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From Pollen to Bee Bread: A *Reservoir* of Functional Yeasts

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Abstract: Nowadays, yeasts are widely used for food and beverage fermentation as well as for their functional traits, as there has been an increase in scientific interest in their contributions to human health. Microbial competition in habitats with adverse abiotic factors could force yeasts to activate competitive tools, such as bioactive compound production. Here, bee pollen, fresh bee bread, and aged bee bread were analyzed as a *reservoir* of potential new functional yeasts. Microbiological analyses of pollen showed a dominance of bacteria and molds, although yeasts were present in all samples and increased in fresh and aged bee bread where osmophilic yeasts appeared. Functional traits such as antioxidant activity; polyphenol and flavonoid production; antimicrobial activity toward molds, yeast, and pathogenic bacteria; phytase activity; and potential probiotic aptitude were studied. Out of fifty-eight isolated yeasts, four showed antioxidant activity higher (around 70%) than Codex® due to having the highest levels of polyphenols or flavonoids. One strain possessed phytase activity, and three strains belonging to *Starmerella* and *Metschnikowia* genera had wide antimicrobial activity. Nine strains exhibited the ability to resist gastrointestinal conditions, and four possessed all probiotic traits tested. All these findings demonstrate the effectiveness of pollen and bee bread as natural sources for new bioactive and functional yeasts.

Keywords: pollen; functional yeasts; fresh bee bread; aged bee bread; functional food

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1. Introduction

Bee pollen is a pellet of field-gathered flower pollen packed by worker honeybees and used as the primary food source for the hive [1,2]. It consists of simple sugars, protein, minerals and vitamins, fatty acids, and a small percentage of other components (average content 54.2%, carbohydrates 21.3%, proteins 5.3%, lipids 2.9%). Bee pollen is stored in brood cells, mixed with saliva, and sealed with a drop of honey. Several studies demonstrate a wide range of bioactivities of bee pollen, such as antimicrobial, antioxidant, antiradical, anticancer, anti-inflammatory, hepatoprotective, anti-atherosclerotic, and immunomodulatory activities [3].

In honeycomb cells, collected pollen is enriched with honey, digestive enzymes, and organic acids that are contained in the secretions of the salivary glands of bees [4–6]. Then, natural fermentation started by lactic acid bacteria that spontaneously inhabit the honeycombs occurs over seven days; the second phase is characterized by the development of a heterogeneous group of microorganisms, including yeasts [1,7], that survive until the end of the process, resisting lactic acid release.

The hive could be considered a selective environment, with a high presence of organic acid and low pH, where specific microbial strains, including yeasts, can colonize, coexist, grow, and evolve with honeybees. These environments act as microbial reservoirs, allowing bees to acquire, deposit, and transfer functional characteristics to microorganisms in future generations [8,9].

The microbial occurrence in bee-collected pollen is very low (about 10^5 CFUs/g of bacteria) compared to other fermented food matrices due to the low water activity [10]. However, microbial bacteria–yeast succession occurs starting from the second day when the concentration of yeasts becomes comparable to that of bacteria; in aged bee bread, the yeast concentration naturally decreases [11].

The main studies regarding new yeast isolation and selection focus on their contribution to increasing the flavor, odor, and texture of fermented foods and beverages. Today, innovative functional traits, healthy effects, and antagonistic activity against spoilage are required. All these advanced features may be associated with the yeast habitat representing a survival strategy based on competition for nutrients, acidification of the growth medium, or the production of bioactive metabolites and/or volatile compounds. The environment around the apiary plays another key role in the nutrition of honeybees: it provides or does not provide specific nutritional resources that vary throughout the seasons, and consequently, the hive microbiota compensate for the supply of essential micro- and macronutrients through fermentation. The microbial community involved in bee bread production seems to produce a large supply of antimicrobial metabolites involved in the bioactivity features of bee bread [12,13].

In this dynamic chain (environment–pollen–bees–bee bread–honey), the possibility of isolating yeasts with specific functional activities becomes high.

The aim of this work was to analyze the microbiota during the hive's natural fermentation process to fill the knowledge gaps regarding microbial composition, focusing on the yeast populations that characterize these ecological niches. This environment represents an effective way to search for new yeast strains that will have positive contributions to human health.

2. Materials and Methods

2.1. Bee Pollen Collected and Bee Bread Sampling

A total of 24 samples of ivy bee pollen (monofloral *Hedera helix* L.) and fresh and aged bee bread (1–4 and 25–30 days hive-stored, respectively) were sterilely collected from locations in Val di Castro and Argignano (43°21′53.6″ N, 13°02′56.5″ W, 930 m a.s.l; 43°32′47.45″ N and 12°95′07.91″ W, respectively) in the Marche region, Italy, during May–September 2024. Samples were immediately transported to the laboratory and processed.

2.2. Processing of Samples

The harvested samples were subjected to microbial analyses through viable cell counts: the samples were weighed (30 g), serial dilution in 0.9% sterile peptone water was carried out, and the samples were homogenized for 1 min and spread on WL nutrient agar plates (Wallerstein Laboratories, Oxoid, Hampshire, UK) supplemented with 0.005% chloramphenicol (Sigma-Aldrich, Saint Louis, MO, USA) to count and isolate yeasts and molds. The plates were incubated at 25 °C for 3–4 days and the yeast population was expressed as colony-forming units (CFUs) per gram of each bee product. Malt yeast agar (Oxoid, Oxford, UK, MYA) was used as a selective medium for osmophilic yeasts, and PCA (Liofhilchem, Roseto degli Abruzzi, Teramo, Italy) was used for mesophilic bacterial growth.

2.3. Isolation and Identification of Yeasts

The yeast isolation was carried out based on macro- and micromorphological characteristics, in proportion to the frequency of each yeast morphotype by plates containing between 30 and 300 colonies. Approximately 10 colonies per plate were purified on YPD agar plates (1% yeast extract, 2% glucose, 2% peptone, 2% agar) and stored long-term in YPD broth with 40% glycerol at −80 °C.

Purified yeast strains were then identified through ITS1-5.8S rRNA-ITS2 region analyses. Briefly, the DNA of each isolate was extracted following the method described by Stringini et al. [14] and amplified by PCR using the primer pairs ITS1 (5′-TCCGTAGGT GAACCTCGCG-3′) and ITS4 (5′-TCCTCCGCTTTATTG ATATGC-3′) [15]. PCR products were separated by horizontal electrophoresis (Bio-Rad, Hercules, CA, USA) in a 1.5% (*w*/*v*) agarose gel using 0.5× TBE buffer and used for identification by sequencing. The genomic sequences obtained were compared with those already present in the data library using the BLAST program [16] and the GenBank database. A total of 18 sequences were included in the NCBI GenBank data library under accession numbers PQ571343 to PQ571359. To exclude the possibility of clones inside the same yeast species, the ISSR-PCR protocol was applied following the procedure reported by Agarbati et al. [17].

2.4. Determination of In Vitro Antioxidant Capacity (DPPH Assay)

The in vitro antioxidant capacities of 48 h pre-grown liquid cultures of each yeast were assessed following the method described by Chen et al. [18]. Briefly, 800 µL of fresh cell solution and 1 mL of DPPH (1,1-Diphenyl-2-Picrylhydrazyl) solution (0.2 mM in methanol) were mixed and left at 25 °C for 30 min. The samples were centrifuged at 2000 g-force for 2 min and the scavenged DPPH was monitored by measuring the decrease in absorbance (A) at 517 nm. The trial was conducted in triplicate and deionized water was used as a blank. The scavenging ability of each strain was defined by solving the following equation: $[1 - A_{517(\text{sample})}/A_{517(\text{blank})}] \times 100\%$.

2.5. Total Polyphenols and Flavonoids Content

Total polyphenol content (TPC) was evaluated using Folin–Ciocalteu reagent [19] on 100 µL of liquid 48 h pre-grown yeast cultures that had been previously diluted 20 times with ultra-pure water following the protocol reported by Bellachioma et al. [20]. Gallic acid was used to create the standard curve (final concentrations in water: 0.05–0.6 mM) whose linear regression value was used to express the results as Gallic acid equivalents (mM).

The same samples were used to determine the total flavonoid content (TFC) using a colorimetric method based on aluminum chloride, as reported by Ganguli [21]. Briefly, 1 mL of each sample was transferred into a 10 mL flask, using deionized water as a blank. Then, 4 mL of deionized water and 300 µL of a 5% $NaNO_2$ solution were added. The mixture was incubated at room temperature for 5 min before adding 300 µL of a 10% $AlCl_3$ solution. After 6 min of incubation at room temperature, 2 mL of a 1 M NaOH solution was added. The reaction mixture was then brought to volume with deionized water and incubated for 15 min at room temperature. Absorbance was measured at 510 nm using a spectrophotometer. The total flavonoid content was calculated using a calibration curve prepared with catechin from 0.17 to 1.4 mM.

2.6. Antimicrobial Activity

To select yeast strains characterized by wide antimicrobial activity, all isolates were tested by simple plate methods for their potential anti-bacterial, anti-mold, and killer activities.

Specifically, to detect anti-bacterial activity, selected yeasts were analyzed for their ability to inhibit the growth of four human pathogenic bacteria genera, namely *Escherichia coli*, *Listeria monocytogenes*, *Salmonella enterica*, and *Staphylococcus aureus*, and also the pathogenic yeast *Candida albicans*, following the double-layer method on the plate [22]. The first layer of media containing yeast smears was covered with a second layer of media containing a suspension of log 5 of pathogens, and the antimicrobial activity of yeasts was represented by a clear zone of pathogen growth inhibition.

The killer activity of all isolated yeasts was tested against two universal sensitive strains belonging to *Saccharomyces cerevisiae* (DiSVA42 strain) and *Brettanomyces bruxellensis* (DiSVA46) by the diffusion plate assay; the plate was seeded with a sensitive strain at a final concentration of 10^6 CFUs mL^{-1} in malt agar pH 4.2. An exponential culture of each yeast was spotted, and after 72 h of incubation at 25 °C, strains were designated as sensitive when the spot grew surrounded by a clear zone of inhibition. The diameter of the inhibition zone was measured with a caliper.

All yeast isolates were then tested for their potential anti-mold activity; mold strains were isolated from overripe fruits. Five strains, almost all constituting the prevailing isolated mold of the sample, were molecularly identified and utilized as sensitive strains: *Penicillium digitatum*, *Penicillium expansum*, *Botrytis cinerea*, *Aspergillus carbonarius*, and *Cladosporidium fulvum*. Briefly, the tested molds were pre-grown on PDA agar (Oxoid) at 25 °C for 60 h. For the positive control, 1×10^6 CFU spores were suspended in normal saline (0.9% NaCl), and two spots of 20 µL were placed on the central axis of a PDA plate 4 cm from each other. The same procedure was applied for the samples, swiping a generous batch of each yeast between the two mold spots. The plates were then incubated at 25 °C and examined daily for 7 days.

All tests were repeated in triplicate.

2.7. Phytase Activity

Phytase activity was evaluated following the protocol proposed by Ogunremi et al. [23] with some modifications: overnight pre-culture of yeasts was inoculated in a 250 mL flask containing 150 mL of minimal salt medium and Na-phytate, then incubated at 30 °C for 24 h in a shaking system (120 rpm). A culture supernatant with extracellular phytase was obtained after centrifugation and used for the phytase assay, as suggested by Kim and Lei [24].

2.8. Preliminary Probiotic Features of Isolated Yeasts

The yeasts were screened for potential probiotic behavior via in vitro assays, and a first selection was made excluding those not able to grow at 37 °C and in the presence of low pH and pepsin (like stomach conditions), and then pH 7.0 and bile salts (like intestinal conditions), following the procedure reported by Agarbati et al. [17]. Only surviving yeast strains were subsequently tested for hydrophobicity, auto-aggregation, adhesion to Caco-2 cells, and biofilm formation to analyze the properties involved in intestinal mucosae interactions. Briefly, the hydrophobicity property was analyzed spectrophotometrically as the ability of hexadecane to catch cells when added to a cell suspension. Water was added to the cell suspension instead of hexadecane as a control [25]. Auto-aggregation was analyzed spectrophotometrically as the ratio between the turbidity of a cell suspension under vigorous agitation and immediately after its vortex [17]. The adhesion of selected yeasts to Caco-2 cells (derived from human colon adenocarcinoma) was carried out by seeding 4.5×10^5 cell/mL^{-1} Caco-2 cells onto 24-well plates and then adding 1 mL of yeast suspension having a concentration of 4.5×10^6 cell/mL. After 1 h of incubation time, non-adherent yeast cells were removed and the adherent yeast cells were collected and enumerated to know their adhesion ability to Caco-2 cells [17]. The yeast's ability to produce biofilm was investigated by applying the protocols proposed by Speranza et al. [26] with inoculation of log 5 cells and shortening the incubation time, reading the results after 3 days. The % of biofilm production was calculated as the ratio between sessile and total cells (sessile cells and free cells).

Probiotic microorganisms must be GRAS for humans; for this reason, the yeast isolates were subjected to safety tests, including hemolytic, gelatinase, and DNase activities [27] following FAO/WHO guidelines.

All tests were carried out with the commercial probiotic *S. cerevisiae* var. *boulardii* (Codex®, Zambon Italia S.r.l., Bresso, Italy) used as a control. The trials were conducted in triplicate.

2.9. Statistical Analysis

An analysis of variance (ANOVA) of experimental data was performed using Duncan tests (JMP® 11 statistical software, statistical discovery from SAS, New York, NY, USA), and the resulting significant differences were determined with the associated *p*-values (<0.05).

3. Results

3.1. Yeast, Bacterial, and Mold Occurrence

A culture-dependent approach using selective or differential media was applied to detect and quantify the microbial community associated with pollen and fresh or aged bee bread samples (Table S1, Supplementary Materials). Within each sample, the variability of bacteria, yeast, and mold populations was compared. Indeed, the logarithm of the total bacterial population varied from 6.8 in pollen to 5.0 in bee bread samples; mold concentrations decreased from logarithm 5.9, 3.7, and 1.0 in pollen, fresh bee bread, and aged bee bread, respectively.

The results of the evaluation of cultivable non-osmophilic yeasts revealed very comparable trends in all samples, but osmophilic yeasts, as expected, were absent in bee pollen (Figure 1).

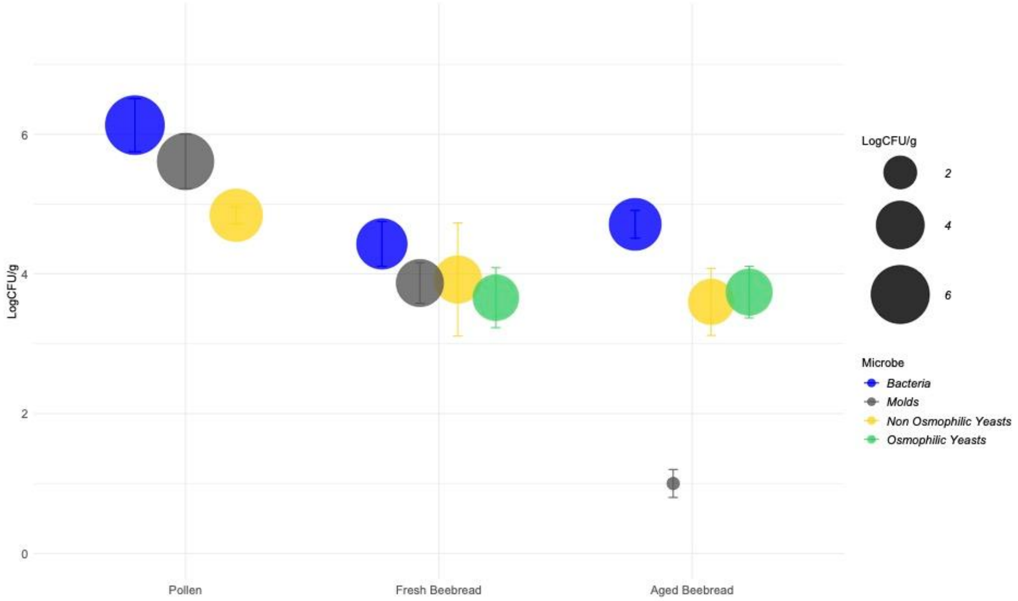

Figure 1. Microbial population occurrence in pollen, fresh bee bread, and aged bee bread. Results quantitatively report bacteria (●), molds (●), and yeasts. The latter is divided into osmophilic (●) and non-osmophilic (●) yeasts.

3.2. Yeast Isolates Identification

All 58 pure cultures obtained from 24 samples (see Table S1, Supplementary Materials) were clustered by matching their micro- and macromorphological characteristics and 26S-rDNA ITS profiles, obtaining 17 groups. A representative yeast from each group underwent sequencing, as shown in Table 1. Four groups were excluded since they belonged to Basidiomycetes (*Bullera alba*, *Cryptococcus aureus*, *Microstroma album*, *Moniliella* spp.). Ascomycetes showed a total of six strains belonging to *Starmerella* spp., recovered in both pollen and bee bread; two cultures of *Zygosaccharomyces* spp. were isolated only in fresh bee bread, and two cultures of *Metschnikowia* spp. and one strain of *Kodameae ohmeri* in bee bread were identified. Yeast-like *Aureobasidium pullulans* were isolated only in aged bee bread. Overall, the results clearly showed less yeast variability in pollen compared with the matrix bee bread.

Table 1. Identification, source, and GenBank accession number of yeast isolates.

Source	Sample Code	Yeast Species Identification	GenBank Accession Number	Grouping
Bee pollen	1BP	*Cryptococcus aureus*	PQ571343	Group 1
Bee pollen	3BP	*Starmerella* spp.	PQ571344	Group 2
Bee pollen	9BP	*Bullera alba*	PQ571345	Group 3
Bee pollen	15BP	*Starmerella* spp.	PQ571346	Group 4
Bee pollen	17BP	*Microstroma album*	PQ571347	Group 5
Fresh bee bread	52BB	*Starmerella magnoliae*	PQ571351	Group 6
Fresh bee bread	54BB	*Zygosaccharomyces pseudorouxii*	PQ571352	Group 7
Fresh bee bread	55BB	*Metschnikowia rancensis*	PQ571353	Group 8
Fresh bee bread	65BB	*Zygosaccharomyces siamensis*	PQ571354	Group 9
Fresh bee bread	67BB	*Starmerella magnoliae*	PQ571355	Group 10
Aged bee bread	18BB	*Starmerella* spp.	PQ571348	Group 11
Aged bee bread	20BB	*Aureobasidium pullulans*	PQ571349	Group 12
Aged bee bread	88BB	*Metschnikowia viticola*	PQ571356	Group 13
Aged bee bread	91BB	*Kodamaea ohmeri*	PQ571357	Group 14
Aged bee bread	93BB	*Starmerella* spp.	PQ571358	Group 15
Aged bee bread	94BB	*Moniliella* spp.	PQ571359	Group 16
Aged bee bread	21H	*Aureobasidium pullulans*	PQ571350	Group 17

3.3. Antioxidant Activity and Total Polyphenols

The antioxidant ability of yeasts is shown in Figure 2a. Although significant differences between the samples and the Codex® are shown, among the 13 yeast isolates, 3BP, 67BB, 20BB, and 21H exhibited the highest percentage of DPPH reduction, at around 70%, whereas 65BB exhibited the lowest value (52.4%). As expected, the unfermented substrate (US) used for yeast growth had the lowest DPPH reduction.

The total polyphenol content (TPC) in 48 h grown yeast cultures is reported in Figure 2b. As can be observed, there are significant differences between the yeast cultures and Codex®, with the exception of 21H, which showed increased TPC. The uninoculated substrate (US) had a significantly lower TPC than all the others.

The total flavonoid content (TFC) was also determined (Figure 2c); as expected, the yeast 67BB, which already showed the highest antioxidant activity, revealed a significantly high TFC, together with 52BB and 91BB. Antioxidants, polyphenols, and flavonoids play an important role, being the main contributors to inhibiting oxidative processes [28].

3.4. Antimicrobial Activity

Table 2 shows the antimicrobial activity of yeast strains based on plate tests against bacteria, yeasts, and molds. Although almost all yeasts showed broad anti-mold activity against all the tested strains, yeasts 67BB, 18BB, and 88BB were the strongest, all isolated from bee bread. An example of the anti-mold activity of 18BB *Starmerella* spp. is reported in Figure 3. Yeasts 67BB, 18BB, and 88BB also showed broader antimicrobial action against the tested strains of pathogenic bacteria. No strain showed a killer phenotype.

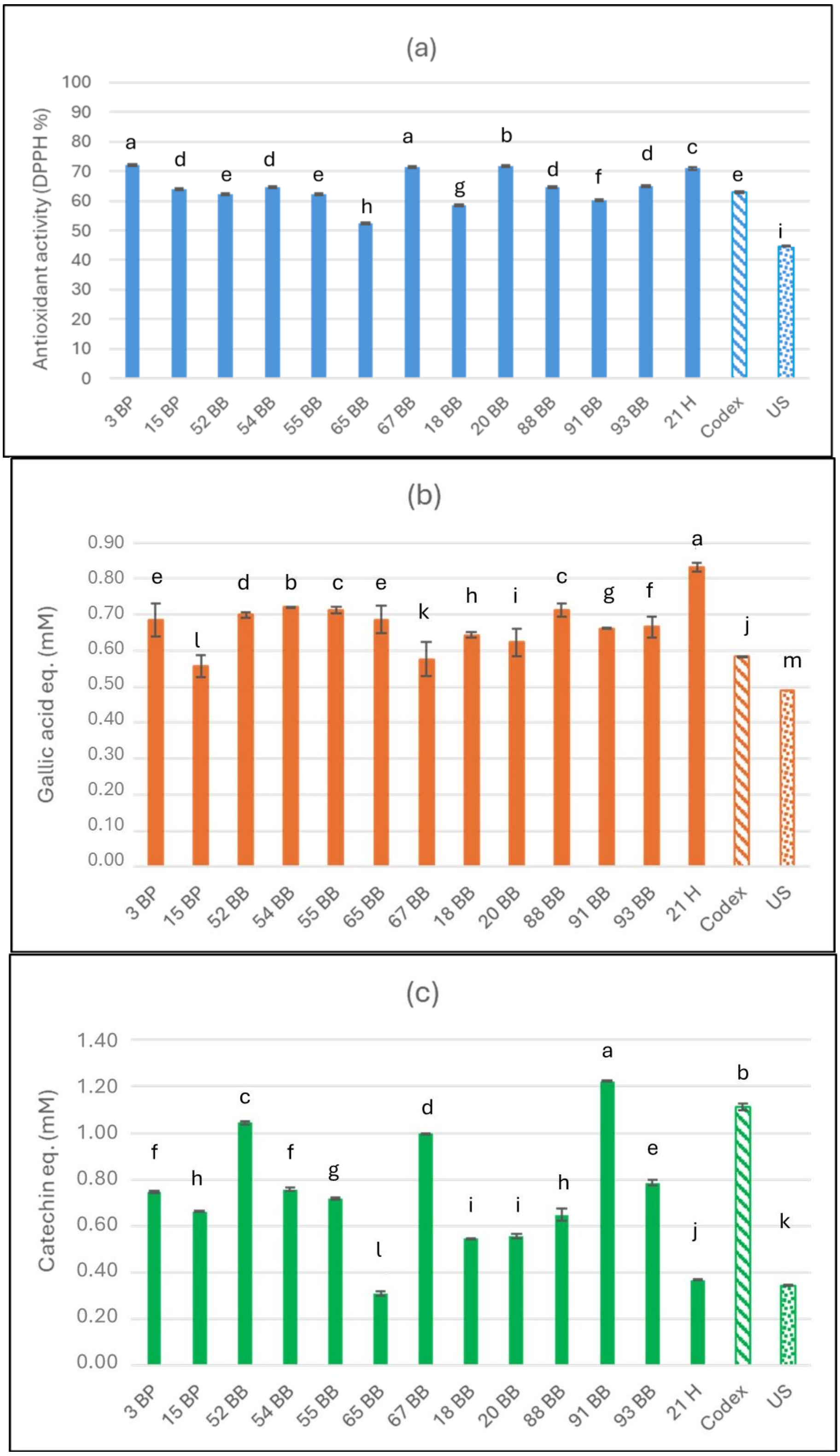

Figure 2. Antioxidant activity (**a**) and total polyphenol (**b**) and flavonoid contents (**c**) of all isolated yeasts. Codex® was used as a positive control and unfermented substrate (US) was used as a negative control. Data means ± standard deviations showing different superscript letters (^{a,b,c,d,e,f,g,h,i,j,k,l,m} within each sample are significantly different according to Duncan tests (p-value < 0.05).

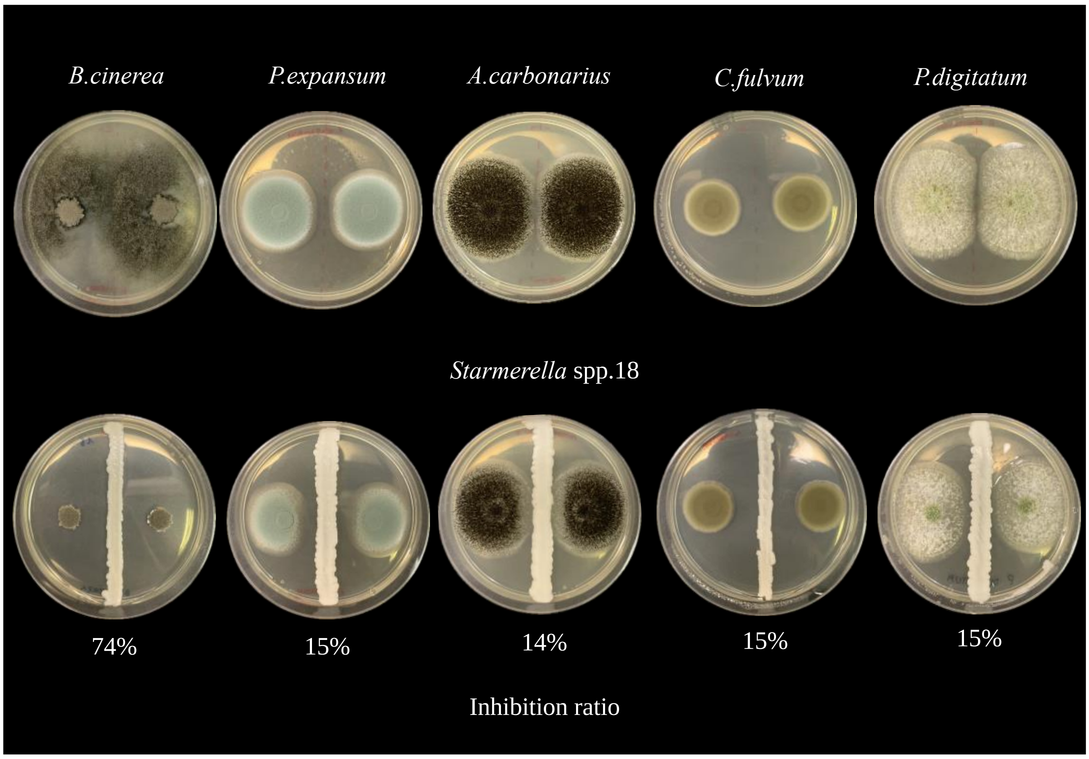

Figure 3. Anti-mold activity shown by *Starmerella* spp. 18BB against *B. cinearea*, *P. expansum*, *A. carbonarium*, *C. fulvum*, and *P. digitatum* (at the bottom) compared with controls (molds without yeast, at the top).

3.5. Probiotic Features, Phytase Activity, and Safety Tests

All 13 identified yeasts belonging to Ascomycetes phyla were analyzed for their main probiotic characteristics. Firstly, they were evaluated for their ability to grow at 37 °C pH 2.0, then in the presence of pepsin, and finally at pH 7.0 and with bile salts. The results reported in Table 3 show that nine of the yeasts analyzed were able to survive in these conditions. The yeast strains 54BB, 62BB, and 65BB were dead at acidic pH and in the presence of pepsin. The yeast 65BB was dead in the presence of bile salts. Subsequently, only the yeasts that passed the first tests were analyzed for their properties linked to intestinal mucosa interactions. Of the nine yeasts that passed the first screening, four of them, 15BP (*Starmerella* spp.), 18BB (*Starmerella* spp.), 67BB (*Starmerella magnoliae*), and 88BB (*Metschnikowia viticola*), showed percentages of hydrophobicity, auto-aggregation, and Caco-2 cell adhesion greater than or equal to 50%, and for this reason, they were selected for biofilm formation and phytase analyses. In particular, the yeast 18BB showed the highest percentage of biofilm formation, much higher than all other yeasts tested and the control strain; only the 91BB strain showed phytase activity.

All the potential new functional yeasts did not show DNase, proteolytic, or hemolytic activities, thus making them safe.

Table 2. Antimicrobial activity of the 13 yeast isolates against sensitive pathogenic bacteria, yeasts, and mold. Pathogenic bacteria inhibition and yeast inhibition are reported as the inhibition halo (mm) of sensitive growth; anti-mold activity is reported as the percentage of mold growth inhibition compared to control.

	Sensitive Strains	**3BP**	**15BP**	**52BB**	**54BB**	**55BB**	**65BB**	**67BB**	**18BB**	**20BB**	**88BB**	**91BB**	**93BB**	**21H**	**Codex®**
Pathogenic bacteria inhibition (mm)	*S. aureus*	0	0	0	0	0	0	0	0	0	0	0	12	0	0
	S. enterica	0	0	12	0	0	0	15	13	0	11	0	0	0	15
	E. coli	0	0	0	0	0	0	13	17	0	13	0	0	0	17
	L. monocytogenes	0	15	0	0	0	12	18	21	0	20	0	0	0	22
killer activity (mm)	*S. cerevisiae DBVPG 6500*	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	B. bruxellensis DBVPG 6706	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Mold reduction (%)	*P. expansum*	3	6	0	0	0	3	7	15	13	6	3	0	16	16
	A. carbonarius	11	15	8	5	8	8	11	14	32	11	16	13	26	18
	P. digitatum	13	10	11	26	18	13	14	15	24	10	16	5	22	11
	C. fulvum	13	15	12	12	7	13	15	15	18	15	18	17	18	11
	B. cinerea	18	55	14	18	10	10	70	74	25	13	10	6	23	72

Table 3. Probiotic features and safety test of the potential new functional yeasts.

Probiotic Features	**3BP**	**15BP**	**18BB**	**20BB**	**21H**	**52BB**	**54BB**	**55BB**	**65BB**	**67BB**	**88BB**	**91BB**	**93BB**	**Codex®**
37 °C pH 2.0 (Log CFU/mL)	5.74 ± 0.04	5.18 ± 0.15	5.73 ± 0.10	0	0	5.88 ± 0.03	0	5.24 ± 0.00	0	5.28 ± 0.03	4.68 ± 0.38	5.37 ± 0.17	6.44 ± 0.09	5.24 ± 0.26
Pepsin (Log CFU/mL)	6.10 ± 0.07	5.48 ± 0.15	5.67 ± 0.03	0	0	5.89 ± 0.03	0	5.47 ± 0.01	0	5.49 ± 0.10	4.53 ± 0.26	5.32 ± 0.31	5.93 ± 0.19	5.47 ± 0.25
Bile salts (Log CFU/mL)	5.74 ± 0.04	5.82 ± 0.08	6.00 ± 0.14	0	0	5.67 ± 0.04	0	5.56 ± 0.23	0	5.36 ± 0.33	4.28 ± 0.03	5.52 ± 0.13	4.20 ± 0.15	4.20 ± 0.15
Hydrophobicity (%)	5.32 ± 0.78	59.86 ± 4.03	50.00 ± 4.17			6.21 ± 1.12		12.40 ± 0.80		50.71 ± 1.94	61.34 ± 4.89	5.09 ± 1.33	11.71 ± 2.48	62.30 ± 0.18
Auto-aggregation (%)	20.7 ± 0.03	74.56 ± 0.01	54.43 ± 0.02			35.54 ± 0.05		72.98 ± 0.02		37.51 ± 0.04	88.18 ± 0.04	81.00 ± 0.01	55.37 ± 0.03	91.99 ± 0.02
Caco-2 adhesion (%)	36.43 ± 0.17	61.46 ± 0.02	89.92 ± 0.58			47.42 ± 0.00		77.33 ± 0.06		63.76 ± 0.15	84.99 ± 0.06	26.50 ± 0.01	31.70 ± 0.03	90.28 ± 0.01
Biofilm formation (%)	0.02 ± 0.00	0.62 ± 0.06	12.69 ± 0.01			1.72 ± 0.03		0.40 ± 0.03		0.26 ± 0.02	0.02 ± 0.03	0.92 ± 0.02	0.03 ± 0.00	0.03 ± 0.03

The results of the ability of yeasts to grow/survive at 37 °C, pH 2.0, and in the presence of pepsin or bile salts are reported as log CFU/mL. Results of hydrophobicity, auto-aggregation, and Caco-2 cell adhesion are reported as percentage values. Quantitative data are reported as mean values ± SD.

4. Discussion

Based on traditional knowledge, yeasts are one of the main players in food biotechnology. However, recently, due to an increase in awareness of their role in the production of nutraceuticals, the ability of potential yeast strains to enhance the functional properties of products has become an important selection criterion [29]. Natural fermented matrices of unexplored ecological niches could be a potential reservoir for new yeast strains with suitable functional attributes.

Considering the growing interest in bee products, bee bread, a niche product produced by and intended for bees, has emerged as a healthy nutraceutical product rich in polyphenols, nutrients (vitamins and proteins), fatty acids, and minerals [30,31]. Recently, due to their high content of phytonutrients such as phenolic compounds, anthocyanins, volatiles, carotenoids, and unsaturated fatty acids (USFAs) with improved lipid profiles, pollen and bee bread have been classified as superfoods [31].

Due to enzymatic transformations, the fermentation process increases the content of new compounds [32]. The transformation is initiated by lactic acid bacteria and terminated by yeasts, whose contribution is still less known. The significant content of lactic acid and several active metabolites resulting from the mixed fermentation process has been widely studied.

The synergy and succession of bacteria and yeasts is the theoretical point on which this study is based: sampling pollen, fresh bee bread, and mature bee bread to isolate yeasts with novel functional attributes as well as probiotic traits.

For this reason, expanding knowledge regarding microorganism colonization and succession in bee bread and the factors involved in bee bread production, with particular attention to yeasts, helps fill the dearth of information in this regard [33]. Moreover, several factors, such as environmental pollution, biocides, and bee diseases, affect the microbial composition of honeybee products. Although there are many environmental stressors implicated in honeybee disease, there has been an intensifying focus on the association of various groups of microorganisms with honeybees and their products, yet these effects are incompletely understood [34].

The first research on the isolation of probiotic yeasts appeared in the literature at the end of 1990 [35,36], and the matrices first used for isolation were principally medical samples such as oropharyngeal biofilms, mammalian intestines, or infant feces. Later, some food-related products were examined, such as dairy products, worldwide naturally fermented foods, fermented table olives, and beverages [37,38]. However, attention was mainly focused on lactic acid bacteria, which were more beneficial. The difficulty of maintaining the stability of probiotic LAB, from production to consumption, has been the key to the food industry's research and development of new probiotic yeast strains.

There is a lack of recent studies that address yeasts in honeybee-collected pollen and bee bread [33]. However, a yeast-species-dependent affinity in the bee agroecosystem was recognized by the addition of different yeast species to artificial nectar fed to bumblebees [39]. Physiologically and ecologically, different groups of yeasts may also be involved in the pollen resources of honeybees, with potentially different effects on bee fitness, and it seems appropriate to address the yeast diversity during the storage and maturation of bee bread using culture-based techniques.

Our findings reveal the presence of *Basidiomycetes* and *Ascomycetes* in the samples analyzed, paying more attention to *Ascomycetes*, as *Metschnikowia*, *Starmerella,* and *Zygosaccharomyces* genera were the most abundant yeasts detected, according to Detry et al. [11], who described the same genera as the most abundant in pollen and fresh and aged bee bread during a study focused on yeast population occurrence in the different stages of bee bread maturation. Yeasts belonging to the *Starmerella* genus are well known for their association with honeybees and their relative products such as pollen and bee bread [11,33,40].

To drive the wellness market toward healthy products and ingredients for food and beverage fortification, antioxidant capacity is a promising feature. Several studies have reported on the natural antioxidant capacity of yeast cells [41–44] by analyzing antioxidant activity for different yeast species, using intact cells and their extracts. Their results demonstrate that intact cells have higher antioxidant capacity than their extracts, which may be due mainly to the high content of (1/3)-β-D-glucans and other β-glucans found in their cell walls, among other cellular compounds.

In this work, six strains belonging to the *Starmerella*, *Kodamea*, and *Aerobasisium* genera showed higher antioxidant power compared to Codex®. Most of the tested strains were isolated from mature bee bread, indicating that this product has a stable and fermented matrix. Due to interactions with other microorganisms in natural environments, yeasts can use competitive approaches that guarantee advantages against other opposing organisms, either through mechanisms of enzyme secretion, toxin production, or the release of volatile organic compounds (VOCs) [45]. In the antimicrobial activity screening, three recovered yeasts (67BB, 18BB, and 88BB) showed high activity against pathogenic bacteria and molds. They were *Starmerella* spp., *S. magnoliae*, and *M. viticola*. A lot of research has indicated *Starmerella* as a model for antimicrobial activity, particularly toward molds, by a mechanism that involves released sophorolipid surfactants that disrupt the cell membranes of sensitive strains [46–48]. These selected antimicrobial yeasts display the greatest probiotic traits such as hydrophobicity, auto-aggregation, and Caco-2 cell adhesion.

Phytate is an anti-nutrient present in cereals. It considerably reduces the bioavailability of other minerals, forming insoluble complexes with numerous cations such as copper, zinc, calcium, magnesium, iron, and potassium. Furthermore, phytate may have a negative influence on the functional and nutritional properties of proteins [49]. Phytate complexes are not available for absorption in the human gut unless they are digested by enzymatic phosphatases, such as phytase, which catalyze the phosphate hydrolysis from phytate. The phytase enzyme is naturally synthesized in plants and some microorganisms. Although yeasts have been reported as useful microorganisms for phytase production [29], in our screening only the *K. ohmeri* 91BB strain demonstrated the functional ability to hydrolyze phytate.

5. Conclusions

Saccharomyces and non-*Saccharomyces* yeasts have favorable technological properties and significant beneficial properties. Yeast applications have numerous advantages in food, feed, fermentation, and the medicinal and agricultural industries. The selected functional strains could be used as supplements in food fermentation and in the manufacture of healthy food products.

Honeybee products are used by humans in traditional, complementary, and integrative medicine. Microorganisms that colonize these honeybee products come from the honeybee's ecosystem, such as their habitat, food, nectar, pollen, etc. In this context, the microbiota of honeybee products could be a suitable reservoir of yeasts with functional and probiotic traits.

In this study, new yeasts with bioactive traits and antagonistic antimicrobial activity against spoilage and preliminary probiotic features were isolated from bee bread and pollen. They could be used in functional food preparation to expand the offer of nutraceutical products on the market to satisfy the requests of consumers who pay attention to healthy food.

Advanced and additional studies on the mode of action or formulation of non-*Saccharomyces* yeasts will be necessary prior to administering these yeasts, considering governmental rules on health and well-being.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/fermentation11050290/s1, Table S1: Source, macro-microscopic characteristics and 26S r-DNA ITS profiles of all 58 yeasts isolated.

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