

Article

Physical and Microbiological Characteristics and Antioxidant Activity of Honey Bee Pollen

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Abstract: This study aimed to evaluate the physical, chemical, and microbiological characteristics of bee-collected pollen, with special consideration to the antimicrobial resistance of the isolated microorganisms to the selected antibiotics. A hierarchy cluster analysis was conducted, in which bee pollen samples were divided into four groups by their colour. The highest antioxidant activity among monofloral bee pollen was found for the autumn raspberry pollen and the lowest for the clover pollen, using the ABTS test. The total phenol content in rapeseed bee pollen was the second-highest among all samples analysed, which might indicate a correlation between high phenol content and strong antioxidant activity. Our study indicated a moderate correlation between bee pollen moisture content and *Enterobacteriaceae* counts, as well as a correlation between moisture content and total bacterial count. Among all bacteria ($n = 34$) isolated from pollen, the highest prevalence was found in *Bacillus* spp. and coagulase-negative staphylococci. The resistance of isolated microorganisms was identified in 18 cases. The high number of antimicrobial resistance cases, i.e., when isolates were resistant to ampicillin (seven cases) and penicillin (eight cases), indicates an environmental effect because, for this study, no antibiotics were used in the apiaries harvesting pollen. This indicates the need for improved safety procedures in bee pollen production for human consumption.

Keywords: monofloral; multifloral; colour; total phenol content; antimicrobial resistance



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1. Introduction

Latvia is an ideal setting for nectar plants in forests, natural meadows, shrubs, and bogs due to its location in a temperate climate zone. Therefore, beekeeping has been an ancient tradition. In addition to producing honey, about 27% of beekeepers produce bee pollen pellets [1], which worker honey bees (*Apis mellifera* L.) collect from flowers of various plants and mix with nectar and bee salivary secretions. Bee pollen is collected in special traps located at the hive entrance. Its main components are carbohydrates such as fructose, glucose, sucrose, and fibre (13–55%); proteins (9–40%); lipids (4–10%); minerals such as K, P, Mg, and Ca. Mărgăoan et al. [2] confirmed that bee pollen differs in composition depending on botanical origin, place of collecting, processing, and storage conditions. Bee pollen is considered a high-value health product, which is discussed in several recent reviews [3–7], indicating its nutritive value and therapeutic properties. Phenols are listed among important bioactive compounds present in bee pollen, which have antioxidant activity [8,9]. The phenolic compounds observed in bee collected pollen belong to the flavonoid group and compounds derived from benzoic and cinnamic acids [10].

According to Végh et al. [7], food safety risks of bee pollen include contamination from environments with pesticides, metals, and mycotoxins, as well as pyrrolizidine alkaloids from specific plants. The microbiological quality of bee pollen, especially the absence of fungi and pathogens, should also be considered an important criterion [11,12]. Bruneau [13] reported that mycelium (*Penicillium verrucosum*, *Aspergillus niger*, *A. carbonarius*, *A. ochraceus*, *A. parasiticum*, and *Alternaria* spp.) can cause the development of toxins in bee pollen. For control of microorganisms, it is suggested to strictly follow hygiene rules, collect pollen every day, and reduce moisture content or freeze it for further storage. Alippi et al. [14] indicated *Bacillus cereus sensu stricto* (50%), *Bacillus megaterium* (40%), and *Bacillus subtilis* (40%) as the most dominant species among aerobic spore-forming bacteria identified in bee pollen samples intended for human consumption in Argentina. Coronel and Pereira [15] also found toxigenic species in bee pollen such as *Aspergillus flavus*, *Alternaria alternata*, *Fusarium graminearum*, etc. which, according to Estevinho et al. [16], make microbial contamination a critical quality parameter for human consumption. Šimunović et al. [17] also confirmed that additional steps should be taken alongside good manufacturing practices to reduce contamination risks. Since worker bee microbiota is dominated by eight bacterial phylotypes [18], Piva et al. [19] suggested that other microorganisms isolated from bees and bee products could be derived from the environment.

Antimicrobial resistance (AMR) is observed when bacteria, viruses, fungi, and parasites acquire new resistance mechanisms and no longer respond to medicines, which may become ineffective. There are various drivers of possible antimicrobial resistance and resistance gene transmission to humans [20]. Antimicrobial-resistant organisms can move between ecosystems—people, food, animals, environment—thus causing particular concern [19]. This becomes an important issue in the case of bee pollen, which is typically collected from the surrounding area.

This study aimed to evaluate the physical, chemical, and microbiological characteristics of bee-collected pollen in Latvia, with special consideration to the antimicrobial resistance of the isolated microorganisms to the selected antibiotics.

2. Materials and Methods

2.1. Bee Pollen Samples

Honey bee samples harvested in the Kurzeme and Zemgale regions of Latvia in 2020 were included in this study. Monofloral pollen samples included bee-collected pollen from autumn raspberry (*Rubus idaeus*), red clover (*Trifolium pratense*), dandelion (*Taraxacum officinale*), fava bean (*Vicia faba* L.), phacelia (*Phacelia tanacetifolia*), rye flower (*Centaurea cyanus* L.), rapeseed (*Brassica napus*), and willow (*Salix babylonica*). Multifloral samples were collected in various districts: MF1 in Broceni, MF2 in Bene, MF in Tervete, MF4 in Saldus, and MF5 in Kuldīga. The harvesting of samples was carried out soon after the blossoming time of the respective plants. Thus, willow and dandelion bee pollen was collected in April–May; red clover, fava bean, rye flower, and rapeseed bee pollen was collected in June–July; autumn raspberry and phacelia bee pollen was collected in July–August. Antibiotics were not used in any of the apiaries that collected bee pollen.

Monofloral pollen samples were supplied by beekeepers. Multifloral samples were purchased from local retail shops where production sites were shown on product packages. The country of origin for all samples was Latvia. The drying and storage of samples were carried out according to each producer's technology.

2.2. Analysis of Bee Pollen Physical Attributes

Bee pollen colour was analysed in the CIE L* a* b* system using colour analyser *Colour Tec PCM/PSM* (Accuracy Microsystems Inc., Vernon Hills, IL, USA). Colour component L* represents colour intensity, where 0 = black, 100 = white; a* values show –a* = green,

+a* = red; b* value: −b* = blue, +b* = yellow. Data were collected in software ColourSoft QCW. Average values of seven measurements for each sample were taken.

The moisture content of bee pollen was analysed by drying samples in Memmert oven (Mettler, Buechenbach, Germany) at 105 ± 2 °C, till constant weight (approximately 2.5 h). Average values of three measurements were taken.

For the determination of water activity, Novasina LabSwift-aw (AG Novasina, Lachen, Switzerland) was used, following the producer's guidelines. Samples were ground prior to the analysis, to obtain a uniform consistency. Water activity was measured in triplicate for each sample.

Ground bee pollen sample (3 g) was mixed with 30 mL of distilled water for pH determination and held for 10 min at room temperature. The pH was recorded using Jenway 3510 pH meter (Barloworld Scientific Ltd., Staffordshire, UK), with a glass electrode calibrated at pH 4 and 7. Triplicate measurements were completed for each sample.

2.3. Determinations of Microbiological Parameters and Antimicrobial Resistance

Microbiological analysis began by analysing the sterile stomacher bag with filter, for which 3 g of sample was diluted with peptone water (maximum recovery diluent (MRD), Oxoid, Hampshire, UK) in a 1:10 ratio. The samples were homogenised in a bag mixer (Interscience, Missillac, France) for 30 s at 600 rpm, with subsequent dilution according to the instructions described in the standard LVS EN ISO 7218 'Microbiology of food and animal feed. General requirements and guidelines for microbiological tests'.

Next, 1 mL of the suspension from each dilution was added to Petri dishes and spread with the following media: plate count agar (PCA, Oxoid, Hampshire, UK) for the determination of total bacterial; mesophilic aerobic and facultative anaerobic microorganisms (MAFAM) and MacConkey II (Biolife, Monza, Italy) for the isolation of *Enterobacteriaceae* bacteria. The plates were incubated for a total bacterial count at 30 ± 1 °C for 72 h and for Gram-negative bacteria, at 37 ± 1 °C for 48 h.

Blood agar was used to observe bacterial growth, for which plates were incubated at 37 ± 1 °C for up to 48 h in aerobic conditions. MALDI MS (Biomérieux, Craponne, France) was used for the identification of bacterial isolates. Briefly, *E. coli* ATCC 8739 on a VITEK MS-DS target slide was used for calibration. A part of a suitable colony from isolates on media was applied to the centre of the sample spot. Isolates were tested in duplicate. For negative control, a VITEK MS-CHCA matrix spot was used. The prepared target slides were introduced to a high-vacuum environment (VITEK MS) for the detection of the protein spectrum of tested isolates. Obtained results were evaluated in MYLA (Biomérieux) software.

The antimicrobial resistance of the selected bacteria isolates was determined according to the disc diffusion method [21] using Muller–Hinton agar (Biolife, Monza, Italy) and antibiotic discs (Oxoid, Hampshire, UK) containing trimethoprim–sulfamethoxazole (25 µg), penicillin G (10 µg), tetracycline (30 µg), ciprofloxacin (5 µg), gentamicin (10 µg), and ampicillin (10 µg). The prepared Petri dishes were incubated at 37 ± 1 °C for 24 h.

The recommendations provided by the manufacturer of antibiotic discs (Oxoid, Hampshire, UK) and the guidelines of the European Committee on antimicrobial susceptibility testing [21] were used to analyse the bacteria antibiotic resistance.

2.4. Analysis of Total Phenol Content and Antioxidant Activity

For the determination of antiradical activity and total phenol content in pollen samples, extracts were prepared according to the following scheme: First, 2.00 ± 0.05 g of each sample was weighed into a centrifuge tube; then, 10 mL of 70% ethanol was added, and samples were placed in an ultrasonic bath for 10 min and centrifuged for 20 min at 3500 rpm in a centrifuge (ELMI CM, Riga, Latvia). After centrifugation, the contents of the tube without precipitate were poured into an opaque glass bottle, and the tube was refilled with 10 mL of 70% ethanol and extracted and centrifuged again. The resulting extracts were

combined, making up to 50 mL with a 70% ethanol solution, and stored in a refrigerator until the analyses were performed.

Next, 2.5 mL of Folin–Ciocalteu reagent [22] was added to 0.5 mL of the previously prepared extract. After 3 min, 2.0 mL of 7.5% Na₂CO₃ solution was added, and samples were left for 30 min. The results were then read on a JENWAY 6300 spectrophotometer (Barloworld Scientific Ltd., Staffordshire, UK) at 765 nm. The total phenol content is expressed as gallic acid equivalent per 100 g of dry weight (GAE mg/100 g dw). For each sample, the total phenol content was determined in nine replicates, and average values were taken.

Two methods were used to determine antiradical activity in bee pollen samples. The ABTS test was performed for the determination of the radical cation of 2,2-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid) (ABTS), in which 5.0 mL of ABTS solution was added to 0.5 mL of the previously prepared extract and aged for 10 min. The absorbance of the solution was read on a JENWAY 6300 spectrophotometer (Barloworld Scientific Ltd., Staffordshire, UK) at 734 nm.

The second method was the DPPH test, in which 3.5 mL of DPPH (2,2-diphenyl-1-picrylhydrazyl reagent) solution was added to 0.5 mL of the previously prepared extract and aged for 30 min. The absorbance of the solution was read on a JENWAY 6300 spectrophotometer (Barloworld Scientific Ltd., Staffordshire, UK) at 517 nm. The activity in the analysed samples was expressed as millimoles of Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) equivalent per 100 g of sample dry weight (mmol TE/100 g dw). Nine replicates were conducted for each pollen sample, with the mean values taken.

2.5. Statistics

For mathematical data processing, means and standard deviations were calculated using MS Excel 2016 software. Significant differences between the samples were determined by using a one-way ANOVA, followed by Tukey's test (there are significant differences if $p < 0.05$). A hierarchical cluster method was used to classify datasets in clusters. Each cluster combined the most closely connected samples. The hierarchy cluster analysis was performed using the SPSS 17.0 statistical program. Correlation analysis was applied to evaluate the strength of a relationship between parameters tested.

3. Results

3.1. Physical Attributes of the Bee Pollen Pellets

The bee pollen samples analysed in this study had different botanical origins; therefore, a rich variety of pollen colours was observed. Among all analysed bee pollen samples, there were different shades of green and brown, different shades of yellow and orange, and different shades of blue-purple and grey. The values of colour components L * a * b * for monofloral and multifloral honey bee-collected pollen are summarised in Table S1. Greater colour diversity was found among the samples of monofloral pollen, as they were obtained from eight different plant species, resulting in bee pollen with significant differences ($p < 0.05$) in colour. A smaller variety of colours was found in the samples of multifloral pollen, as they mostly consisted of three colours—orange, yellow and green—with the largest percentage attributed to yellow and green pollen.

Cluster analysis was used to group and more accurately determine the colour diversity of the bee pollen samples (Figure 1).

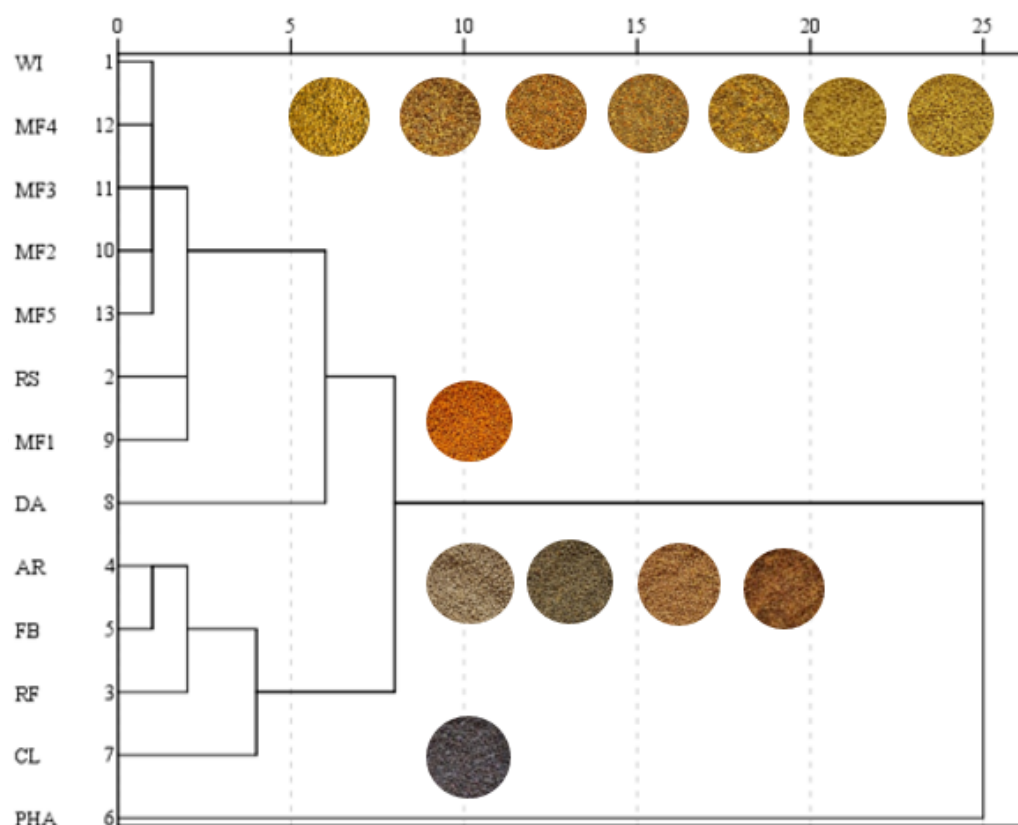


Figure 1. Dendrogram showing average linkage between groups of honey bee-collected pollen based on its colour: plants of origin for monofloral pollen: AR—autumn raspberry, CL—clover; DA—dandelion; FB—fava beans; PHA—phacelia; RF—rye flower; RS—rapeseed; WI—willow; plants of origin for multifloral pollen: MF1—Broceni; MF2—Bene; MF3—Tervete; MF4—Saldus; MF5—Kuldiga.

Cluster analysis allocated bee pollen samples in the following four groups:

- The first cluster consisted of all multifloral samples studied and two monofloral (WI and RS) bee pollen samples, in which the pollen was in different hues of yellow, green and slightly orange;
- The second cluster consisted of a dandelion pollen sample (DA), which was dominated by an orange-yellow hue;
- The third cluster consisted of samples of the monofloral pollen dominated by brown, bluish-grey hue, from AR, RF, FB, and CL;
- In the fourth cluster, there was one sample (PHA), the colour of which was dark purple.

Physicochemical parameters of products are essential to ensure their storage and safety for consumers. Therefore, the following parameters were analysed for bee pollen: moisture content, water activity, and pH (Table 1).

The moisture content of products is an important quality indicator, which affects the shelf life. The higher the product moisture, the shorter the shelf life, because the increased moisture of the product creates a favourable environment for the development of microorganisms. According to the Regulations of the Cabinet of Ministers of the Republic of Latvia (LR) No. 461 (12 August 2014) and the Guidelines for the primary production of apiculture products, the moisture content of dried pollen must be between 7.0% and 8.0%. The moisture content of the monofloral bee pollen analysed in the current study was from $6.62 \pm 1.24\%$ to $10.89 \pm 0.43\%$, whereas for multifloral pollen, it was $6.67 \pm 0.22\%$ to $8.61 \pm 0.96\%$.

Table 1. Moisture content, water activity, and pH of honey bee-collected monofloral and multifloral pollen.

Samples		Moisture Content (%)	Water Activity	pH
Monofloral	AR	9.99 ± 0.43 a,b	0.363 ± 0.001 b	5.827 ± 0.013 a
	CL	6.62 ± 1.24 c,d	0.322 ± 0.001 c	4.756 ± 0.014 g
	DA	7.45 ± 0.25 c	0.298 ± 0.002 e	4.927 ± 0.025 f
	FB	9.47 ± 0.44 b	0.294 ± 0.005 e	5.607 ± 0.008 c
	PHA	10.89 ± 0.43 a	0.383 ± 0.003 a	4.738 ± 0.006 g
	RF	9.93 ± 0.71 a,b	0.389 ± 0.000 a	4.751 ± 0.016 g
	RS	7.32 ± 1.00 c,d	0.228 ± 0.001 h	5.018 ± 0.016 e
	WI	6.80 ± 0.68 c,d	0.310 ± 0.002 d	5.822 ± 0.016 a,b
Multifloral	MF1	7.35 ± 1.17 c,d	0.187 ± 0.002 i	5.037 ± 0.005 e
	MF2	6.67 ± 0.22 d	0.258 ± 0.002 g	5.735 ± 0.025 b
	MF3	8.61 ± 0.96 b,c	0.324 ± 0.001 c	5.296 ± 0.014 d
	MF4	7.69 ± 0.46 c	0.286 ± 0.004 f	5.783 ± 0.018 a,b
	MF5	7.82 ± 0.15 c	0.251 ± 0.001 g	4.872 ± 0.020 f

Plants of origin for monofloral pollen: AR—autumn raspberry; CL—clover; DA—dandelion; FB—fava beans, PHA—phacelia, RF—rye flower, RS—rapeseed, WI—willow; plants of origin for multifloral pollen: MF1—Broceni; MF2—Bene; MF3—Tervete; MF4—Saldus; MF5—Kuldiga. The same letters in column indicate no significant difference ($p < 0.05$).

The water activity of the monofloral pollen analysed differed significantly ($p < 0.05$) and ranged from 0.228 ± 0.001 to 0.389 ± 0.001 , while for multifloral pollen, it ranged from 0.187 ± 0.002 to 0.324 ± 0.001 (Table 1).

pH is another bee pollen quality indicator. In the literature, it has been reported that too low pH (below 4.00) can contribute to the activity of unfavourable microorganisms in pollen. The biochemical changes that occur in pollen as soon as it enters the hive can lead to a lowering of the pH. According to the Regulations of the Cabinet of Ministers of the Republic of Latvia (LR) No. 461 (12 August 2014) and the Guidelines for the primary production of apiculture products, the pH of dried pollen must be in the range of 4.3–7.0. The average pH values of the analysed monofloral bee pollen were from 4.738 ± 0.006 to 5.827 ± 0.013 , while for multifloral bee pollen, it was from 4.872 ± 0.020 to 5.783 ± 0.018 , both of which fall within the specified range.

Although none of the average pH values of all bee pollen samples analysed exceeded the permissible limits, significant differences ($p < 0.05$) were found between the values of monofloral pollen. The reasons behind differences in pH could be biochemical processes occurring in the pollen, differences in its chemical composition, and storage conditions.

3.2. Microbiological Characteristics of Bee Pollen and Antibiotic Resistance of Isolated Microorganisms

The collection site, environmental pollution, treatment and storage conditions, excessive moisture content, and increased water activity may have adverse effects on bee pollen microbial quality. Therefore, it is important to monitor microorganism content to ensure pollen suitability for human consumption.

In monofloral bee pollen samples, the highest *Enterobacteriaceae* count (82 cfu/g) was found in phacelia pollen, while the lowest count was in willow pollen (14 cfu/g). In multifloral samples, the highest *Enterobacteriaceae* count (81 cfu/g) was found in MF2 pollen, while the lowest count was in MF5 pollen (21 cfu/g). There were no differences ($p > 0.05$) in the average *Enterobacteriaceae* bacteria count between the tested monofloral (48 cfu/g) and multifloral pollen (50.2 cfu/g).

Among analysed monofloral samples, the highest total bacterial count (7.35×10^2 cfu/g) was found in autumn raspberry bee pollen, while the lowest was found in willow bee pollen (0.55×10^2 cfu/g). In multifloral samples, the highest total bacterial count (3.25×10^2 cfu/g) was found in MF2 pollen, while the lowest count was in MF5 pollen (0.8×10^2 cfu/g). Overall, there were differences ($p < 0.05$) in the total average bacte-

rial count between tested monofloral and multifloral pollen samples, with values of 2.71×10^2 and 2.02×10^2 cfu/g, respectively.

The further identification of microorganisms revealed a variety of species present in the honey bee-collected samples (Figure 2). Among all bacteria ($n = 34$) isolated from pollen, the highest prevalence was found in *Bacillus* spp. (20/34) and coagulase-negative staphylococci (6/34). The most often identified species were *Bacillus altitudinis/pumilus* (7/13), *Lysinibacillus fusiformis* (5/13), *Bacillus cereus* (4/13), and *Staphylococcus epidermidis* (4/13). The distribution of microorganisms in the samples studied is presented in the supplementary data (Supplementary Table S2).

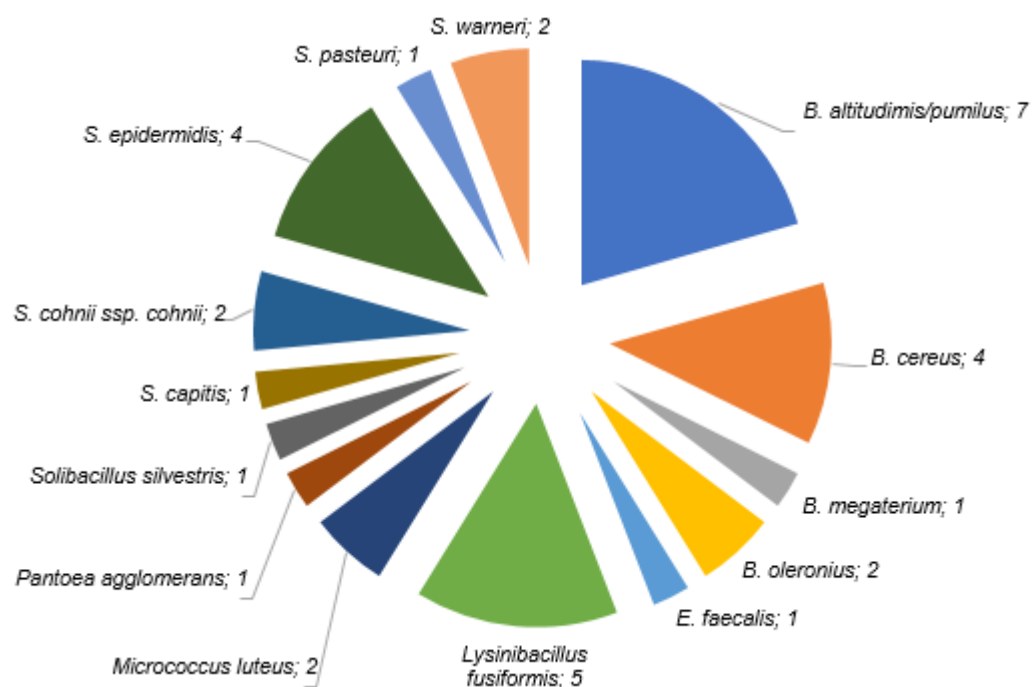


Figure 2. Prevalence of microorganisms isolated from bee pollen samples ($n = 13$). Numbers indicate the cases.

The resistance of *Lysinibacillus fusiformis*, *Staphylococcus warneri*, *Staphylococcus epidermidis*, *Staphylococcus capitis*, *Staphylococcus cohnii ssp. Cohnii*, *Bacillus oleronius*, *Micrococcus luteus*, and other microorganisms isolated from pollen samples was evaluated against antimicrobials such as ampicillin, gentamicin, tetracycline, ciprofloxacin, penicillin, trimethoprim-sulfamethoxazole, which are popular active ingredients in antibiotics used to treat a variety of infectious diseases. Figure 3 summarises the information on bee pollen microbial resistance to various antibiotics.

Resistance to the analysed antibiotics was identified in 18 cases (Supplementary Table S3).

Lysinibacillus fusiformis was a more frequently isolated bacterium (6/18); in two samples, it showed resistance to CIP, with one showing resistance also to P, and the other to AMP. In five pollen samples, we isolated *Staphylococcus epidermidis*, which showed resistance to AMP and P (2/5) and to SXT (1/5). However, only in three pollen samples did we isolate *Staphylococcus warneri*, which possessed antibiotic resistance to AMP (3/3), P (2/3), and Te (1/3).

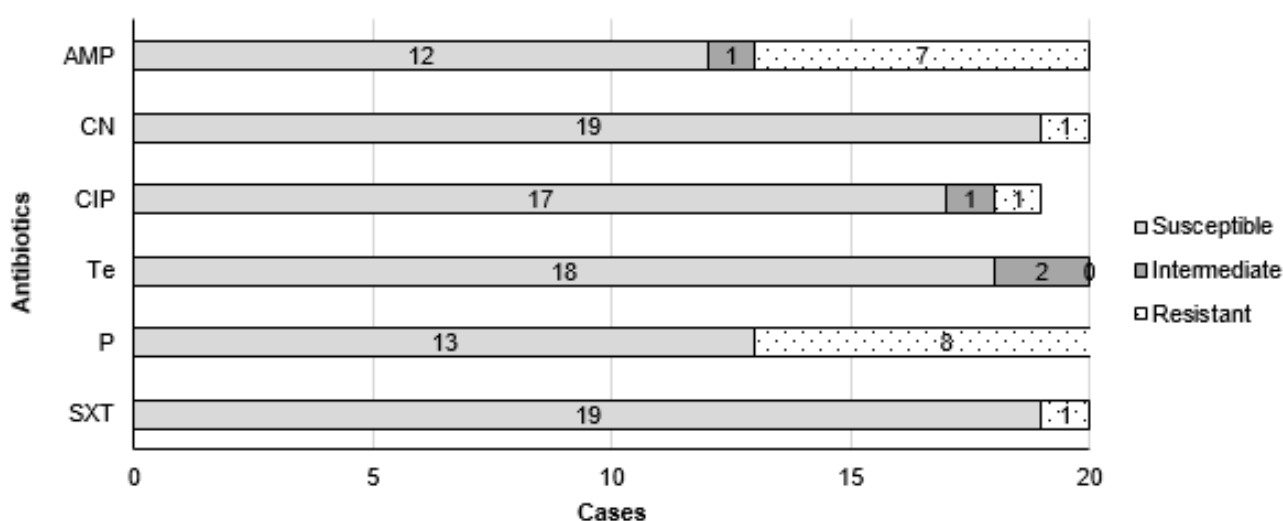


Figure 3. Antibiotic resistance of microorganism species isolated from honey bee pollen samples: AMP—ampicillin; CN—gentamicin; CIP—ciprofloxacin; Te—tetracycline; P—penicillin G; SXT—trimethoprim–sulfamethoxazole.

3.3. Total Phenols and Antiradical Activity in Honey Bee Pollen

Analyses of the results obtained (Table 2) confirmed that the content of total phenolic compounds in the pollen was from 4.71 ± 0.19 to 32.13 ± 1.26 mg GAE/g dw in monofloral pollen samples, whereas it ranged between 16.13 ± 0.52 and 26.13 ± 0.15 mg GAE/g dw in the multifloral bee pollen samples analysed.

Table 2. Total phenol content and antioxidant activity of monofloral and multifloral bee pollen.

Samples *		TPC (mg GAE/g dw)	DPPH (mmol TE/100 g dw)	ABTS (mmol TE/100 g dw)
Monofloral	AR	32.13 ± 1.26 a	0.42 ± 0.01 h	2.21 ± 0.07 a
	CL	4.71 ± 0.19 i	1.01 ± 0.02 c	0.40 ± 0.02 i
	DA	8.21 ± 0.31 h	0.51 ± 0.01 g,h	0.94 ± 0.02 g
	FB	18.40 ± 0.14 e,f,g	0.64 ± 0.01 e	0.72 ± 0.03 h
	PHA	19.48 ± 0.59 d,e	1.08 ± 0.02 c	1.83 ± 0.08 c
	RF	21.39 ± 0.44 c,d	0.58 ± 0.00 e,f,g	1.65 ± 0.05 d
	RS	29.94 ± 0.77 a	1.58 ± 0.03 a	2.05 ± 0.04 b
	WI	19.37 ± 0.80 d,e,f	1.44 ± 0.06 b	1.68 ± 0.01 d
Multifloral	MF1	17.13 ± 0.66 f,g	0.53 ± 0.02 f,g	1.14 ± 0.02 f
	MF2	19.38 ± 0.20 d,e,f	0.86 ± 0.03 d	1.17 ± 0.05 f
	MF3	26.13 ± 0.15 b	1.00 ± 0.00 c	1.41 ± 0.02 e
	MF4	22.06 ± 0.91 c	0.64 ± 0.01 e	1.40 ± 0.01 e
	MF5	16.13 ± 0.52 g	0.60 ± 0.01 e,f	0.88 ± 0.01 g

TPC—total phenol content; plants of origin for monofloral pollen: AR—autumn raspberry; CL—clover; DA—dandelion; FB—fava beans; PHA—phacelia; RF—rye flower; RS—rapeseed; WI—willow; plants of origin for multifloral pollen: MF1—Broceni; MF2—Bene; MF3—Tervete; MF4—Saldus; MF5—Kuldiga. The same letters in column indicate no significant difference ($p < 0.05$).

The DPPH and ABTS test results of the pollen analysed are summarised in Table 2. There were significant differences ($p < 0.05$) between antiradical activities of the studied pollen samples, regardless of the determination method used.

Correlation analysis indicated a strong correlation between total phenol content and ABTS antioxidant activity ($r = 0.84$). However, no correlation was established between TPC and DPPH antioxidant activity ($r = 0.16$).

The ABTS test showed higher antioxidant activity, which can be explained by the fact that this method determines antiradical activity at different pH values and is especially suitable for ethanol-based extracts. In comparison, the DPPH method is much more

sensitive at lower pH values and to extracts containing ethanol; in addition, the two methods have different reaction pathways, with different reagents used in the analyses.

4. Discussion

Analysis of the bee pollen samples' colours (Supplementary Table S1) and their diversity indicated that the predominant pollen samples were yellow or orange, so it was very difficult to determine the exact botanical origin of pollen by colour alone. Pollen from different plant species can be very similar or even identical in colour. This was also confirmed by some of the pollen analysed in the current study, such as bee pollen from willow (WI) and rapeseed (RS), as well as samples of multifloral pollen with a very similar colour profile. For this reason, further examination under a microscope is required for accurate identification of the plant species from which the pollen is collected, as described by Campos et al. [23].

According to the information presented in a study by [24], there are plants in nature whose pollen colour is particularly specific, and their origin can be easily determined without more in-depth research. These types of plants include phacelia, the bee pollen of which is dark blue; the clover, the pollen of which is dark brown; raspberry, having grey pollen; lastly, fava beans, the bee pollen of which is recognizable by its green hue. Such colour relationships were precisely the ones also found in our study.

The highest moisture content was found in PHA ($10.89 \pm 0.44\%$), AR ($9.99 \pm 0.43\%$), and RF ($9.93 \pm 0.71\%$) bee pollen, which could be explained by high relative air humidity during the bee pollen collection period and drying process, which has been identified in the literature as one of the main determinants of moisture content in pollen. Moisture content (up to 8.0%), which complies with Regulation No. 461 of the Latvian Cabinet of Ministers on optimal pollen moisture, was found in clover (CL), dandelion (DA), rapeseed (RS), and willow (WI) pollen. A comparison of the results of other studies revealed that the optimal moisture content of dried bee pollen varied from country to country—up to 8% in Argentina, 10% in Bulgaria, and 6% in Switzerland and Poland [25]. Swiss scientists have found that if the moisture content of dried pollen is higher than 6%, it can negatively affect the quality of the pollen—mould can form, which can result in an unpleasant taste and aroma for pollen. Studies by [26], based on changes in moisture content depending on the duration of pollen storage, revealed that proper packaging and storage of pollen is a key factor, and if this process is carried out correctly, storage time does not contribute to moisture content changes in pollen.

After evaluating the moisture content in the analysed multifloral bee pollen, it was found that there were significant differences between the moisture content of the analysed samples ($p < 0.05$), although they did not exceed the optimal limits specified in the Regulations of the Cabinet of Ministers of the Republic of Latvia. The highest moisture content ($8.61 \pm 0.96\%$) was found in Tervete (MF3) pollen, while the lowest moisture content ($6.67 \pm 0.22\%$) was found in Bene (MF2) pollen. Among all analysed multifloral pollen samples, Tervete (MF3) pollen was found to have a slightly higher moisture content than that specified in the Regulations of the Cabinet of Ministers of the Republic of Latvia (LR) No. 461 (12 August 2014) and in the Guidelines for Primary Production of Beekeeping Products (7–8%), but this was within the measurement error.

The differences between all of the analysed moisture content indicators of bee pollen samples can be explained by the fact that they were collected in different parts of Latvia, with different relative humidity during the harvest period; in addition, drying methods and packaging materials (polyethylene (PE) pouches vs. glass containers) also differed.

Water activity (a_w), along with moisture content, is one of the factors that characterise and affect the quality of the pollen. Among the monofloral pollen studied, the highest water activity, with a value of 0.389 ± 0.000 , was found in bee pollen from rye flower (RF), phacelia (PHA), with 0.383 ± 0.003 , and autumn raspberries (AR), with 0.363 ± 0.001 , all of which were slightly above or close to the permissible norm. In comparison, the lowest water activity (0.228 ± 0.001) was found in rapeseed pollen (RS). Of all the analysed multifloral

pollen samples, the highest water activity (0.324 ± 0.001) was found for the Tervete district collected bee pollen (MF3) sample, whereas the lowest water activity (0.187 ± 0.002) was determined for the bee pollen (MF1) sample collected in the Broceni District. The water activity of the analysed multifloral bee pollen samples was not higher than the permissible norm (0.38), but it differed significantly ($p < 0.05$) between the analysed pollen samples.

These differences could be partly explained by the moisture content of the bee pollen pellets; there was a moderate correlation ($r = 0.68$) between the moisture content and water activity of the samples analysed. Red clover bee pollen sample (Table 1) had low moisture content (6.62 ± 1.24) and high water activity (0.322 ± 0.001), while fava bean pollen demonstrated the opposite trend, having high moisture content (9.47 ± 0.44) and low water activity (0.294 ± 0.005). This mainly may be due to the ability of specific pollen to bind water. If the water binding ability is low, more free water is present, which results in higher water activity. These differences can also be explained by the weather conditions during the harvest season and the place of harvest, as well as the treatment and storage conditions (including the duration of storage) until the time of the experiments. The optimal water activity of dried bee pollen should not exceed 0.38 [27]. With this amount of water activity, the potential risk of microbiological contamination of bee pollen by various yeasts and fungi is reduced, and pollen is safe for longer storage. As pollen is very hygroscopic, external environmental factors can significantly affect its quality.

According to Luo et al. [28], moisture content and microbiological safety should be the key parameters in bee-collected pollen quality control. Our study indicated a moderate correlation between ($r = 0.64$) bee pollen moisture content and *Enterobacteriaceae* counts, as well as between moisture content and total plate count. This confirms the need for sufficiently low water content, requirements of which is less than 4% in Brazil, less than 6% in Switzerland and Poland, not exceeding 8% in Uruguay, and a maximum of 10% in Bulgaria [11].

Microbiological analyses proved that in the analysed bee pollen samples, *Enterobacteriaceae* ranged from 14.0 cfu/g in willow pollen to 82.0 cfu/g in phacelia pollen, with the highest value of total plate count detected in autumn raspberry pollen (7.35×10^2 cfu/g). In comparison, the indicated values by Campos et al. [11] are within the permissible range, below 100 cfu/g and 10^5 cfu/g for *Enterobacteriaceae* and total plate count, respectively. This confirms the suitability of pollen studied for human consumption. There are no international legal norms established for microbiological control of pollen. Although national standards have been established in several countries, in Latvia, there are no established specific norms or total microorganisms (MAFAM) and *Enterobacteriaceae* in bee pollen.

The high number of AMR cases with isolates that were resistant to ampicillin (7 cases) and penicillin (8 cases) points to an environmental effect because these antibiotics are not normally used in apiaries. This is in agreement with the study of [29], who reported about 80% resistance of *Staphylococcus aureus* to penicillin. In our study, *S. epidermidis*, *S. warneri*, *S. pasteurii*, *S. capitis*, and *Lysinibacillus fusiformis* showed AMR to both ampicillin and penicillin, while *Pantoea agglomerans* was resistant only to penicillin. *Enterococcus faecalis* was the only strain showing resistance to gentamicin and ciprofloxacin. None of the isolates studied demonstrated resistance to tetracycline.

Antimicrobial resistance can be explained by the resistance of bacteria to antimicrobials, to which they were previously susceptible. Antimicrobials, called antibiotics, are important in human and veterinary medicine for treating bacterial infections [19,20]. Various antibiotics containing penicillin, ampicillin, gentamicin, and tetracycline are prescribed for the treatment of various diseases (pneumonia, bronchitis, infected wounds, sepsis, urological diseases, conjunctivitis, etc.). If microorganisms having antimicrobial resistance are present in a food product or its raw materials and further enter the human body, it may reduce the effectiveness of the treatment of infectious diseases, i.e., a particular antimicrobial becomes ineffective and does not produce the desired results. Although pollen is recommended for daily use to strengthen immunity, caution should be taken, as antimicrobial resistance was observed in some pollen samples in this study.

Bee pollen is now considered to be one of the functional food items, with particular emphasis on the fact that it is rich in biologically active substances and has the ability to act as a natural antioxidant with beneficial effects on human health [3]. Therefore, it is important to determine the total amount of phenols and antiradical activity in pollen samples in studies, thus assessing which of the pollens analysed are the most valuable sources of antioxidants and are particularly recommended in the diet.

Polyphenols are among the main bioactive compounds in bee pollen that determine their antiradical activity. Phenols act as natural antioxidants that fight free radicals. The content of phenolic compounds in pollen can vary significantly due to the diversity of plant botanical origins, as well as the period during which the bee harvested them [5,10]. The profile of phenolic compounds in bee pollen can serve as an important quality indicator. Previous studies of antioxidant research in pollen have shown some correlation between phenol content and antioxidant capacity in pollen [10].

Among monofloral bee pollen studied, the highest phenol content (Table 2) was detected in pollen collected from autumn raspberry and rapeseed, whereas the lowest was detected in clover pollen. The differences in the total phenol content could be related to both the season when the pollen was collected and the botanical origin of the plant, which indicates that the pollen of this species is characterised by a low content of phenolic compounds when pollen is exposed to low temperature [30]. Based on a comparison of the total phenol content of monofloral bee pollen with that of multifloral samples, it could be concluded that phenol content in multifloral pollen was slightly higher. None of the multifloral bee pollen samples analysed had a total phenol content of less than 15 mg GAE/g dw, while few monofloral pollen samples had a phenol content of less than 10 mg GAE/g dw.

Antiradical activity in pollen samples was determined using two methods—DPPH and ABTS methods—both of which are especially suitable for the determination of antioxidant activity in natural plant extracts. The highest antioxidant activity (2.21 ± 0.07 mmol TE/100 g dw) among monofloral pollen was found for the autumn raspberry pollen (AR), whereas the lowest (0.40 ± 0.02 mmol TE/100 g dw) was found for the clover pollen (CL), using the ABTS test. The total phenol content in rapeseed pollen was the second-highest among all samples analysed, which might indicate a correlation between high phenol content and strong antioxidant activity. The results obtained by the DPPH test confirmed the highest antioxidant activity for rapeseed (RS) bee pollen and the lowest for autumn raspberry (AR) bee pollen.

5. Conclusions

This study confirmed a large variation in total phenolic content depending on the bee pollen type, indicating the type-specific biological value of pollen. Higher variation was observed in monofloral samples than in multifloral pollen. A strong correlation between TPC and ABTS antioxidant activity was established.

Among all bacteria ($n = 34$) isolated from bee-collected pollen, the highest prevalence was found in *Bacillus* spp. (20/34) and coagulase-negative staphylococci (6/34). Resistances to the analysed antibiotics were identified in 18 cases. The results of this study draw attention to the environmental issues where bees collect pollen because bacterial isolates demonstrated resistance to antibiotics (eight cases against penicillin and eight cases against ampicillin). No antibiotics were used in apiaries that provided bee pollen samples. Therefore, resistance was developed elsewhere, which may raise concerns about the safety of pollen consumption in the human diet.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/app12063039/s1>. Table S1: Colour of bee pollen samples, Table S2: Diversity of microorganisms isolated from bee pollen samples, Table S3: Antimicrobial resistance detected in pollen samples.

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