**ABSTRACT**

Bee collected pollen (BCP) is a well-known functional food, used by consumers as a nutritional supplement and also by apitherapists for its beneficial biological properties.

Bee bread (BB) is produced via solid state fermentation of BCP, which takes place in the cells of the honeycomb where BCP is stored. It is the main source of proteins, fats, vitamins and minerals for the bees and, like BCP, it also contains a plethora of phytochemicals (polyphenols, organic acids, fatty acids, phytosterols etc). BB is also considered a functional food with therapeutical applications in folk medicine and apitherapy.

Research on BB has been rather limited until now but research interest is growing regarding the antimicrobial properties of both BCP and BB, due to emerging microbial resistance to antibiotics. Both BCP and BB exert antimicrobial properties against various pathogens, such as bacteria and fungi. Like other bee products, the lack of antimicrobial resistance in BCP and BB can be attributed to the synergism of more than one antimicrobial compounds within these products. BCP and BB exert targeted action against pathogens and affect the host microbiome in a prebiotic manner.

The antimicrobial, antioxidant and nutritional properties of BCP and BB, are directly related to their composition, which is highly dependent on their botanical origin. Greek flora is renowned for its high biodiversity, representing 6,760 taxa, 1,442 of which endemic. The foremost aim of the research presented in this thesis, was to assess for the first time the bioactivity of Greek BB samples collected from different parts of Greece and further venture to correlate them to botanical origin.

In this study, 18 BB samples were collected during different seasons from different locations in Greece, as well as 2 BCP samples and their botanical origin was determined before bioactivity was tested. Of note, BB and BCP samples were used as mere water suspensions in order to assess the properties they exhibit when consumed as food (true bioavailability/true bioactivity).

Samples were analyzed for their antibacterial properties, antioxidant activity, total phenolic content (TPC) and total flavonoid content (TFC). The antimicrobial activity of each sample was tested against *Staphylococcus* *aureus*, *Pseudomonas* *aeruginosa*, *Klebsiella* *pneumoniae* and *S. enterica* *ser*. Typhimurium. The results showed that all samples exert inhibitory and most of them bactericidal activity against at least two pathogens.

In addition, all samples demonstrated significant antioxidant activity with the *Castanea sativa* monofloral sample exhibiting the highest antioxidant values. Interestingly, no strong correlation was observed between antioxidant and antimicrobial activity, but statistical analysis revealed corelations between specific plant families and antimicrobial - antioxidant activity.

BB samples were further assessed for their ability to eradicate or inhibit the formation of biofilms, which are estimated to be involved in 80% of human body infections. BB samples tested against *Staphylococcus* *aureus* and *Acinetobacter* *baumanii* showed significant ability to both inhibit the formation and eradicate biofilms. The inhibition of biofilm formation depends on BB concentration and tested pathogen. Overall higher inhibition rates are observed against *A.* *baumannii*. For the eradication of *A.* *baumannii* biofilm, higher BB concentrations are required compared to those required for the eradication of *S. aureus* biofilm.

Antiviral activity of BB samples was tested *in vitro* against Enterovirus D68. A cell culture assay coupled with comparative real-time PCR analysis was performed using different sample concentrations to assess antiviral activity. The MTT method was applied to calculate sample toxicity levels in cell culture. The results demonstrate that greek BB and BCP exhibit strong antiviral activity against EV-D68, with IC50 values ranging from 0.048 to 5.45 mg/ml. This was the first research worldwide to investigate the antiviral activity of BCP and BB and, following these encouraging results, we proceeded to assess the antiviral activity of BCP, BB and artificially fermented pollen against influenza A virus (IAV) H1N1.

In this second experiment, the protein, aqueous and n-butanol fractions of the samples were also tested to shed light into the nature of the antiviral agents. In addition, the antiviral activity of artificially fermented bee pollen with microorganisms isolated from BB, was evaluated. Antiviral activity against IAV (H1N1) was evaluated *in vitro* by cell culture assay and comparative real-time PCR analysis. IC50 values ranged from 0.022 to 10.04 mg/ml and selectivity index (SI) values ranged from 1.06 to 338.64. Artificially fermented bee pollen samples AF5 and AF17 exhibited higher SI values than unfermented pollen, and protein fractions showed the highest SI values. In addition, chemical profiling of BCP and BB samples, analyzed using NMR and LC-MS, revealed the presence of specific metabolites (flavonoids) that may contribute to antiviral activity. The results indicate that the significant anti-IAV activity of the tested products could be attributed to chemical composition (particularly to yet unidentified protein compounds) and possibly to substances produced by the microbiome. Further research into the antiviral properties of Greek BCP and BB will further elucidate the mode of action and could lead to new treatments against IAV or other viral diseases.

The antimicrobial activity of strains isolated from BB microbiome, inoculated into double pasteurized pollen, as raw material for artificial solid state fermentation, was also evaluated. The technique of double pasteurization, with an intermediate incubation step, was chosen in order to eliminate the sporogenic bacteria, and this is the first research to use this technique in BCP fermentation.

The aim of the experiment was to produce a food with better antimicrobial activity than the original BCP and at the same time το reveal possible contribution of BB microbiome to antimicrobial activity. Artificially fermented pollen regained its antibacterial activity against pathogens which was lost after double pasteurization, indicating that BB microbiome contributes to antimicrobial activity. The type of starter culture and incubation conditions both influence the antibacterial activity of artificially fermented pollen.

Finally, in terms of bioactivity, artificially fermented BCP with BB microorganisms was evaluated for the first time, compared to the BCP that served as the raw material for fermentation, on the longevity of bees reared in cages in the laboratory, with promising results.

Apart from bioactivity, an equally important research objective of this thesis was the investigation of BB microbiome, during maturation (BCP from pollen trap, and 1- day, 11 -day, 27- day and 41- day-old BB) and to identify the most relatively abundant microbial groups using both Next Generation Sequencing (NGS) and microbial culture techniques. There are few studies on BB microbiome despite the abundant evidence regarding its biosynthetic and antimicrobial activity.

Our data demonstrate that BCP and BB microbiomes include both environmental and bee microbial communities. At the beginning of BB maturation microbial diversity is greater. Bacterial diversity increases steadily from day 1 to day 27, then gradually decreases (day 27 to day 41). The diversity of fungi gradually decreases from day 1 to day 27. These findings indicate that during BB maturation, microbial diversity fluctuates dynamically resulting in a rather stable microbial community structure in mature BB. During maturation, changes that affect microbial growth (production of microbial metabolites, microaerophilic or anoxic conditions, acidity, variable nutrient substrates and water availability), may occur, favouring specific communities over others.

Proteobacteria are the dominant phylum in BB representing over 60% of the total unique operational taxonomic units (OTUs). Their abundance increased rapidly within 24 h, reaching nearly 70%, and gradually decreased from day 11 to day 41, returning to approximately the initial relative abundance. The relative abundance of Firmicutes, which was the second most abundant phylum, increased by up to 20% in mature BB. The genus *Lactobacillus* increased rapidly within the first 24 hours, indicating the contribution of lactobacilli to the fermentation process especially in the early stages. It is worth noting that lactobacilli are also found in the mature 41-day-old BB, testifying to their adaptation to this environment. The activity of lactobacilli is important for BB preservation due to their antimicrobial activity.

Regarding fungi, Ascomycota was the most abundant phylum, accounting for almost 100% of the relevant OTUs in 27-day-old MPS. Zygosaccharomyces was the most abundant genus accounting for 99.58% of the relevant OTUs in 27-day-old MPS. Basidiomycota is the second most abundant fungal group, which gradually decreased from 20% of the relevant OTUs in 1-day pollen to 0% in 27-day BB.

Bacterial strains isolated from BB were tested for their antibacterial activity against the pathogens *S. aureus*, *P. aeruginosa*, *S.* Typhimurium and *K. pneumoniae*. Of 309 bacterial isolates, 42 showed antibacterial activity against MRSA strain 1552 of S. aureus, 34 against carbapenem-resistant P. aeruginosa strain 1773, 47 against *S*. Typhimurium and 43 against *K. pneumoniae*. Four isolates exerted antibacterial activity against two of the pathogens, 38 against three pathogens and 23 against all four pathogens. Regarding yeasts, strains of *Z.* *siamensis* showed antibacterial activity against *S.* Typhimurium. Strains that exerted antibacterial activity against at least two pathogens and simultaneously produced various enzymes were selected for identification. Seven strains that exerted antibacterial activity against one pathogen and three strains that exerted no antibacterial activity but were able to produce various enzymes were identified.

*Apilactobacillus* and *Fructobacillus* strains exerted high antibacterial activity against *Gram-*negative pathogens but lower antibacterial activity against *Gram-*positive *S. aureus*. *Bacillus* isolates showed antibacterial activity mainly against *S. aureus*, although some exerted antibacterial activity against *Gram*-negative pathogens.

Bacteria isolated from BB showed a positive phenotype regarding the secretion of cellulases, amylases and proteinases. Furthermore, this is the first study to reveal positive phenotypes regarding the secretion of hemicellulases, Coomassie Brilliant Blue G or R dye-degrading enzymes, and Malachite Green dye-degrading enzymes from bacteria isolated from BB. Of 309 bacterial strains isolated from BB, 41 produced hemicellulases, 13 cellulases, 39 amylases, 132 proteinases, 85 Coomassie brilliant blue G or R dye-degrading enzymes, and 72 Malachite Green dye-degrading enzymes. All identified strains produced more than one of the above enzymes.The production of lignin-degrading enzymes (laccases or oxidases) by BB bacteria provides them with the ability to degrade exine (the outer layer of pollen grains). Similarly, the production of cellulases is crucial for the degradation of intin (the inner layer of pollen grains) which is mainly made of cellulose. The ability of BB bacteria to break down pollen grain walls facilitates the release of nutrients and bioactive compounds contained in pollen grains.

Finally, taking into account that bees have been facing multiple challenges, from climate crisis to pesticides and pathogens such as *Vairimorpha (Nosema) ceranae*, we tested whether any alterations in the structure and abundance of microbial communities in honeybee gut and BB microbiomes could serve as biomarkers of honey bee survival. The study implemented a meta-taxonomic approach to investigate whether *V. ceranae* affects the gut microbiome (bacteria and fungi) of adult *A. mellifera* honeybees as well as the BB microbiome stored in *A. mellifera* colonies experiencing severe *V. ceranae* infection (>2,500,000 spores per bee) compared to colonies showing very low spore counts (<40,000 per bee). The analysis revealed an overall decrease in microbial diversity reflected in the number of observed OTUs, both in bacteria and fungi, in both bee gut samples and BB samples. Further analysis showed that *Podosphaera* spp. was absent in BB samples collected from colonies with high spore counts, while the relative abundance of *Blumeria* spp. decreased significantly. Interestingly, the relative abundance of *Rosenbergiella* spp. increased in BB samples collected from colonies with high spore counts. Although further research is needed, overall reduced microbial diversity and relative abundance of certain microbial groups may serve as biomarkers of bee colony collapse.

In conclusion Greek BB is a highly bioactive functional food which can also serve as a source of novel antibacterial and antiviral compounds. BB can form the basis for producing innovative functional foods or drinks. Furthermore, the artificial fermentation of BCP with BB microorganisms is an important achievement on its own, since BB is much harder to collect than BCP. Artificially fermented pollen could further be used for the development of innovative functional foods/drinks and also prebiotic/probiotic preparations for honey bees that could enhance bee immunity, health and survival.