.5–4.0 mm; + + + +, halo of inhibition radius of 4.0–6 mm; + + + + +, halo of inhibition radius > 6 mm.

(44.99 ± 8.61 mg kg^{-1} dry weight). Data were confirmed by gel-filtration chromatography analysis of the peptide profiles, which showed a marked increase of the peaks area after fermentation of BCP (Fig. 3A). Compared to the raw-BCP, the main differences found in started- and unstarted-BCP were due to peptides with apparent molecular masses < 150,000 Da.

Total FAA were found in raw-BCP at the concentration of 10.97 ± 0.85 g kg^{-1} (dry weight). The FAA found at the highest concentration were proline, serine, γ-aminobutyric acid (GABA), histidine, and alanine (Fig. 4). Total FAA significantly ($P < 0.05$) increased during fermentation in both started- and unstarted-BCP to 25.29 ± 1.16 and 22.99 ± 3.15 g kg^{-1} (dry weight), respectively. The most substantial increases ($P < 0.05$) were found for leucine, lysine, alanine, valine, phenylalanine, arginine, tyrosine, and glutamic acid (Fig. 4).

A multi-step protocol, which mimics the *in vivo* gastrointestinal digestion, was used to estimate the *in vitro* protein digestibility of BCP. Digestibility of protein of raw-BCP was 62.1 ± 0.80% of the total proteins. Fermentation by the selected mixed starter significantly ($P < 0.05$) increased the digestibility of protein of BCP (75.14 ± 0.71%) (Fig. 3B) and to a lesser extent ($P > 0.05$) during spontaneous fermentation of BCP (Fig. 3B).

Compared to the amount of water and methanol soluble free phenolics found in raw-BCP (8.43 ± 0.85 and 45.73 ± 4.81 g gallic acid equ kg^{-1} dry weight, respectively), substantial increases ($P < 0.05$) were found in started-BCP (15.98 ± 0.64 and 69.53 ± 4.52 g gallic acid equ kg^{-1} dry weight, respectively) (Fig. 5). An increase, but not statistically significant ($P > 0.05$) was also found in unstarted-BCP (Fig. 5).

Table 4

Antifungal activity[a] of selected lactic acid bacteria strains grown in pollen extract (PE) at 30 °C for 24 h. Cell-free supernatants was recovered and used to assay the inhibitory spectrum of PE. Unfermented PE was used as control. The growth of moulds was assayed on Potato Dextrose Agar at 25 °C for 8 days.

	Aspergillus versicolor CBS 117286	*Aspergillus niger* DPPMAF3	*Penicillium roqueforti* DPPMA1	*Penicillium polonicum* CBS 112490	*Penicillium albocoremium* CBS 109582	*Aspergillus parasiticus* CBS 971.97	*Penicillium paneum* CBS 101032	*Penicillium bialowiezense* CBS 110102
Unfermented PE	–	–	–	–	–	–	–	–
Lactobacillus kunkeei PF12	+ + +	+ +	+ +	–	+ +	–	–	–
L. kunkeei PF16	+ + +	–	–	–	+ +	+	–	–
L. kunkeei PL13	+ + +	–	–	+	+ +	+ +	+ +	–
L. kunkeei PL28	–	–	–	–	–	–	–	–
L. kunkeei BV61	–	–	–	–	–	–	–	–
L. kunkeei PF18	–	–	–	–	–	–	–	–
L. kunkeei B17	–	–	–	–	–	–	–	–
L. kunkeei BIII60	–	–	–	–	+ +	–	–	–
L. kunkeei PF15	–	–	–	–	–	–	–	–
L. kunkeei PL9	–	–	–	–	–	–	–	–
L. kunkeei PLA21	–	–	–	–	–	–	–	–
Fructobacillus fructosus B4	–	–	+ +	–	–	+ +	–	–
L. kunkeei PFB7	–	–	–	–	–	–	–	–
L. kunkeei PL27	–	–	–	–	–	–	–	–
L. kunkeei PFA2	–	–	–	–	+ +	–	–	–
L. kunkeei PFA3	–	–	+ +	–	–	–	–	+
L. kunkeei PL3	–	–	–	–	–	–	–	–
L. kunkeei PLA6	–	–	–	–	–	–	–	–
L. kunkeei PL33	–	–	–	–	–	–	–	–
L. kunkeei PL15	–	–	–	–	–	–	–	–
L. kunkeei PFA7	–	–	–	–	–	–	–	–
L. kunkeei PL24	–	–	–	–	–	–	–	–
L. kunkeei PLA14	–	–	–	–	–	–	–	–
L. kunkeei PF6	–	+ +	+	–	–	+ +	–	–
L. kunkeei PL31	–	–	–	–	–	–	–	–
L. kunkeei PLA13	+ + +	–	–	–	–	–	–	–
Lactobacillus plantarum PLB16	–	–	+	–	+ +	–	–	–
L. plantarum PLB15	–	–	–	–	–	–	–	–
L. kunkeei B4I	–	–	–	–	–	–	–	–
L. kunkeei PF7	–	–	–	–	–	–	–	–
L. kunkeei PFA15	–	–	–	–	–	–	–	–
L. kunkeei PLB30	–	–	–	–	–	–	–	–
L. kunkeei PFA5	–	–	–	–	–	–	–	–
L. kunkeei PLA16	–	–	–	–	–	–	–	–
L. plantarum PLB1	–	–	–	–	–	–	–	–
L. kunkeei PFA35	–	–	–	–	–	–	–	–
L. kunkeei B7	–	–	–	–	–	–	–	–
L. kunkeei PLA8	–	–	+	–	+ +	–	–	–
L. kunkeei PFB13	+ +	–	+	–	–	–	–	–
L. kunkeei PFA4	–	–	+	–	–	–	–	–

[a] The percentage of hyphal radial growth inhibition was calculated from the mean values as follows: percent inhibition = [(mycelial growth under control conditions - mycelial growth in the presence of cell free PE)/mycelial growth under control conditions] x 100. Inhibitory activity was scored as follows: −, no inhibition; +, hyphal radial growth inhibition < 25%; + +, hyphal radial growth inhibition 25–50%; + + +, hyphal radial growth inhibition 50–75%; + + + +, hyphal radial growth inhibition > 75%.

3.6. Microbial stability of fermented bee-collected pollen

Microbial stability of raw-, started- and unstarted-BCP was investigated during storage at room temperature (25 °C) for 50 days (Fig. 6). A decreasing trend was found for lactic acid bacteria, although the reduction was significantly ($P < 0.05$) slower in started-BCP compared to raw- and unstarted-BCP. Yeasts were found at the highest ($P < 0.05$) cell densities in raw-BCP during the first 20 days of storage, and in unstarted-BCP during the last 20 days of storage. Moulds were detected up to 20 days of storage, where the highest ($P < 0.05$) cell density was found in raw- and unstarted-BCP. Cell density of *Pseudomonas* spp. and *Aeromonas* spp. was the highest ($P < 0.05$) in raw-BCP. Coliforms, enterobacteria, and *Staphylococcus* spp. were not found in any samples.

The susceptibility of BCP to be colonized by moulds was assessed by monitoring the conidial germination of *Aspergillus parasiticus* CBS 971.97. Raw- and unstarted-BCP were the only samples to be colonized by *A. parasiticus* CBS 971.97 after 10 days of storage.

4. Discussion

An increasing interest towards honeybee products as bee bread (BB) and bee-collected pollen (BCP) was recently raised due to their nutritional and medicinal properties, which are almost globally recognized (Kieliszek et al., 2018). Biochemical processes underlying the maturation of BB are not full deepened, as well the real role of lactic acid bacteria is also questioned (Herbert and Shimanuki, 1978; Gilliam, 1979b, 1997; Loper et al., 1980; Vásquez and Olofsson, 2009; Anderson

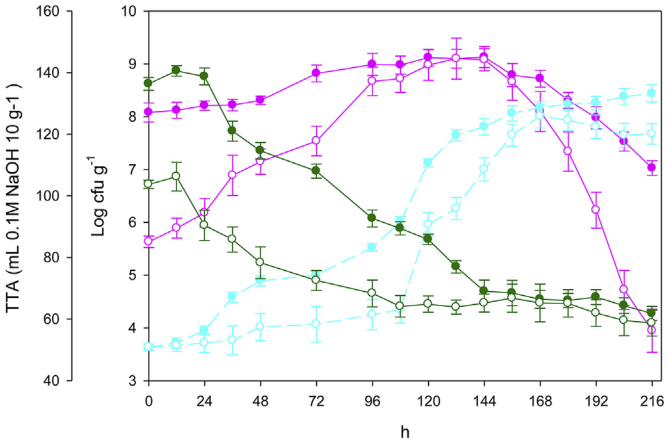

Fig. 2. Cell density of lactic acid bacteria (pink lines) and yeasts (green lines), and total titratable acidity (TTA) (cyan lines) values during fermentation of bee-collected pollen (BCP) carried out at 30 °C for 216 h. Values refer to started-BCP (full symbols) and unstarted-BCP (empty symbols). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

et al., 2011, 2014; Mattila et al., 2012; De Grandi-Hoffman et al., 2013; Lee et al., 2015; Carroll et al., 2017), and few reports addressed these issues with targeted experiments. To our knowledge, this study is the first to show a complete and detailed overview describing inter- and intra-species differences including phenotypic traits of lactic acid bacteria, which are part of the peculiar widespread niche composed of flowers, fresh BCP, BB and honeybee crop. Useful glimpses of this complex ecosystem were gleaned under biologically relevant conditions using reliable growth model systems, as pollen extract and fresh BCP. Furthermore, the effects of lactic fermentation on BCP were shown, which reflected both on the microbial ecology of BCP and on its nutritional value. A systematic comparison between fresh and fermented BCP under controlled conditions allowed this investigation. Lastly, a protocol to ferment BCP was successfully set up, which included the inoculum of selected lactic acid bacteria and yeast strains, simulating the microbial consortium underlying the conversion of bee collected pollen into bee bread.

Lactic acid bacteria isolated from ivy flowers, BCP, 15 and 30 days hive-stored BB were identified. Moreover, lactic acid bacteria strains previously isolated from honeybee gastrointestinal tract were included, since the honeybee foregut (crop) represents an interface with the pollination environment, colony pollen stores and other colony members, and bacteria from the crop are similar to those found in the pollen stores (Anderson et al., 2011, 2013; Corby-Harris et al., 2014). Except for flowers, where *Lactococcus lactis* was the only species detected, all the others niches considered in this study were dominated by *Lactobacillus kunkeei* and, to a lesser extent, by *Fructobacillus fructosus.* Even if identified in BCP and 15 days hive-stored BB, *Lactobacillus curvatus*, *Lc. lactis*, *Lactobacillus plantarum*, and *Leuconostoc citreum*, were not detectable in BB after 30 days of storage in the hive. As BCP represents an extreme environment for bacteria, due to the harsh pH values, osmotic stress, and the high level of antimicrobial compounds (e.g., phenolics), we speculated that BCP exerts a selective pressure in favor of highly adapted species such as the case of *L. kunkeei* (Anderson et al., 2011, 2013). Thus, our findings suggest an emergent microbial community co-evolved during BCP storage into the hive. Aiming to highlight the niche specialism of *L. kunkeei*, the growth performances of autochthonous lactic acid bacteria were investigated under *in vitro* conditions using the pollen extract as model system. To obtain a growth medium strictly mimicking the conditions of the habitats of origin, the pollen extract was produced through a multi-step sequential chemical extraction. *L. kunkeei* strains showed the highest cell density increases, followed by strains of *F. fructosus* and *L. plantarum*, whereas *L. curvatus*, *Lc. lactis*, and *Leuc. citreum* strains were the less performing. Our results reveal that although a large microbial diversity characterized flowers and fresh BCP, most of lactic acid bacteria species disappeared throughout the BB maturation, giving way to *L. kunkeei* and *F. fructosus* to dominate long stored BB and honeybee crop (Anderson et al., 2011, 2013). Adaptation of lactic acid bacteria strains to the harsh conditions of BCP seemed most related to species-specific, and, more in deep, to strain-specific features. In fact, growth performances of some *L. kunkeei* and *F. fructosus* strains were low and comparable to that of less competitive species. The high intra-species diversity found at phenotypic level in *L. kunkeei* and *F. fructosus* strains was confirmed also through RAPD-PCR genotyping (Rangberg et al., 2012; Tamarit et al., 2015; Asenjo et al., 2016; Filannino et al., 2016; Linjordet, 2016). We speculate that most adapted *L. kunkeei* and *F. fructosus* strains share

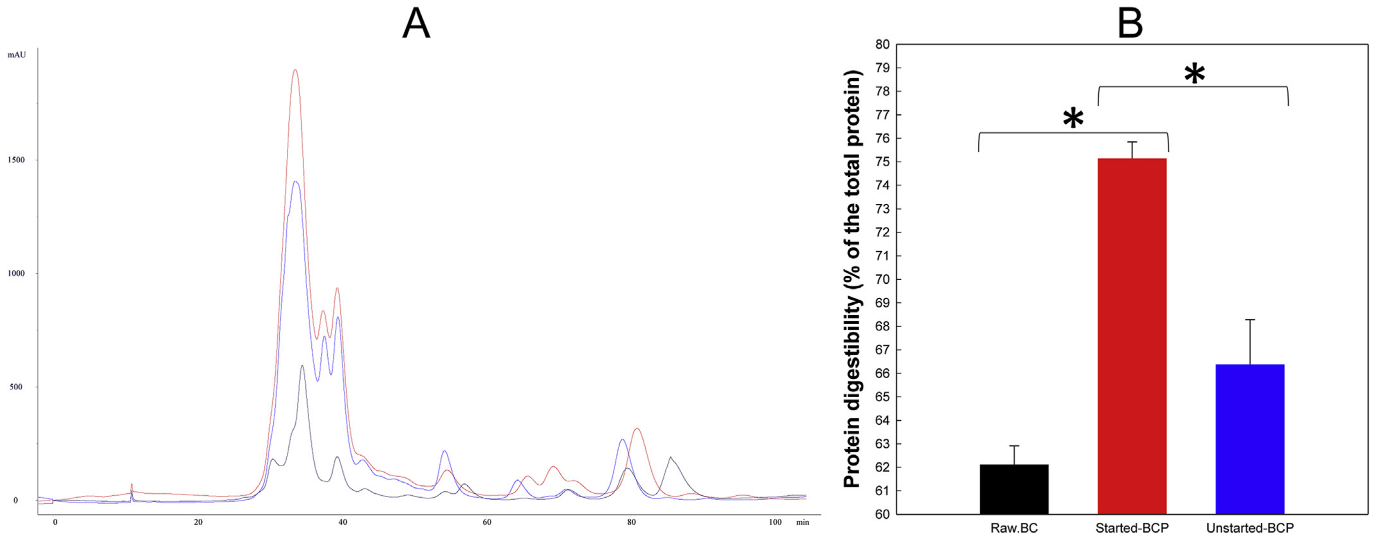

Fig. 3. (A) Gel-filtration chromatography profiles of peptide (214 nm) in raw bee collected pollen (raw-BCP) (black line), started-BCP (red line) and unstarted-BCP (blue line) after fermentation at 30 °C for 216 h. Chromatograms are representative of three biological replicates analyzed in triplicate. (B) *In vitro* protein digestibility of raw-BCP (black bar), started-BCP (red bar) and unstarted-BCP (blue bar) after fermentation at 30 °C for 216 h. Data are the means (± SD) of three independent experiments analyzed in triplicate. Data were subjected to Student's *t*-test. The asterisks indicate values statistically different ($P < 0.05$). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

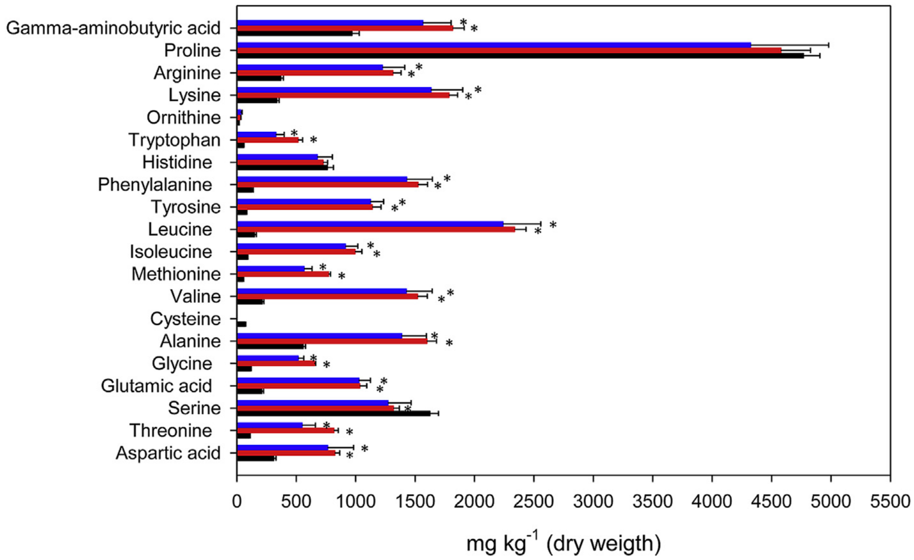

Fig. 4. Concentration of free amino acids (FAA) (mg kg^{-1} dry weight of bee collected pollen) in raw bee collected pollen (raw-BCP) (black bars), started-BCP (red bars) and unstarted-BCP (blue bars) after fermentation at 30 °C for 216 h. Data are the means (± SD) of three independent experiments analyzed in triplicate. Data were subjected to Student's *t*-test. The asterisks indicate values statistically different ($P < 0.05$) compared to the raw-BCP. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

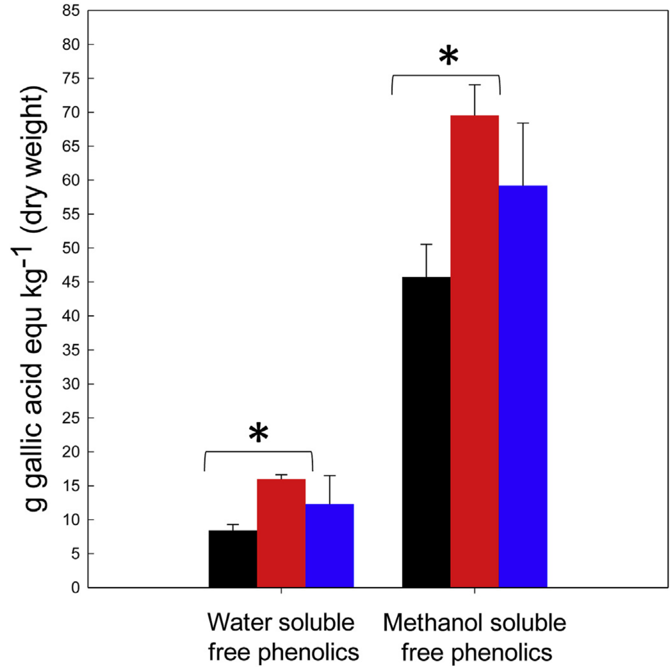

Fig. 5. Waters and methanol soluble free phenolics concentrations (g gallic acid equ kg^{-1} dry weight of bee collected pollen) in raw bee collected pollen (raw-BCP) (black bars), started-BCP (red bars) and unstarted-BCP (blue bars) after fermentation at 30 °C for 216 h. Data are the means (± SD) of three independent experiments analyzed in triplicate. Data were subjected to Student's *t*-test. The asterisks indicate values statistically different ($P < 0.05$). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

metabolic traits that determine their capacity to growth and survive in mature BB and honeybee crop.

Focusing on the effect of lactic fermentation on the ecology of the BCP, a strong antimicrobial activity was observed *in vitro* testing fermented pollen extract towards Gram-positive and -negative bacteria, most of them potentially pathogenic for humans. Antifungal activity was also exerted by the fermented pollen extract towards fungal species belonging to genera *Aspergillus* and *Penicillium* recognized as potentially toxigenic (Gilliam et al., 1989; González et al., 2005; Loncaric et al., 2009; Brindza et al., 2010; Hani et al., 2012; Nardoni et al., 2016; Kostić et al., 2017; Mauriello et al., 2017). The widest inhibition spectra were found after fermentation with some strains of *L. kunkeei* whereas no inhibition was observed testing the unfermented pollen extract or the cell free extract obtained after growing in synthetic medium. All these evidences suggest that acidification has no role, but rather the antimicrobial activity was due to specific metabolites produced mainly by some *L. kunkeei* strains during growth on BCP, which likely gave them the capacity to outcompete other microbial groups contaminating BCP. Furthermore, our finding support the role of *L. kunkeei* in stabilizing BCP, and call into question previous studies, which attributed the BB stability only to added honey, nectar, honeybee secretions and to inherent properties of pollen (Anderson et al., 2014).

We set up a biotechnological protocol to ferment BCP, under controlled conditions, with the dual purpose of showing the role of lactic bacteria in BCP transformation and stabilization, as well ensuring a sustainable and standardized production of fermented BCP, which could satisfy the growing market demand. A mixed inoculum of selected *L. kunkeei* PF12, PL13, and PF15 strains, and *Hanseniaspora uvarum* AN8Y27B ensured an effective and standardized fermentation process. Pectin-degrading enzymes of *H. uvarum* were essential for breakdown of pollen walls, resulting in the release of nutrients (Engel et al., 2012). This assumption highlights the strict relationship between lactic bacteria and others microbial groups during BB maturation (Gilliam, 1979b). Previous studies regarding BCP fermentation have been undertaken, but several drawbacks emerged, as the uncontrolled fermentation and the low viability of lactic acid bacteria, due to the harsh features of BCP (Salazar-González and Díaz-Moreno, 2016). This is the first study considering the use of autochthonous *L. kunkeei* strains. The niche specialism of this species and our criteria for strains selection ensured high viability of starters both during fermentation as well during storage at room temperature. As doubts have been raised, whether the lactic acid bacteria may lead to significant physical-chemical and biochemical changes on BCP during hive-storage (Anderson et al., 2014), we compared the nutritional values and the microbial stability of BCP fermented under the conditions of this study to that of raw BCP. BCP fermented by selected mixed starters showed higher levels of peptides, FAA, and free phenolics, as well higher *in vitro* protein digestibility. The same biochemical changes have been already described during natural BB maturation (Human and Nicolson, 2006; Zuluaga et al., 2015), thus confirming the ability of our protocol to emulate the natural fermentation process of BB. Under the condition of our study, similar changes were also observed in spontaneously fermented BCP, but to a lesser extent and to a lower level of reproducibility. This trend was likely due to the use of fresh non-sterile BCP, therefore harboring the autochthonous microbiota of BCP able to carry

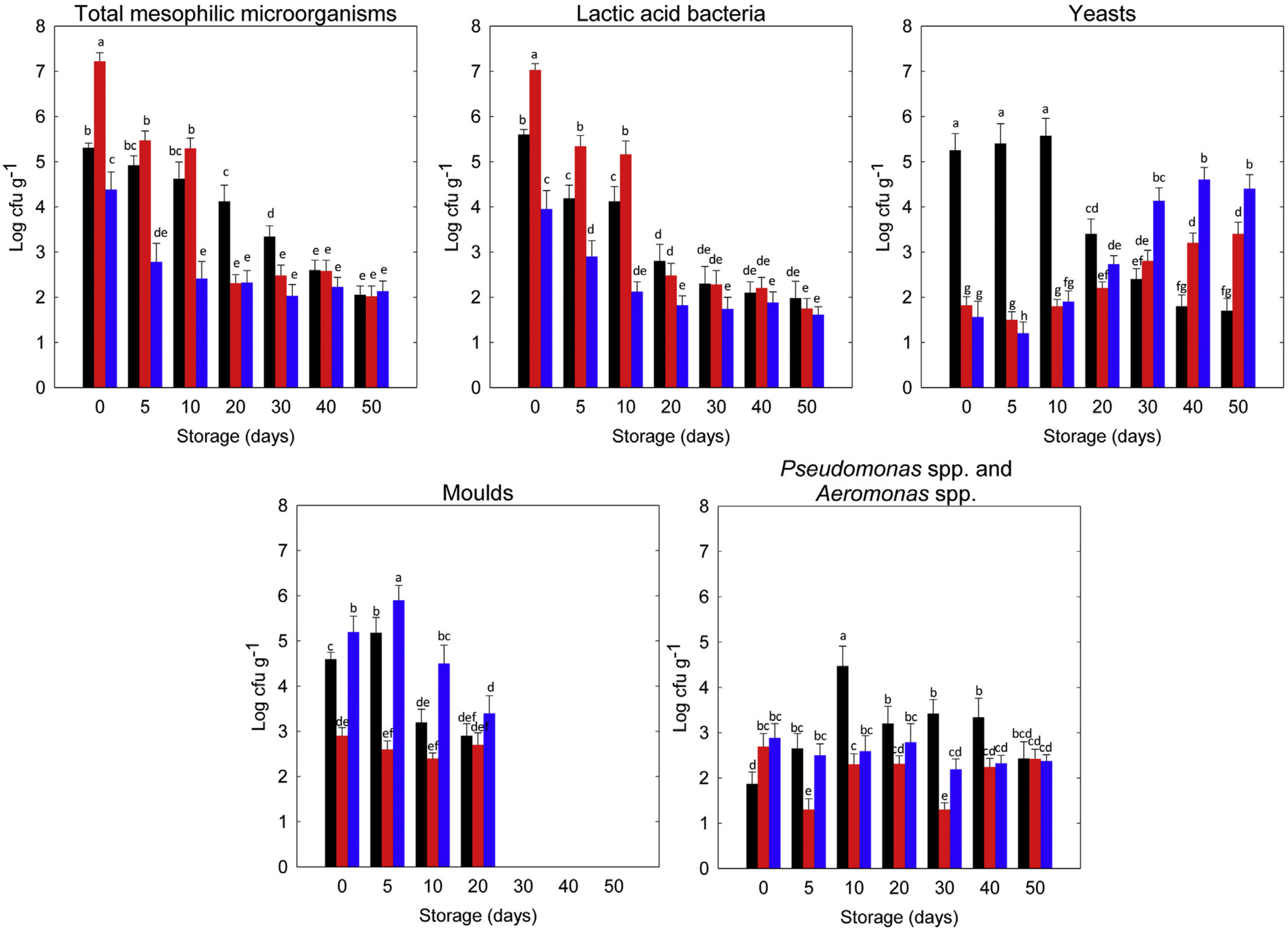

Fig. 6. Cell density of total mesophilic microorganisms, lactic acid bacteria, yeasts, moulds, *Pseudomonas* spp. and *Aeromonas* spp. found raw collected pollen (raw-BCP) (black bars), fermented started-BCP (red bars) and unstarted-BCP (blue bars) during storage at room temperature (25 °C) for 50 days. Data are the means (± SD) of three independent experiments analyzed in triplicate. Data were subjected to one-way ANOVA followed by Tukey's procedure at $P < 0.05$. Bars with different superscript letters differ significantly ($P < 0.05$). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

out a spontaneous fermentation. However, during the spontaneous fermentation, the maturation process was slower and less intense than in started-BCP. Raw and spontaneously fermented BCP were also more exposed to the uncontrolled growth of yeasts, moulds, and other bacterial groups.

Focusing on the consumption of fermented BCP by humans, the use of the selected starters increased the digestibility and bioavailability of nutrients (e.g., proteins, polysaccharides), and bioactive compounds (phenolics) naturally occurring in BCP. Several studies emphasized that raw BCP, due to the pollen grain structure, is not easily digestible by mono-gastric organisms, such as humans (Cook et al., 2003; Mutsaers et al., 2005; Human and Nicolson, 2006; Hesse et al., 2009; Campos et al., 2010; Zuluaga et al., 2014; Urcan et al., 2017; Kieliszek et al., 2018; Zhou et al., 2018). Furthermore, our biotechnological protocol ensured a product microbiologically stable and safe. Several authors recommend a more comprehensive microbiological risk assessment of BCP (Hani et al., 2012; Nardoni et al., 2016; Mauriello et al., 2017). Toxigenic moulds in trapped BCP represents a potential risk for human health, and attention should be paid to all stages of the post-harvest process (Medina et al., 2004; González et al., 2005; Nardoni et al., 2016). Under the conditions of our study, only BCP fermented by the selected starters was compliant with the standards of pollen quality proposed by the International Honey Commission (yeast and moulds < 4 Log cfu g^{-1}). Furthermore, started BCP was clearly less susceptible to the growth of toxigenic moulds such as *Aspergillus parasiticus* (Kostić et al., 2017).

5. Conclusions

Summarizing, our study clearly linked the BB maturation process to an active role of autochthonous lactic acid bacteria in consortium with yeasts, highlighted the niche specialism of *L. kunkeei*, and developed a fermentation protocol of BCP, almost emulating the natural fermentation process of BB, which led to a stable, safe, and standardized fermented product with an increased nutritional value for humans. Aiming at confirm the improved nutritional and functional features of fermented BCP according our protocol, further trials will be performed through *ex-vivo* and *in-vivo* experiments.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fm.2019.02.007.

References

Denisow, B., Denisow–Pietrzyk, M., 2016. Biological and therapeutic properties of bee pollen: a review. J. Sci. Food Agric. 96, 4303–4309. https://doi.org/10.1002/jsfa.

7729.

Akeson, W.R., Stahmann, M.A., 1964. A pepsin pancreatin digest index of protein quality evaluation. J. Nutr. 83, 257–261. https://doi.org/10.1093/jn/83.3.257.

Altschul, S.F., Madden, T.L., Schäffer, A.A., Zhang, J., Zhang, Z., Miller, W., Lipman, D.J., 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res. 25, 3389–3402. https://doi.org/10.1093/nar/25.17.3389.

Anderson, K.E., Sheehan, T.H., Eckholm, B.J., Mott, B.M., DeGrandi–Hoffman, G., 2011. An emerging paradigm of colony health: microbial balance of the honey bee and hive (*Apis mellifera*). Insectes Soc. 58, 431–444. https://doi.org/10.1007/s00040-011-0194-6.

Anderson, K.E., Sheehan, T.H., Mott, B.M., Maes, P., Snyder, L., Schwan, M.R., Walton, A., Jones, B.M., Corby-Harris, V., 2013. Microbial ecology of the hive and pollination landscape: bacterial associates from floral nectar, the alimentary tract and stored food of honey bees (*Apis mellifera*). PLoS One 8, e83125. https://doi.org/10.1371/journal.pone.0083125.

Anderson, K.E., Carroll, M.J., Sheehan, T.I.M., Mott, B.M., Maes, P., Corby-Harris, V., 2014. Hive-stored pollen of honey bees: many lines of evidence are consistent with pollen preservation, not nutrient conversion. Mol. Ecol. 23, 5904–5917. https://doi.org/10.1111/mec.12966.

Anđelković, B., Jevtić, G., Mladenović, M., Marković, J., Petrović, M., Nedić, N., 2012. Quality of pollen and honey bee bread collected in spring. J. Hyg. Eng. Des. 1, 275–277.

Asenjo, F., Olmos, A., Henriquez-Piskulich, P., Polanco, V., Aldea, P., Ugalde, J.A., Trombert, A.N., 2016. Genome sequencing and analysis of the first complete genome of *Lactobacillus kunkeei* strain MP2, an *Apis mellifera* gut isolate. PeerJ 4 e1950. https://doi.org/10.7717/peerj.1950.

Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72, 248–254. https://doi.org/10.1016/0003-2697(76)90527-3.

Brindza, J., Gróf, J., Bacigálová, K., Ferianc, P., Tóth, D., 2010. Pollen microbial colonization and food safety. Acta Chim. Slovaca 3, 95–102.

Campos, M.G., Bogdanov, S., de Almeida-Muradian, L.B., Szczesna, T., Mancebo, Y., Frigerio, C., Ferreira, F., 2008. Pollen composition and standardisation of analytical methods. J. Apicult. Res. 47, 154–161. https://doi.org/10.1080/00218839.2008.11101443.

Campos, M., Frigerio, C., Lopes, J., Bogdanov, S., 2010. What is the future of Bee-Pollen? J. ApiProduct ApiMed. Sci. 2, 131–144. https://10.3896/IBRA.4.02.4.01.

Carroll, M.J., Brown, N., Goodall, C., Downs, A.M., Sheenan, T.H., Anderson, K.E., 2017. Honey bees preferentially consume freshly-stored pollen. PLoS One 12, e0175933. https://doi.org/10.1371/journal.pone.0175933.

Celano, G., De Angelis, M., Minervini, F., Gobbetti, M., 2016. Different flour microbial communities drive to sourdoughs characterized by diverse bacterial strains and free amino acid profiles. Front. Microbiol. 7, 1770. https://doi.org/10.3389/fmicb.2016.01770.

Church, F.C., Swaisgood, H.E., Porter, D.H., Catignani, G.L., 1983. Spectrophotometric assay using *o*-phthaldialdehyde for determination of proteolysis in milk and isolated milk proteins. J. Dairy Sci. 66, 1219–1227. https://doi.org/10.3168/jds.S0022-0302(83)81926-2.

Coda, R., Rizzello, C.G., Nigro, F., De Angelis, M., Arnault, P., Gobbetti, M., 2008. Long-term fungi inhibitory activity of water-soluble extract from *Phaseolus vulgaris* cv. Pinto and sourdough lactic acid bacteria during bread storage. Appl. Environ. Microbiol. 74, 7391–7398. https://doi.org/10.1128/AEM.01420-08.

Collin, S., Vanhavre, T., Bodart, E., Bouseta, A., 1995. Heat treatment of pollens: impact on their volatile flavor constituents. J. Agric. Food Chem. 43, 444–448. https://doi.org/10.1021/jf00050a035.

Cook, S., Awmack, C., Murray, D., Williams, I., 2003. Are honey bees' foraging preferences affected by pollen amino acid composition? Ecol. Entomol. 28, 622–627. https://doi.org/10.1046/j.1365-2311.2003.00548.x.

Corby-Harris, V., Maes, P., Anderson, K.E., 2014. The bacterial communities associated with honey bee (*Apis mellifera*) foragers. PLoS One 9, e95056. https://doi.org/10.1371/journal.pone.0095056.

De Angelis, M., Corsetti, A., Tosti, N., Rossi, J., Corbo, M.R., Gobbetti, M., 2001. Characterization of non-starter lactic acid bacteria from Italian ewe cheeses based on phenotypic, genotypic, and cell wall protein analyses. Appl. Environ. Microbiol. 67, 2011–2020. https://doi.org/10.1128/AEM.67.5.2011-2020.2001.

De Angelis, M., Siragusa, S., Berloco, M., Caputo, L., Settanni, L., Alfonsi, G., Amerio, M., Grandi, A., Ragni, A., Gobbetti, M., 2006. Selection of potential probiotic lactobacilli from pig feces to be used as additives in pelleted feeding. Res. Microbiol. 157, 792–801. https://doi.org/10.1016/j.resmic.2006.05.003.

De Grandi-Hoffman, G., Eckholm, B.J., Hua-Huang, M., 2013. A comparison of bee bread made by Africanized and European honey bees (*Apis mellifera*) and its effects on hemolymph protein titers. Apidologie 44, 52–63. https://doi.org/10.1007/s13592-012-0154-9.

De Melo, A.A.M., Estevinho, M.L.M.F., Almeida-Muradian, L.B., 2015. A diagnosis of the microbiological quality of dehydrated bee-pollen produced in Brazil. Lett. Appl. Microbiol. 61, 477–483. https://doi.org/10.1111/lam.12480.

Dias, L.G., Tolentino, G., Pascoal, A., Estevinho, L.M., 2016. Effect of processing conditions on the bioactive compounds and biological properties of bee pollen. J. Apicult. Res. 55, 357–365. https://doi.org/10.1080/00218839.2016.1248109.

Ellis, A., Hayes, G., 2009. An evaluation of fresh versus fermented diets for honey bees (*Apis mellifera*). J. Apicult. Res. 48, 215–216. https://doi.org/10.3896/IBRA.1.48.3.11.

Engel, P., Martinson, V.G., Moran, N.A., 2012. Functional diversity within the simple gut microbiota of the honey bee. Proc. Natl. Acad. Sci. U. S. A 109, 11002–11007. https://doi.org/10.1073/pnas.1202970109.

Filannino, P., Di Cagno, R., Addante, R., Pontonio, E., Gobbetti, M., 2016. Metabolism of fructophilic lactic acid bacteria isolated from *Apis mellifera* L. bee-gut: a focus on the phenolic acids as external electron acceptors. Appl. Environ. Microbiol. 82, 6899–6911. https://doi.org/10.1128/AEM.02194-16.

Gilliam, M., 1979a. Microbiology of pollen and bee bread: the genus *Bacillus*. Apidologie 10, 269–274.

Gilliam, M., 1979b. Microbiology of pollen and bee bread: The yeasts. Apidologie 10, 43–53.

Gilliam, M., 1997. Identification and roles of non-pathogenic microflora associated with honey bees. FEMS Microbiol. Lett. 155, 1–10. https://doi.org/10.1111/j.1574-6968.1997.tb12678.x.

Gilliam, M., Prest, D.B., Lorenz, B.J., 1989. Microbiology of pollen and bee bread: taxonomy and enzymology of molds. Apidologie 20, 53–68.

Goebel, B.M., Stackebrandt, E., 1994. Cultural and phylogenetic analysis of mixed microbial populations found in natural and commercial bioleaching environments. Appl. Environ. Microbiol. 60, 1614–1621.

González, G., Hinojo, M.J., Mateo, R., Medina, A., Jiménez, M., 2005. Occurrence of mycotoxin producing fungi in bee pollen. Int. J. Food Microbiol. 105, 1–9. https://doi.org/10.1016/j.ijfoodmicro.2005.05.001.

Hani, B., Dalila, B., Saliha, D., Daoud, H., Mouloud, G., Seddik, K., 2012. Microbiological sanitary aspects of pollen. Adv. Environ. Biol. 6, 1415–1420.

Herbert, E.W., Shimanuki, H., 1978. Chemical composition and nutritive value of bee-collected and bee-stored pollen. Apidologie 9, 33–40.

Hesse, M., Halbritter, H., Zetter, R., Weber, M., Buchner, R., Frosch-Radivo, A., Ulrich, S., 2009. Pollen Terminology. An illustrated hand book. Springer, Wien.

Human, H., Nicolson, S.W., 2006. Nutritional content of fresh, bee-collected and stored pollen of *Aloe greatheadii* var. *davyana* (*Asphodelaceae*). Phytochemistry 67, 1486–1492. https://doi.org/10.1016/j.phytochem.2006.05.023.

Kaplan, M., Karaoglu, Ö., Eroglu, N., Silici, S., 2016. Fatty acid and proximate composition of bee bread. Food Technol. Biotechnol. 54, 497–504. https://doi.org/10.17113/ftb.54.04.16.4635.

Kieliszek, M., Piwowarek, K., Kot, A.M., Błażejak, S., Chlebowska-Śmigiel, A., Wolska, I., 2018. Pollen and bee bread as new health-oriented products: A review. Trends Food Sci. Technol. 71, 170–180. https://doi.org/10.1016/j.tifs.2017.10.021.

Kostić, A.Ž., Petrović, T.S., Krnjaja, V.S., Nedić, N.M., Tešić, Ž.L., Milojković-Opsenica, D.M., Barać, M.B., Stanojevic, S.P., Pešić, M.B., 2017. Mold/aflatoxin contamination of honey bee collected pollen from different Serbian regions. J. Apicult. Res. 56, 13–20. https://doi.org/10.1080/00218839.2016.1259897.

Lee, F.J., Rusch, D.B., Stewart, F.J., Mattila, H.R., Newton, I.L., 2015. Saccharide breakdown and fermentation by the honey bee gut microbiome. Environ. Microbiol. 17, 796–815. https://doi.org/10.1111/1462-2920.12526.

Linjordet, M.S., 2016. A comparative analysis of lactic acid bacteria isolated from honeybee gut and flowers, with focus on phylogeny and plasmid profiling. Norwegian University of Life Sciences, Oslo, Norway. http://hdl.handle.net/11250/2398654.

Loncaric, I., Heigl, H., Licek, E., Moosbeckhofer, R., Busse, H.J., Rosengarten, R., 2009. Typing of *Pantoea agglomerans* isolated from colonies of honey bees (*Apis mellifera*) and culturability of selected strains from honey. Apidologie 40, 40–54. https://doi.org/10.1051/apido/2008062.

Loper, G.M., Standifer, L.N., Thompson, M.J., Gilliam, M., 1980. Biochemistry and microbiology of bee-collected almond (*Prunus dulcis*) pollen and bee bread. I-Fatty Acids, Sterols, Vitamins and Minerals. Apidologie 11, 63–73.

Markiewicz-Żukowska, R., Naliwajko, S.K., Bartosiuk, E., Moskwa, J., Isidorov, V., Soroczyńska, J., Borawska, M.H., 2013. Chemical composition and antioxidant activity of beebread, and its influence on the glioblastoma cell line (U87MG). J. Apicult. Sci. 57, 147–157. https://doi.org/10.2478/jas-2013-0025.

Maruyama, H., Sakamoto, T., Araki, Y., Hara, H., 2010. Anti-inflammatory effect of bee pollen ethanol extract from *Cistus* sp. of Spanish on carrageenan-induced rat hind paw edema. BMC Complement Altern. Med. 10, 30. https://doi.org/10.1186/1472-6882-10-30.

Masoud, W., Jespersen, L., 2006. Pectin degrading enzymes in yeasts involved in fermentation of Coffea arabica in East Africa. Int. J. Food Microbiol. 110, 291–296. https://doi.org/10.1016/j.ijfoodmicro.2006.04.030.

Mattila, H.R., Rios, D., Walker-Sperling, V.E., Roeselers, G., Newton, I.L., 2012. Characterization of the active microbiotas associated with honey bees reveals healthier and broader communities when colonies are genetically diverse. PLoS One 7, e32962. https://doi.org/10.1371/journal.pone.0032962.

Mauriello, G., De Prisco, A., Di Prisco, G., La Storia, A., Caprio, E., 2017. Microbial characterization of bee pollen from the Vesuvius area collected by using three different traps. PLoS One 12, e0183208. https://doi.org/10.1371/journal.pone.0183208.

McFrederick, Q.S., Wcislo, W.T., Taylor, D.R., Ishak, H.D., Dowd, S.E., Mueller, U.G., 2012. Environment orkin: whence do bees obtain acidophilic bacteria? Mol. Ecol. 21, 1754–1768. https://doi.org/10.1111/j.1365-294X.2012.05496.x.

Medina, Á., González, G., Sáez, J.M., Mateo, R., Jiménez, M., 2004. Bee pollen, a substrate that stimulates ochratoxin A production by *Aspergillus ochraceus* Wilh. Syst. Appl. Microbiol. 27, 261–267. https://doi.org/10.1078/072320204322881880.

Mutsaers, M., van Blitterswijk, H., van't Leven, L., Kerkvliet, J., van de Waerdt, J., 2005. Bee products properties, processing and marketing. Agromisa Foundation, Wageningen, The Netherlands.

Nagai, T., Nagashima, T., Myoda, T., Inoue, R., 2004. Preparation and functional properties of extracts from bee bread. Nahrung 48, 226–229. https://doi.org/10.1002/food.200300421.

Nardoni, S., D'Ascenzi, C., Rocchigiani, G., Moretti, V., Mancianti, F., 2016. Occurrence of moulds from bee pollen in Central Italy-A preliminary study. Ann. Agric. Environ. Med. 23, 103–105. https://doi.org/10.5604/12321966.1196862.

Podrižnik, B., Božič, J., 2015. Maturation and stratification of antibacterial activity and total phenolic content of bee bread in honey comb cells. J. Apicult. Res. 54, 81–92. https://doi.org/10.1080/00218839.2015.1090774.

Quiroga, E.N., Sampietro, A.R., Vattuone, M.A., 2001. Screening antifungal activities of selected medicinal plants. J. Ethnopharmacol. 74, 89–96. https://doi.org/10.1016/S0378-8741(00)00350-0.

Rangberg, A., Diep, D.B., Rudi, K., Amdam, G.V., 2012. Paratransgenesis: an approach to improve colony health and molecular insight in honey bees (*Apis mellifera*)? Integr. Comp. Biol. 52, 89–99. https://doi.org/10.1093/icb/ics089.

Rizzello, C.G., Nionelli, L., Coda, R., De Angelis, M., Gobbetti, M., 2010. Effect of sourdough fermentation on stabilisation, and chemical and nutritional characteristics of wheat germ. Food Chem. 119, 1079–1089. https://doi.org/10.1016/j.foodchem.2009.08.016.

Salazar-González, C., Díaz-Moreno, C., 2016. The nutritional and bioactive aptitude of bee pollen for a solid-state fermentation process. J. Apicult. Res. 55, 161–175. https://doi.org/10.1080/00218839.2016.1205824.

Sanger, F., Nicklen, S., Coulson, A.R., 1977. DNA sequencing with chainterminating inhibitors. Proc. Natl. Acad. Sci. U. S. A 74, 5463–5467. https://doi.org/10.1073/pnas.74.12.5463.

Schillinger, U., Lücke, F.K., 1989. Antibacterial activity of *Lactobacillus sake* isolated from meat. Appl. Environ. Microbiol. 55, 1901–1906.

Singleton, V.L., Rossi, J.A., 1965. Colorimetry of total phenolics with phosphomolybdic-phosphotungstic acid reagents. Am. J. Enol. Vitic. 16, 144–158.

Siragusa, S., Di Cagno, R., Ercolini, D., Minervini, F., Gobbetti, M., De Angelis, M., 2009. Taxonomic structure and monitoring of the dominant population of lactic acid bacteria during wheat flour sourdough type I propagation using *Lactobacillus sanfranciscensis* starters. Appl. Environ. Microbiol. 75, 1099–1109. https://doi.org/10.1128/AEM.01524-08.

Soares de Arruda, V.A., Santos Pereira, A.A., Silva de Freitas, A., Barth, O.M., de Almeida-Muradian, L.B., 2013. Dried bee pollen: B complex vitamins, physicochemical and botanical composition. J. Food Compos. Anal. 29, 100–105. https://doi.org/10.1016/j.jfca.2012.11.004.

Sobral, F., Calhelha, R.C., Barros, L., Dueñas, M., Tomás, A., Santos-Buelga, C., Vilas-Boas, M., Ferreira, I.C., 2017. Flavonoid composition and antitumor activity of bee bread collected in northeast Portugal. Molecules 22, 248. https://doi.org/10.3390/molecules22020248.

Tamarit, D., Ellegaard, K.M., Wikander, J., Olofsson, T., Vasquez, A., Andersson, S.G., 2015. Functionally structured genomes in *Lactobacillus kunkeei* colonizing the honey crop and food products of honeybees and stingless bees. Genome Biol. Evol. 7, 1455–1473. https://doi.org/10.1093/gbe/evv079.

Torriani, S., Felis, G.E., Dellaglio, F., 2001. Differentiation of *Lactobacillus plantarum, L. pentosus*, and *L. paraplantarum* by recA gene sequence analysis and multiplex PCR assay with recA gene-derived primers. Appl. Environ. Microbiol. 67, 3450–3454. https://doi.org/10.1128/AEM.67.8.3450-3454.2001.

Urcan, A., Mărghitaş, L.A., Dezmirean, D.S., Bobiş, O., Bonta, V., Mureşan, C.I., Mărgăoan, R., 2017. Chemical composition and biological activities of beebread-review. Bull. Univ. Agric. Sci. Vet. Med. Cluj. Napoca 74, 6–14. https://doi.org/10.15835/buasvmcn-asb:12646.

Vanderplanck, M., Moerman, R., Rasmont, P., Lognay, G., Wathelet, B., Wattiez, R., Michez, D., 2014. How does pollen chemistry impact development and feeding behaviour of polylectic bees? PLoS One 9, e86209. https://doi.org/10.1371/journal.pone.0086209.

Vásquez, A., Olofsson, T.C., 2009. The lactic acid bacteria involved in the production of bee pollen and bee bread. J. Apicult. Res. 48, 189–195. https://doi.org/10.3896/IBRA.1.48.3.07.

Vásquez, A., Olofsson, T.C., Sammataro, D., 2009. A scientific note on the lactic acid bacterial flora in honeybees in the USA—a comparison with bees from Sweden. Apidologie 40, 26–28. https://doi.org/10.1051/apido:2008063.

Xiao, W., 2006. Yeast protocols. Humana Press, Totowa. https://doi.org/10.1007/978-1-4939-0799-1.

Yang, K., Wu, D., Ye, X., Liu, D., Chen, J., Sun, P., 2013. Characterization of chemical composition of bee pollen in China. J. Agric. Food Chem. 61, 708–718. https://doi.org/10.1021/jf304056b.

Zhou, W., Yan, Y., Mi, J., Zhang, H., Lu, L., Luo, Q., Li, X., Zeng, X., Cao, Y., 2018. Simulated digestion and fermentation in vitro by human gut microbiota of polysaccharides from bee collected pollen of Chinese wolfberry. J. Agric. Food Chem. 66, 898–907. https://doi.org/10.1021/acs.jafc.7b05546.

Zuluaga, C.M., Serrato, J.C.B., Quicazán, M.C., 2014. Valorization alternatives of Colombian bee-pollen for its use as food resource – a structured review. Vitae 21, 237–247.

Zuluaga, C.M., Serrato, J.C.B., Quicazán, M.C., 2015. Chemical, nutritional and bioactive characterization of Colombian bee-bread. Chem. Eng. Trans. 43, 175–180. https://doi.org/10.3303/CET1543030.