NATIONAL UNIVERSITY OF SCIENCE AND TECHNOLOGY

POLITEHNICA BUCHAREST

Faculty of Chemical Engineering and Biotechnologies

PhD Thesis - Summary

Multifunctional magnetic systems based on bioactive compounds

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Sisteme magnetice multifuncționale pe bază de compuși bioactivi

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BUCHAREST

2024

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Keywords: dysbiosis, bee products, phenolic compounds, drug delivery systems, colorectal cancer

PART I. State-of-the-Art Review

CHAPTER 1. Gut Microbiota: Impact on Nutrition and Health

1.1. Introduction

The human microbiota is constituted of bacteria, fungi, archaea, viruses, and protozoans that colonize many areas of the body, such as the skin, mouth, mammary gland, urogenital, respiratory system, and gastrointestinal tract (GIT), and bring to the host a dower of a lot of cells and genes than its own. Also, microbiota is the primary key to maintaining and sustaining health [1, 2]. Determinant factors such as nutrition, lifestyle, age, hormonal changes, or hereditary genes significantly influence microbiota balance, and any change (dysbiosis) can cause gradual alteration of the existent equilibrium or even diseases. The immune system is the first affected by changes in human homeostasis, especially by GIT dysbiosis. Furthermore, gut microbiota imbalance is associated with ATB resistance, chronic inflammation, neurological and cardiovascular disorders, and cancer [1, 2].

The GIT, with its epithelial barrier, has a total area of 400 m^2 and represents a complex ecosystem, which contains > 10^{14} microorganisms (MO) concerning > two thousand species and twelve different phyla, and their microbiome presents up to five hundred more genes than human DNA [3, 4]. The primary part of gut microbiota phyla includes *Actinomycetota* (*Actinobacteria)*, *Bacteroidota* (*Bacteroide*s), *Bacillota* (*Firmicutes),* and *Pseudomonadota* (*Proteobacteria)*. In addition, the GIT microbiota composition is influenced by numerous factors like ethnic-geographical conditions, method of delivery, host genetics, diet, medical history, immune system, body mass index, blood levels, age, sanitary and socio-economic conditions, sleep, drugs (antibiotics-ATBs, antihistamines, metformin, etc.), lifestyle, etc. [5].

Microbiota composition differs from GIT according to environmental conditions from the mouth, esophagus, stomach, small intestine, and large intestine [6-9]. The human colon predominantly comprises *Bacteroides*, *Firmicutes*, and *Proteobacteria* at the phylum level [10, 11]. The biodiversity of gut microbiota, especially of the fecal microbiome, is considered a biomarker of health, and any dysbiosis significantly affects intestinal and immunological homeostasis. Furthermore, the microbiota imbalance is reflected in the concentration of MO and the loss of the intestinal epithelium's thickness, which becomes more susceptible to pathogens and inflammatory processes [12].

1.2. GIT microbiota and oxidative stress

The gut microbiota generates various metabolites associated with many biological processes, especially immune system regulation. Moreover, GIT microbiota homeostasis is directly linked to the REDOX equilibrium, and any disturbance generates inflammatory processes [13].

Furthermore, dysbiosis is correlated with oxidative stress, and prolonged exposure to ROS generates several intestinal diseases, such as enteric infections, inflammatory bowel diseases (Crohn's disease, ulcerative colitis), and colorectal cancer (CRC) [14].

Overall, the multifunctionality of gut microbiota is a key feature in maintaining a healthy life. The gut microbiota regulates the proliferation of the MO in the GIT, nutrient transport, immune system, vitamin, enzymes, short-chain fatty acids (SCFAs) production, and host metabolism, degrades proteins and carbohydrates [15].

1.3. Gut microbiota interactions in colorectal cancer

Gut microbiota composition plays a crucial role in colorectal carcinogenesis. The microbial communities from the gut present many functions, and alteration of the metabolism and immune function are determinant factors in the development of CRC [16, 17].

The diversity of gut microbiota is changed for patients diagnosed with CRC. Mostly, potential probiotic bacteria species, such as *Bifidobacterium*, *Lactobacillus*, *Treponema*, *Faecalibacterium* spp., *Ruminococcus,* etc., are in low levels, while the *Fusobacteria*, *Akkermansia* spp, *Porphyromondaceae*, *Methanobacterials,* and *Coriobacteridae* are in high quantities [18]. Plus, *Fusobacterium* spp., *Clostridium septicum*, *Escherichia coli*, *Shigella* spp., *Streptococcus bovis*, *Bacteroides fragilis*, *Enterococcus faecalis*, *Helicobacter pylori* are involved in CRC development [19].

Furthermore, levels of microbial metabolites are considered biomarkers for CRC patients. The evaluation of the intestinal microbial composition, molecular functions, genetic factors, and association with CRC patients' patterns could serve as biomarkers for the risk or screening of CRC [20, 21].

1.4. Gut microbiota modulation

Different treatment approaches comprising probiotics, prebiotics, symbiotics, postbiotics, ATBs, and fecal microbiota transplantation for modulation of gut microbiota and balance of the REDOX equilibrium state in GIT are applied [22, 23].

CHAPTER 2. Magnetic Nanoparticles

2.1. Generalities

The history of magnetic materials started thousands of years ago, but the discovery of nano-sized magnets, known as magnetic nanoparticles (MNPs), started with the beginning of nanotechnology [24]. The MNPs display various properties than traditional bulk magnets as a result of the nano-scale dimensions, and the most studied MNPs with ferrimagnetic properties are magnetite (Fe_3O_4), maghemite (γ-Fe_2O_3), and hematite (α-Fe_2O_3) [25, 26]. The magnetic and tunneling magnetoresistance properties of magnetite are significantly linked to their size, shape, and synthesis process [25, 26].

2.2. Synthesis of MNPs

In the past years, researchers have had a great interest in developing different methods for obtaining NPs with regular shapes, stability, magnetic properties, biocompatibility, etc. The MNPs can be synthesized using many techniques, which can be divided into conventional (physical, chemical, or biological) and non-conventional, and each route gives different properties. The most widely applied are chemical methods due to versatility and various routes, such as coprecipitation, thermal decomposition, sol-gel, laser-ablation, hydrothermal, microemulsion, etc. [26-29].

2.3. Biomedical applications of MNPs

Magnetic nanoparticles are more utilized in biomedical applications because of their biological compatibility, physicochemical properties, and ease of manipulation when using a magnetic field. The use in specific biological applications depends on the properties of the MNPs, especially the particle size distribution, the shape, and the interaction between the particles, which are properties due to synthesis methods [30, 31]. The conditions that MNPs must meet to be applied in biomedical applications are minimal toxicity, chemical stability in biological fluids, compatibility with the body, and high magnetization saturation [32].

The *in vivo* applications are represented by the diagnostic methods (nuclear magnetic resonance) and by therapeutic applications (transport and drug delivery systems -DDSs to a target place in the body, magnetic hyperthermia -MH, etc.) [30, 33]. The background of biomedical applications consists of MNPs interacting with an EMF and/ or light [34]. Recent studies have established that using nanotechnology and intelligent magnetic DDSs denotes an alternative in antimicrobial and anticancer treatment due to biocompatibility with the human body and the possibility of targeting the desired place with a magnetic field [35, 36].

CHAPTER 3. Colorectal Cancer Treatment

3.1. Chemotherapeutics

The treatment of colorectal cancer is different depending on the stage, site, and number of tumors (metastasis), and the immunologic status of the patient. Presently, the standard treatment involves surgery, chemotherapy, and radiotherapy [37].

Some of the most used chemotherapeutic drugs are fluoropyrimidines (5-fluorouracil and capecitabine), oxaliplatin, and irinotecan (IRI). 5-fluorouracil (5-FU) has been used in chemotherapy for more than 60 years, and in the latest period, 5-FU is used in combination with other drugs to enhance the antitumoral properties [38]. However, chemotherapeutic drugs generate several side effects that affect the entire body [39, 40]. Nevertheless, due to the drug resistance, ineffective treatment, and severe effects of chemotherapeutic drugs for CRC patients, it is necessary novel approaches, such as combined or/ and targeted therapy.

3.2. Natural compounds

In the last period, researchers have given great attention to the therapeutic properties of natural compounds in different forms of cancer. Considering the drug's resistance against cytostatic drugs, the combination with other compounds is intensively investigated [41]. Simultaneously administering the pharmaceutic drug with a natural compound/ extract rich in bioactive compounds could enhance the efficacity of the antitumoral drug and its bioavailability, reduce dosage, and alleviate adverse effects [41].

3.3. Nanotherapeutics

Efficacy and adverse effects during cancer treatment require novel approaches, and for this purpose, using nanotechnology has gained significant interest in recent years. The advantages of using NPs include enhancing drug solubility, stability, bioavailability, biocompatibility, diminishing adverse effects, and increasing survival rates [42]. Furthermore, the intensive development of nanocarriers and nanomaterials is of interest due to the delivery of bioactive compounds/ biomolecules at targeted sites, drastically reducing the adverse effects of chemotherapeutics [42].

Nanocarriers have the capacity to transport bioactive agents through passive targeting of irregular architecture and hypervascular system of tumors, improving the uptake of tumors and therapeutic index. MNPs exhibit various applications, including CRC therapy, because of their biocompatibility, effective surface area, targeted transport, etc. [43].

PART II. Original Contributions

CHAPTER 4. General and Specific Objectives of the Research

The reason for addressing the issue of microbiota dysbiosis and applying nanotechnology comes from the disadvantages related to conventional methods of delivering bioactive compounds. Human microbiota homeostasis is the foremost key to maintaining and sustaining health, and a prolonged imbalance is associated with severe pathologies, such as ATBs resistance, chronic inflammation, neurological diseases, etc. Moreover, recent data related that prolonged exposure to oxidative stress/ inflammation is correlated with disturbance of the immune system and development of colorectal cancer, respectively.

Additionally, due to the severe effects and resistance of chemotherapeutics drugs, the gut microbiota is highly damaged, and it is required novel approaches for the recovery of gut microbiota and colorectal cancer therapy. One of them is represented by the simultaneous administration of cytostatic drugs with phytochemicals because natural compounds could improve the efficacity of the drug and its bioavailability, reduce dosage, and alleviate adverse effects.

However, nanotechnology could enhance the therapeutic properties of bioactive compounds by using DDSs and targeted therapy because nanocarriers can transport bioactive agents through passive targeting of irregular architecture and hypervascular systems of tumors. On the other hand, NPs like magnetic NPs gained special attention due to their stability, bioavailability, biocompatibility, effective surface area, and targeted transport with a magnetic field.

Consequently, this work has been focused on the study of the chemical and biological properties of a low-cost and eco-friendly source of biologically active compounds (bee products) and developing DDSs based on the use of MNPs and the combination of chemotherapeutic drugs with extracts rich in phenolic compounds.

Based on this general goal, three objectives were classified, such as:

1. To obtain bee pollen extracts and evaluate their chemical composition, antioxidant, and biological properties.
2. To obtain bee bread extracts and assess their chemical, antioxidant, and antimicrobial properties.
3. To develop drug delivery systems based on magnetite nanoparticles as nanocarriers for bioactive compounds.

Each of the previously established general objectives will be detailed in terms of specific synthesis activities, physicochemical characterizations, evaluations from a biological point of view, and the investigation of the potential for using developed nanocarriers in the *in vivo* evaluations.

The first objective was to assess the chemical and biological properties of BP samples acquired from beekeepers from Romania. Initially, the BP extracts (BPEs) were obtained from raw BP collected in a pollution-free area. Secondly, the composition of BP/ BPEs was evaluated using the spectrophotometric method (total phenols and total flavonoids) and chromatography analysis (UHPLC-DAD-ESI/MS and GC-MS). Additionally, antioxidant capacity and antimicrobial and cytotoxic properties were assessed. Regarding the antimicrobial activity, the qualitative and quantitative assays were initially evaluated, followed by a semiquantitative assay of adherence to an inactive substrate and the antimicrobial effect of BPEs on the adherence ability of microbial strains with pathogenic potential. Moreover, to determine the capability of BPEs to modulate gut microbiota, the prebiotic effect of the BPEs on the adherence capacity and the growth of bacterial strains with probiotic potential were assessed. In addition, the synergistic antimicrobial impacts of BPEs and soluble compounds of lactic strains (*L. rhamnosus* MF9 and *E. faecalis* 2M17) on the adhesion capability to sensible cellular substrates of selected pathogenic strains were evaluated.

The second objective is to determine bee bread samples' chemical composition, antioxidant, and antimicrobial properties. Firstly, the palynological analysis was performed utilizing SEM and a light microscope, and the chemical composition of BB samples was determined using FT-IR Spectroscopy. Secondly, the BBEs were prepared, and the PC richness was assessed using spectrophotometry and chromatographic methods. Antioxidant activity was also performed via spectrophotometric analysis. Moreover, the antimicrobial properties of the BBEs were assessed for both standard and clinical pathogenic strains. Additionally, inhibitory effects on microbial adherence ability to the inert substrate generated by BBEs were performed.

The final objective was the development of magnetite-based drug delivery nanocarriers to improve the antitumoral activity of 5-FU and diminish the adverse effects against gut microbiota. Initially, the MNPs were synthesized through a spray-assisted coprecipitation method and loaded with BP/ BB extracts and the antitumoral drug. The developed nanocarriers were morphologically and structurally characterized. Moreover, the bioactive compounds' release profiles were evaluated for both the antitumoral agent and the PCs. Similarly, the antioxidant activity was performed through three methods. Furthermore, the antibacterial and antitumoral properties of nanocarriers were evaluated. Additionally, the influence of loaded MNPs on selected bacteria with probiotic potential isolated from newborn feces was assessed.

CHAPTER 5. Chemical Composition, Antioxidant and Biological Properties of Bee Pollen Extracts

This study evaluated the chemical and biological properties of bee pollen samples from Romania. Firstly, the bee pollen alcoholic extracts (BPEs) were obtained from raw bee pollen harvested by *Apis mellifera carpatica* bees. The chemical composition of BPE was obtained by determination of total phenol content and total flavonoid content, UHPLC-DAD-ESI/MS analysis of phenolic compounds, and GC-MS analysis of fatty acids, esters, and terpenes. Additionally, the antioxidant activity was evaluated using the Trolox Equivalent Antioxidant Capacity method. Furthermore, the biological properties of BPE were assessed.

Materials and Methods

Materials

The BP samples came from pollen harvested by bees in 2020, from plant species of spontaneous flora throughout the spring, at different time intervals depending on climatic conditions and the diversity of flowering plants.

Bee Pollen Extract (BPE) Preparation

The extractions were performed by heating 2 g fresh BP at 40 °C with ethanol 70% (*v*/*v*) [44-46]. This method contains more steps, such as vortexing, ultrasonication, and centrifugation.

Results and Discussions

Chemical Composition of BPE

Bee pollen has a complex composition that depends on many factors [47]. The classes of compounds of interest, in terms of antioxidant and antimicrobial activity, are mainly PCs, terpenes, and FAs, which will be presented below.

Determination of TPC, TFC, and TEAC

The TPC, TFC, and TEAC results are presented in **Table *1*** as mean value ±SD.

Table 1. Total phenol content, total flavonoid content, and antioxidant activity.

Sample	TPC (GAE) [1]	TFC (QE) [2]	TEAC [3]
P1	**15.51 ± 0.01** ^{c,d}	**0.27 ± 0.01** ^{d}	**0.06 ± 0.02** ^{b}
P2	**16.15 ± 0.02** ^{c,d}	**0.30 ± 0.01** ^{d}	**0.07 ± 0.01** ^{b,d}
P3	13.24 ± 0.01 ^{d}	0.20 ± 0.02 ^{d}	0.04 ± 0.02 ^{d}
P4	**14.46 ± 0.01** ^{d}	**0.26 ± 0.01** ^{d}	**0.05 ± 0.01** ^{b}
P5	10.77 ± 0.02 ^{d}	0.19 ± 0.01 ^{d}	0.03 ± 0.01 ^{a,d}

[1] TPC expressed as mg gallic acid/g BP; [2] TFC expressed as mg quercetin/g BP; and [3] TEAC expressed as mmol Trolox/g BP.

According to the results shown in the previous table, P1, P2, and P4 have the highest TPC and TFC levels. The BPE antioxidant activity data (TEAC method) correlates with TPC and TFC results. It can be observed that P1, P2, and P4, which have a higher content of both phenols and flavonoids, also have the best antioxidant activity, which correlates with other studies [48, 49].

Phenolic Compound Profile by UHPLC-DAD-ESI/MS

The results presented in **Table *2*** show the identification and quantification of 20 phenolic compounds analyzed by UHPLC-DAD-ESI/MS in all BPE samples.

Table 2. The phenolic acid and flavonoid concentrations from BP (µg/g).

Phenolic Compound	Sample				
	P1	**P2**	**P3**	**P4**	**P5**
Phenolic Acids					
gallic acid	0.015	0.087	0.157	0.075	**2.310**
3,4-dihydroxybenzoic acid	0.254	0.401	0.454	0.209	**0.549**
4-hydroxybenzoic acid	7.603	**19.770**	8.455	4.458	2.685
chlorogenic acid	0.733	2.441	6.481	**46.939**	0.275
caffeic acid	0.404	0.471	0.734	**1.167**	0.275
syringic acid	0.090	ND	ND	ND	**0.325**
p-coumaric acid	1.227	1.151	1.153	**1.256**	1.036
ferulic acid	**2.978**	**2.894**	0.961	1.271	1.199
cinnamic acid [#]	0.898	0.227	**2.236**	1.286	0.400
Flavonoids					
epicatechin	0.868	1.029	0.070	**1.286**	0.137
catechin	**1.257**	0.959	0.332	0.224	0.587
rutin	45.662	1.691	**135.301**	69.451	ND
myricetin	**0.943**	0.506	0.052	0.224	0.012
quercetin	3.577	1.639	3.214	**7.883**	**5.981**
kaempferol	2.155	1.308	7.337	8.676	**26.472**
isorhamnetin	3.502	0.558	12.141	5.086	**40.220**
apigenin	0.015	0.052	0.070	0.165	**2.735**
pinocembrin	0.644	**2.197**	0.559	1.227	0.412
galangin	ND	**0.506**	0.017	0.239	0.175
chrysin	0.464	**1.970**	0.384	0.853	0.387

* ND (not detected) Naringenin, hesperidin, pinostrombin, and resveratrol were not detected in the bee pollen samples. [#] *p*-coumaric, ferulic, caffeic, and chlorogenic acids are derivatives of cinnamic acid (which does not contain phenolic groups), which was still included in the table.

According to these results, each BPE sample had its phenolic profile. Some phenolic acids were present in significant quantities, while flavonoids were sometimes detected in larger amounts than phenolic acids.

BPE Analysis by GC-MS

Based on the GC-MS analysis, 28 compounds were detected and quantified. Some terpenes were presented as minor constituents (less than 1% compound/ BP sample). FA is identified in higher percentages, such as stearic acid methyl ester (88.31% for P4), linolenic acid (43.42% -P5; 43.23% -P3), linoleic acid (19.40% -P1), and palmitic acid (13.53% -P2). The results confirm that the chemical profile variability of BP samples depends on the plant species from which bees harvested [50-52]. In addition, some BP could be a valuable source of polyunsaturated FAs.

Biological Activity of BPE

Qualitative Evaluation of Antimicrobial Activity

In our study, all BPEs tested have shown antimicrobial activity against pathogenic strains, and Gram-positive bacteria were more sensitive than Gram-negative bacteria and yeasts. The most sensitive Gram-negative strains were *E. coli* ATCC 25922 and *P. aeruginos*a ATCC 25785. The most sensitive yeast strain was *C. krusei*.

Quantitative Evaluation of Antimicrobial Activity

The MIC value is represented by the lowest concentration of the tested BPEs that inhibited microbial growth. The results are presented in **Table *3***, expressed as μg/mL.

Table 3. Determination of MIC values (μg/mL).

Strain	MIC				
	P1	**P2**	**P3**	**P4**	**P5**
Gram-Positive Bacteria					
1. *Enterococcus faecalis* ATCC 19433	**1250**	2150	4290	2510	3000
2. *Staphylococcus aureus* ATCC 25422	1250	1080	2150	**630**	**380**
Gram-Negative Bacteria					
1. *Enterobacter cloacae*	2510	2150	**540**	2510	**750**
2. *Escherichia coli* ATCC 25923	1250	**540**	**540**	1250	1500
3. *Pseudomonas aeruginosa* ATCC 25853	**630**	**540**	**270**	1250	1500
Yeasts					
1. *Candida albicans* ATTC 1688	5010	2150	4290	**630**	**750**
2. *Candida famata*	**1250**	2150	**1070**	2510	3000
3. *Candida glabrata*	**630**	**540**	1070	1250	3000
4. *Candida guillermondii*	**630**	2150	2150	1250	**750**
5. *Candida krusei*	2510	1080	**270**	1250	3000
6. *Candida lusitaniae*	1250	2150	**270**	2510	3000

S. aureus ATCC 25422 was the most sensitive Gram-positive bacteria to the influence of BPE samples, with the lowest values of MIC for P4 and P5 samples. The most effective antimicrobial extracts were obtained from raw pollen derived from *C. monogyna*, *T. officinale*, *Salix* sp., *Malus* sp., and *Prunus* sp. (P1, P2, and P3). Previous studies [53-57] show that antimicrobial activity correlates with these samples' antioxidant activity.

Semiquantitative Assessment of Microbial Adherence to the Inert Substratum

The influence of the BPE on the pathogenic microbial strains' adherence to the inert substratum is presented in **Table *4***.

Table 4. Determination of minimal concentration for biofilm eradication (MBEC) values

Strain	**MBEC** (μg/mL)				
	P1	**P2**	**P3**	**P4**	**P5**
Gram-Positive Bacteria					
1. *Enterococcus faecalis* ATCC 19433	**1250**	2.150	2150	2510	3000
2. *Staphylococcus aureus* ATCC 25422	1250	1080	2150	**1250**	**750**
Gram-Negative Bacteria					
1. *Enterobacter cloacae*	1250	1080	**1070**	2510	**1500**
2. *Escherichia coli* ATCC 25923	2510	**270**	**540**	1250	1500
3. *Pseudomonas aeruginosa* ATCC 25853	**630**	**540**	270	1250	1500
Yeasts					

1. *Candida albicans* ATTC 1688	2510	2150	2150	**630**	**750**
2. *Candida famata*	**1250**	2150	**1070**	2510	3000
3. *Candida glabrata*	**630**	**540**	1070	1250	3000
4. *Candida guillermondii*	**630**	2150	2150	1250	**750**
5. *Candida krusei*	2510	1080	**270**	1250	1500
6. *Candida lusitaniae*	630	2150	**1070**	2510	3000

According to the MIC and MBEC values (**Table *3*** and **Table *4***), *S. aureus* ATCC 25422, *P. aeruginosa* ATCC 25853, and *C. glabrata* were the most sensitive tested strains. The BPEs had a moderate influence on the yeast's growth. The MIC and MBEC assays confirmed the qualitative antimicrobial assays and correlated with the results of the chemical composition analysis

Evaluation of the Inhibitory Effect of the BPE Samples on the Ability of the Tested Microbial Strains to Attach to the Cellular Substrate

The bioactive compounds' antimicrobial effect from BPEs on microbial strains' adherence capacity is presented in the figures below.

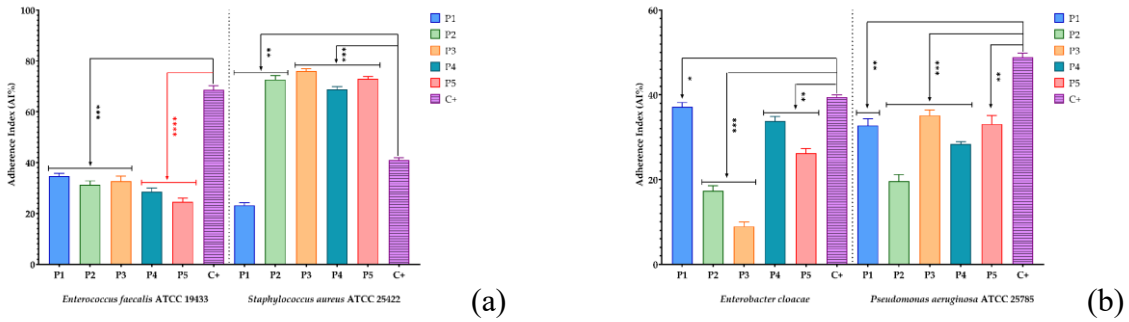

Figure 1. Graphic representation of AI% values representing the influence of BPE on adherence capacity of Gram-positive bacteria (a) and Gram-negative bacteria (b); (P1–P5)-BPE samples; (C+)-bacterial adherence control.

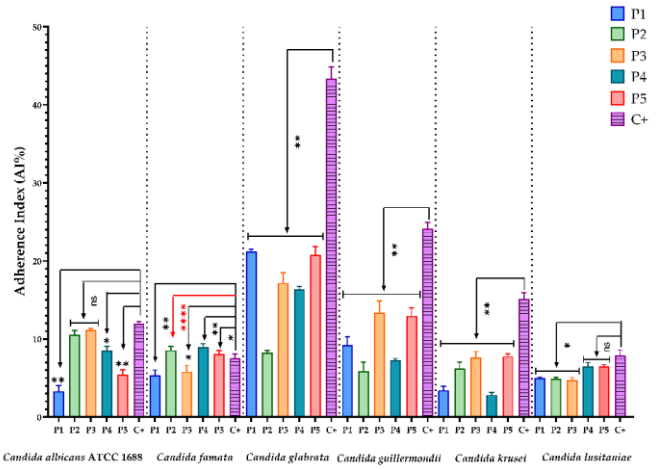

Figure 2. Graphic representation of AI% values representing the influence of BPE on the adherence capacity of yeast strains; (P1–P5)-BPE samples; (C+)-yeast adherence control.

Experimental data show that for Gram-negative bacterial strains and yeasts, all BPEs reduced the adherence capacity compared to the control (**Figure *1***, and **Figure *2***). One exception was *C. famata,* with only P1 and P3 samples expressing this effect. Regarding Gram-positive bacterial strains, the same inhibitory effect was observed for *E. faecalis* ATCC 19433. Still, for *S. aureus*

ATCC 25422, only P1 inhibits the ability to adhere to the inert substratum. The biological activity of bee pollen depends on the chemical composition profile, mostly flavonoids, phenolic acids, FA, phytosterols, and enzymes [58], or *Lactobacillus* strains from raw BP [59-61]. The BPE's antimicrobial activity is deeply linked to phenolic content [62].

Evaluation of the Prebiotic Effect of the BPE Samples on the Ability of Two Microbial Strains with Probiotic Potential to Adhere to a Cellular Substrate

Prebiotics' relationship with gut microbiota is essential in improving human health. They can modulate the gut microbiota's composition, growth, and population [63].

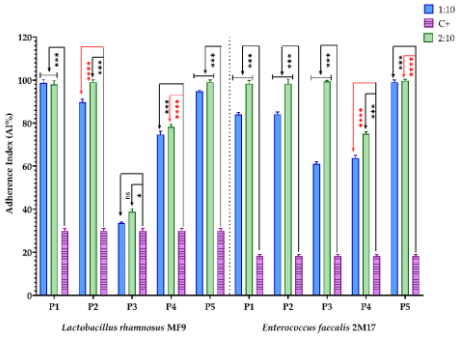

Figure 3. The influence of BPE on the adherence capacity of the bacterial strains with probiotic potential; (P1–P5)-BPE samples; (C+)-bacterial adherence control.

Compared to the control, all BPEs stimulated the adherence capacity (Figure 3) of the microbial strains with probiotic potential, *L. rhamnosus* MF9, and *E. faecalis* 2M17.

Assessment of the Prebiotic Effect of the BPE on the Growth of Two Bacterial Strains with Probiotic Potential

BP can be considered an essential source of prebiotic compounds, and the effect of the BPE samples on the growth of *L. rhamnosus* MF9 and *E. faecalis* 2M17 is presented in **Figure *4***.

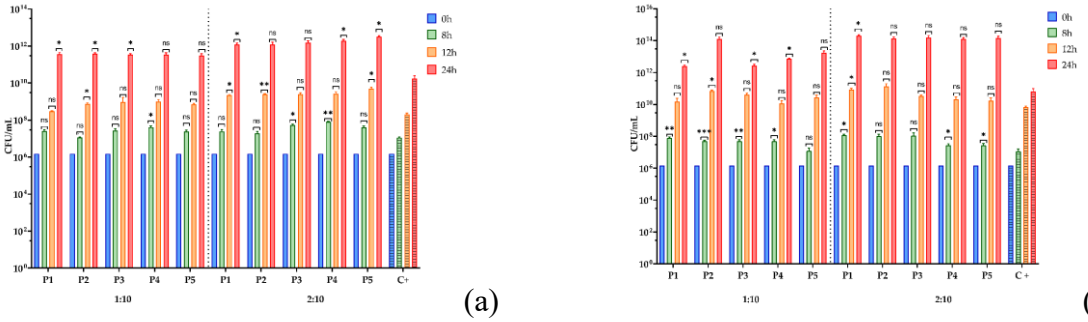

Figure 4. The influence of BPE on the bacterial growth of *L. rhamnosus* MF9 (a) and *E. faecalis* 2M17 (b); (P1–P5)-BPE samples; (C+) growth control.

Regarding the prebiotic effect of the BPE on the growth curve for tested probiotic strains, all BPEs stimulated the growth of the microbial strains with probiotic potential compared to the control, at both dilutions 1:10 and 2:10.

Assessment of the Synergic Influence of BPE and Probiotic Soluble Compounds on the Capacity of Some Pathogenic Strains to Adhere to the Cellular Substratum

Besides the assessment of the antimicrobial effect of the BPE and soluble compounds of lactic strains on the adherence capacity to the sensitive cellular substrate of some pathogenic strains, it can be considered that the BPE, together with the soluble compounds of *L. rhamnosus* MF9 and *E. faecalis* 2M17, inhibited without exception the adherence capacity of the *C. guillermondii* and *E. cloacae*, compared to the control at 1:10 (**Figure *5***).

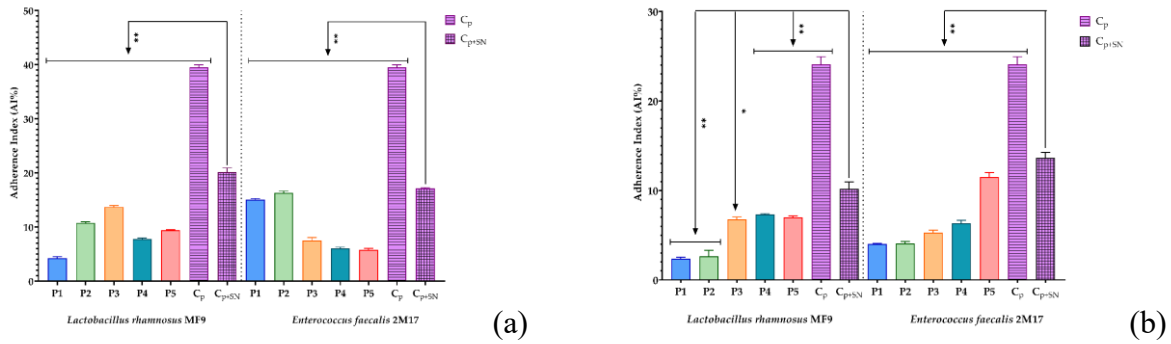

Figure 5. Graphic representation of AI% values representing the synergic influence of BPE and probiotic Soluble Compounds (SN) on the adherence capacity of *Enterobacter cloacae* (a) and *Candida guillermondii (b)*; (P1–P5)-BPE samples, pathogenic strains adherence control (Cp), pathogenic strains with probiotic supernatant adherence control (C_{P+SN}).

According to the results obtained in this study, the BPEs, rich in compounds with prebiotic effects, strongly stimulated the growth of probiotic strains. Furthermore, when they were in contact with the soluble compounds of the two lactic strains, they determined a synergistic inhibitory effect on the multiplication process of the two clinical strains with pathogenic potential. To our knowledge, no data is available in the literature to confirm this.

Cytotoxic Activity of BPEs

The in vitro tumor cell viability and proliferation in the presence of BPEs were examined using the MTT method (**Figure *6***a), and the cytotoxic potential of all BPEs on tumor cells was evaluated by LDH assay that indicates the number of dead cells in the culture (**Figure *6***b).

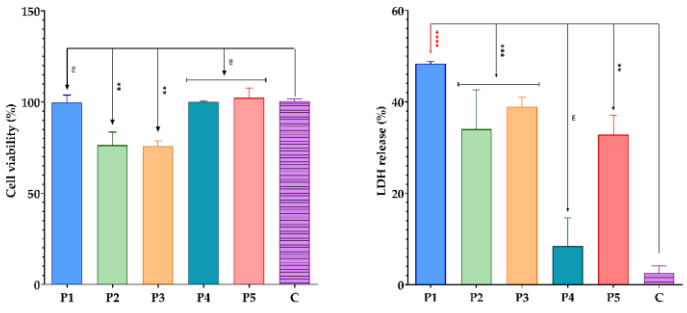

Figure 6. (**a**) MTT assay. (**b**) LDH assay; (P1–P5) BPE samples, C-cell culture control.

The MTT analysis showed that all BPEs did not stimulate the proliferation of Hep-2 cells (***Figure 6***). P3 has the lowest percentage of cell viability, showing an inhibitory effect, followed by P2. These results are confirmed by the increased LDH level in the presence of these two samples (***Figure 6***b). The P5 sample stimulated the proliferation of Hep-2 cells, but the stimulatory effect was insignificant. Among the tested BPEs, P4 had the lowest level of cytotoxicity, as shown by the quantification of LDH. Conversely, P1 has the highest LDH levels (**Figure *6***a) but did not significantly influence the viability and the proliferation of tumoral cells, these results being contradictory. Otherwise, P2 and P3 presented the best cytotoxic effects on tested tumor cells and correlated with the previous results; they can be considered potential tumor proliferation inhibitory agents.

Conclusions

BP is an excellent source of PCs responsible for antioxidant activity and FAs, including polyunsaturated and terpenic compounds. P1, P2, and P4 samples had higher phenol and flavonoid content and the best antioxidant activity. Each BPE sample had its own phenolic profile. Several phenols were present in significant quantities: 4-hydroxybenzoic acid (P2), chlorogenic acid (P4), ferulic acid (P1, P2), and gallic acid (P5). Regarding flavonoids, rutin (P3 > P4 > P1), quercetin (P4 > P5), kaempferol (P5 > P4 > P3), and isorhamnetin (P5 > P3) were prevalent. Some terpenes were identified as minor constituents (globulol, methyleugenol, etc.). FA was in higher percentages, such as stearic acid methyl ester (P4), linolenic acid (P3, P5), and linoleic acid (P1).

All BPEs presented antimicrobial activity against pathogenic strains. Gram-positive bacteria and yeasts were more sensitive than Gram-negative bacteria. Antibacterial activity seems to be related to the chemical composition of BP, and a synergistic effect may be responsible for antimicrobial activity in some cases.

PCs from the studied extracts could be a rich source of prebiotic compounds, given the stimulating effect of the growth of microbial strains with probiotic potential. Furthermore, a synergistic antimicrobial effect of the BPEs was observed along with soluble compounds of *L. rhamnosus* MF9 and *E. faecalis* 2M17 against two clinical pathogenic strains. Additionally, all BPEs did not stimulate the proliferation of Hep-2 cells, and P2 and P3 samples presented a higher inhibitory effect. Likewise, the antitumor activity of P2 and P3 is confirmed by cytotoxicity assay.

These results indicate the potential of BP to be used as an antimicrobial, prebiotic, and tumor proliferation inhibitory agent that, in association with probiotic compounds, maintains and even improves gut homeostasis by promoting the recovery of intestinal microbiota (rebiosis), fighting or preventing bacterial infections, and inhibiting the onset of tumor processes.

CHAPTER 6. Chemical, Antioxidant and Antimicrobial Properties of Bee Bread Extracts

The present study illustrates twelve BB samples' palynological analysis, chemical composition, and antioxidant and antimicrobial activities. First, botanical origin analysis using scanning electron microscopy (SEM) and a light microscope (LM) was performed. Chemical composition was determined using FTIR spectroscopy, and the PCs were evaluated using spectrophotometric (total phenolic compounds and total flavonoids) and chromatographic methods. Additionally, antioxidant capacity was determined using a spectrophotometric assay. The antimicrobial activity of the BBEs was qualitative and quantitatively evaluated on some pathogenic strains (standard and clinical). In addition, the novelty of this study consists of the inhibitory activity of microbial (bacterial and fungi) adhesion capacity to the inert substratum induced by BBEs, as well as their antifungal activity on *Candida krusei* and *Candida kefyr*, which are for the first time reported.

Materials and Methods

Materials

The BB samples were provided by Romanian beekeepers between the spring and summer of 2022 and were deposited at −45°C. The apiaries were distributed in seven districts of Romania: Arges, Calarasi, Giurgiu, Prahova, Sibiu, Valcea, and Teleorman. The BBs come from pollen harvested by *Apis mellifera carpatica* bees from wild flora and house plants, and for these reasons, a palynological analysis was carried out to establish its botanical origin accurately.

Bee Bread Extract (BBE) Preparation

The extractions were performed using a method described in a previous study [46].

Results and Discussions

Palynological Analysis

BBs' taxonomic/botanical assignments were performed using LM and SEM, and **Figure *7*** and **Figure *8*** represent the comprehensive results.

The data results of the botanical identification presented in **Figure *7*** indicate that 31 families were identified and that there is a detailed interconnection between the plant families and BB samples. For example, **BB8** presented pollen grains from an extensive list of plant families (24), and **BB6** and **BB7** were from 22. Likewise, in **BB1**, **BB3**, **BB5**, and **BB9** samples, 21 plant families were identified. **BB10** was the least varied sample, containing pollen grains from 9 plant families, with the *Salicaceae*, *Primulaceae*, and *Fabaceae* as the dominant families.

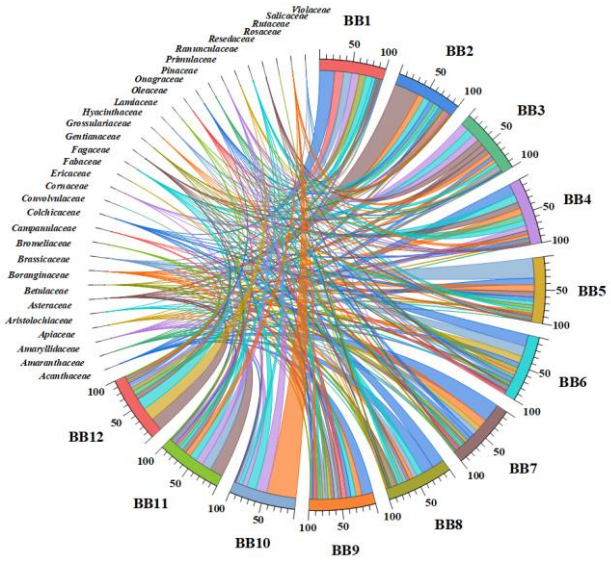

Figure 7. Chord diagram based on the relationship between botanical families and BB samples.

Multivariate statistical analysis was performed on the plant families identified in BBs to differentiate and group/cluster the BB samples based on the palynological analysis. The PCA plot of the plants' taxonomic assignments from BP (**Figure *8***a) showed a clear discrimination of **BB12** and some provenience families, which are correlated with the relative abundance. Particularly, **BB1** and **BB4** are grouped on the left side of **Figure *8***a, and *Resedaceae*, *Rosaceae*, *Hyanthaceae*, and *Grossulariaceae* are the representative/specific families from which the pollen of these samples originates. The other families are associated with **BB5**, **BB6**, **BB7**, **BB8,** and **BB9**.

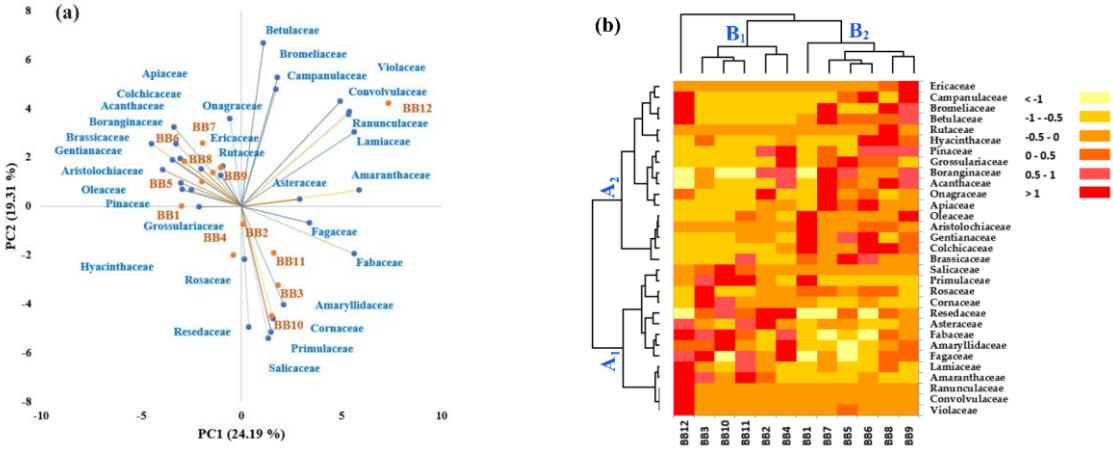

Figure 8. Discrimination of bee bread samples based on a palynological analysis. (**a**) Principal component analysis and (**b**) hierarchical cluster and heat map analysis.

According to **Figure *8***b, the data obtained confirm the previous results. As in **Figure *8***a, the BB samples were grouped into two main groups (B_1 and B_2), and this highlighted a discrimination of **BB12**. The heat map combined with the clusters expressed a snapshot of the botanical origin of BB samples and was represented by a main cluster, which was fused in two subclusters (A_1 and A_2).

Chemical Composition of BBE

The complex composition of BB can vary widely due to many factors, such as plant and bee species, geographical area, seasonal changes, fermentation strains, beekeeper activities, etc. [64, 65]. The alcoholic extracts (BBEs) were analyzed to establish the samples' chemical compositions.

FTIR Spectroscopy

The FTIR spectra for all BB samples were measured between 4000 and 400 cm^{-1}, as illustrated below. **Figure *9*** shows that all BB samples presented similar FTIR spectra, which were characterized in previous studies [45, 66, 67]. Also, all BB samples shared comparable adsorption bands with minor spectral differences. The FTIR spectra related the presence of water, the functional group of amines I and II, amides (which suggests the presence of amino acids and proteins), carbohydrates, lipids, PCs, and FAs.

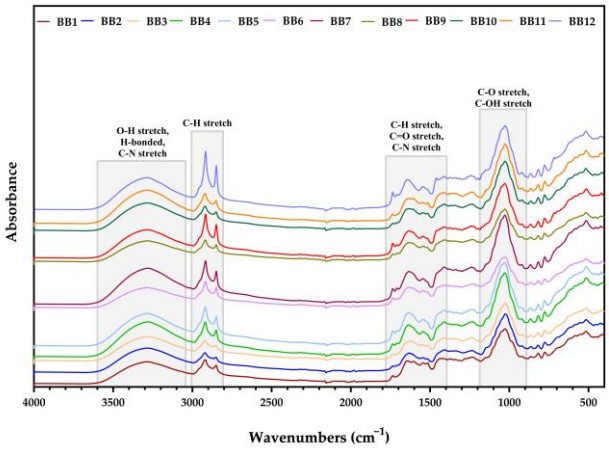

Figure 9. FTIR spectra for BB.

Determinations of TPC, TFC, and TEAC

TPC, TFC, and TEAC results are presented in **Table *5*** as mean value ± SD.

Table 5. Total phenols content, total flavonoids content, and antioxidant activity.

Sample	TPC (GAE) [1]	TFC (QE) [2]	TEAC [3]
BBE1	12.40 ± 0.010	**0.87 ± 0.502**	**0.07 ± 0.020**
BBE2	7.10 ± 0.005	0.52 ± 0.031	0.02 ± 0.010
BBE3	12.50 ± 0.005	**1.86 ± 0.516**	0.03 ± 0.005
BBE4	**14.40 ± 0.010**	0.53 ± 0.020	0.04 ± 0.006
BBE5	11.40 ± 0.005	0.50 ± 0.052	0.04 ± 0.009
BBE6	13.60 ± 0.005	0.60 ± 0.051	**0.06 ± 0.010**
BBE7	11.20 ± 0.025	0.45 ± 0.035	0.04 ± 0.004
BBE8	**18.30 ± 0.029**	0.52 ± 0.090	**0.05 ± 0.020**
BBE9	**15.80 ± 0.047**	0.59 ± 0.030	**0.05 ± 0.008**
BBE10	**18.30 ± 0.051**	0.70 ± 0.050	**0.05 ± 0.050**
BBE11	**14.90 ± 0.017**	**0.95 ± 0.011**	0.03 ± 0.030
BBE12	11.20 ± 0.015	**0.85 ± 0.500**	0.02 ± 0.005

[1] TPC expressed as mg gallic acid equivalents/g BB; [2] TFC expressed as mg quercetin/g BB; [3] TEAC expressed as mmol Trolox/g BB.

The results presented in **Table *5*** show that **BBE8** and **BBE10** presented the highest values of TPC. Correlating the TPC and TFC results with TEAC values is also sometimes difficult. Still, even if phenolic acids and flavonoids are the main compounds to determine antioxidant activity, other biomolecules can influence this [68-73]. The TPC, TFC, and antioxidant activity depend upon the plant species from which they are derived.

Phenolic Compound Profiles by UHPLC-DAD-ESI/MS

A total of 24 compounds, including 9 phenolic acids, 13 flavonoids and derivatives, and stilbene *t*-resveratrol, were unambiguously identified, and sesquiterpene abscisic acid was quantified in the BBE. According to the quantitative data, flavonoids were quantified in higher amounts than phenolic acids, which is in concordance with the TPC and TFC contents of BB and literature data [74]. Moreover, the extracts contained other PCs, like rutin, hesperidin, resveratrol, and abscisic acid (ABA). Overall, the samples had significant quantities of phenolic acids, like *p*-coumaric acid, gallic acid, caffeic acid, and cinnamic acid. High contents could be observed for quercetin, kaempferol, isorhamnetin, and ABA.

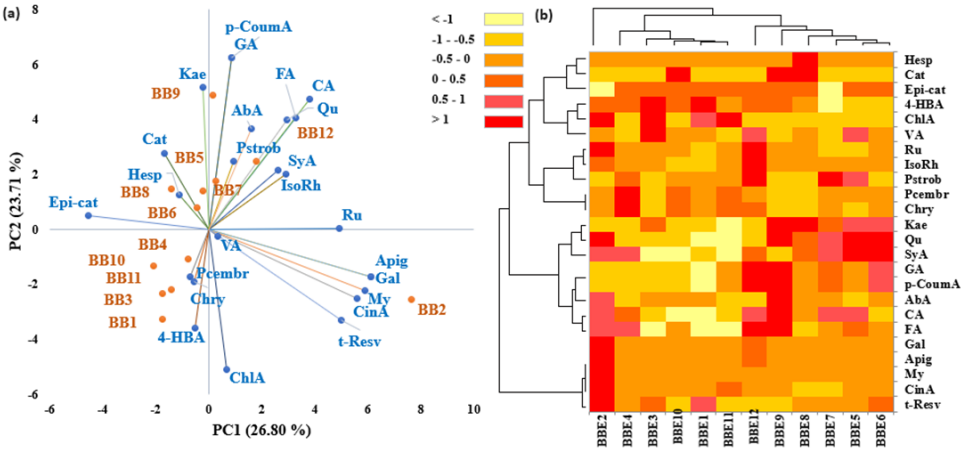

Figure 10. Discrimination of bee bread samples based on quantitative phenolic compound biomarkers: (**a**) principal component analysis and (**b**) hierarchical cluster and heat map analysis.

Multivariate statistical analysis, including principal component analysis (PCA) and heat map analysis (HMA), was applied to the phenolic quantitative data to differentiate between BB samples with different origins (**Figure *10***). From the PCA analysis, a clear discrimination of the BB2 sample was observed, which could be correlated with the botanical origin because this sample presented the highest percentage of the *Asteraceae* family plant species (**Figure *7***). Furthermore, apigenin (Apig), galangin (Gal), myricetin (My), cinnamic acid (CinA), t-resveratrol (t-Resv), and chlorogenic acid (ChlA) represent specific PCs for **BB2** and are distributed on the right-downside area of **Figure *10***a. Also, the right side of the figure indicates specific PCs for **BB7** and **BB12**, like

rutin (Ru), isorhamnetin (IsoRh), syringic acid (SyA), pinostrombin (Pstrob), abscisic acid (AbA), ferulic acid (FA), caffeic acid (CA), quercetin (Qu), gallic acid (GA), and *p*-coumaric acid (*p*-CoumA). Corresponding to the palynological analysis, the **BB12** sample is specific to BP of plant species of *Amaranthaceae*, *Betulaceae*, *Bromeliaceae*, *Concolvulaceae*, *Lamiaceae*, *Onagraceae*, and *Violaceae*. The **BB7** sample also has plant pollen from the mentioned families, while it presents a high content of pollen from the *Acanthaceae* family.

According to botanical origin (**Figure *7*** and **Figure *8***), **BB3**, **BB10,** and **BB11** present significant contents in pollen from plant species of *Amaryllidiceae*, *Cornaceae, Fabaceae*, *Fagaceae*, *Primulaceae*, *Resedaceae*, and *Salicaceae* families. Linking these results with **Figure *10***a, the pinocembrin (Pcembr), chrysin (Chry), and 4-hydroxybenzoic acid (4-HBA) are distributed to **BB1**, **BB3**, **BB4**, **BB10**, and **BB11**, which are clustered on the left downside of the PCA graph. Kaempferol (kae), catechin (cat), epicatechin (Epi-cat), and hesperidin (Hesp) represent specific PCs for **BB5**, **BB6**, **BB8,** and **BB9**, which are grouped on the left side of **Figure *10***a. Likewise, *Acanthaceae*, *Apiaceae*, *Asteraceae*, *Boranginaceae*, *Brassicaceae*, *Colchicaceae*, *Oleaceae*, *Onagraceae*, and *Pinaceae* were the families distinctive for these samples. In particular, Hesp is linked to the *Ericaceae* plant species and is present only in **BB8** and **BB9**, which also have a significant content of phenolic acids and flavonoids.

The hierarchical clusters heat map confirms the PCA results, which highlight the discrimination of **BB2**. As seen in **Figure *10***b, the other BB samples are clustered into two main groups, corresponding to the left downside of the PCA graph and the upside.

The heat map of the PC profiles indicates a principal cluster, which corresponds to *t*-resv, CinA, My, Apig, and Gal, and the **BB2** sample, respectively. The main cluster is divided into two sub-clusters, which are distributed into other groups at the same distance.

Biological Activity of BBE

Qualitative Assessment of the Antimicrobial Activity

Antimicrobial activity was qualitatively assessed by determining the diameters of the growth inhibition zones that appeared around the spot (of BBE samples) and expressing them as mean values ± SDs (**Figure *11***).

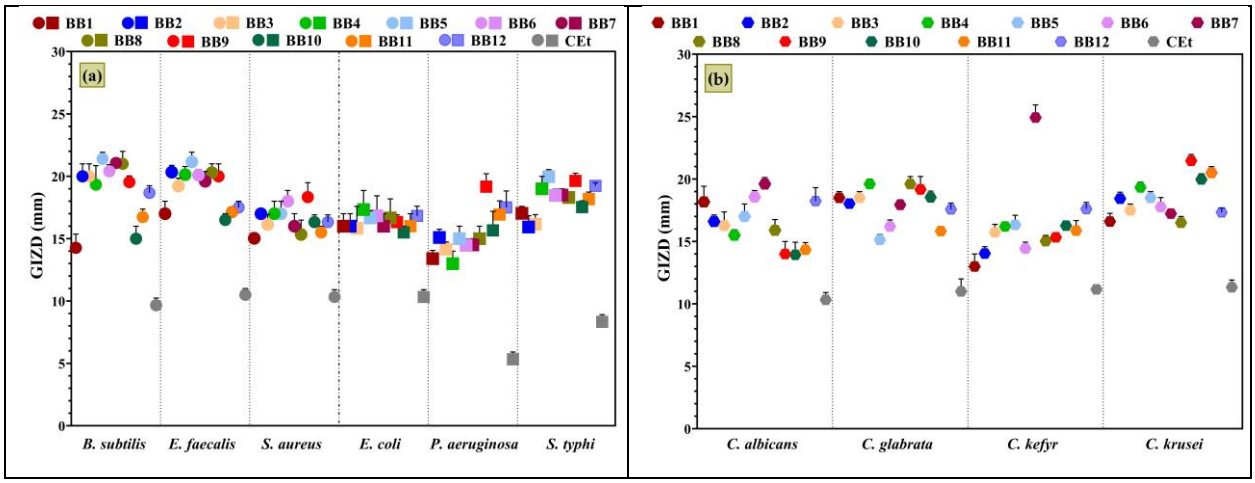

Figure 11. The growth inhibition zone diameters (GIZDs) of BBEs on selected pathogenic strains: (**a**) Gram-positive and Gram-negative bacteria; (**b**) yeasts.

BBE samples presented a significant antimicrobial effect on the growth of all microbial strains tested, and *B. subtilis*, *E. faecalis*, *S. typhi*, *C. krusei*, and *C. glabrata* were the most sensitive strains.

The extract with the highest antibacterial activity on Gram-positive bacteria was **BBE5**, followed by **BBE8**, **BBE1**, **BBE2**, **BBE6,** and **BBE9**. The data obtained are linked to the botanical origins because these samples are also clustered in **Figure *8***.

In the case of Gram-negative bacteria, **BBE5**, **BBE4**, **BBE9,** and **BBE12** showed the highest inhibitory effect. The antimicrobial profiles for yeasts differed, but **BBE7** and **BBE9** presented the most heightened sensitivity. Still, according to our knowledge, there are no data to compare our results regarding the antimicrobial activity of BBE against *C. kefyr* and *C. krusei*.

Quantitative Evaluation of Antimicrobial Activity

The MIC value was characterized by the smallest concentration of the tested BBEs that inhibited microbial development. The results are shown in **Figure *12*,** expressed as µg/mL.

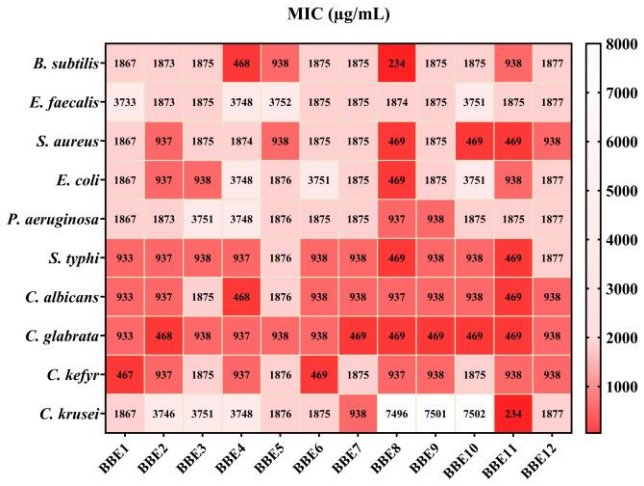

Figure 12. Quantitative evaluation of the antimicrobial activity of BBEs.

Figure *12* highlights the most significant antimicrobial activity of BBEs on the pathogenic strains. *S. typhi* and *C. glabrata* were the most sensitive tested strains and, in contrast, *E. faecalis* was the most resistant to BBE. The lowest MIC value was obtained for **BBE8**, followed by **BBE4** on *B. subtilis*. Also, the extracts showed significant inhibition of *S. aureus* development, and **BBE8**, **BBE10**, **BBE11**, **BBE2,** and **BBE12** induced the highest sensitivity.

Overall, **BBE8**, **BBE9**, **BBE2,** and **BBE4** presented the highest antimicrobial activity, correlated with chemical composition. Moreover, according to the statistical assays, the mentioned samples stood out from the others. In agreement with the PCA assays, **BBE2** was associated with precise taxonomic assignments (of plants with pollen contribution) and PCs. Also, **BBE12** is linked with FA, CA, and Qu, and with specific botanic families (**Figure *8***).

Semiquantitative assay of the microbial adherence to the inert substratum

The BBE's influence on the tested microbial strains' adherence to the inert substratum is displayed in **Figure *13***, which represents the minimal biofilm eradication concentration (MBEC) values.

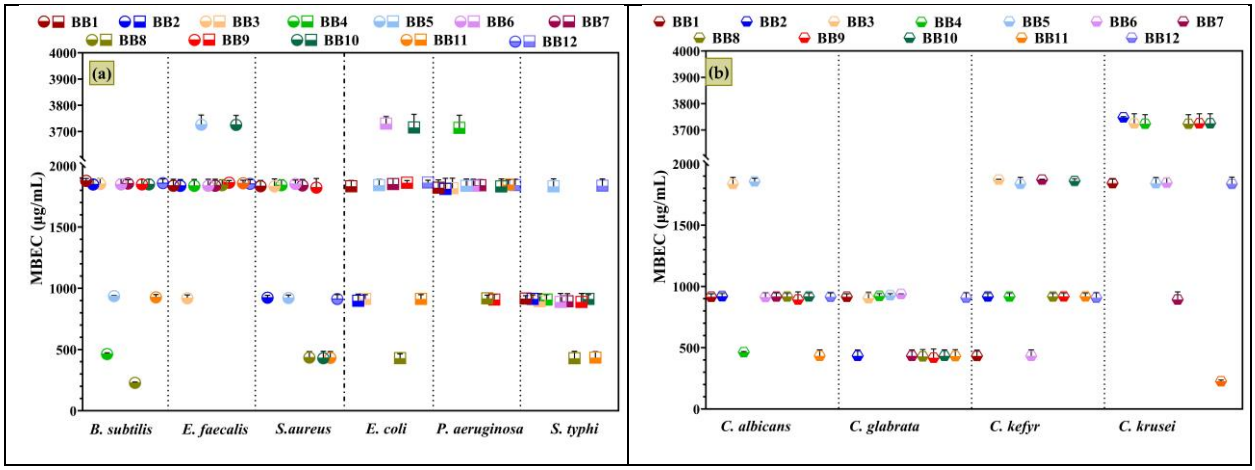

Figure 13. Graphical chart of the MBEC values: (**a**) Gram-positive and Gram-negative bacteria; (**b**) yeasts.

Figure *13*'s data confirm the qualitative (**Figure *11***) and MIC results (**Figure *12***) and are correlated with the chemical composition and botanical origin of BBs. Consequently, *S. typhi* and *C. glabrata* depicted the highest sensitivity in the presence of BBE samples. Also, BBEs significantly inhibited the adherence of *C. albicans* and *C. kefyr*. For the other strains, BBE samples showed similar antimicrobial profiles, but with some exceptions. For example, **BBE8**, with the highest TPC content, exhibited the strongest antibiofilm activity against *B. subtilis*. Also, a great inhibitory effect was displayed against *S. aureus*, *S. typhi*, *C. albicans*, and *C. kefyr*. Furthermore, **BBE2**, **BBE9,** and **BBE12**, which had a high amount of PCs, displayed significant antibiofilm effects on the tested strains.

Conclusions

Bee bread is a promising source of PCs and antioxidants. The main objective of this study was to investigate the relationship between botanical origin, chemical composition, antioxidant activity, and the effect on selected pathogenic strains.

The palynological analysis revealed a high relative abundance of pollen from plants belonging to *Salicaceae*, *Asteraceae*, *Brassicaceae*, and *Acanthaceae* families. In total, thirty-one families were identified.

The flavonoid concentrations were much higher than the phenolic acids in all bee bread extracts analyzed. The extracts contained mainly rutin, hesperidin, and resveratrol, as well as a high content of quercetin, kaempferol, isorhamnetin, and abscisic acid. There were also significant quantities of *p*-coumaric acid, gallic acid, caffeic acid, and cinnamic acid. The **BBE2**, **BBE8**, **BBE9**, and **BBE12** samples had the highest levels of phenolic acids, flavonoids and heterosides. **BBE2** and **BBE9** presented the highest concentrations of phenolic compounds, which ranged from 9209.73 and 18,889.74 μg PC/g BB.

The antimicrobial activity of bee bread extracts is strongly linked to chemical composition, antioxidant activity, and pollen botanical origin. The bee bread extracts' phenolic profiles are complex and different, and it is challenging to attribute the inhibitory growth effect to a single compound or a pollen type from a specific botanic family precisely. Furthermore, a synergistic effect between bioactive compounds is most probably responsible for the biological properties of bee bread. The bee bread extracts presented a significant antimicrobial impact on the growth of all microbial strains tested. *S. typhi* and *C. glabrata* were the most susceptible tested strains. Also, *C. albicans* and *C. kefyr* were sensitive to the influence of BBEs.

The present study reported the bee bread extracts' antibiofilm effects/ inhibitory activity on microbial adhesion capacity to the inert substratum of bacteria or fungi for the first time. Likewise, the samples (**BBE8** and **BBE9**) that had pollen grains dominant from the *Acanthaceae*, *Colchicaceae*, and *Ericaceae* botanical families presented significant quercetin and kaempferol amounts and displayed great antimicrobial effects against *P. aeruginosa* and *C. glabrata*. In addition, the sensitivity of *B. subtilis*, *S. aureus*, and *C. glabrata* is linked to pollen from *Brassicaceae* plant families (**BBE5**). Significant antimicrobial activity was correlated with pollen from plants belonging to the *Salicaceae* and *Asteraceae* families (**BBE10** and **BBE2**, respectively).

Rich in PCs and with significant antimicrobial properties, BB can be a valuable source of natural nutrients and bioactive compounds that enhance human health. Further studies should evaluate the pre- and probiotic potential of BB as well as the cytotoxic action to complement the existing data.

CHAPTER 7. Development of Magnetite-based Drug Delivery Nanocarriers

The gut microbiota dysbiosis that often occurs in cancer therapy requires more efficient treatment options to be developed. In this concern, the present research approach is to develop DDSs based on MNPs as nanocarriers for bioactive compounds. First, MNPs were synthesized through the spraying-assisted coprecipitation method, followed by loading BP or BB extracts and an antitumoral drug (5-fluorouracil/ 5-FU). The loaded-MNPs were morphologically and structurally characterized through TEM, SAED, SEM, XRD, FT-IR, DLS, and thermogravimetric analysis. UV-Vis spectroscopy was applied to establish the release profiles and antioxidant activity. Furthermore, the antibacterial and antitumoral activity of loaded-MNPs was assessed.

Materials and Methods

Materials

All reagents were purchased from Sigma-Aldrich (Darmstadt, Germany). All strains tested in this paper were provided from the Microorganisms Collection of the Department of Microbiology, Faculty of Biology of the University of Bucharest.

Synthesis of Magnetic Nanoparticles and Loading with Bioactive Compounds

The spraying-assisted coprecipitation method synthesized the Fe3O4 NPs stabilized with anhydrous trisodium citrate [75, 76]. After that, 1 g of the previously obtained magnetic NPs were loaded with 10 mL BPEs/ BBEs by grinding until the solvent was evaporated. The BPEs and BBEs used were previously characterized [46, 77]. The 5-FU was solubilized in ethanol and loaded on the surface of the Fe_3O_4@BPEs/ BBEs following the same method.

Results and Discussions

Magnetic-Based Systems Characterization

In the present study, the synthesized citrate-coated MNPs were first loaded with the BPEs and BBEs, which presented the highest concentration of PCs and antioxidant and antimicrobial properties determined previously [46, 77].

Transmission Electron Microscopy

The morphology of the citrate-coated MNPs was evaluated through the TEM, HR-TEM, and SAED assessments (**Figure *14***). As can be observed, the NPs have a quasi-spherical shape and possess an increased tendency to agglomerate due to magnetic dipole moment interaction between particles. The distributions are considerably narrow, suggesting that the size of the magnetic nanoparticles is between 5 and 10 nm (average size particle of 7.0 ± 2 nm), which could

further demonstrate their usability in the desired applications. Furthermore, the SAED results confirm that patterns are linked with the Miller indices characteristic for Fe_3O_4 [78, 79].

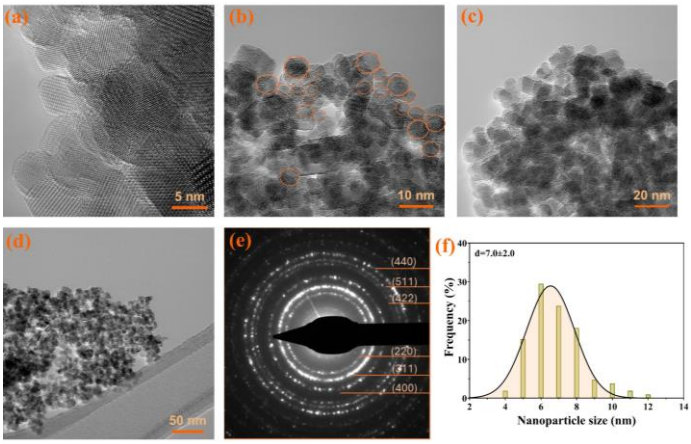

Figure 14. The TEM images, SAED diffraction pattern, and size distribution for citrate-coated Fe_3O_4 NPs

X-ray diffraction

The structure of the obtained nanocarriers was determined via X-ray diffraction, which confirmed the cubic structure of MNPs for all samples (**Figure 15**). Consequently, adding bioactive compounds from extracts and 5-FU does not conduct the formation of secondary iron oxides.

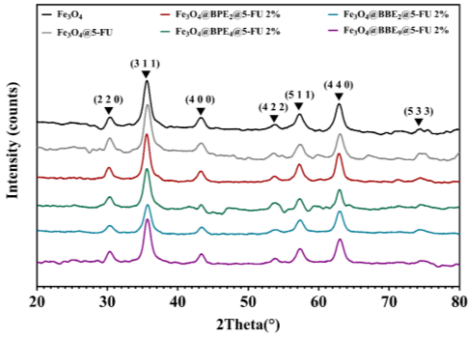

Figure 15. Diffractograms for magnetite and loaded-MNPs

Figure *15* displays the characteristic peaks of tetrahedral and polyhedral magnetite crystals [75, 80]. The crystallite sizes of developed MNPs range from 6.19 ± 0.89 to 8.31 ± 2.49 nm. Based on the presented results, it can be concluded that the ethanol used in the extract preparation and for 5-FU solubilization did not influence the magnetic structure of the MNPs.

FT-IR Spectroscopy

The FT-IR analysis was performed to establish the bonds and functional groups present within the obtained MNPs.

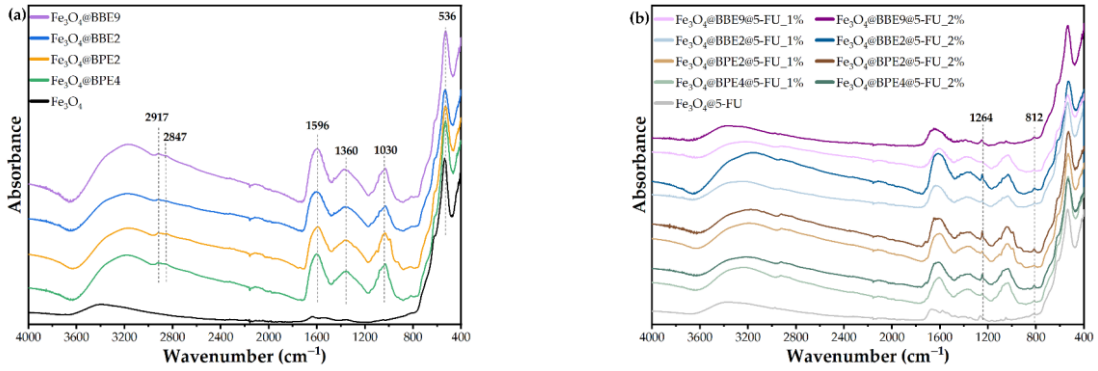

Figure 16. FT-IR spectra for magnetite and loaded-MNPs

The FT-IR spectrum of the bare Fe_3O_4 sample (**Figure 16a**) exhibits distinctive peaks consistent with the molecular structure of magnetite (Fe_3O_4). The prominent absorption band at 536 cm^{-1} indicates the Fe–O bond stretching vibrations, a defining characteristic of magnetite. Moreover, the broad peak centered around 3400 cm^{-1} is attributed to O–H stretching vibrations, likely from absorbed water or hydroxyl groups on the sample's surface. Fe_3O_4 NPs coated with BPEs and BBEs similarly displayed the characteristic peaks of Fe–O stretching vibrations at the same wavelengths. Nevertheless, the broad peaks attributed to O–H vibrations shifted to shorter wavenumbers, around 3180 cm^{-1}, and could be ascribed to O–H stretching vibrations from both the extracts and the residual water molecules on the surface of the NPs. Precisely, additional peaks in the coated nanoparticles arise from specific functional groups present in the BP and BB extracts, such as at 2917 cm^{-1} and 2847 cm^{-1}, corresponding to symmetric and asymmetric stretching of C–H groups present in carbohydrates and lipids or the large peak at 1030 cm^{-1} corresponding to C–O and C–OH vibrations in carbohydrates and PCs [74].

Concerning the samples loaded with 5-FU, **Figure 16b** shows the preservation of the above-mentioned signals, common to magnetite, BP, and BB extracts. Absorption bands at 1264 cm^{-1} and 812 cm^{-1}, corresponding to C–H stretching (in-plane) and C–F stretching in 5-FU [81], were observed in the magnetite sample loaded with the active substance (Fe_3O_4@5-FU). The signals were further distinguished for the drug-loaded samples coated with the extracts, and it was noticeable that the intensity of these bands varied with concentrations, visibly more intense in the case of samples loaded at higher concentrations of 5-FU (2% versus 1%).

DLS assay

The stability of the nanocarriers was evaluated through zeta potential, hydrodynamic diameter, and polydispersity index (PDI) measurements (**Figure 17**). The hydrodynamic diameter increases when it is loaded with 1% 5-FU but decreases when it is added with 2% anticancer agent. It can be assumed that the 5-FU is hydrophilic, and its addition at small concentrations increases

the MNPs' hydrophilicity. In contrast, 5-FU overlaps the functional groups from BPEs/ BBEs at higher concentrations and decreases the interaction with the solvent (PBS). For this reason, the Fe_3O_4@5-FU 2% sample presented a higher hydrodynamic diameter value. According to zeta potential values for Fe_3O_4@5-FU 2%, Fe_3O_4@BBE_2@5-FU 1%, and Fe_3O_4@BBE_9@5-FU 1% and to the higher hydrodynamic diameter values may be due to the agglomeration of NPs. Considering the PDI values that are lower than 0.6, they indicate a thin/ narrow particle distribution. The smaller PDI values indicate the long-term stability and uniformity [82-84].

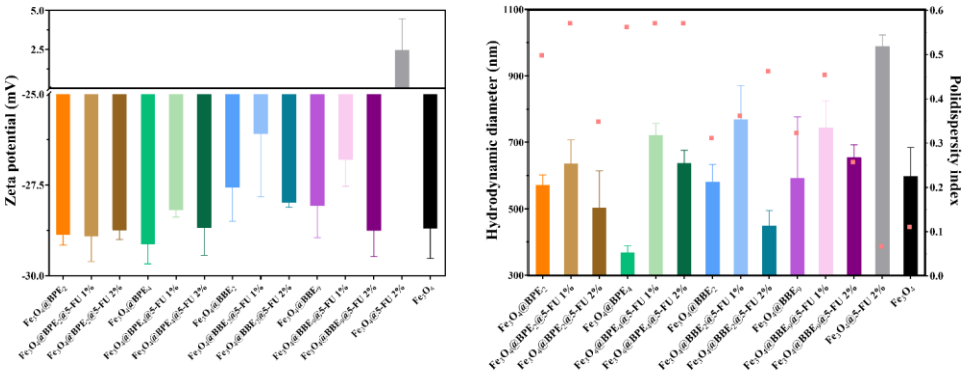

Figure 17. The zeta potential, hydrodynamic diameter (illustrated as columns), and the PDI (illustrated as dots) values for obtained MNPs

Bioactive Agents Release Behavior

The drug and PCs release profiles (**Figure 18**) were assessed in PBS (pH = 7.4) at 37°C.

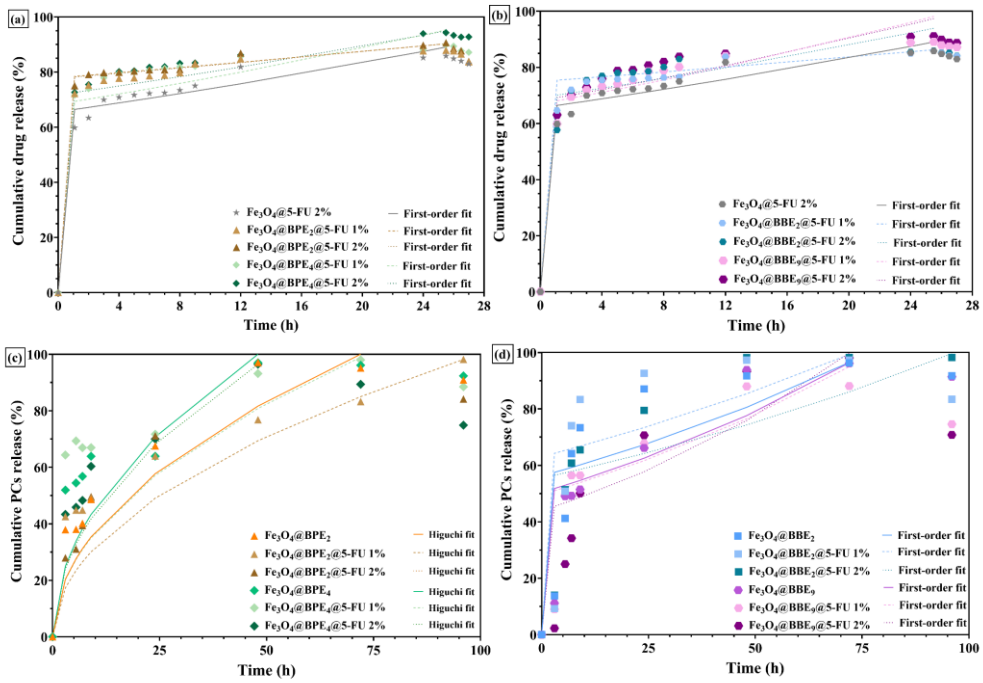

Figure 18. Antitumoral drug and PCs release profiles of nanocarriers loaded with BPEs (a,c) and BBEs (b,d).

The 5-FU drug release profiles of nanocarriers loaded with BPEs and BBEs showed that the antitumoral drug releases rapidly in the first hour but at a lower rate for samples without extracts (**Figure 18 a** and **b**). Also, it can be observed that in two hours, the 5-FU has a maximum/ fast release rate (~75% released), followed by a plateau. The nanocarriers with 5-FU and extracts showed a higher percentage of drug released after 24-25.5 h, and a slower release of drug molecules/ degradation can be observed immediately. It could be assumed that biomolecules from extracts facilitated the antitumoral drug to release in higher amounts in the first hours, which can be helpful for the death of tumoral cells [85, 86]. Conversely, the PCs' release profiles (**Figure *18* c** and **d**) illustrate similar patterns with prolonged release rates. In both cases, the PCs are gradually released from MNPs, reaching a plateau after 48 h, maintained up to 96 h, followed by a decrease that suggests the PCs' degradation. The higher release time of PCs compared to 5-FU is attributed to the flavonoids in extracts.

Antioxidant Activity

Different concentrations of MNPs were assessed to establish the antioxidant concentration that scavenges 50% of the initial DPPH radical, which is known as IC_{50} (**Figure 19**). The lower IC50 values correspond to higher antioxidant activity [87].

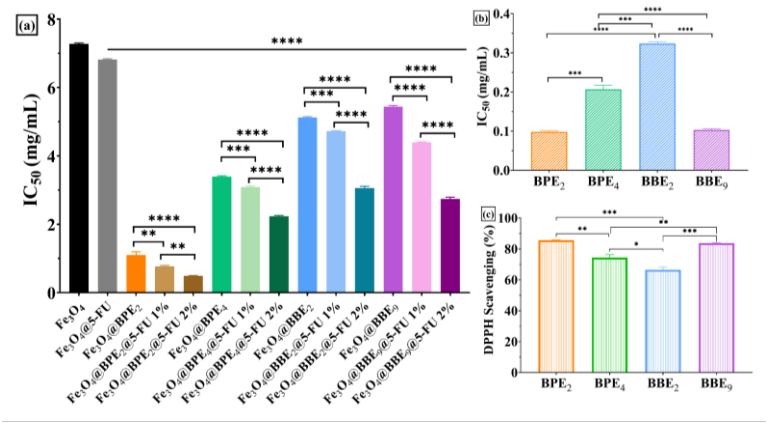

Figure 19. Antioxidant activity of developed MNPs through DPPH assay. (a) IC_{50} values for nanocarriers, (b) IC_{50} values for extracts and (c) DPPH% for extracts.

The developed MNPs presented the capacity to scavenge the DPPH radical, as expected, considering the previous studies on BPE and BBE antioxidant activity (TEAC method) [46, 77]. **Figure *19*** highlights the lower IC_{50} values for MNPs loaded with BPEs and BBEs than Fe_3O_4@5-FU 2% or Fe_3O_4 samples, which indicate that obtained nanocarriers are more potent antioxidants that could induce lower toxicity for patients [88]. Additionally, **Figure *19*a** displayed a decrease of IC_{50} values simultaneously with the concentration of 5-FU loaded on the MNPs, which implies a synergic effect between the antitumoral drug and PCs from extracts. Similar results are reported for Fe_3O_4 loaded with PCs (gallic acid, quercetin, plant extracts) [88-90].

Antibacterial Activity

Minimum Inhibitory Concentration Assay

Secondly, the qualitative antimicrobial assessment was continued with a qualitative assay by determining the MIC values (**Figure 20**).

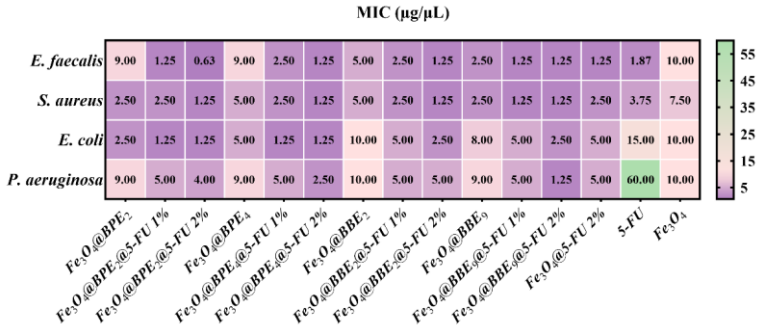

Figure 20. Heat map of MIC values. Data results marked in intense purple indicate the significantly lowest MIC values and highest sensitivity of strains.

The qualitative data were confirmed by MIC assay, and Gram-positive bacteria represented the most sensitive strains. In contrast, MNPs with BBEs presented moderate inhibition on Gram-negative bacteria, and *P. aeruginosa* was the most resistant. Additionally, for most of the MNPs, the MIC values were lower than Fe_3O_4@5-FU 2% and 2% 5-FU. The addition of extracts significantly inhibited the development of the strains, which can be explained by the antioxidant activity of the BPEs and BBEs, as well as by the PCs' release behavior. From our knowledge, there are no references to compare our results regarding the positive impact of MNPs on the antibacterial properties of antineoplastic agent, especially the synergic effects between extracts, 5-FU, and MNPs.

Semiquantitative Assay of the Bacterial Adherence to the Inert Substratum

Additionally, the influence of loaded-MNPs on the pathogenic strains' adherence to the inert substratum was assessed. The MBEC values were graphically represented in **Figure 21**. The antibiofilm data confirms the qualitative and quantitative/ MIC results (**Figure 20**). Therefore, *S. aureus* and *E. faecalis* were the most sensitive strains in the presence of developed MNPs. Likewise, loaded-MNPs significantly inhibited the adherence of *E coli* and had moderate activity on *P. aeruginosa*.

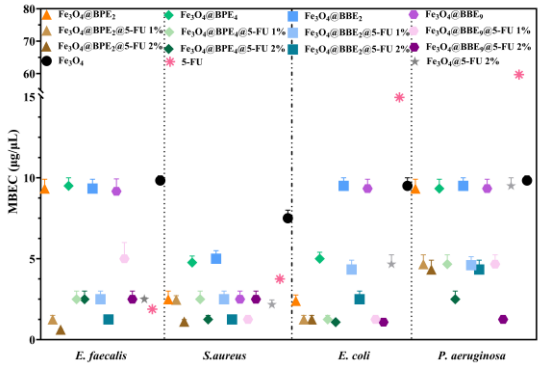

Figure 21. Graphical chart of MBEC values: (a)- *E. faecalis*, (b)- *S. aureus*, (c)-*E. coli*, (d)-*P. aeruginosa*.

The BPEs-loaded MNPs presented similar antibacterial profiles, and the strongest antibiofilm activity was exhibited by MNPs loaded with BPE_4. Regarding these MNPs properties, it could be explained as the improvement of 5-FU anti-adherence activity. However, according to our knowledge, there are no reference data to compare our results on the enhancement of 5-FU antibacterial activity when is loaded on the MNPs surface with BPEs or BBEs.

Influence of bioactive compounds against a probiotic bacteria

The effect on the growth of *L. rhamnosus* MF9 under the influence of loaded-MNPs after 24 h is represented in **Figure 22**.

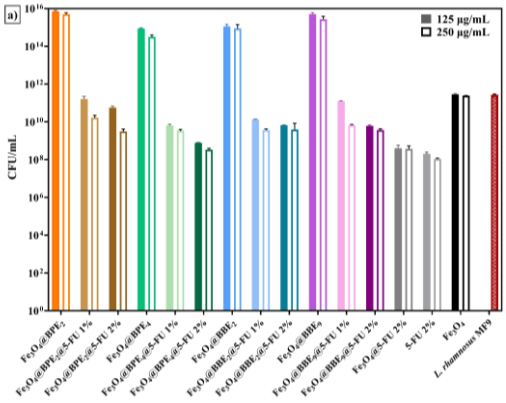

Figure 22. The influence of the MNPs on the growth of *L. rhamnosus MF9* (after 24h). a) Graphic representation of CFU/mL values

The Fe_3O_4 NPs do not influence the growth of *L. rhamnosus* MF9 but are significantly stimulated (more than four logarithmic units) by all BPEs and BBEs-loaded MNPs (**Figure 22a**). Also, **Figure *22*a** confirms the impact of 5-FU on the probiotic bacteria's growth. At 125 µg/mL concentrations of MNPs, the *L. rhamnosus* MF9 growth was negatively influenced by the samples with 2% antineoplastic agent, less Fe_3O_4@BPE_2@5-FU 2%.

According to antioxidant properties, the MNPs loaded with 5-FU 1% showed the highest antioxidant activity, and it could be correlated with the capacity of these samples to alleviate the

oxidative stress induced by the anticancer drug against gut microbiota. In addition, the prolonged PCs' release profiles (**Figure 18c** and **d**) suggest an enhanced possibility of microbiota recovery.

The Antiproliferative Assay

The *in vitro* tumor cell viability and proliferation of HT-29 cells in the presence of synthesized MNPs were examined using an MTT assay (**Figure 23**).

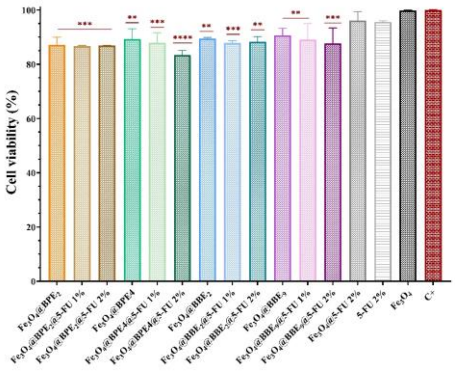

Figure 23. The influence of loaded-MNPs on the cell viability, and the proliferation of the colorectal tumoral cells (C+).

Figure 23 displays the significant differences between magnetite and loaded-MNPs. Fe_3O_4@BPE@5-FU/ Fe_3O_4@BBE@5-FU and Fe_3O_4@BPE/Fe_3O_4@BBE samples present a lower cell viability than Fe3O4@5-FU 2%. Likewise, adding 5-FU improves the inhibition of tumoral cell proliferation, which could be explained by a synergic inhibitory effect between PCs from extracts and 5-FU. Otherwise, this study aimed to investigate the effects of 5-FU and extracts, especially their influence on gut microbiota. For this reason, it used small concentrations of 5-FU and MNPs, and it does not expect significant antitumoral activity.

Conclusions

The present study aimed to develop drug delivery systems based on citrate-stabilized MNPs, which act as nanocarriers for PCs from extracts and as an anticancer drug. Results demonstrated the achieving of MNPs with narrow size distributions and an average of NPs sizes ~ 10 nm. Moreover, it was shown that the antitumoral drug and PCs had fast release rates in the first 24 h from MNPs. Furthermore, the PCs had a prolonged release behavior, and loading MNPs with extracts increased the antioxidant and antibacterial properties. Additionally, the non-toxic properties of magnetite and prebiotic potential of developed DDS on *L. rhamnosus* MF9 were shown, which underline the role of the antioxidants (PCs) intake on gut microbiota. The moderate antiproliferative activity on a colorectal tumoral cell line at small concentrations of MNPs and 5-FU suggests the potential of DDS to alleviate the adverse effects of anticancer drugs and improve the gut balance.

CHAPTER 8. General Conclusions and Originality

General conclusions

The thesis examines the foremost pathologies caused by intestinal microbiota imbalances, especially in the case of colorectal cancer. Also, strategies to improve the treatment efficiency for colorectal cancer and the maintenance/ recovery of the intestinal microbiota are studied. The specified objectives/ purposes within the thesis were organized to focus on any limitations present in previous studies or, on the other hand, to introduce original notions that were not found earlier through the literature review.

The PhD thesis was structured in primary parts and, afterward, divided into different chapters. The first part is focused on an extensive review of recent scientific literature data, intending to introduce the thesis's primary subject, specifically the impact of gut microbiota on nutrition and human health. The main approaches for gut microbiota modulation and the advantages of natural compounds and nanotechnology in CRC treatment were also discussed. The second part of the thesis presents the original/ experimental part, enumerating the objectives and their fulfillment by displaying the obtained results as published articles.

Chapter 1 presented a summary of human microbiota, emphasizing the importance of the determinant factors that significantly influence microbiota balance, especially by GIT dysbiosis.

Chapter 2 illustrated an overview of the synthesis, characterizing, and biomedical applications of MNPs, respectively, and the capacity to use them as nanocarriers for biologically active compounds.

Chapter 3 underlined the traditional/ conventional drugs for CRC treatment and associated effects. Moreover, it presented the potential of natural compounds and nanotherapeutics for improving efficacity, alleviating adverse effects, and reducing the chemoresistance of antitumoral drugs.

The second part presented the main stated objective of the thesis, which is divided into three parts (Chapters 5-7): (i) obtaining bee pollen extracts and evaluating their chemical composition, antioxidant, and biological properties; (ii) obtaining bee bread extracts and assess their chemical, antioxidant and antimicrobial properties, and (iii) developing DDSs based on MNPs as nanocarriers for bioactive compounds.

Chapter 5 established five raw bee pollen samples' chemical composition and biological properties. Besides, antimicrobial and antitumoral properties were studied in the prebiotic effects/ the capacity of bee pollen extracts rich in PCs and fatty acids to modulate the development of two

probiotic strains, which indicate the potential of BPEs to maintain and enhance the recovery of gut microbiota.

Chapter 6 investigated the relationship concerning the botanical source, chemical composition, antioxidant activity, and antimicrobial influences on tested pathogenic strains of twelve bee bread samples, which denoted that BB is a promising source of PCs and natural nutrients that exhibited significant antimicrobial properties.

Chapter 7 aimed at the development of DDSs based on citrate-coated MNPs, which acted as nanocarriers for PCs from BBEs/ BPEs and 5-fluorouracil. This study closely connects the previous chapters because it valorizes/highlights the therapeutic potential of extracts rich in bioactive compounds. Furthermore, the developed nanocarriers presented the capability to alleviate adverse effects induced by 5-FU through prolonged release of PCs that stimulated the development of lactic bacteria.

Original contributions and perspectives

The novelty of the first study involved the synergistic antimicrobial effect of BPEs and soluble compounds of lactic strains (*L. rhamnosus* MF9 and *E. faecalis* 2M17) against the adhesion capability of selected clinically isolated - pathogenic strains (*C. guillermondii* and *Enterobacter cloacae*). Moreover, the inhibitory effects of BPES on the ability of microbial strains to attach to the cellular substrate (Hep-2 cell lines) were first reported. Overall, the BPEs significantly inhibited the adherence of potentially pathogenic strains tested (*E. faecalis* ATCC 19433, *S. aureus* ATCC 25422, *E. cloacae*, *P. aeruginosa* ATCC 25785, *C. albicans* 1688, *C. famata*, *C. glabrata*, *C. krusei,* and *C lusitanie*). Considering the richness of BP in PCs and fatty acids, and the relationship between antioxidant activity, chemical composition, cytotoxic effects on a tumoral cell line, the prebiotic effect, and the impact on pathogenic strains, BP has a potential prebiotic and antitumoral agent for the GIT microbiota.

Perspective: developing DDSs by incorporating/ loading BPEs in nanomaterials, such as porous silica, magnetite, pH-sensitive polymers, etc., and guarantee a target delivery at the desired tissue (gastrointestinal tract, for example).

The second objective was based on the correlation between antimicrobial and antioxidant properties, chemical composition, and palynological analysis of BB samples. This study's novelty involves the inhibitory effects of BBEs against microbial adhesion ability to the inert substrate. Moreover, the BBEs induce antifungal sensitivity, and for *C. krusei* and *C. kefyr*, it is reported for the first time.

Perspectives: evaluation of the prebiotic and probiotic potential of BB, as well as the antitumoral properties, respectively. The second perspective consists of designing intelligent DDSs, which act as nanocarriers, and utilizing combined and targeted therapy for bioactive compounds from BBEs in several disorders (chronic inflammation, IBD, cardiovascular, gut dysbiosis, etc.).

The novelty of the last objective involved the development of magnetite-based drug delivery nanocarriers to enhance the antitumoral efficacity of 5-FU and diminish the adverse effects against gut microbiota. Besides, designing the DDS highlighted the antibacterial, prebiotic, and antiproliferative activities of BPEs and BBEs. Also, MNPs enhance the antimicrobial activity of bioactive compounds. Moreover, another element of novelty consists of the sensitivity of bacterial strains to adhere to the inert substratum induced by the developed nanocarriers.

Perspectives: Optimizing the dosage of the 5-FU and BPEs/ BBEs formulations with the best release, antioxidant, and antimicrobial profiles in order to conduct *in vivo* assessments. Another perspective represents using magnetically developed nanocarriers in targeted therapy by applying an external magnetic field at different frequencies and with a controlled release of bioactive compounds in the target tissue/ tumor. Additionally, another viewpoint is to evaluate the anti-inflammatory properties of developed nanocarriers.

Furthermore, due to the high levels of PCs of bee products and their biological associated effects, another perspective is to develop DDS based on MNPs with pure/ synthetic PCs and antitumoral drugs with the purpose of obtaining more efficient and reproducible pharmaceutical formulations.

List of publications (As the first author and related to the thesis topic):

1. **C.-I. Ilie,** E. Oprea, E.-I. Geana, A. Spoiala, M. Buleandra, G. Gradisteanu Pircalabioru, I. A. Badea, D. Ficai, E. Andronescu, A. Ficai, L.-M. Ditu, *Bee Pollen Extracts: Chemical Composition, Antioxidant Properties, and Effect on the Growth of Selected Probiotic and Pathogenic Bacteria*, Antioxidants **2022**, 11, 959. https://doi.org/10.3390/ antiox11050959, **Q1, IF = 7.00.**
2. **C.-I. Ilie**, A. Spoiala, E.-I. Geana, C. Chircov, A. Ficai, L.-M. Ditu, E. Oprea, *Bee Bread: A Promising Source of Bioactive Compounds with Antioxidant Properties — First Report on Some Antimicrobial Features*, Antioxidants **2024**, 13, 353. https://doi.org/10.3390/antiox13030353, **Q1, IF = 6.00**
3. **C.-I. Ilie**, A. Spoiala, C. Chircov, G. Dolete, O.-C. Oprea, B.-S. Vasile, A. S. Crainiceanu, A.-I. Nicoara, I. C. Marinas, M. S. Stan, L.-M. Ditu, A. Ficai and E. Oprea**,** *Antioxidant, Antitumoral, Antimicrobial and Prebiotic Activity of Magnetite Nanoparticles Loaded with Bee Pollen/ Bee Bread Extracts and 5-Fluorouracil*, Antioxidants **2024**, **Q1, IF = 6.00**
4. **C.-I. Ilie**, A. Spoială, D. Ficai, A.-I. Nicoară, O.-C. Oprea, V.-A. Surdu, R. D. Trușcă, E. Andronescu, L.-M. Dițu, A. Ficai - *Magnetic platforms based on magnetite and polyphenols with antimicrobial activity*, U.P.B. Sci. Bull., Series B, Vol. 84, Iss. 4, 45-58, **2022**

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