Abstract

Honey is a natural product that is consumed all over the world, due to its beneficial properties such as the high nutritional value and the positive effects on human health. The antibacterial activity of honey is of high importance and has been confirmed by numerous studies.

It has been suggested that microorganisms commonly detected in honey, contribute to its overall antimicrobial activity, due to production of antimicrobial compounds. The isolation and characterization of bacterial colonies from 46 Greek honey samples of diverse botanical and geographical origins as well as the determination of the antibacterial activity against five important nosocomial and foodborne pathogens is presented and discussed in **Chapter 2**. In total, 2014 bacterial colonies were isolated and then tested for their antibacterial potential. 16% of the isolated colonies inhibited the growth of *Staphylococcus aureus*, 11.2% exhibited inhibition of *Pseudomonas aeruginosa* and *Acinetobacter baumannii*, 10.2% inhibited the growth of *Salmonella* Typhimurium and 12.4% inhibited the growth of the pathogenic bacterium *Citrobacter freundii*. In total, 316 of the isolated colonies that inhibited the growth of more than two of the tested pathogens, were grouped by Restriction Fragment Length Polymorphism (RFLP) analysis, according to the restriction profile of their amplified 16S rRNA gene. Fifty of these colonies were identified by 16S rRNA gene sequencing. The majority of these isolates (62%) were identified as *Bacillus* spp. Only 10% of them were found to be Gram-negative bacteria. In addition, genes encoding polyketide synthases and non-ribosomal peptide synthetases were identified in several strains. These enzymes are responsible for the synthesis of secondary metabolites of great biotechnological interest and it is possible that they may contribute to the observed antimicrobial activity of the tested strains.

Twentyone of the identified bacterial strains were tested regarding their biosynthetic potential (e.g. enzyme production). Phenotypic tests were performed in order to detect amylase, proteinase, hemicellulase, cellulase and pigments degradation activity. Molecular tests were performed for the detection of genes encoding hemicellulase, cellulase and laccase. 33.3% of strains were tested positive in 2 out of 8 phenotypic tests, while 14.3% (all *Bacillus* spp.) tested positive in 7 out of 8 tests. 71.4% of strains tested positive at least in 1 out of 3 molecular tests. Most of the strains (66.7%) where reported to possess genes encoding cellulase.

The probiotic potential of 33 identified bacterial strains was tested. Safety tests of these strains and their resistance in conditions simulating those of the gastrointestinal tract were performed. 66.7% of the strains were resistant to acidic conditions (pH 2.5). The same percentage, though regarding different strains, was resistant in the presence of bile salts. Pancreatin enhanced the growth of all strains. 84.8% of the strains were susceptible to all the antibiotics, while roughly half of them (48.5%) were identified as γ-hemolytic strains. 45.5% were able to adhere to eukaryotic cells. Overall, 39.4% of the tested strains were found to be safe for consumption. CTB31 *Bacillus* sp. (*B. amyloliquefaciens*/*B. velezensis*) met all the criteria of a probiotic strain by definition.

These results demonstrate that honey microbiome contributes to its overall antimicrobial activity and it is a potential source of secondary metabolites, acting against important nosocomial and foodborne pathogens. In addition, bacteria isolated from Greek honey exhibited enzymatic potential, producing enzymes of great biotechnological interest

and also possible probiotic potential, justifying further research of honey microbiome, with prospective use in biotechnological applications.

Pine honey is a unique type of honey produced exclusively in Eastern Mediterranean countries such as Greece and Turkey. **Chapter 3** deals with the characterization of the microbial diversity of four geograpgically diverse Greek pine honeys, by implementing Next Generation Sequencing (NGS). The aim was to investigate whether a core microbiome could be identified to all of e pine honeys, as well as the elucifation of possible differences depending on the geographical origin. DNA from the four honey samples was isolated by various extraction methods and extraction using the Wizard kit was selected. The quality of the extracted DNA was checked by amplification of the V3-V4 regions of 16S rRNA gene for bacteria and the ITS regions (ITS1-ITS4) for fungi. Next generation sequencing (NGS) was conducted for all the samples via Illumina MiSeq PE250 platform. *Clostridium* genera and non-cultivable bacteria of the Gemmatimonadaceae family appeared as dominant in all samples. Six common genera were identified in all 4 samples. Samples NGS64 and NGS83 (Rhodes and Chania) shared 6 common genera. Samples NGS20 and NGS99 (Evia and Thassos) appeared to share 3 genera, while NGS20 and NGS83 (Evia and Chania) shared only one. Finally, 5 genera were detected only in NGS64 Rhodes, 4 only in NGS83 Chania, 3 only in NGS99 Thassos and 2 genera were detected only in NGS20 Evia. A putative correlation of the bacteriome of the samples with their geographical origin was demonstrated. Regarding the fungal ITS regions, unfortunately, due to low concentration of total DNA of the samples, results were obtained only for NGS64 Rhodes. Overall, further research analyzing a higher number of samples is essential in order to lead to safe conclusions and clearly characterize the microbiota of Greek pine honeys.

The antioxidant and anti-inflammatory properties of pine honey are well studied, however much less is known about its antibacterial activity. Chapter 4 presents the study carried out in this thesis upon the mechanisms of antibacterial activity of pine honey against the pathogenic bacterium *P. aeruginosa* PA14 at molecular level by analyzing the total transcriptome of the bacterium through RNA sequencing (RNA-seq). *P. aeruginosa* PA14 was exposed to a sub-inhibitory concentration of pine honey (0.5 MIC) for a short period of time (45 min). The sequencing of the samples conducted through the platform Illumina NovaSeq6000, 2x150bp paired-endreads. The quality of the data obtained from the sequencing was checked using the FASTQC software. The *P. aeruginosa* PA14 genome database was used for sequence analysis and the HISAT and DESeq2 software packages (version 1.18.0) were used for the molecular analysis of the differential gene expression. Pine honey induced the differential expression (>2-fold change and $p \leq 0.05$) of 463 genes, 274 of which were down-regulated and the remaining 189 were up-regulated. Gene ontology analysis revealed that pine honey affected a wide range of biological processes of the pathogen bacterium. According to the Gene Ontology analysis, the most affected and specifically down-regulated genes, were shown to be involved biological process as the redox process, transmembrane transportation, proteolysis, signal transduction, biosynthetic processes, phenazine biosynthesis, bacterial chemotaxis and antibiotic biosynthesis. The biological processes up-regulated in response to pine honey treatment were those related to the regulation of DNA template transcription, iron transportation and phosphorylation. Pathway analysis showed that pine honey treatment affected the 2-component regulatory system, the ABC transporter system, quorum sensing, bacterial chemotaxis, biofilm formation, and the SOS response. Overall, the results demonstrated that multiple

mechanisms of action are involved in the antibacterial activity of pine honey *against P. aeruginosa*.

Proteins are found in low concentrations in honey, nevertheless they could be useful tools to access the authenticity and geographic origin of honey. In addition, honey contains antimicrobial peptides that contribute to its overall antibacterial activity. The protein profile of 90 honey samples of various botanical and geographical origins was determined and reported in Chapter 5. Samples were diluted 50% w/v, total protein concentration was quantified, followed by the electrophoresis of 10 μg/sample on polyacrylamide gel under denaturing conditions (SDS-PAGE). The profile was studied after Coomassie staining. Honey samples of the same botanical origin showed a remarkably similar protein profile. Geographical origin did not appear to affect this profile. In addition, some protein bands were common to all honey samples regardless of botanical origin, although in some cases their intensity differed.

The attempt to isolate proteins/oligopeptides with antimicrobial activity from Greek honeys is described further in the Chapter 5. Initially, the minimum inhibitory concentration (MIC) of various honey samples against the pathogens *Pseudomonas aeruginosa Staphylococcus aureus*, *Salmonella* Typhimurium, *Klebsiela pneumoniae* and *Acinetobacter baumanii* was determined before and after the addition of proteinase K,. Thirtynine samples exhibited antibacterial activity linked to the protein content against *S. aureus*, 17 against *P. aeruginosa*, 28 against *S.* Typhimurium, 33 against *K. pneunoniae* and 17 against *A. baumanii*. The proteins of these samples were isolated by ammonium sulfate precipitation and then were quantified. MIC of the protein samples was determined against the pathogens. 14/39 protein fractions exhibited MIC values ≤ 300μg/ml against *S. aureus*, 1/17 against *P. aeruginosa*, 4/28 against *S.* Typhimurium, 2/33 against *K. pneumoniae* and 4/17 samples against A*. baumanii*. After adding proteinase K, an increase in the MIC values was observed in 14 samples for *S. aureus*, 1 for *P. aeruginosa*, 4 for *S.* Typhimurium, 2 for *K. pneumoniae* and finally, in 4 samples for *A. baumannii*.

These samples were up to 10-fold overconcentrated and electrophoresed on SDS-PAGE without denudation. Then the proteins were fixed on the gel, followed by washing steps with ddH_2O. The gel was then overlaid with soft agar containing S. aureus. Unfortunately, no inhibition zone was observed in any of the samples. A zone of inhibition of growth of S. aureus was only detected in one sample (Velanidia & Anthi, Agrafa, 2020).

Functional (meta)genomic analysis can de used to identify genes and operons encoding bioactive molcecules of high biotechnological interest. In Chapter 6 functional (meta)genomics analysis was implemented in order to isolate genes or gene clusters encoding bioactive molecules that inhibit the growth of pathogens. One honey sample (No41 cotton, Larissa, 2019) with strong antibacterial activity was selected. This honey was the source of isolated bacterial strains that exhibited strong antibacterial activity against five pathogenic bacteria. The total DNA of the honey sample was extracted and then used for the construction of a clone library, using the CopyControl Fosmid Library Production Kit with pCC1FOS Vector. The same procedure was carried out for 7 identified bacteria with confirmed antibacterial activity against 5 pathogenic bacteria that have been isolated from honey. Total genomic DNA was isolated by each of these 7 strains and was used to construct another clone library. The two libraries were screened through gel overlay assay, to detect clones encoding bioactive compounds exerting antimicrobial activity against various pathogenic bacteria.

In total, 940 clones from the library obtained from the total genomic DNA of the 7 identified bacterial strains (7144 clones) were screened for their antibacterial potential against the pathogens *S. aureus*, *P. aeruginosa*, *S.* Typhimurium and *A. baumannii*. 17 clones exhibited antibacterial activity against at least one out of the four tested pathogens. Also, 8 clones out of 48 coming from the total DNA library (honey sample 41) showed antibacterial activity against at least one out of the four pathogens. Digestion with *BamHI* restriction enzyme was performed on 23 clones (7 for the PL library and 16 for FOS41), to control the quality control of the two libraries.

Two clones that showed antibacterial activity against *S.* Typhimurium were selected to determine the complete sequence of their fosmids via Illumina HiSeq PE 150 sequencing platform. Complete assembly of the two fosmids was not possible and the analysis did not determine the organism of origin. There was no identification of genes or gene clusters involved in the synthesis of antimicrobial compounds and molecules against the pathogenic bacterium *S.* Typhimurium.