Effect of honey bee forage plants in Tunisia on diversity and antibacterial potential of lactic acid bacteria and bifidobacteria from Apis mellifera intermissa and its products

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Abstract

Lactic acid bacteria and bifidobacteria (LAB & Bifido), isolated from the gastrointestinal tract of *Apis mellifera intermissa* (BGIT), honey (H), propolis (P) and bee bread (BB) of hives set in different vegetations (wildflowers, caraway, orange blossom, *Marrubium vulgare, Eucalyptus* and *Erica cinerea*), were subjected to analysis of their antibacterial potential. Isolates able to inhibit *Staphylococcus aureus* were selected and identified with MALDI-TOF MS leading to 154 strains representing 12 LAB & Bifido species. *Lactiplantibacillus plantarum*, *Pediococcus pentosaceus* and *Enterococcus faecalis* were predominantly found in all matrices. BGIT showed the highest LAB & Bifido diversity with exclusive occurrences of five species (including *Bifidobacterium asteroides* and *Limosilactobacillus fermentum*). Honey was the second origin harboring an important variety of LAB species of which *Apilactobacillus kunkeei* and *Enterococcus mundtii* were characteristic of both H and BGIT. Principal components analysis revealed associations between antibacterial activities of LAB & Bifido, matrices and honey bee forage plants. Inhibition trends of *S. aureus* and *Citrobacter freundii* were highlighted with: *Lactiplantibacillus plantarum* from BGIT, P, H of bees feeding on *Erica cinerea*; *Pediococcus pentosaceus* from BGIT, P, BB associated with *Erica cinerea*; and *Bifidobacterium asteroides* from BGIT/orange blossom system. However, *Enterococcus faecium* associated with BGIT/*Eucalyptus* system antagonized *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa* and *Acinetobacter baumannii*. Our findings highlighted noteworthy effects of bee forage plants on the antibacterial activity of LAB & Bifido. Our approach could be useful to identify multiple conditions promoting antibacterial potency of LAB & Bifido under the combined effects of feeding plants and living matrices.

Introduction

Lactic acid bacteria and bifidobacteria (LAB & Bifido) are dominant members of the gut microbiome in humans and animals and are also frequently found in dairy products and fermented food (Ramos et al. 2020, Ağagündüz et al. 2021). They are good candidates for innovative and promising alternative therapeutics against antibiotic-resistant pathogens (Nataraj et al. 2020; Yadav et al. 2022). The antimicrobial potential of LABs includes a variety of bioactive compounds (such as organic acids, acetaldehyde, diacetyl, enzymes, carbohydrates, short-chain fatty acids, and hydrogen peroxide, and bacteriocin-like substances (BLS)) which display various antagonistic activities against a wide range of pathogens (Yadav et al. 2022; Zhang et al. 2021; Hernández-González et al. 2021). The bioactive properties of LAB & Bifido are related to complex and multifactorial processes implying coevolution with their host under the influence of multiple environmental conditions (Nowak et al; 2021).

LAB & Bifido share several morphological and physiological similarities: both are Gram-positive, usually catalase-negative, commonly non-motile and non spore-forming bacteria. Also, they mainly produce lactic acid as a major end-product during carbohydrate fermentation (Bottacini et al. 2014; De Filippis et al. 2020). Phylogenetically, LAB belong to the Firmicutes phylum, Bacilli class, Lactobacillales order. They are grouped in over 50 genera, including, among others, *Lactobacillus*, *Latilactobacillus*, *Lactiplantibacillus*, *Limosilactobacillus*, *Apilactobacillus*, *Streptococcus*, *Pediococcus*, *Leuconostoc*,

Enterococcus, and *Weisella*. Regarding bifidobacteria, the *Bifidobacterium* genus is affiliated with the phylum Actinobacteria, class Actinobacteria, order Bifidobacteriales, and family Bifidobacteriaceae. Currently, 48 recognized species belong to the *Bifidobacterium* genus, such as *Bifidobacterium asteroides*, *Bifidobacterium bifidum*, *Bifidobacterium bombi*, and *Bifidobacterium gallinarum* (Bottacini et al. 2014).

The honey bee (*Apis mellifera*), a social insect whose microbiota has some similarities to that of mammals, but with a simpler composition is proposed as a useful model organism for gut microbiota study (Zheng et al. 2018; Nowak et al. 2021). It produces various products, including honey, propolis and bee bread which have been used for centuries in traditional medicine to prevent disease and promote healing, as well as in diets and complementary nutrition (Pasupuleti et al. 2017; Cornara et al. 2017). The honey bee, one of the most important pollinating insects, has strongly colonized many ecosystems around the world with substantial implications for natural ecosystems (Klein et al. 2007; Villalba et al. 2020; Nowak et al. 2021). Accordingly, this insect has a great impact on the way of life of humans and the environment. However, it is threatened by various biotic and abiotic factors such as infection by pathogens, use of pesticides, climate change and lack of forage (Goulson et al. 2015; Neov, et al. 2019). Restoring gut homeostasis by supplementing the gut microbiota with appropriate LAB as probiotics may improve the health status of honey bees. This is a promising approach that requires a better knowledge of its microbiota (Iorizzo et al. 2022).

The honey bee subspecies *Apis mellifera interemissa*, also recognized as the Tunisian bee or Punic bee, has a capital role in the evolution of the domestic bees of the central Mediterranean region (Chouchaine et al. 2015). The present study was undertaken (i) to explore the diversity of LAB & Bifido isolated from the gastrointestinal tract of the bee *Apis mellifera intermissa* (BGIT), propolis, bee bread and honey collected from hives kept in different vegetations in northeast and center of Tunisia, and (ii) to assess their antibacterial potential. Multiple anti-pathogenic systems implying antibacterial activities of the LAB & Bifido associated with different matrices and conditioned by different forage plants were highlighted by principal component analysis

Materials And Methods

Sampling

Adult worker of *Apis mellifera interemissa*, propolis, honey and bee bread were sampled during the four seasons, between 2018 and 2019, from seven hives located in different vegetations and geographical regions in Northeastern and Central Tunisia: wildflowers (April 2018, region of Grombalia 36°36'10.5"N, 10°29'49.9"E), caraway (April 2018, Bni-Khiar 36°27'39.0"N, 10°46'57.0"E), orange blossom (May 2018, Bni-Khalled 36°38'57.5"N, 10°35'30.4"E), *Marrubium vulgare* (June 2018, Al-Waslatiyah 35°51'18.4"N, 9°34'42.8"E), *Eucalyptus* (July 2018, Takelsa 36°47'43.9"N, 10°37'38.8"E), *Erica cinerea* (November 2018, Al-Haouaria 37°03'15.0"N, 11°00'40.6"E), and overwintering season (January 2019, Ain-Tebournouk 36°34'03.3"N, 10°27'56.0"E). Overwintering honey bees consumed stored food and did not need supplies for their survival, since winter is relatively short in Tunisia. From each hive, worker honey bees were

collected and placed in sterile and humidified bottles; propolis, honey and bee bread were aseptically collected in 50 mL conical tubes using appropriate materials. Samples were then transported to the laboratory in iceboxes.

Lactic acid bacteria and bifidobacteria isolation

Ten randomly selected adult worker honey bees were euthanized and externally disinfected with 70% ethanol and they were aseptically dissected under an AP-7 stereo microscope (Novex, The Netherlands) using sterile materials. The gastrointestinal tract (crop to rectum) of each honey bee was isolated, macerated and suspended in 10 mL of sterile 0.9% NaCl solution. Propolis, honey and bee bread (1 g each) were separately suspended and homogenized in 10 mL of saline solution. Then, 100 µL aliquots from each prepared suspension were spread onto pre-solidified de Man, Rogosa and Sharpe (MRS) (Condalab, Madrid, Spain) medium (pH 6.5) supplemented with 2% fructose (Fluka, Switzerland) and 0.1% L-cysteine (Sigma Aldrich, St. Louis, MO, USA) (MRSs). Plates were then incubated under either aerobic conditions for 24 h at 30°C, or anaerobic conditions using anaerobic jars containing GENbox anaer gas packs (bioMérieux SA, France) for 72 h at 35°C (Olofsson et al. 2016). Afterwards, 10-30 colonies per plate were selected based on the differences in morphology, color and size, and were repeatedly streaked on agar plates to obtain pure isolates. Next, the non-staining KOH method was applied to determine the Gram type of bacteria (Gregersen 1978). The isolates were then tested for catalase activity using hydrogen peroxide (Pharmagreb, Tunis, Tunisia). Gram-positive and catalase-negative bacteria were chosen for further characterization.

Screening for anti-*Staphylococcus aureus* activity

LAB & Bifido isolated on MRSs agar were screened for inhibitory activity against a clinical *S. aureus* strain (D315, isolated from a pus sample in 2018 at the Traumatology and Great Burned Center, Tunis) according to the agar well diffusion assay on trypto-casein medium (Solabia, Pantin, France) (Liasi et al. 2009). To this end, 100 µL of *S. aureus* culture, grown at 37°C in trypto-casein-soy (TS) broth and standardized to 0.5 McFarland, were mixed with 25 mL of pre-solidified TS medium and allowed to solidify in Petri plates. Then, wells of 6 mm diameter were punched aseptically and filled with 100 µL of cell-free supernatant (CFS) from each LAB & Bifido isolate. CFS was prepared as previously described (Rabaoui et al. 2023): 10 mL of LAB & Bifido culture were centrifuged at 9000 xg for 20 min at 4°C (MPW-350 R centrifuge, MPW MED. Instruments, Warsaw, Poland) and the CFS was filtered through a 0.22 mm syringe filter (Sartorius, Germany). The plates were then incubated at 37°C for 24 h. Anti-*S. aureus* activity of LAB & Bifido isolates was ascertained by measuring the zone of growth inhibition (IZ) around the well. Antibacterial activity was categorized as weak (IZ < 11 mm), moderate (11 ≤IZ ≤16 mm) or strong (IZ >16 mm) (Truc et al. 2019).

Determination of the fermentative type

A carbon dioxide (CO_2) production test was conducted in order to identify the glucose metabolism by homofermentative or heterofermentative pathway (Mulaw et al. 2020). For that aim, 10 mL tubes of MRS

broth containing 1% glucose with inverted Durham bells were inoculated with 50 µL of 1% overnight fresh bacterial culture and then incubated at 35°C for 48-72 h. The presence of gas (i.e. CO_2) in the Durham bell is a clear indicator of a heterofermentative metabolism.

Identification of bacteria by MALDI-TOF MS

Sample preparation

Isolate preparation for MALDI-TOF MS was carried out according to the extended direct transfer method as previously described (Gaudreau et al. 2018). From a 24 h to 72 h culture grown on 5% sheep blood agar, a single colony was smeared, with a toothpick, as a very thin layer on a polished steel MSP 96-spot target (Bruker Daltonik, Bremen, Germany) and then air dried at room temperature for 3 min. Subsequently, the preparation was automatically performed by the MALDI Biotyper Galaxy (Bruker Daltonik). Thus, the spot was overlaid with 1 µL of 70% formic acid and left to dry for 5 min at ambient temperature. Afterwards, 1 µL of Bruker HCCA matrix solution (10 mg α-cyano-4-hydroxycinnamic acid in 50% acetonitrile, 2.5% trifluoroacetic acid and 47.5% water) was deposited onto the spot and allowed to dry for 3-5 min at room temperature.

MALDI-TOF MS anlaysis

Bacterial spectral capture and analysis were performed with the Microflex LT/SH mass spectrometer (Bruker Daltonik). Generated spectra were assigned a score based on similarity with spectra from the manufacturer's reference database library (MBT-Version 6-RUO 6903). Mass spectra were acquired in linear positive ion mode, at a laser frequency of 60 Hz, and analyzed in a mass/charge ratio (m/z) range of 1,960 - 21,200 Da. For each spectrum, a minimum of 240 laser shorts from different points of the isolate spot were collected and analyzed. ATCC 25922 THL strain of *Escherichia coli* was used as bacterial test standard (Bruker Daltonik), on each target plate, for calibration and quality control.

MALDI-TOF MS data interpretation and processing

The MALDI-Biotype software processes the raw spectra of the respective strain to generate the Main SPectra (MSP). Then, it compares the peak list of the MSP against each reference entry of the database and expresses the microorganism identification as a logarithmic score between zero (no similarity) and three (high similarity), using a pattern-matching algorithm. Interpretation was performed using identification score cutoff values recommended by the manufacturer: a score of 2.000 to 3.000 indicates a high precision identification at the species level, a score of 1.700 to 1.999 indicates reliable genus-level identification, while a score of <1.700 was considered as unreliable identification. The MALDIquant and MALDIquantForeign packages were used to import and process the MALDI-TOF mass spectra within the R software (Gibb and Strimmer 2012; R. Core Team 2021).

Antagonistic activity of the screened LAB & Bifido isolates

The antagonistic activity of the screened LAB & Bifido isolates was tested against clinical strains isolated in 2018 at the Traumatology and Great Burned Center, Tunis: *Escherichia coli* (E644), *Klebsiella pneumoniae* (E625), *Pseudomonas aeruginosa* (E314), *Citrobacter freundii* (E619), from urine samples, and *Acinetobacter baumannii* (D343), from pus sample (referred hereafter as indicator strains), according to the agar well diffusion assay as mentioned above. The bacteria were grown on TS agar medium at 37°C for 18 h.

Preliminary identification of the antimicrobial compounds

The isolates were investigated for their antimicrobial compounds. To this end, the CFSs were subjected to different treatments. First, CFS pH adjusted to six using 1M NaOH highlights the antimicrobial contribution of organic acids. In a separate experiment, the neutralized CFSs were treated with catalase (1 mg/mL, Sigma), at 25°C for 30 min, to exclude the effect of hydrogen peroxide. In addition, to verify the proteinaceous nature of the antimicrobial compound (i.e. a bacteriocin-like substance (BLS)), CFSs were subjected to the action of proteinase K (Biomatik, Ontario, Canada), pepsin, trypsin, and α-chymotrypsin (Sigma). The effects of lysozyme (Biomatik), a-amylase and lipase (Sigma) were also analyzed. All enzymes were used at a final concentration of 1 mg/mL according to the manufacturer's instructions. The enzymatic reaction was performed at 37°C for 3 h and stopped by heating the mixture at 100˚C for 5 min. The antibacterial activity of the treated CFSs was performed according to the agar well diffusion as described above. Untreated CFSs were used as positive controls. The residual antibacterial activity (RA) was estimated as follow:

RA (%) = (IZ of treated CFS/IZ of untreated CFS) x 100

Statistical analyses

Processed MALDI-TOF mass spectra (R. Core Team 2021) were classified by hierarchical cluster analysis using Euclidean distances and Ward's aggregation algorithm. The R factoextra package was used to build the dendrogram (Kassambara 2017).

One way ANOVA tests were applied to analyze significant differences (with p-value <0.05) between the mean values for different assays with the isolates or their CFS, using XLSTAT statistical software (version 23.5.1232).

Frequency distribution analysis of the bacterial species was carried out for different pair cases combining sample origins and forage plants. Variability plots associating numbers of LAB & Bifido isolates with combined abscissa axes (LAB & Bifido isolates, origins, honey bee forage plants, clusters) were carried out using Gauge Chart command of JMP statistical software (SAS Institute).

Global trend analysis of inhibition levels of pathogenic bacteria by systems combining LAB & Bifido isolates, their origins and honey bee forage plants was carried out by principal component analysis (PCA) (Jolliffe 2002; Semmar 2011).

Results

Isolation and characterization of LAB & Bifido

Seven hundred eighty LAB & Bifido grown on MRSs, with Gram-positive and catalase-negative properties, were obtained. They formed creamy or off white, raised, and smooth colonies. Most of strains (82.7%) were homofermentative and 63.84% were anaerobic bacteria. Nearly half (45.38%) of isolates were recovered from bee GIT, 11.92% from propolis, 18.46% from honey, and 24.23% from bee bread. Isolates originated from the hive set in *Erica cinerea* plantation held the highest percentage (23.59%) followed by isolates sampled from the hive kept in wildflowers (17.95%).

Antagonistic activity of the LAB & Bifido against *S. aureus*

The anti-*S. aureus* activity screening of the 780 isolates showed that 24.74% (193/780) of them exhibited moderate to strong antibacterial effects. Among the selected 193 LAB & Bifido, 41% exhibited strong antagonistic effect against *S. aureus*, with average inhibition zones ranging from 16.66±0.01 to 21.33±0.01 mm. Out of the 193 isolates, 50%, 10.36%, 14%, and 17% were isolated from the honey bee GIT, bee bread, propolis and honey, respectively. They were predominantly recovered from samples of hive set in *Erica cinerea* plantation (31.6%), followed by wildflowers (15.02%), caraway (14%), *Marrubium vulgare* (11.4%), overwintering hive (10.36%), orange blossom (8.8%), and *Eucalyptus* (7.2%).

MALDI-TOF MS identification and clusteranalysis analysis of the LAB & Bifido isolates

All LAB & Bifido grown on blood agar plates for MALDI-TOF MS identification were non-haemolytic (no halo formation surrounding the colonies). The spectral scores of the 193 isolates obtained by the BioTyper software allow highly reliable identification at bacterial species level of 79.8 % (154 isolates) of them (log score ≥ 2). These 154 LAB & Bifido species were identified as: *Bifidobacterium asteroides* (13 isolates), *Pediococcus pentosaceus* (49), *Weissella paramesenteroides* (1), 42 isolates belong to 4 species of genus *Enterococcus* (*Enterococcus durans* (1), *Enterococcus mundtii* (3), *Enterococcus faecalis* (21) and *Enterococcus faecium* (17)), *Lactiplantibacillus plantarum* (37), *Limosilactobacillus fermentum* (3), *Apilactobacillus kunkeei* (7), *Latilactobacillus curvatus* (1), and *Lacticaseibacillus rhamnosus* (1). The remaining 39 LAB isolates (20.2%) achieved score values ranging from 1.7 to 1.99 and hence were classified to the genus level: *Bifidobacterium* spp. (13 strains), *Leuconostoc* spp. (2 strains), *Pediococcus* spp. (4), and lactobacilli (20 isolates).

Phyloproteomic relationships between the 193 LAB & Bifido isolates presented on a dendrogram generated from MALDI-TOF MS peak data analysis are shown in Fig. 1. Twenty-seven groups of closely related bacteria were obtained. At a dendrogram height of at least at least 50, three large clusters were distinguished: a first cluster that comprised *Bifidobacterium asteroides*, *Lactiplantibacillus plantarum*, *Pediococcus pentosaceus*, *Apilactobacillus kunkeei, Weissella paramesenteroides, Latilactobacillus curvatus, Enterococcus mundtii, Leuconostoc* spp., lactobacilli, *Limosilactobacillus fermentum* and *Enterococcus faecium.* A second cluster included *Enterococcus*

faecalis, *Enterococcus faecium*, *Enterococcus durans* and *Lactiplantibacillus plantarum* species and a third cluster was composed of bacterial isolates belonging to *Pediococcus pentosaceus* species. Bacterial isolates of the same species were clustered within the same cluster for *Bifidobacterium asteroides*, *Lactiplantibacillus plantarum*, *Pediococcus pentosaceus* and *Apilactobacillus kunkeei*. On the other hand, it is noteworthy that some isolates belonging to same species or genus are grouped in separate clusters. Thus, *Pediococcus pentosaceus* isolates were classified in the first and third clusters, separated at a cophenetic distance of 81.20, whereas *Lactiplantibacillus plantarum* and *Enterococcus faecium* isolates were found separated in the first and second clusters at a cophenetic distance of 57.77. In addition, four sub-clusters of *Lactiplantibacillus plantarum* isolates were observed in the first cluster while two other sub-clusters of the same species were seen at a cophenetic distance of 29.51 within the second cluster. Two species, *Weissella paramesenteroides* and *Latilactobacillus curvatus*, turned out to be separated at a very small cophenetic distance (19.03).

Distribution of LAB & Bifido by origin and honey bee forage plant

Fig. 2 showed that among the 12 identified LAB & Bifido species, *Lactiplantibacillus plantarum, Pediococcus pentosaceus*, and *Enterococcus faecalis* species were detected in the four matrices (e.i. BGIT, honey, bee bead, and propolis). The highest LAB & Bifido diversity was detected in BGIT with eleven identified species, of which five were exclusive to this origin: *Bifidobacterium asteroides*, *Latilactobacillus curvatus, Limosilactobacillus fermentum, Lacticaseibacillus rhamnosus,* and *Enterococcus durans. Pediococcus pentosaceus*, *Lactiplantibacillus plantarum*, and *Enterococcus faecalis* isolates were predominantly isolated from BGIT (40.81% (20/49), 59.45% (22/37), and 47.61% (10/21), respectively. Our results revealed that honey was the second origin harboring an important variety of LAB species (6 species), of which *Enterococcus mundtii* and *Apilactobacillus kunkeei* were found only in honey and BGIT. The only *Weissella paramesenteroides* strain isolated in the present study was found in bee bread with co-occurrence of *Enterococcus faecalis* (2), *Lactiplantibacillus plantarum* (2), and *Pediococcus pentosaceus* (12) which were also seen in other origins. Isolates of *Enterococcus faecium* were detected in samples collected from BGIT (82.35 %, 14/17), propolis (5.88 %, 1/17), and honey (11.76 %, 2/17).

Out of the 12 LAB & Bifido species identified in our study, *Pediococcus pentosaceus* was associated with all hives, except that set in *Eucalyptus* plantation (Fig. 3). Seven species were isolated from samples of hive set in wildflowers field. *Enterococcus durans*, *Enterococcus mundtii* and *Weissella paramesenteroides* species were distinctive of this hive. LAB & Bifido diversity (6 identified species) was also found in *Marubium vulgare* plantation. Among them, *Apilactobacillus kunkeei*, *Bifidobacterium asteroides*, *Enterococcus faecalis*, and *Pediococcus pentosaceus* species were shared with hives set in caraway and orange blossom plantations. Compared to other species, *Lactiplantibacillus plantarum* isolates were predominantly (46.66 %, 7/15) detected in the hive placed in *Marubium vulgare* plantation, while *Latilactobacillus curvatus* species was distinctive of this hive. Five LAB & Bifido species were identified in samples from the overwintering hive and hives kept in caraway, orange blossom and *Erica cinerea* plantations. These hives shared *Enterococcus faecalis* and

Pediococcus pentosaceus species, whereas *Lacticaseibacillus rhamnosus* was distinctive of the hive set in *Erica cinerea* plantation. Only three LAB species (*Enterococcus faecium*, *Lactiplantibacillus plantarum*, and *Limosilactobacillus fermentum*) were associated with the hive placed in *Eucalyptus* plantation.

Moreover, by considering isolate prevalence, 71% (12/17) of *Enterococcus faecium* isolates detected in our study were found in samples from hive kept in *Eucalyptus* plantation. More than half (53.06 %, 26/49) and 24.48 % (12/49) of the *Pediococcus pentosaceus* isolates were found samples of hives placed in *Erica cinerea* and wildflowers plantations, respectively. Most of *Enterococcus faecalis* (62%, 13/21) and *Bifidobacterium asteroides* (38%, 5/13) isolates were associated with hive set in caraway plantation. *Lactiplantibacillus plantarum* isolates were mainly (43.24 % (16/37)) associated with hive kept in *Erica cinerea*.

Antagonistic activity of LAB & Bifido against pathogenic bacteria

The antibacterial activity of the 154 isolates against five Gram-negative pathogenic bacterial strains (*E. coli*, *K. pneumoniae*, *A. baumannii*, *C. freundii* and *P. aeruginosa*) showed that almost all isolates (97.4%) exhibited inhibitory activity towards at least three pathogens. The majority (87.60%) of the isolates displayed antagonistic effects towards all tested pathogens, but the degree of antagonism varied among the LAB & Bifido isolates, with IZs ranging from 11 to 24 mm. The LAB & Bifido strongly antagonized (IZ > 16 mm) *C. freundii*, *P. aeruginosa*, *A. baumannii*, *E. coli*, and *K. pneumoniae* with percentage values of 71.42%, 38.31%, 26.6%, 23.37%, and 9.74%, respectively (Fig. 4). The following species, *Lactiplantibacillus plantarum* (IZs 12-22 mm), *Enterococcus faecium* (13-21 mm), *Limosilactobacillus fermentum* (12-20 mm), *Enterococcus mundtii* (13-18 mm), *Weissella paramesenteroides* (12-18 mm), *Enterococcus durans* (13-17 mm), *Latilactobacillus curvatus* (13-22 mm), and *Lacticaseibacillus rhamnosus* (12-20 mm) displayed the largest spectrum of inhibitory activity, where 100% of the isolates inhibited all tested pathogenic bacteria. In the case of *Bifidobacterium asteroides* (IZ 13-22 mm), *Pediococcus pentosaceus* (12-21 mm), *Enterococcus faecalis* (12-21 mm) and *Apilactobacillus kunkeei* (12-22 mm), 92.3% (12/13), 89.8% (44/49), 57.14% (12/21) and 42.86% (3/7) of isolates exhibited antagonistic activity against all pathogens, respectively.

Characterization of the antimicrobial compounds

The antibacterial activity completely disappeared for 92.85% (143/154) of the isolates when the CFS was adjusted to pH 6 with NaOH, indicating that their inhibitory potential depends primarily on the presence of organic acids. However, a *small but meaningful reduction* in the antibacterial activity after adjusting the CFS to pH 6 (RA range from 65.23±0% to 86.88±0.57%) was detected for 11 isolates (7.14%) (Table 1). Besides, no inhibition effect of H_2O_2 was detected in all neutralized CFSs, as their antibacterial potency was preserved after catalase treatment.

Table 1 outlines the effects of additional CFS treatments for further characterization of the antibacterial compounds produced by the eleven isolates. After CFS treatment with the four proteolytic enzymes,

complete inactivation of the inhibitory effect was observed for *Enterococcus faecium* $P_{OB}3$, *Bifidobacterium asteroides* $BGIT_{MV}16$ and *Apilactobacillus kunkeei* $BGIT_{MV}22$ isolates. The remaining CFSs were affected by enzymatic proteolysis to a variable degree (RA ranging from 0% to 89.77±0.33%). These findings suggest that a proteinaceous compound (i.e. BLS) was strongly involved in the antibacterial activity of the 11 isolates, with a small contribution of organic acids. After treatment with lipase, the BLS lost its antibacterial effect either entirely (for *Pediococcus pentosaceus* $BGIT_C15$, *Enterococcusfaecium* $P_{OB}3$, *Bifidobacterium asteroides* $BGIT_{MV}16$, *Apilactobacillus kunkeei* $BGIT_{MV}22$ isolates) or partially (for the seven remaining isolates, RA ranging from 37.51±0.57% to 79.96±0.4%). Moreover, after treatment with α-amylase and lysozyme, the activity of BLS completely disappeared from the CFS of *Enterococcus faecium* $P_{OB}3$ and *Bifidobacterium asteroides* $BGIT_{MV}16$ isolates, whereas it was significantly reduced for *Apilactobacillus kunkeei* $BGIT_{MV}22$ and *Bifidobacterium asteroides* $BGIT_{MV}5$ isolales (RA ranging from 56.07±0.33% to 89.69±0.33%). Compiled results of the lipase and glycosidase assays suggest that the BLS requires lipid (for all 11 isolates) and carbohydrate moieties (for 4 isolates).

Principal component analysis of antibacterial activity

Initially, ANOVA highlighted significant differences between the mean values of the LAB & Bifido antibacterial activities toward the six pathogenic species ($p < 0.05$).

PCA provided six principal components (P1-P6) of which the first four covered 91% of total variance (Fig. 5-7). In the first correlation circle (Fig. 5a), the six pathogenic bacteria were separated into two positive correlation groups associating *E. coli*, *K. pneumoniae*, *A. baumannii*, and *P. aeruginosa* along the first principal component (P1) vs *C. freundii* and *S. aureus* along the second one (P2). This indicated specific states of pathogens associated with different honey bee forage plant/LAB & Bifido systems. Moreover, the first loading plot (P1P2) showed projections of the six pathogenic bacteria along left side of P1 ($P1^-$) (Fig. 5a). According to the individual scores' plot, a subset of points corresponding to honey bee feeding on caraway showed opposite projections to pathogens along P1 (i.e. $P1^+$) (Fig. 6a vs Fig. 5a). These points were associated with some types of LAB including mainly isolates of *Enterococcus faecalis* (from propolis), *Pediococcus pentosaceus* (from propolis) and *Apilactobacillus kunkeei* (from honey) (Fig. 6b and Fig. 6c). This finding indicated that honey bee foraging on caraway was relatively less favorable for these three LAB species against *K. pneumonia*, *A. baumannii*, *E. coli* and *P. aeruginosa* (projected in $P1^-P2^-$ which is opposite to $P1^+P2^+$ containing the three LAB species) (Fig. 5a vs Fig. 6 a and Fig. 6b).

The whole population showed compact aspect oriented between the opposite quadrants $P1^-P2^+$ and $P1^+P2^-$ (Figs. 6a-6c). As $P1^-P2^+$ was occupied by *S. aureus* and *C. freundii* (Fig. 5a), compact aspect of LAB & Bifido indicated some gradient of anti-pathogenic effects toward these two-pathogens (Figs. 6d-6f): the score plot $P1^-P2^+$ was essentially occupied by isolates of *Lactiplantibacillus plantarum* (from propolis, BGIT, and honey) and *Pediococcus pentosaceus* (from propolis, bee GIT, and bee bread) (Figs.

6e and 6f) that were associated with honey bee feeding on *Erica cinerea* (Fig. 6d). This highlighted high anti-*S. aureus* and anti-*C. freundii* effects of the LAB & Bifido associated with *Erica cinerea* system. Also, the same quadrant ($P1^-P2^+$) revealed that isolates of *Bifidobacterium asteroides* and *Lactiplantibacillus plantarum* (from BGIT) associated with orange blossom and *Marubium vulgare* systems, respectively, exerted anti-*S. aureus* and -*C. freundii* effects (Figs. 6d-6f).

The opposite quadrant $P1^+P2^-$ revealed, on the contrary, less favorable anti-pathogenic system against *S. aureus* and *C. freundii* (Figs 6j-6l): the most extreme projection concerned *Pediococcus pentosaceus* and *Enterococcus faecium* isolates sampled from BGIT of overwintering honey bees. The $P1^+P2^-$ subspace was also occupied by *Pediococcus pentosaceus* and *Apilactobacillus kunkeei* isolates sourced from honey of honey bee feeding on orange blossom. Also, *Apilactobacillus kunkeei* (isolates from BGIT), *Pediococcus pentosaceus* (from honey) and *Lactiplantibacillus Plantarum* (from propolis and BGIT), associated with wildflowers, displayed relatively low inhibition effects against *S. aureus* and *C. freundii*. Moreover, the quadrant $P1^+P2^-$ showed projection (along P2) of *Enterococcus faecium* (from BGIT) associated with overwintering honey bees, indicating another unfavorable anti-*S. aureus* and-*C. freundii* system.

In the quadrant $P1^-P2^-$, the wide occupation (many projected points) of *Enterococcus faecium*, and *Lactiplantibacillus plantarum* isolates sampled from BGIT associated with *Eucalyptus*-based forage, highlighted its relatively high anti-*E. coli*, -*K. pneumoniae*, -*A. baumannii*, and -*P. aeruginosa* effects (Figs 6g-6i). These same anti-pathogens trends were also concerned with some points corresponding to *Lactiplantibacillus plantarum* isolates originated from propolis and associated with overwintering honey bees.

Further structured information was provided by the second factorial plot P3P4 making to reach 91% of explained total variance (Fig. 7). The second correlation circle showed frank opposition between *C. freundii* and *S. aureus* ($P3^+P4^+$ vs $P3^-P4^-$, respectively) (Fig. 5b). By this way, the second correlation circle (P3P4) highlighted specific states concerning pathogenic species which previously showed common aspects in P1P2. Moreover, along P3, *C. freundii* and *P. aeruginosa* showed some opposition, whereas they shared some projection subspace along $P4^+$. This indicated the presence of both common and specific anti-pathogens systems that were further highlighted by individuals' factorial plots (Fig. 7). Distant parts of P3P4 (representing original trends) were occupied by some LAB points associated with some honey bee forage plants and origin matrices (Figs. 7a-7c): by zooming, quadrant $P3^-P4^+$ showed anti-*P. aeruginosa* effect of *Enterococcus faecalis* isolates originated from propolis of bees feeding on caraway (Fig. 5b vs Figs 7d-7f). However, this effect was observed to a lesser measure and in another way for *Enterococcus faecalis* sampled from BGIT/orange blossom system. Also, other less common anti-*P. aeruginosa* systems concerned isolates of *Pediococcus pentosaceus* (from propolis) and *Apilactobacillus kunkeei* (from honey) associated with caraway.

Regarding the quadrant $P3^-P4^-$, it revealed antagonistic effects against *S. aureus* of (i) *Pediococcus pentosaceus* isolates from bee bread/*Erica cinerea* system. In the same quadrant, *Pediococcus pentosaceus* isolates (associated with BGIT, overwintering honey bees) and *Enterococcus faecalis* (associated with *Marrubium vulgare* and bee bread) showed weak activities against *C. freundii* which was projected in opposite space $P3^+P4^-$ (Figs 7g-7i). Relatively low anti-*P. aeruginosa* effects (Fig. 5b) of *Apilactobacillus kunkeei* and *Pediococcus pentosaceus* isolates (sampled from honey/orange blossom system) were highlighted in the quadrant $P3^+P4^-$ (Figs 7j-7l). Analogous weak anti-pathogen pattern was also revealed in this quadrant by some points of *Enterococcus faecalis* isolates (from propolis and honey) and *Bifidobacterium asteroides* in caraway feeding bees.

Discussion

The honey bee has been part of human culture since ancient times and its products have an extensive history of use (Topal et al. 2021). Although multiple investigations focused on the honey bee microbiota (Zheng et al. 2018), several studies have examined the microbiological communities of bee products. According to the MALDI-TOF MS manufacturer's score interpretation criteria, almost three-quarters of the LAB & Bifido isolates in this study achieved reliable species level scores. This result is in agreement with those of Gaudreau et al. (2018) who reported that 72% (13/18) of bacteria growing as biofilms were identified at the species level. Moreover, Dušková et al. (2012) described higher success rates of species-level assignment (93%) for lactobacilli strains using the MALDI-TOF MS method compared to PCR assay (77%).

Overall, *Lactiplantibacillus plantarum*, *Pediococcus pentosaceus* and *Enterococcus faecalis* species were predominantly detected, particularly in the BGIT. It is well established that among Gram-positive bacteria, lactobacilli dominate the gut microbiota of honeybee workers (Nowak et al. 2021). However, strains of *Enterococcus faecalis* were identified from the intestinal tracts of *Apis mellifera* L. honey bees in Egypt (Mahmoud Elzeini et al. 2021). El-Sohaimy et al. (2020) showed that *Lactiplantibacillus plantarum* strains were mainly (62.5%) isolated from the stomach of three different types of Egyptian bees: *Apis mellifera lamarckii*, Carniolan bees (*Apis mellifera carnica*) and hybrid Carniolan bees, followed by *Pediococcus pentosaceus* (12.5%), *Lactiplantibacillus pentosus* (12.5%), and *Latilactobacillus sakei* (12.5%). Also, Iorizzo et al. (2020) reported the predominance of *Lactiplantibacillus plantarum* in the gut of *Apis mellifera* in Italy. According to a chineese study (Luo et al. 2020) the abundance of lactobacilli and other species was high in *Apis cerana* from different areas. Almost half of the *Apilactobacillus kunkeei* isolates identified in our study were detected in BGIT. A Japanese study found that *Apilactobacillus kunkeei* made up more than half (57.1%) of lactobacilli in the *Apis mellifera* stomach (Asama et al. 2015). On the other hand, *Bifidobacterium asteroides* was found in low abundance among our collection, as expected (Kwong & Moran 2016).

Concerning bee products, our results revealed that honey harbored an important variety of LAB species. Asama et al. (2015) detected *Apilactobacillus kunkeei* as the predominant species identified not

only in honey (98.8%), but in other bee products: bee pollen (98.6%), bee bread (99.5%), and royal jelly (99.7%). To the best of our knowledge, this is the first report on the detection of *Enterococcus mundtii* in honey bee and its products. This bacterium has been described in cow's milk, plant, soil, and dairy products (Dapkevicius et al. 2021), and recently in the slime of Tunisian edible snails (Rabaoui et al. 2023). On the other hand, *Pediococcus pentosaceus*, *Lactiplantibacillus plantarum* and *Enterococcus faecalis* strains were found in all bee products from at least five hives. Belhadj et al. (2014) identified *Lactiplantibacillus plantarum*, *Limosilactobacillus fermentum*, *Lactococcus lactis*, *Pediococcus acidilactici*, *Pediococcus pentosaceus*, and unidentified lactobacilli from bee pollen collected in different Algeria areas. Nevertheless, all isolated bacteria (34) from bee pollen and bee bread provided by Polish apiaries, and screened for their antagonistic activity against pathogenic bacteria, belong to the genus *Bacillus* (Pełka et al. 2021**)**. In the study conducted on LAB isolated from honey samples from Malaysia, Libya, Saudi Arabia, and Yemen, *Lactiplantibacillus plantarum* (from Al-Seder honey), *Latilactobacillus curvatus* (from Al-Hanon honey), *Pediococcus acidilactici* (from Tualang honey) and *Pediococcus pentosaceus* (from Al-Maray honey) were identified (Bulgasem et al. 2016). The only *Weissella paramesenteroides* strain isolated in the present study was found in bee bread from the hive set in wildflowers. *Weissella paramesenteroides* occurs in a great variety of habitats, such as soil, plants, milk, fermented food, human, and animal feces (Abriouel et al. 2015). However, detection of this bacterium in beehive and its products has been scarcely reported. Thus, *Weissella paramesenteroides* was identified in the BGIT of four different stingless bee species (*Melipona beecheii*, *Scaptotrigona pectoralis, Plebeia jatiformis* and *Plebeia llorentei*) native to the subtropical region in Mexico (Torres-Moreno et al. 2021).

LAB & Bifido, like other bacteria, are well known to produce antimicrobial substances, defend themselves. Several studies have reported that these compounds could protect the larvae and adult bees from honey bee diseases and preserve honey and bee bread (Vásquez and Olofsson 2009; Ramos et al. 2020). Overall, the LAB & Bifido of our collection showed moderate to strong antibacterial activities. Based on the study of Belhadj et al. (2010), LAB (such as *Lactiplantibacillus plantarum* and *Enteococcus* sp.) from honey bee collected pollen displayed antagonistic activity against *S. aureus*, *Bacillus cereus*, *E. coli*, and *Salmonella typhi*. Butler et al. (2016) showed that a mixture of the 13 LAB & Bifido symbionts (including for example *Apilactobacillus kunkeei* and *Bifidobacterium asteroides* strains) in a matrix of heather (*Calluna vulgaris*) honey had antimicrobial activity against all tested bacterial strains (*S. aureus*, *P. aeruginosa* and *E. coli*). The growth of all tested mastitis pathogens (including *S. aureus*, *K. pneumoniae* and *P. aruginosa*), was inhibited by a honey and LAB & Bifido combination (Piccart et al. 2016).

The present investigation highlights the role of organic acids as a predominant protective mechanism against all bacterial pathogens, whereas 7.14% of the isolates produced BLS. This is in line with the findings of Imade et al. (2021) who reported that, among isolates of LAB inhibiting the growth of *S. aureus* ATCC 25923 and *E.coli* ATCC 25922, less than 5% LAB showed bacteriocin activity. According to Janashia et al. 2016, none of 102 LAB strains, isolated from honey bee intestines and bee bread, produced detectable bacteriocins. The 11 BLS activities detected in the present study require lipid moiety;

four of them need also carbohydrate. However, deep understanding of interaction properties between protein-lipid and/or carbohydrate require structural study of the BLS. Antibacterial activity against *S. aureus*, as well as against other Gram-positive and negative bacterial pathogens, was reported for complex bacteriocins such as enterocin B3A–B3B (from *E. faecalis* strain) (Al-Seraih et al. 2017) and plantaricin TF711 (from *Lactiplantibacillus plantarum* strain) (Hernández et al. 2005).

Numerous studies have explored the biological activities and chemical compositions of bee products from different geographic regions, and from different seasons and flower distribution (Shehata et al. 2020; Mahmood et al. 2021). In addition, compounds secreted by LAB & Bifido have been frequently repoted (Hernández-González et al. 2021; Yadav et al. 2022). However, very little is known about the correlation between antibacterial properties of LAB & Bifido from honey bees and their products on one hand and the bee-foraging flora on the other hand. The synthesis of different antibacterial trends highlighted by PCA led to conclude that LAB & Bifido, associated with different honey bee forage plants and origins, showed variable spectrum of antagonistic effects towards pathogenic bacteria. For the positive inhibition trends (as summarized in Fig. 8a): (i) *Lactiplantibacillus plantarum* was concerned with the most antibacterial effects (against the six pathogens), followed by *Pediococcus pentosaceus* (5 pathogens) and *Enterococcus faecium* and *Bifidobacterium asteroides* (4 pathogens); (ii) *Enterococcus faecalis* showed more specific effects against some bacterial pathogens: *S. aureus* and *P. aeruginosa*. *Pediococcus penstosaceus*, and *Enterococcus faecalis* showed antibacterial effects associated with different (specific) honey bee forage plants and origines. However, *Enterococcus faecium* from BGIT displayed a large antibacterial pattern under the same honey bee forage plant (*Eucalyptus*). On the other hand, specific antibacterial activity was associated with some bee foraging plants. Thus, LAB & Bifido sampled from hive set in caraway were essentially implied in anti-*P. aeruginosa* activity. Concerning negative inhibition trends, *S. aureus* and *C. freudii* were relatively less inhibited by several systems combining LAB & Bifido with bee foraging plant and origins (Fig. 8b). Indeed, weak anti- *S. aureus* and -*C. freudii* effects were exhibited by: (i) *Pediococcus pentosaceus*, *Lactiplantibacillus plantarum*, *Apilactobacillus kunkeei* (all associated with honey bees feeding on wildflowers) and *Enterococcus faecium* (overwintering honey bees) were sampled from propolis, BGIT and honey, (ii) *Pediococcus pentosaceus* and *Apilactobacillus kunkeei* from honey/orange blossom system. Finally, other weak antibacterial systems specifically concerned some LAB & Bifido sourced from honey bees foraging on caraway.

In conclusion, our study highlited both specific and polymorphic antibacterial activites of different LAB & Bifido species associated with different origins (BGIT, honey, propolis, and bee bread) and honey bee forage plants. Special emphasis should be placed firstly on the relationship between honey bee forage plant and the high diversity of the peptide mass fingerprints of isolates of the same species which were gathered in different clusters using MSP. Secondly, in light of our findings on the LAB & Bifido antibacterial activities, an additional interest for research consists in identifying potential probiotics among these isolates.

Declarations

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Data availability statement

Data generated or analyzed during this study are provided in full within the published article.

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Authors' Contributions

Houda Ben-Miled designed the study, performed experiments and drafted the manuscript. Nabil Semmar realized the statistical analysis (distribution analysis, principal component analysis) of antibacterial activity and contributed to the manuscript writing and revision. Miguel Sautié Castellanos completed biostatistical analyses and contributed to manuscript writing. Kamel Ben-Mahrez contributed to the manuscript revision. Marie-Odile Benoit-Biancamano contributed to study supervision, and manuscript writing and edition. Samia Réjiba supervised the work, wrote and edited the manuscript. All authors read and approved the final manuscript.

Competing interests

The authors declare there are no competing interests.

Consent to publish

Not applicable.

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Table

Table 1 is available in the Supplementary Files section.

Figures

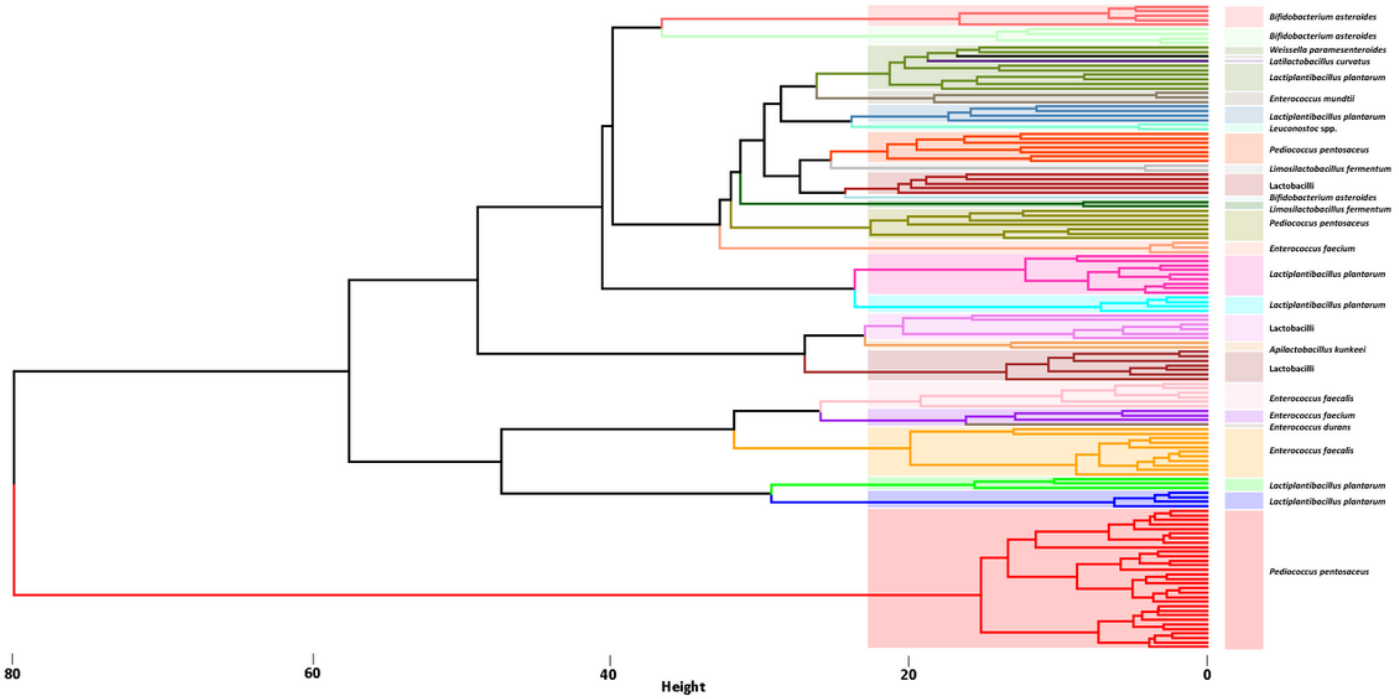

Fig.1

Figure 1

Dendrogram generated from the hierarchical cluster analysis of MALDI-TOF MS spectra of the 193 LAB & Bifido isolates, which belong to at least 10 genera.The distance levels of each subdivision are shown in arbitrary units.

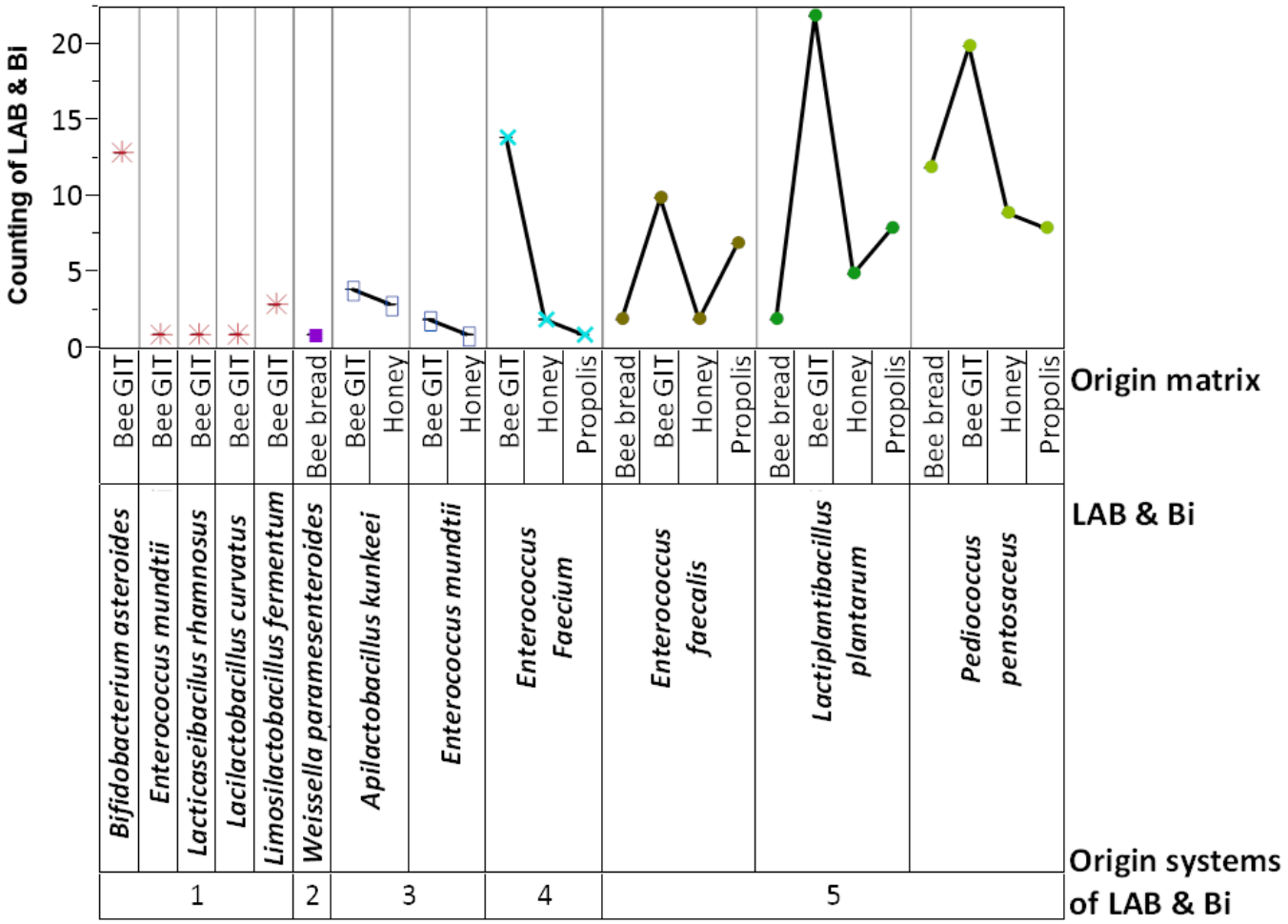

Figure 2

Distribution analysis of frequencies and modes of clustering of LAB & Bifido in association with the four sample origins.

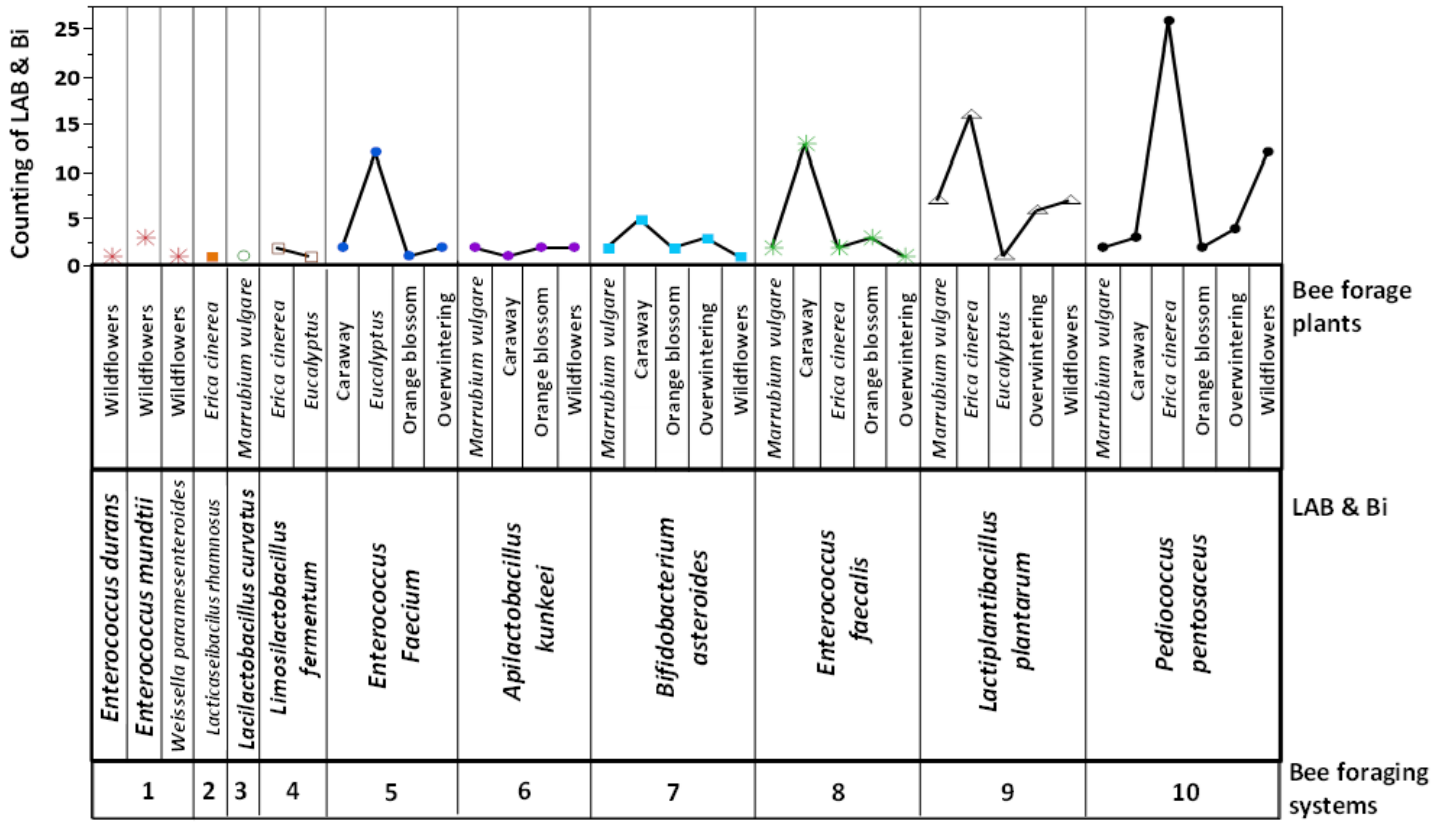

Figure 3

Distribution analysis of frequencies and modes of clustering of LAB & Bifido in association with the seven bee forage plants.

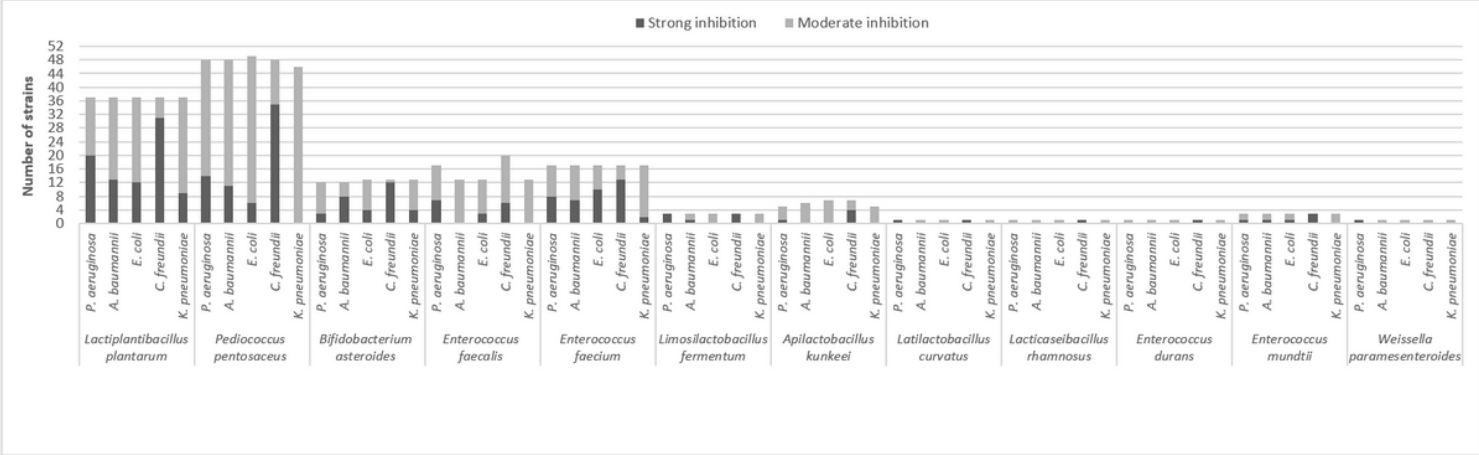

Figure 4

Distribution of the antibacterial activity against five pathogenic bacteria by species of LAB & Bifido.

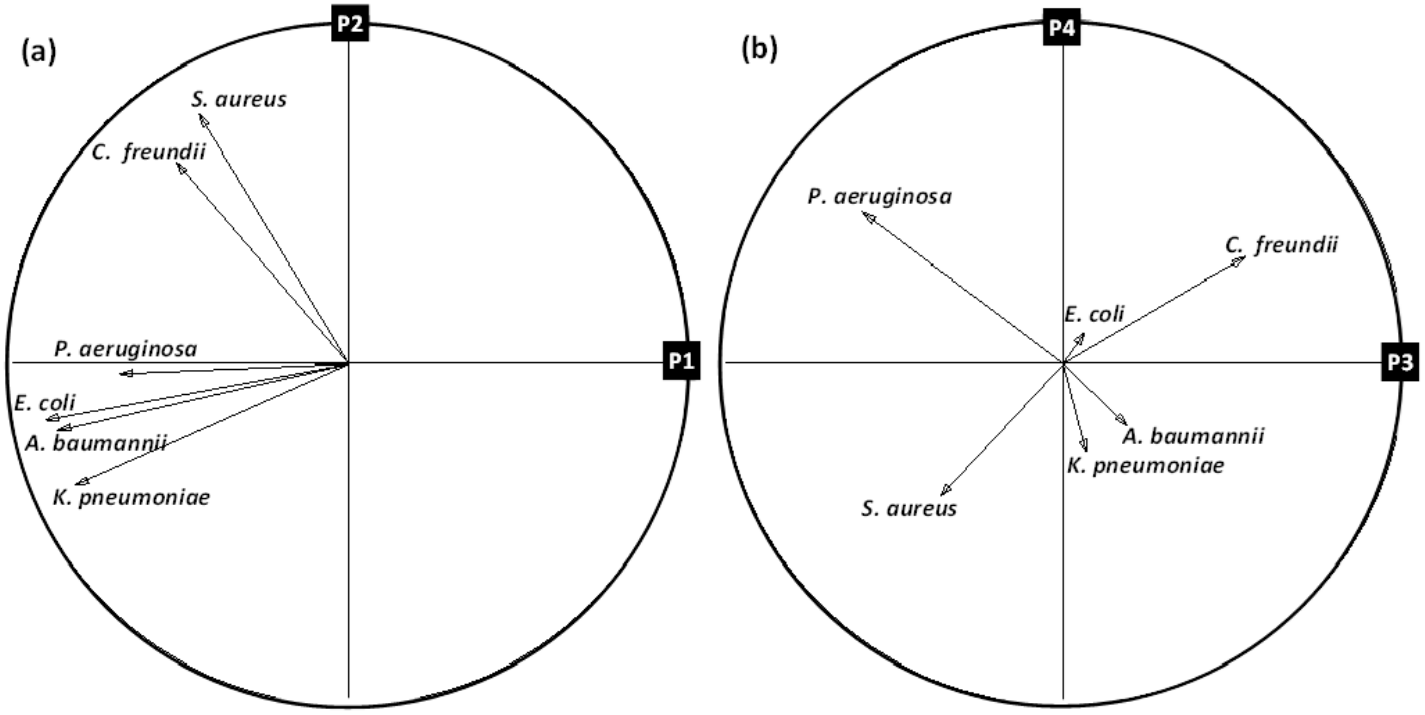

Figure 5

Correlation circles between pathogenic bacteria variables given by PCA associated with the first (P1P2) (**a**) and second (P3P4) (**b**) factorial plots, respectively.

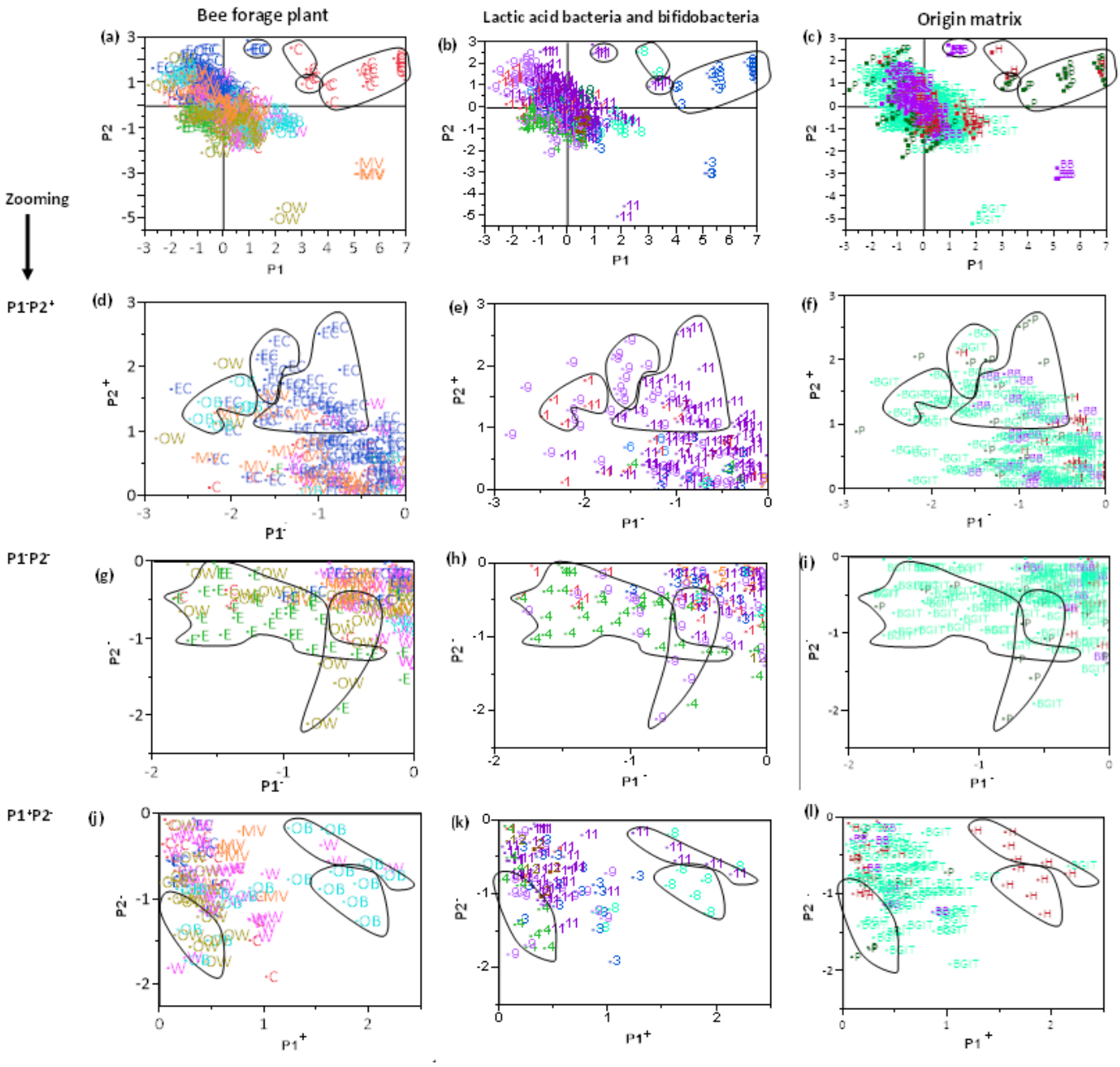

Figure 6

Factorial plots P1P2 of individuals given by principal component analysis highlighting topological variations of honey bee forage plants (**a, c, e, g**), LAB & Bifido (**b, d, f, h**) and origin matrices (i-l). Different quadrants of scores (individuals) plot were zoomed (**c-h**). C, caraway; E, *Eucalyptus*; EC, *Erica cinerea*; MV, *Marrubium vulgare*; OB, orange blossom; OW, overwintering honey bees; W, widflowers; BB, bee bread; BGIT, honey bee gastrointestinal tract; H, honey; P, propolis; 1, *Bifidobacterium asteroides;* 2, *Enterococcus durans*; 3, *Enterococcus faecalis*; 4, *Enterococcus faecium*; 5, *Enterococcus mundtii*; 6, *Latilactobacillus curvatus*; 7, *Limosilactobacillus fermentum*; 8, *Apilactobacillus kunkeei*; 9, *Lactiplantibacillusplantarum*; 10, *Lacticaseibacillusrhamnosus*; 11, *Pediococcus pentosaceus*; 12, *Weissella paramesentroides.*

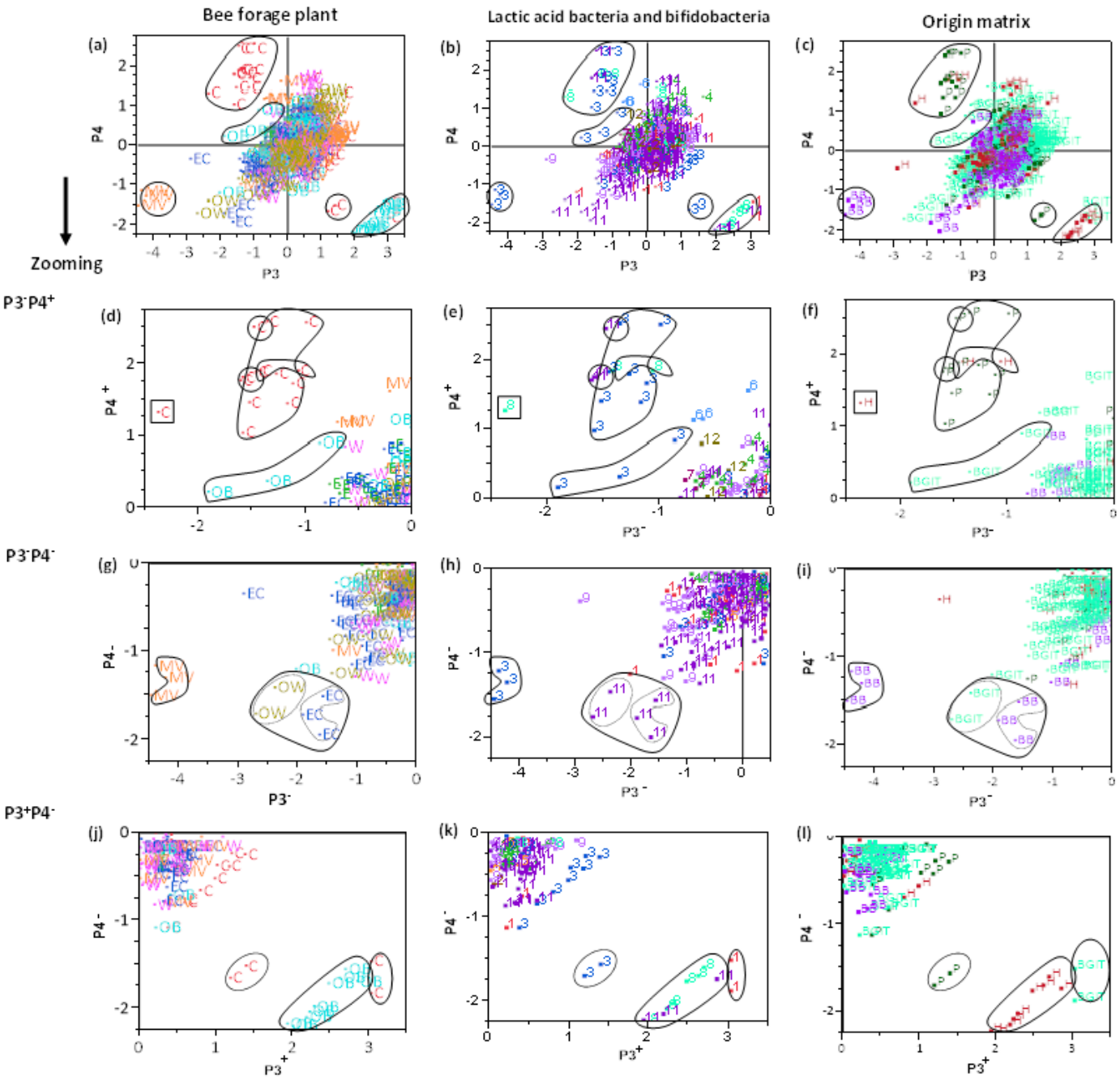

Figure 7

Factorial plots P3P4 of individuals given by principal component analysis highlighting topological variations of honey bee forage plants (**a, c, e, g**), LAB & Bifido (**b, d, f, h**) and origin matrices (**i-l**). Different quadrants of scores (individuals) plot were zoomed (**c-h**). C, caraway; E, *Eucalyptus*; EC, *Erica cinerea*; MV, *Marrubium vulgare*; OB, orange blossom; OW, overwintering honey bees; W, widflowers; BB, bee bread; BGIT, honey bee gastrointestinal tract; H, honey; P, propolis; 1, *Bifidobacterium asteroides;* 2, *Enterococcus durans*; 3, *Enterococcus faecalis*; 4, *Enterococcus faecium*; 5, *Enterococcus mundtii*; 6, *Latilactobacillus curvatus*; 7, *Limosilactobacillus fermentum*; 8, *Apilactobacillus kunkeei*; 9, *Lactiplantibacillus plantarum*; 10, *Lacticaseibacillusrhamnosus*; 11, *Pediococcus pentosaceus*; 12, *Weissella paramesentroides*.

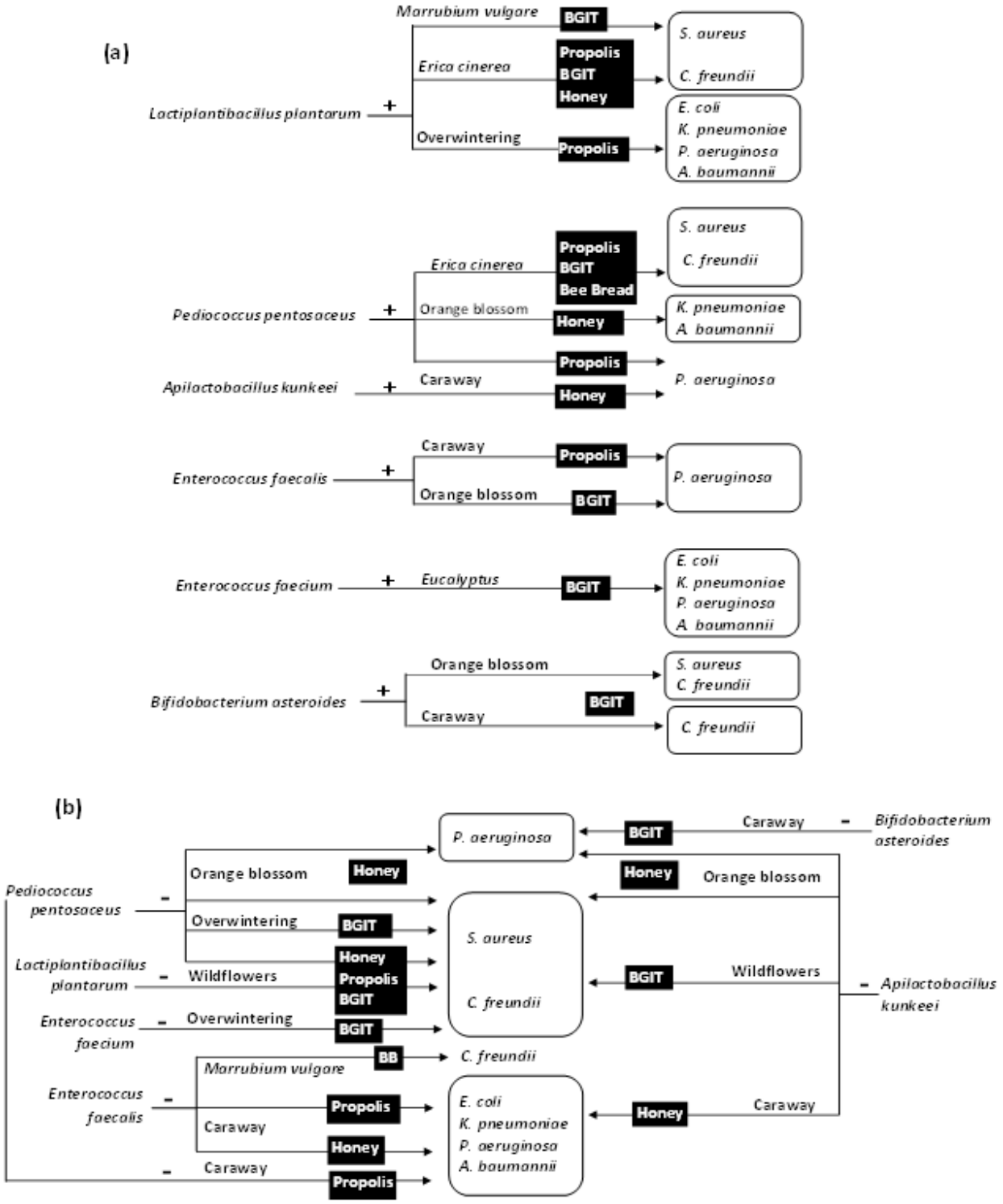

Figure 8

Synthesis on positive (**a**) and negative (**b**) anti-pathogenic effects of different LAB & Bifido species associated with different honey bee forage plants and issued from different origin matrices (highlighted in black). BGIT, honey bee gastrointestinal tract.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Table1.docx