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## Honey as a Functional Food

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### Abstract

The most well-known functional properties of honey are its antioxidant and antimicrobial activities. The bioactive components of honey are affected by the flora from which it is produced and by geographical variations. Phenolic compounds promote, among other activities, high antioxidant action, being capable of minimizing intracellular oxidative damage associated with cellular aging, apoptosis and neurodegenerative diseases. A living cell system would provide a better platform for determining antioxidant activity, since the bioactive honey compounds can act modulating antioxidant defense gene expression. Indeed, phenolic compounds, amino acids and reducing sugars are among the substances responsible for honey antioxidant activity. Most of phenolic compounds also exert antimicrobial activity against a number of pathogens and spoilage microorganisms. The antimicrobial activity of honey is also due to the action of enzymes. In addition, honey was found to contain lactic acid bacteria (LAB), which itself produce a myriad of active compounds that remain in variable amounts in mature honey. In addition, these antioxidant compounds might play a key role as prebiotic, protecting and stimulating growth of probiotic bacteria. Oligosaccharides present in honey are well-known prebiotic substances stimulating growth, activity and protecting probiotic bacteria during passage through the gastrointestinal tract and during storage of the products. This chapter describes the main bioactive components of honey, especially with respect to the phenolic compounds and their antioxidant activity and assay methods.

**Keywords:** oligosaccharides, antioxidants, prebiotic

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### 1. Introduction

Honey is a complex product that can be easily digested and assimilation and is produced from the nectar, a sugary liquid of flowers, due to action of bee enzymes (diastase, invertase and glucose oxidase) [1].

The great majority of the dry weight of honey (95–98%) consists of carbohydrates, mainly glucose and fructose, but also sucrose, maltose and other oligosaccharides. A minor portion (2–5%) is made up of various secondary metabolites, such as polyphenols and flavonoids, minerals, proteins, amino acids, enzymes, organic acids, minerals, vitamins, fatty acids, pollen and other solid particles from the process of obtaining honey [1, 2]. It also contains traces of fungi, algae, yeasts and lactic acid bacteria (LAB) [3].

Prebiotics are substances that beneficially affect the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon, the probiotic bacteria. Honey is often used as a sweetener but its use in medical preparations date from ancient cultures [4, 5]. Such functional properties includes antibacterial, antioxidant, antitumor, anti-inflammatory, antibrowning and antiviral [6, 7]. More recently, it was also found to be prebiotic and even a source of probiotic microorganisms [8, 9].

Antioxidant activity is defined as the capability of a compound to protect an organism from oxidant attack. Two widely used methods to verify this capability are the diphenylpicrylhydrazyl (DPPH) and the 2,2'-azinobis (3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) assays. Both of them share the same mechanism of the reduction of the stable free radical but not measure the effect of an antioxidant on cell survival [9]. The biological yeast-based method can also measure the ability of a compound to induce cellular resistance to the damaging effects of oxidants [10, 11].

This chapter describes the main bioactive components of honey, with emphasis on phenolic compounds, antioxidant activity and assay methods.

## 2. Honeybee composition

Honeybees exist before human inhabits the Earth. It is formed due to action of honeybee's enzymes (diastase, invertase and glucose oxidase) on nectar or secretions of flowers. Honey is composed of various sugars, mainly glucose and fructose, but also sucrose, maltose and other oligosaccharides. In addition, honey contains proteins, amino acids, enzymes, organic acids, minerals and pollen. Besides, it can also contains traces of fungi, algae, yeasts and other solid particles from the process of obtaining honey [12] and lactic acid bacteria (LAB) [3].

Overall, honey contains acids, such as gluconic, succinic, malic, acetic, citric and butyric acid. Gluconic acid is found in greater amounts and is produced by action of glucose oxidase enzyme on the glucose to produce gluconic acid and hydrogen peroxide. Eighteen free amino acids occur in honey. Proline is the most abundant. Honey has small amount of vitamins that are negligible in the nutritional point of view. Therein includes ascorbic acid, niacin, pantothenic acid, riboflavin and thiamine. The minerals found in honey are potassium, sodium, calcium, magnesium, chlorine, iron, copper, manganese, phosphorus, sulfur and silica. Its content level of minerals is very variable and depends on the nectar source. Besides honey contains small amount of vegetable substances that contribute to the aroma and taste.

Honey has a set of five biologically active enzymes: Enzyme invertase (responsible for sucrose hydrolysis), diastase (which digests starch produced by plants), glucose oxidase (responsible for the production of acid and hydrogen peroxide), catalase (which uses hydrogen peroxide as substrate) and acid phosphatase. All these enzymes are derived from the glandular secretions of the honeybee. Hydroxymethylfurfural (HMF) can be found in low amounts in honey, which is produced by the decomposition of fructose in the presence of free acids, a process that occurs constantly in honey. The production of HMF depends on the temperature/time that the honey is subjected, particularly during pasteurization and storage [12].

### 3. Honey as probiotic source

Probiotic was originally defined by Parker [13] as “organisms and substances which contribute to intestinal balance.” Later, Fuller [14] redefined as “viable microbial supplement which beneficially affects the host by improving the intestinal microbial balance, having specific effect in preventing pathological condition.” Fuller's definition showed the need for the viability of probiotics in the food matrices and after passing the gastrointestinal tract. Probiotic definition has been expanded, not restricting to the health effects on the indigenous microbiota. According to Schaafsma [15], “oral Probiotics are microorganisms which upon ingestion in certain numbers, exert health effects beyond the inherent basic food nutrition.”

The honey relationship with probiotic microorganisms is already in the generation of honeybees, when honeybees to be fed with honey over the 21 days of generation are stimulated immunologically due to probiotics contained in honey [16].

For a long time, researchers believed that the source of lactic acid bacteria in the honey was pollen and secretions of flowers that arrived to honey transported by honeybees. However, later studies proved that the lactic acid bacteria are present in the stomach of the honeybees; therefore, it is a source of lactic acid bacteria. The colonization mechanism is not fully clarified yet [8].

In the honey production process, the enzyme glucose oxidase is responsible for the transformation of the glucose in galacturonic acid. This causes the natural acidification of honey and therefore its preservation. Then, the majority of pathogenic and spoilage microorganisms are inhibited [12]. Due to honey acidity, yeasts and lactic acid bacteria are the predominant microorganisms. Among the lactic acid bacteria, there are probiotic microorganism, especially those belonging to *Lactobacillus* and *Bifidobacterium* genus.

Within the most isolated species of *Lactobacilli* genus are those belonging to the species *L. apis*, *L. insects*, *L. alvei*, *L. plantarum*, *L. pentosus*, *L. parabuchneri*, *L. kunkeei*, *L. kefir* [17], and *Lactobacillus acidophilus*. Among *Bifidobacterium* genus, novel species were identified, *B. asteroides* and *B. coryneform* [8]. LAB symbionts within honeybees are responsible for many of the antibacterial and therapeutic properties of honey [3].

Olofsson et al. [3] reported that 13 lactic acid bacteria symbionts from the honey stomach of honeybees (*Apis mellifera*) were also found in large concentrations in fresh honey as well as having a wide spectrum of antimicrobial activity against various honeybee pathogens and bacteria and yeasts from flowers. According to these authors, many of the unknown healing and antimicrobial properties of honey are linked with these LAB symbionts. Every single member of the LAB microbiota of honeybees produces different bioactive metabolites. Organic acids were produced by all tested strains but in different amounts. Lactic, formic and acetic acids were produced as well as a wide variety of other interesting metabolites such as benzene and 2-heptanone and also putative lactic acid bacteria proteins in different honey types, suggesting their importance in honey production and antimicrobial activity.

#### 4. Honey as prebiotic

The most well-known properties of honey are its antioxidant and antimicrobial contents. Different types of honey contain different characteristics and properties. Hence, the different sources of honey reflect its content and characteristics.

Prebiotics are substances that beneficially affect the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon, the probiotic bacteria. Traditionally, prebiotics were related to nondigestible oligosaccharides and polysaccharides substances, which beneficially affect the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the intestinal colon [18, 19]. However, this concept should be expanded to other substances, present in honey, which selectively benefit probiotic bacteria by stimulating its growth or activity. Most of the antioxidant compounds present in honey affect the viability of a series of undesirable microorganisms but does not affect probiotic bacteria or, in many cases, even stimulate their growth or activity [20–22].

Honey oligosaccharides had a potential prebiotic activity. These compounds selectively stimulate the growth of beneficial microorganisms, such as *Lactobacillus* and *Bifidobacterium* [23, 24]. Sanz et al. [24] conducted a study on how honey oligosaccharide affects the bacteria population in human gut intestinal track (GIT) and found honey that contain higher amount of oligosaccharide resulted in large amount of beneficial bacteria's growth.

The main oligosaccharides found in honeys surveyed in Brazil were the disaccharides, turanose, nigerose, melibiose, sucrose, isomaltose and four trisaccharides, maltotriose, panose, melezitose and raffinose [25]. Sanz et al. [24] found the highest amounts of maltulose and turanose (0.66–3.52 and 0.72–2.87 g/100 g of honey, respectively) in samples of honey from different regions of Spain and commercially available nectar and honeydew honeys. The trisaccharides, melezitose and panose, were the most abundant oligosaccharides from New Zealand honeys [26]. The fructooligosaccharides (FOS) quantified from wild Malaysian honeys were inulobiose, kestose and nystose [23].



Both lactobacilli and bifidobacteria are benefited in environments with low redox potential, and the presence of antioxidant compounds in honey is important in this regard. Flavonoids, amino acids and phenolic acids are the main antioxidant compounds in honey. Most valuable and superior antioxidant compounds of honey such as some phenolic compounds and glutathione are unstable over time and thermolabile. Thus, its final quality is compromised when raw honey goes through conventional thermal processing.

The main criteria for selection of probiotics are resistance to gastrointestinal conditions [14, 27]; characterization of genus, species, strain and its origin [27]; antimicrobial activity, adhesion to the intestinal epithelium, interaction between probiotics and intestinal microbiota of the host; absence of history of pathogenicity and infectivity; metabolic activity of bile salts; lack of hemolytic activity; absence of genes that convey resistance to antibiotics [28]; potential for reducing biofilm formation by pathogenic microorganisms and resistance to lysozyme besides technological properties [29]. As safety criteria, besides being nonpathogenic, the cultures must have no history of disease, do not deconjugate bile salts or produce toxins, shall not adduce antibiotic resistance genes and do not translocate or induce them, and preferably to be of human origin [27].

We studied the effect of adding 5% of honey to fermented milks on the survival of *Lactobacillus paracasei* and *Lactobacillus rhamnosus* of human origin (isolated from fecal samples of infants), after simulated gastrointestinal tract conditions. The resistance of the examined strains under conditions simulating the gastrointestinal tract was tested as previously described [30] and modified by adding lysozyme (100 µg/mL) to intestinal juice. The production process is shown in **Figure 1**.

Honey did not affect the survival of *L. paracasei* but avoid the reduction of *L. rhamnosus* number. Adding honey (5% w/v) in fermented milk positively affects the survival of *L. rhamnosus* during simulated gastrointestinal conditions. In the presence of honey, the population of *L. rhamnosus* after simulated intestinal condition was more than one log cycle higher than control without honey (**Figure 2**).

Similar response was observed with the commercial *L. casei*-01 (Christian Hansen), which was not affected by the presence of honey, differently of *Bifidobacterium* strains [20].

*Bifidobacterium* are more sensitive to acids than *Lactobacillus* genus. In fermented milk, *Bifidobacterium longum* was more sensitive than *Bifidobacterium brevi* during storage at 10, 20 and 30°C for 10 days. The same was observed with the pH reduction in smoothie yogurt, *B. brevi* was not affected, whereas *Bifidobacterium longum* lost viability during pH reduction from 6.5 to 3.8 [31].

Indeed, the honey has prebiotic effect by stimulating the growth and activity of probiotic bacteria. Besides, because of osmotic constitution and composition of the honey, it acts as protectant to the passage of probiotic bacteria throughout gastrointestinal tract. In fact honey has three functions related to probiotics aspects: it may contain probiotic microorganisms itself, prebiotic substances and protective function to probiotics during the transit by gastrointestinal conditions.

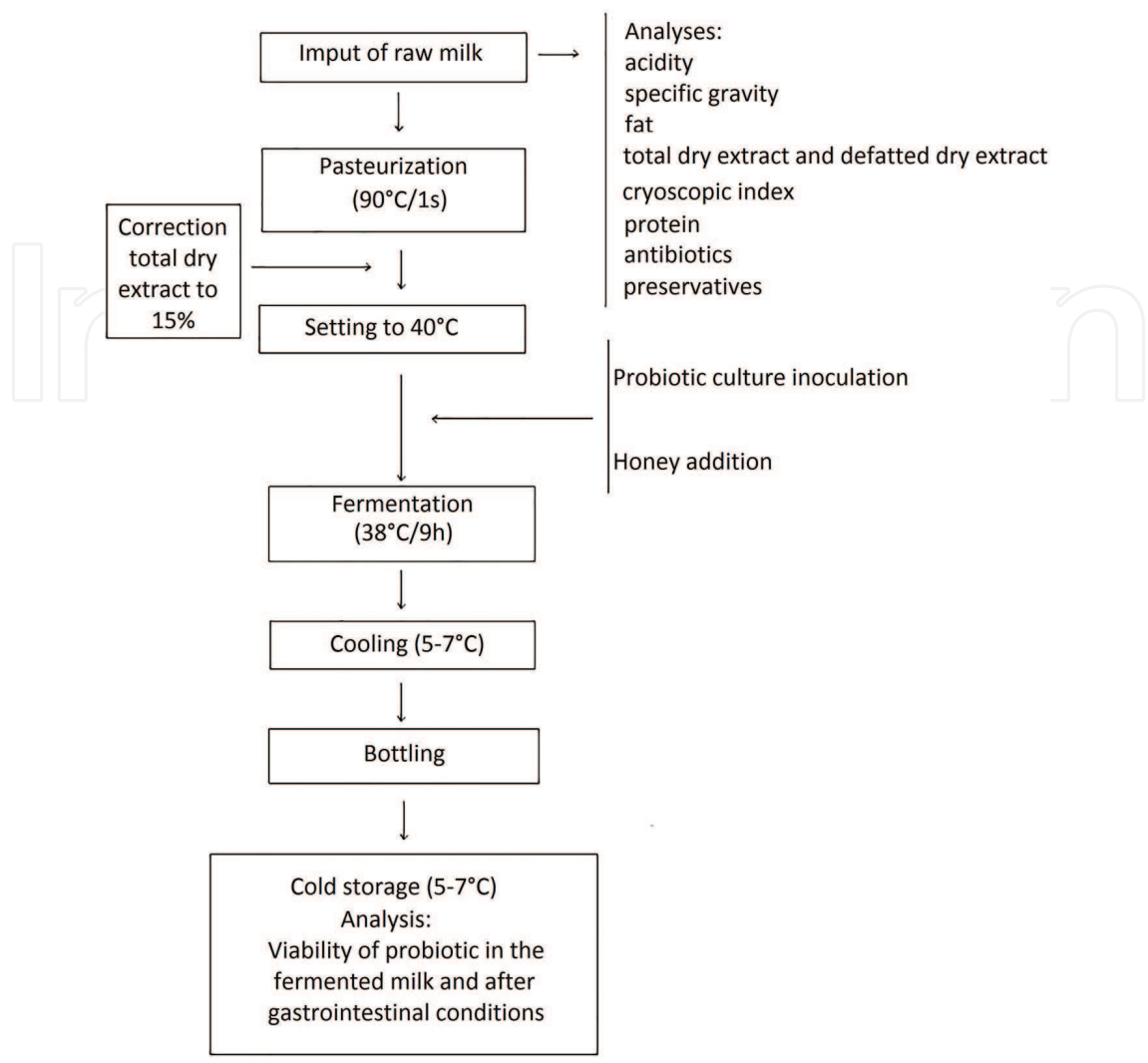


Figure 1. Production of probiotic fermented milk added with 5% of honey.

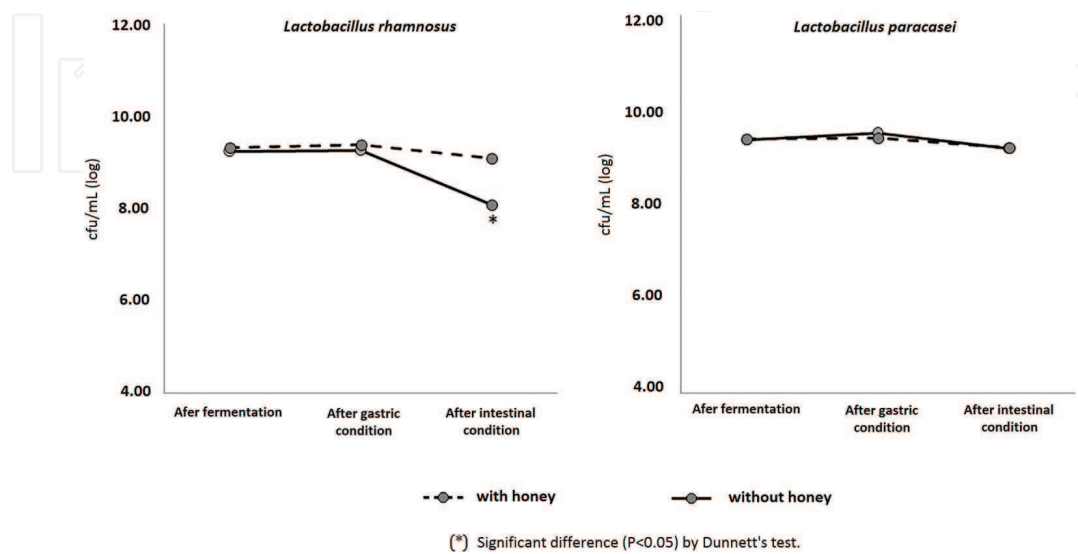


Figure 2. Survival of *Lactobacillus rhamnosus* and *Lactobacillus paracasei* after gastric and internal condition.

Favarin et al. [30] found that suspending free cells of two *Bifidobacterium* strains in honey solutions resulted in a protective effect, equivalent to the plain microencapsulation with sodium alginate 3% and concluded that microencapsulation and the addition of honey improved the ability of *Bifidobacterium* to tolerate gastrointestinal conditions *in vitro*.

## 5. Antioxidants of honey

During recent years, functional foods have attracted growing attention because of consumer's increasing concerns about their health, which has stimulated research effort into such foods [6]. An example, which emphasizes the importance of diet to health, is the French paradox, first observed in French population and found later also in other Mediterranean populations. Epidemiological studies revealed that antioxidant-rich diet is correlated with the increased longevity and decreased incidence of cardiovascular diseases observed in these populations despite their high fat diet, low exercise and smoking habits. It is well known that antioxidants can contribute to prevention of other illnesses, including neurodegenerative diseases, cancer and diabetes [32, 33].

Oxidative stress is an imbalance between oxidative and antioxidant molecules. The reactive species ( $O_2^{\cdot-}$ ,  $\cdot OH$ ,  $H_2O_2$  and others) have low stability and high reactivity resulting in low steady-state concentrations and high diversity of reactions they can participate in. Because of that, oxidative damage induces in biomolecules, as carbohydrates, proteins, lipids, and nucleic acids, which may alter its function, causing cells damage. As a consequence might flaw tissues and organs, leading to diseases [34]. Despite of their great capacity for damaging cells, other agents play important role, such as real players in many normal functions of living organisms, for instance in signalization of immune system cells [35].

Antioxidants are agents responsible for inhibition and reduction of injuries caused by reactive species in cell. Our genome encodes antioxidant enzymes to protect against oxidative damage, such as superoxide dismutase, catalase and glutathione peroxidase. Indeed, low molecular weight molecules as tocopherol, ascorbic acid and polyphenols can help on this process.

Free radicals can also affect food quality by reducing its nutritional content, color loss, unpleasant odors and flavors, promoting the development of food spoilage and, consequently, abbreviating their shelf life. Many synthetic antioxidants have been used in the food industries, but recent researches have mentioned their disadvantages and possible toxic properties for human and animal health [6, 34].

Honey and other bee products, whereby royal jelly and propolis may be used as functional foods because of their naturally high antioxidant potential, which could contribute to the prevention of certain illnesses [36–38]. Ancient Egyptians, Chinese, Greeks and Romans used honey in combination with vegetable or animal fat but also as part of all sorts of ointments [38]. The use of honey in modern medicine was strongly declined due to discovery of new drugs, but the search for more natural treatments boosts again search of honey and other products of bees [39].



Honey is a supersaturated solution of sugars (70–75%), of which fructose (38% w/w) and glucose (31% w/w) are the main contributors, 20–25% of water and about 3–5% for various substances [22, 38]. Hundreds of bioactive substances have already been found in honeys from different regions. This wide variation occurs when honeybees collect nectar from plants, incorporating secondary metabolites product of vegetables. This metabolism is rather variable and primarily depends on the botanical and geographical origin of the floral source, although certain external factors also play a role, such as seasonal and environmental factors and its processing [22, 40].

Honey antioxidant activity appeared to be a result of the combined effect of a range of compounds. Phenolic compounds (flavonoids and phenolic acids), as well as non-phenolic (ascorbic acid, carotenoid-like substances, organic and amino acids, and proteins including certain enzymes such as glucose oxidase and catalase) can contribute to honey antioxidant activity [40, 41].

The honey phenolic compounds are the main antioxidant compounds of honey. They are the phenolic acids and flavonoids, which are considered potential markers of the honey botanical origin. The phenolic acids are divided in two subclasses: the substituted benzoic acids and cinnamic acids. The flavonoids present in honey are divided into three classes with similar structure: flavonols, flavones and flavanones. These are important due to their contribution to honey color, taste and flavor and also due to their beneficial effects on health [21].

Large amount of research in honey also reports strong correlation between the total phenolic content and the antioxidant activity of honey extracts. Because of that, several literature reports have sought to identify and isolate them. Despite the relevant importance of polyphenolic compounds, which are recognized as the major constituents and responsible for the health-promoting properties of honey, their identification and quantification are of great interest for understanding their contributions to the overall bioactivity of honey [40].

## 6. Evaluation of the phenolic content

Analytical procedures used to determine polyphenols in a honey sample include their extraction from the matrix as well as their separation and quantification. The determination begins with an extraction step by means of solvents, which are mostly mixtures of water-alcohol in different proportions. Aqueous ethanol solutions (25–70 % v/v) are used in some work for 12–24 hours under stirring [42, 43]. While the methanolic extraction is used in different proportions with water [1, 44], there is still work using combined techniques of aqueous extraction, with heating or acidification, and subsequent ethanol extraction [40, 45]. Few studies conduct extraction with other solvents such as ethyl acetate [46].

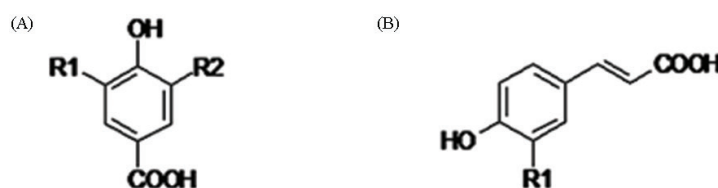
The filtered or centrifuged extracts and different profiling techniques can be used for the determination of phenolic compounds. Liquid chromatography is considered to be the most useful separation technique for the analysis of polyphenols in different samples.

Coupled with various detection techniques, such as a diode array detector (DAD) [1, 21, 40, 47] and/or mass spectrometry, it enables both identification and quantification of polyphenols [42, 45, 46]. Since phenolic components can vary greatly, the suitable technique is liquid chromatography coupled with various types of mass detection, LC-MS enables high selectivity, sensitivity and universality when analyzing various polyphenolic components in their complex matrices.

Determination of a polyphenolic profile of honey is a complex task, so it is essential to develop separation and detection techniques, which would enable an unambiguous determination of as many components as possible. Tandem mass spectrometry is the detection method of choice when a comprehensive analysis of nontarget analyte is needed [46].

A wide variety of compounds isolated from honey and propolis come from flora, region and climate differences, where the nectar or sap was collected [12, 48, 49]. The phenolic compounds extracted, isolated and characterized can be classified into two major groups: phenolic acids and flavonoids.

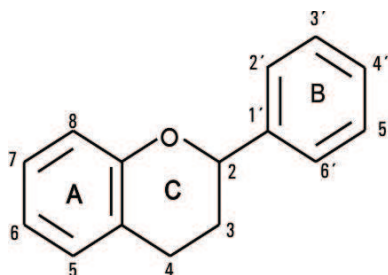
The group of phenolic acids is divided into two main groups: derivatives of hydroxybenzoic acid (**Figure 3A**) and the hydroxycinnamic acid derivatives (**Figure 3B**). The benzoic acid derivatives include salicylic acid, gentistic, p-hydroxybenzoic, protocatechuic, vanillin, gallic, syringic and others. These are the most simple phenolic compounds found in foods [49, 50].



**Figure 3.** (A) General structure of derivatives of benzoic acid (benzoic acid, R1 = R2 = H). (B) General structure of the derivatives of hydroxycinnamic acid (hydroxycinnamic acid R1 = H) [12].

Hydroxycinnamic acid derivatives include p-coumaric, caffeic, ferulic, among others. They may also be in conjugated form between themselves or with other organic compounds. This is the case of chlorogenic acid, which is the combination of quinic acid and caffeic acid [49, 50]. All cited phenolic acids have been described in honey samples in different concentrations according to the flora collected by honeybees [40, 46].

Flavonoids are compounds that possess the diphenylpropane skeleton: two benzene rings linked through oxygen containing a pyran or pyrone ring [46] (**Figure 4**). Flavonoids are a group of substances comprising classes of flavonols, flavones, flavonones, isoflavones, anthocyanins and catechins. In plants, flavonoids are involved in pigmentation of fruits and flowers and the regulation of plant growth and plant protection against oxidative agents [32, 52]. In samples of honey and propolis naringenin, chrysin, rutin, morin, kaempferol, myricetin, hesperidin, apigenin, among others [40, 45, 46, 51] are found.



**Figure 4.** Structure of the major chemical types of flavonoids [51].

## 7. Phenolic profile of honey

Regions characterized by a hot and humid climate with very high exposure to sunlight (as in northeast Brazil) are particularly known to exert a marked influence on the polyphenolic content of plants. Sun-exposed plants such as juazeiro (*Ziziphus joazeiro* Mart.) can contain much more total phenolics than the same varieties or other when grown in the shady locations [53].

Assays made with honey collected in the central and southern region of Amazonas state in Brazil found that total phenolic content of methanolic extracts from the honey samples ranged from 17.0 to 66.0 mg galic acid equivalent (GAE)/g of extract and also high antioxidant profile. Gallic, 3,4-dihydroxybenzoic, 4-hydroxybenzoic, vanillic, salicylic, syringic, coumaric, trans,trans-abscisic, cis,trans-abscisic and cinnamic acids, catechol and flavonoids, taxifolin, naringenin and luteolin were identified. Concentrations ranged from 0.02 to 67.0 mg/mL of extracts, varying with the sample [54].

Brazilian honeys from the semiarid region, which were composed of 24 monofloral honeys produced by *Meliponini*, native species of bee, were found to present strong antioxidant activity. The total phenolic content varied from 0.31 to 1.26 mg GAE/g with differences ( $p \leq 0.05$ ) among samples from distinct floral sources. The scavenging activity of DPPH radicals varied from  $11.2 \pm 1.3\%$  to  $46.9 \pm 1.9\%$ . Phenolic compounds p-coumaric, ellagic and 3,4-hydroxybenzoic acid and the flavonoids rutin, catechin, chrysin and naringenin were detected in higher amounts in *Ziziphus joazeiro* Mart. honeys than in the other honeys produced by the same bee species [40].

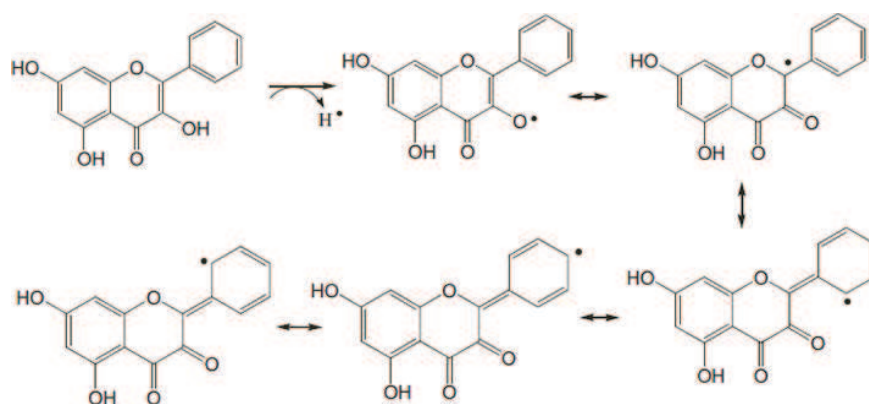
Fifty eight polyfloral honey samples, from different regions in Serbia, were studied to determine their phenolic profile, total phenolic content and antioxidant capacity. It was reported that the phenolic content ranged from 0.03 to 1.39 mg GAE/g and the radical scavenging activity of DPPH radicals ranged from 1.31 to 25.61% [44], an antioxidant capacity lower than that found in honey from high sunlight incidence regions.

All these studies found strong correlation between total phenolic content or total flavonoid content and radical inhibition capacity, indicating that phenolics and flavonoids are the primary factors responsible for the antioxidant properties of the studied honeys. Consequently, these results reinforce the influence of the botanical source on honey antioxidant properties.

Honey phenolic composition is not predictable, since it is highly related with the flora where honeybees collected nectar. Thus, the profile of phenolic compounds can be used to determine honey flora origin. For instance, a study in honeys produced in arid regions in northeast Brazil showed a high quantity of rutin in honeys from *Ziziphus spina-christi*, suggesting that it is a marker in honeys from the *Ziziphus* species [40]. Samples originated from Vojvodina and Zlatibor regions were clearly distinguished from those from the rest of Serbia because of the presence of dicaffeoylquinic acid, ellagic acid, caffeic acid phenethyl ester and chlorogenic acid, among others [45].

## 8. Mechanisms of action of phenolic compounds

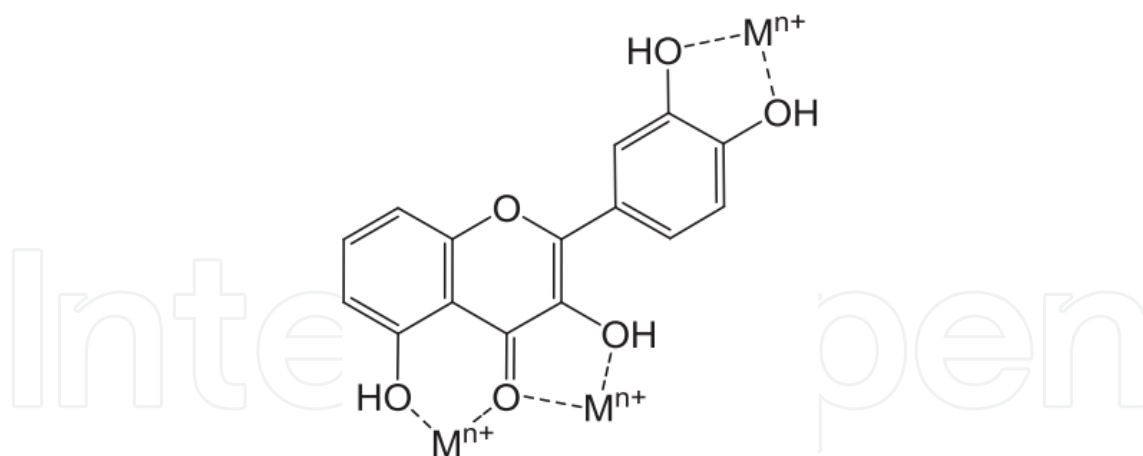
Several mechanisms have been proposed to explain the observed antioxidant activity of phenolic compounds. The first is the direct removal of radicals through the formation of more stable compounds from radical supply of hydrogen (**Figure 5**). The various possible resonance hybrids in flavonoids and phenolic acids structure make them less reactive, limiting the deleterious power of other reactive species [55].



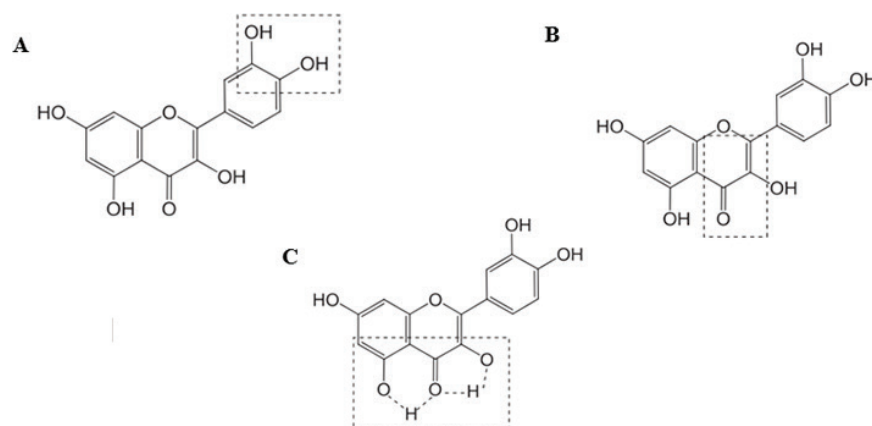
**Figure 5.** Radical stabilizing resonance structures by monoelectric oxidation of hydroxyl group in galangin [55].

Another mechanism of action of its antioxidant activity is their metal chelating propriety (**Figure 6**), which removes ions such as  $\text{Fe}^{2+}$ , which catalyzes the formation of free radicals by Fenton and Waber-Heiss reactions and which are propagators responsible by reactive oxygen species; decreasing, so the intracellular oxidative stress [56].

The *in vitro* activity of phenolic compounds depends on their structure. In flavonoids, the hydroxyl groups are in the ortho position (**Figure 7A**), especially in ring B; the presence of double bond to oxygen in the ring C (**Figure 7B**) and hydroxyl groups at positions 3 and 5 (**Figure 7C**) were found to increase the antioxidant capacity, since they contribute to stabilizing resonance structures [32, 57]. The presence of glycosides, however, reduces the antioxidant activity. The antioxidant activity of glycosidated conjugate rutin decrease about 50% when compared to quercetin [32].



**Figure 6.** Possible flavonoid coordinating points with metals [32].



**Figure 7.** (A) Hydroxyl ortho position; (B) the presence of double-bonded oxygen in the 5-position of ring C; (C) the presence of hydroxyl at positions 3 and 5 [32].

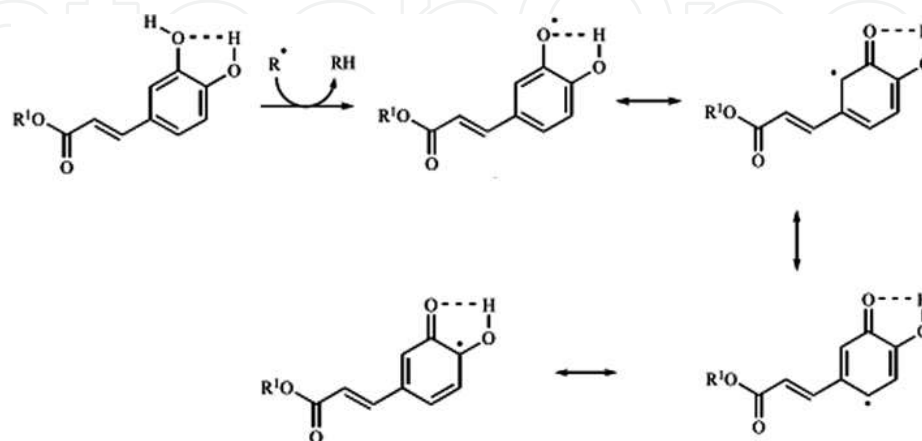
Phenolic acids have increased activity in the presence of hydroxyl groups in the ortho position (**Figure 8**) or carbonyl groups in the ortho hydroxyls, as with syringic acid [57]. Moreover, in general, the hydroxycinnamic acids have shown *in vitro* activities higher than the hydroxybenzoic acids [58].

However, tests on biological models show that the flavonoids and other phenolic compounds act modulating the expression and activity of enzymes related to antioxidant defences [59, 60]. Phenolic compounds have the ability to induce phase II enzymes, such as quinone reductase NADPH and GST, as well as inhibiting enzymes related to carcinogenesis such as protein activation 1 (AP1), nuclear factor (NF)- $\kappa$ B and MAP-kinases [32, 60].

It is also important to emphasize that phenolic compounds also have pro-oxidant activity, dependent on its concentration. The presence of hydroxyl groups in the ortho position can also produce radicals or hydrogen peroxide, in the presence of copper ions and oxygen



molecules [61, 62]. The flavonoid rutin and morin at concentrations above  $100 \mu\text{g mL}^{-1}$  were able to produce hydrogen peroxide and damage DNA through comet assay in human lymphocytes. However, this effect was not observed with naringenin, and hesperidin in the same concentration, which do not have hydroxyl groups in ortho position on ring B [63]. The generation mechanism of hydrogen peroxide or radicals can explain the antimicrobial action of flavonoids and their toxic effects at higher concentrations to microorganisms [32].



**Figure 8.** Radical sequestration mechanism of hydroxycinnamic acid including resonance stabilization radical by intramolecular hydrogen bonding [58].

*In vivo* testing confirms the antioxidant activity observed *in vitro*. Phenolic extracts from two monofloral Cuban honeys were able to inhibit erythrocytes oxidative damage. This study indicated that honey contains relevant antioxidant compounds responsible, at least in part, for its biological activity and that uptake of its flavonoids may provide defence and promote cell functions in erythrocytes [64]

A study was undertaken to determine whether replacing sucrose in the long-term diet with honey, which has high antioxidant content, could decrease deterioration in brain function during ageing. Rats were fed *ad libitum* for 52 weeks on a powdered diet that was either sugar-free or contained 7.9% sucrose or 10% honey. Apparently, long-term feeding of honey, sucrose and a sugar-free diet may have some effects on anxiety and spatial memory in rats, with honey-fed rats exhibiting less reduction in spatial memory and decreased anxiety at the completion of the study [65].

Manuka honey, derived from the *Leptospermum scoparium* tree, was investigated about its protection effect against oxidative damage and improvement of the process of skin wound healing, using human dermal fibroblast cells. Up to 16 compounds were identified in this honey, with leptosperin derivatives and methyl syringate as the major ones. It protected against apoptosis, intracellular ROS production and lipid and protein oxidative damage. Manuka honey also protected mitochondrial functionality, promoted cell proliferation and activated the AMPK/Nrf2 signaling pathway, associated with antioxidant defence, as well as the expression of the antioxidant enzymes such as SOD and CAT [37].



## 9. Antioxidant activity assays

Various assays have been applied to determine honey antioxidant activity. The most common ones are colorimetric assays, DPPH (1,1 diphenyl-2-picrylhydrazyl), ABTS (2,2'-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid)), FRAP (ferric reducing antioxidant power) and TEAC (Trolox equivalent antioxidant capacity), based on electron transfer, and ORAC (oxygen radical absorbance capacity) assay, based on hydrogen atom transfer and other techniques as voltammetric assays [34, 41, 46]. The total phenolic content is commonly spectrophotometrically determined with a Folin-Ciocalteu method, sometimes with modification and total flavonoid contents is generally measured by colorimetric assay with aluminum chloride [40, 54].

At the present time, no single available assay for testing the antioxidant capacity provides all the desired information. An evaluation of the overall antioxidant capacity may require multiple assays to generate an "antioxidant profile" encompassing reactivity towards both aqueous (DPPH and ABTS) and lipid/organic radicals (ORAC) directly through radical quenching and radical-reducing mechanisms (DPPH, ABTS, FRAP and ORAC) and indirectly through metal complexing (FRAP) [64].

Gorjanović et al. [41] evaluate hydrogen peroxide sequestration capacity of single bioactive compounds isolated from honey by voltammetric technique. As result, the flavonoids showed the highest hydrogen peroxide scavenging activity among the compounds, followed by phenolic acids. Activity of predominant honey sugars, fructose, glucose and maltose was found to be three orders of magnitude lower than tested flavonoids, but their contribution to total activity is significant due to their quantity. High hydrogen peroxide scavenging activity has been attributed to some amino acids, aromatic and basic ones, whereas non-polar amino acids, such as proline, the most prevalent amino acid in honey (0.40–2.2 mg/kg), possess low activity. Although phenolics are minor honey constituents, their antioxidant activity is high enough to correlate between honey hydrogen peroxide scavenge and total phenolic content [41].

Antioxidants *in vitro* assays do not consider physiological conditions such as concentration of intracellular metabolites nor does consider metabolic factors such as bioavailability and enzymatic transformations [58]. The *in vivo* assay, using yeast cells, specifically the specie *Saccharomyces cerevisiae*, represents an alternative to evaluate antioxidant activity. Yeasts are unicellular eukaryotic organisms widely studied and have great similarity with higher mammalian cells, especially in regard to the antioxidant defence system [66]. Because of this, it becomes an interesting biological model to evaluate biological activity related to natural extracts and molecules [66].

The use of *S. cerevisiae* cells as a study model has other important advantages. Its genome is completely elucidated, thereby facilitating the production of genetically modified strains for further studies; adding to this, its low cost of cells maintenance, ease of handling in the laboratory, rapid growth and low rate of spontaneous mutations [67]. Moreover, preliminary studies in yeast substituting the use of guinea pigs, rats and mice, certainly speeds up research work.

Furthermore, the *in vivo* assays measure the effect of an antioxidant on cell survival [9]. The biological yeast-based method can also measure the ability of a compound to induce cellular resistance to the damaging effects of oxidants [10, 11]. The determination of the lipid membrane integrity is an important parameter in verifying oxidative damage.

Lipid membrane peroxidation constitutes a primary cytotoxic event that triggers a sequence of lesions in the cell. Changes in membranes lead to disorders related to membrane permeability by changing the ionic flow and the flow of other substances, which results in the loss of selectivity for intake and/or outtake of nutrients and toxic substances to the cell, DNA damage and changes in the cell cycle [68, 69]. The Thiobarbituric Acid Reactive Substances (TBARS) assay method [70] measures the extent of lipid degradation by quantifying malondialdehyde (MDA) formed from the oxidation of triacylglycerols. In this method, the reagent thiobarbituric acid generates adduct with malondialdehyde, which is detectable spectrophotometrically at 532 nm. Besides the aforementioned method, cell viability assays are also employed in assessing oxidative damage in yeast, which evaluates the stress tolerance increase caused by treatment with antioxidant compounds [71]; mitochondrial function assays, since many apoptotic processes start in this organelle [72]; measurement of intracellular reactive oxygen species formation, using 2,7-dichlorofluorescein as indicator [71, 73]; protein carbonylation tests [74, 75], which is also formed as consequence of oxidative damage; assessment of energetic metabolism and enzymatic activity associated with the stress response [67, 74], among other methods.

Propolis, as well as honey, is a product of bees derived from the collection of plant fluids and alike contains phenolic compounds in its composition. Sá et al. [76] evaluated the antioxidant capacity of propolis extracts using a wild-type (BY4741) *S. cerevisiae* and antioxidant-deficient strains ( $\Delta$ ctt1,  $\Delta$ sod1,  $\Delta$ gsh1,  $\Delta$ gtt1 and  $\Delta$ gtt2), either to 15 mM menadione or to 2 mM hydrogen peroxide during 60 min. They observed that all strains, except the mutant  $\Delta$ sod1, acquired tolerance when previously treated with 25  $\mu$ g/mL of alcoholic propolis extract. Such a treatment reduced the levels of ROS generation and lipid peroxidation, after oxidative stress. However, cells were drastically affected by direct exposure to  $H_2O_2$ , after propolis treatment, survival increased almost three times. The increase in Cu/Zn-Sod activity by propolis suggests that the protection might be acting synergistically with Cu/Zn-Sod.

The antioxidative activities of propolis and its main phenolic compounds, caffeic acid, p-coumaric acid, ferulic acid and caffeic acid phenethyl ester (all 0.05 g/L), were investigated in the yeast *S. cerevisiae*. After 1 h of yeast cells exposure, their intracellular oxidation was measured using 2,7-dichlorofluorescein. Yeast cells exposed to 96% ethanolic extracts of propolis in DMSO showed 42% decreased intracellular oxidation compared with nontreated cells, with no significant differences seen for the individual phenolic compounds [72].

It is concluded that honey and other bee products possess proven *in vivo* and *in vitro* antioxidant activity, and this property can be the foundation of the functional properties assigned to them.

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