Royal Jelly: an ancient remedy with remarkable antibacterial properties

Filippo Fratini[1,2], Giovanni Cilia[1], Simone Mancini[1], Antonio Felicioli[1,2]

[1]Department of Veterinary Sciences, Viale delle Piagge 2, University of Pisa (Italy)
[2]Interdepartmental Research Center "Nutraceuticals and Food for Health", Via del Borghetto 80, University of Pisa (Italy)

corresponding author: filippo.fratini@unipi.it +390502216959, +390502216941

Abstract

Royal Jelly (RJ), a honeybee hypopharyngeal gland secretion of young nurse and an exclusive nourishment for bee queen, has been used since ancient times for care and human health and it is still very important in traditional and folkloristic medicine, especially in Asia within the apitherapy.

Recently, RJ and its protein and lipid components have been subjected to several investigations on their antimicrobial activity due to extensive traditional uses and for a future application in medicine.

Antimicrobial activities of crude Royal Jelly, Royalisin, 10-hydroxy-2-decenoic acid, Jelleines, Major Royal Jelly Proteins against different bacteria have been reported. All these beehive products showed antimicrobial activities that lead their potential employment in several fields as natural additives. RJ and its derived compounds show a highest activity especially against Gram positive bacteria.

The purpose of this Review is to summarize the results of antimicrobial studies of Royal Jelly following the timescale of the researches. From the first scientific applications to the isolation of the single components in order to better understand its application in the past years and propose an employment in future studies as a natural antimicrobial agent.

Keywords: Royal Jelly; antimicrobial activity; honeybees; Major Royal Jelly Proteins; 10-hydroxy-2-decenoic; natural peptides

1. Introduction

Royal Jelly (RJ) is a glandular secretion white-yellowish (Fig. 1), gelatinous-viscous sour taste, with a slight characteristic smell of phenol (which gives it its characteristic flavour) produced from the hypopharyngeal and mandibular salivary glands of young nurse (bees aged between 5 and 14 days) (Chauvin, 1968; Fujita *et al.*, 2013). RJ is the exclusive nourishment for all bee larvae, from hatching to the third day of life; those larvae which are selected to develop into queens are fed with RJ until the fifth day of larval life (the time at which the cell is operculated), and then RJ remains a dedicated feed for the queen bee alimentation for the duration of her life. Furthermore, RJ also has a significant impact on the life span: a worker bee lives around 45 days, while a queen bee could live up to five years during which is able to spawn in a day the equivalent of her weight in eggs (approximately 2000-3000 eggs per day for several years).

Storage conditions of RJ for its human employment is a critical point for maintain unchanged its properties; RJ is light and heat susceptible and undergoes oxidation to a direct contact with air

(Bogdanov *et al.*, 2004; Buttstedt *et al.*,2013; Kheyri *et al.*,2012; Sabatini *et al.*, 2009; Scarselli *et al.*, 2005; Zhang *et al.*, 2012).

1.1 Historic background

The first historical notes about human employment of RJ date back to ancient Greece; Greeks thought that the "ambrosia", the nectar which gave immortality to the gods of Olympus, was composed in part by RJ. At that time it was already consumed without knowing its specific effects, and historians reported that the honeycombs were shredded with inside honey, larvae, propolis, pollen and RJ and eaten fresh (Cassignau, 1991; Mraz, 1995). Aristotle was the first to have discovered the function of RJ in the bees society and, by studying its effects in queen bee, he attributed to the consumption of RJ an increase of physical strength and, above all he supposed its role in an improvement of intellectual capacity; the breakfast of his school was exclusively made with honey and RJ (Domerego, 2001; Molan, 1999).In ancient Egypt, RJ was used like a cosmetic, which reached its zenith of notoriety with Cleopatra, as one of her personal beauty secrets. Furthermore, in that period RJ became a symbol of strength and majesty of the Pharaohs, which usually ate RJ (Emonet, 2001; Levet, 2008).In Asia, specifically in China, RJ is used in traditional medicine since ancient time. This product of beekeeping, which was produced exclusively in the sovereign gardens, was correlated with the longevity and the sexual force, even in old age, of ancient dynasties of China (Cherbuliez and Domerego, 2003;Contessi, 2010).Jan Swammerdam (1637-1680), a Dutch naturalist, microscopist and entomologist, was the first to described the compound of nourishment in the royal cell and discovered that the "beehive chief" is a queen and not a king as supposed until the seventeenth century (Contessi, 2010; Viel, 2003).The French scientist René Antoine de Réaumur (1683-1757) coined the term "Royal Jelly" to name the feed of queen bee and he related the assumption of RJ with the exceptional growth of the queen (Cherbuliez and Domerego, 2003; Molan, 1999).In 1852 Reverend Langstroth, known as the father of American beekeeping, was the first to analysed chemically RJ, however he used methods did not guarantee a scientifically significant information (Domerego, 2001; Levet, 2008). Langstroth also proposed during the fifties the use of RJ as a commercial product, especially in areas where the production of honey was not profitable (Contessi, 2010; Viel, 2003). The use of RJ as a functional product and health enhancer was investigated since the early 60s, with the development of the "Apitherapy". From then on, particularities and properties of RJ were discovered and RJ reached a widely used in therapy for both men and bee itself (Contessi, 2010; Molan, 1999)

2.Composition

RJ is an acid colloid (3.6-4.2 pH) composed mainly by water, sugar, proteins, lipids, vitamins and some mineral salts (Melliou and Chinou, 2005; Ramadan and Al-Ghamdi, 2012;Vecchi *et al*., 1993). The major component is water, ranged from 60% to 70% (Caboni *et al.*, 2004; Melliou and Chinou, 2005), followed by carbohydrates from 11% to 23%(Sabatini *et al*., 2009; Sesta, 2006), proteins from 9% to 18%, (Melliou and Chinou, 2005; Ramadan and Al-Ghamdi, 2012; Simuth, 2001), lipids from 4% to 8%, (Malka *et al.*,2009; Nagai *et al*., 2005;Sabatini *et al.*, 2009) and there are present in low amount vitamins and mineral salts with other unknown substances present in traces and all together could range from 0.8-3%)(Fig.2) (Caboni *et al.*,2004; Lercker *et al.*,1992; Scarselli *et al*., 2005, Simuth *et al*., 2004; Zhang *et al.*, 2012.

RJ composition could vary with seasonal and regional conditions of feeding (Antinelli *et al.*, 2003; Attalla *et al.*, 2007; Biondi *et al*., 2003; Chen and Chen, 1995; Sabatini *et al.*, 2009), with metabolites and changes in the physiology of nurse bees as well as with the larval age (Abd-Alla *et al.*, 1995; Brouwers *et al*., 1987; Lercker *et al.*, 1985, 1993), with bees genetic and race (Liu *et al.*, 2008; Malka *et al.*, 2009; Sano *et al*., 2004; Zheng *et al.*, 2011), and above all could be modified from the storage conditions postharvest (Caboni *et al.*,2004; Li *et al*. 2008; Liu *et al.*, 2008; Ragab and Ibrahim, 1999; Zheng *et al.*,2011). Several researches correlated these variations of the composition of RJ to its antimicrobial activity (Abd-Alla *et al.*,1995; Li *et al.*, 2008; Liu *et al.*, 2008; Ragaband Ibrahim, 1999; Zheng *et al.*, 2011).

Pollen grains are always presents in RJ as contaminant and could are very useful as indicators of geographical origin and may enriched RJ with some proteins from plants origin (Biondi *et al*., 2003; Chen and Chen, 1995; Scarselli *et al*., 2005;Simuth *et al*., 2004).

2.1 Carbohydrates

Carbohydrates represent about 30% of dry matter (Sabatini *et al*., 2009; Sesta, 2006) and they may be important indicators of the authenticity of RJ, through the analysis of minor sugars contained(Daniele and Casabianca, 2012; Lercker *et al.*,1981;Serra Bonvehi, 1992).The most abundant sugars, as in honey, are fructose, glucose and sucrose (Ramadan and Al-Ghamdi, 2012; Wytrychowski *et al.*,2012), but small traces of oligosaccharides such as maltose , trehalose, melibiose , ribose and other sugars can also be found (Finke, 2005; Kheyri *et al.*, 2012; Lercker *et al.*,1981, 1985, 1986; Simuth, 2001).

2.2 Proteins

Proteins can reach about the 50% of dry matter of RJ (Bilikova *et al.*, 2002; Buttstedt *et al.*, 2014; Furusawa *et al.*, 2008; Scarselli *et al.*, 2005). During the last 20 years RJ was deeply investigated and several proteins have been identified.

Important protein components of RJ are those belonging to a family named Major Royal Jelly Proteins (MRJPs), or named apalbumins, that represent the 83-90% of protein component (Scarselli *et al*., 2005; Simuth, 2001). In this protein family have been identified eight proteins (MRJP 1-8) with molecular masses ranged from 49-87 KDa (Albert and Klaudiny, 2004; Albert *et al.*, 1999a; Albert *et al.*, 1999b; Hanes and Simuth, 1992; Malecova *et al.*, 2003; Moriyama *et al.*, 2015).

The MRJPs plays an essential nutritional role in the diet of the queen bee (Tamura *et al*., 2009); MRJP 1, MRJP 4 and MRJP 5 represent the main intake of essential amino acids, as well MRJP 2, and MRJP 5 are the most important nitrogen reserve for its growth (Albert *et al.*, 1999b; Schmitzova,1998); moreover, MRJP 3 is a polymorphic protein, and this might also explain the role of MRJP 3 as nitrogen supply(Albert *et al*., 1999b).

The MRJPs could also play an important role in the production of other bee products, especially in formation of pollen-pellet and pollen-bread (Simuth, 2001); and it was demonstrated that they have a major role in the differentiation between queen bee and worker (Buttstedt and Erler, 2013).

MRJP 1 is also recovered in the honeybee neurons, this suggests another unknown function of the protein in addition to nurturing (Peixoto *et al*., 2009).

MRJP 1 and MRJP 2 have been characterized like major allergens in the RJ, and, *in vivo*, stimulate mouse macrophages TNF-alpha production (Rosmilah *et al*., 2008; Simuth *et al*., 2004).

Other proteins, present in lower amount than MRJPs, are Royalisin, Jelleines, Aspimin and Royalactina.

Several researches showed the antibacterial properties of Royalisins and proposed their uses as potential antimicrobial natural peptides (Bilikova *et al*., 2011; Fujiwara *et al*., 1990). Royalisins are amphipathic proteins (both hydrophobic and hydrophilic properties) composed of 51 residues, with net charge +2; the origin is unknown, but it is supposed that they could derive directly from honeybee. The peculiarity of its structure lies on its high content of cysteine (6 residues) and three intramolecular disulphide bridges which can give a compact structure exhibiting high stability at low pH and high temperature. Royalisins has extensive sequence homology with the sapecin (protein constituted from 40 amino acids, taken from embryonic *Sarcophaga peregrine* cells and phormicins from *Phormia terra novae* larvae (Fujiwara *et al.*, 1990).

As Royalisins, Jelleines, showed their antimicrobial effects in *in vitro* tests (Fontana *et al.*, 2004; Romanelli *et al.*, 2011). Jelleines formation could be the result of tryptic digestion of MRJP 1 by specific proteases. The Jelleines, despite having the structural base of the antimicrobial peptides and are characterized by hydrophobic residues, which influence the interactions with bacterial membranes, do not show similarity with other known antimicrobial peptides, including those produced by bees after a possible infection (apidaecine, abaecine, hymenoptaecine).Jelleine I (PFKISIHL-NH_2) differ to Jelleine II (TPFKISIHL-NH_2) only for a Thr (T) residue from C-terminal portion. This modification seems to vary the antibacterial activities of the two peptides, with a higher activity of Jelleine I than Jelleine II. Moreover, the removal of the residue Leu (L) at the N-terminus of Jelleine II with the formation of Jelleine IV (TPFKISIH-NH_2) determines the complete loss of antimicrobial activity. However, a significant decrease of activity was showed also when a residue Thr (T) C-terminal was replaced with Glu(E) as reported in the different sequence between Jelleine II and Jelleine III (EPFKISIHL-NH_2).Because of the presence of an Arg (R) residue in position 373 and a Thr(T) residue in position 374 of the primary sequence of the MRJP-1 it can be supposed that the Jelleine II can be the product of digestion with trypsin of MRJP-1 (produced by the hypopharyngeal glands and secreted in the RJ). An action of exo-proteinase both on C-terminal to N-terminal tryptic fragment could lead, respectively, to the formation of Jelleines I and IV (Cabrera *et al*., 2014; Fontana *et al.,* 2004).

Apismin was also found, highly expressed, in the honeybee head and it was demonstrated its capacity to strongly bind MRJP 1 (Bilikova *et al*., 2002).Royalactina seems to induce the differentiation of the queen bee as well MRJP 1 (Kamakura, 2011).

Recently apolipophorin III-like protein was identified for the first time in RJ(Han *et al.,* 2011). ApolipophorinIII-like protein is a lipid binding protein that may form protein-lipid complexes in order to carry lipids into aqueous environments (Fujita *et al.,* 2012; Kim and Jin, 2015). ApolipophorinIII-like protein may contribute additional to the antibacterial properties of RJ and could also play a significant role in the development of immune responses of honeybee larvae (Fujita *et al.,* 2012; Han *et al.,* 2011).

Glucose oxidase enzyme (GOx) was also detected in RJ (Li *et al*., 2008; Sano *et al*., 2004). GOx that catalyses the oxidation of glucose to hydrogen peroxide was also detected in honey where showed a high antibacterial activity (Sagona *et al*., 2015).

Therefore, MRJPs, Royalisin, Jelleines, Apismin, Royalactina, apolipophorinIII-like protein and glucose oxidase, present in RJ may contribute each other in virtue of their different chemical structure, to the development of queen bee and to the efficient immune systems of honeybees, and provided as well an effective protective action of RJ both *in vivo* and *in vitro* uses.

2.3 Lipids

The lipids are present from 3 to 19 % of the RJ dry matter (Melliou and Chinou, 2005; Nabas *et al*., 2014; Ramadan and Al-Ghamdi, 2012).

Approximately 90% of lipids is constituted by fatty acids; the rest are neutral lipids, steroids, hydrocarbons and phenols (Nabas *et al*., 2014; Ramadan and Al-Ghamdi, 2012).

The fatty acids of RJ have 8–10 carbon atoms, usually either hydroxy fatty acids or dicarboxylic acids, unlike organic acids of most animal and plant materials (Kodai *et al*.,2007; Noda *et al*.,2005; Ramadan and Al-Ghamdi, 2012).

The analysis of the lipid components can be a criterion of the genuineness of the RJ, because an adulteration with honey or sugars, decrease the protein and lipid component, increase the concentration of minor sugars and makes RJ insoluble in alkaline medium (Boselli *et al*., 2003; Lercker *et al.,*1981; Li and Chen, 2003).

The main acid of fatty acid fraction is 10-hydroxy-2-decenoic (10-HDA) (Terada *et al.*,2011; Kitahara *et al.*,1995; Genc and Aslan, 1999), an unsaturated acid that seems to be involve in the antibacterial activity of RJ (Bloodworth *et al.*,1995; Nagai and Inoue, 2005).

10-HDA also showed to have an important biological role in the development of the colony strategies (Wu *et al*., 1991).Moreover, the 10-HDA content has been adopted as a marker for quality and freshness analysis of RJ (Antinelli *et al*., 2003; Ferioli *et al*., 2007).

Also the octanoic acid, present in lower amount than 10-HDA, seems to cover more than only a nutritional function; recently research showed that the octanoic acid is involved in the repellence action of queen cells again *Varroa destructor* (Nazzi *et al*., 2009).

2.4 Vitamins

RJ is very abundant in B group vitamins, mainly vitamin B5 followed by vitamins B1, B2, B6, B8, B9 and B12 (Li *et al.*, 2013; Viuda-Martos *et al.*, 2008). Vitamin PP and vitamin C are present only in small amounts(Liu *et al.*, 2008; Melliou and Chinou, 2005; Nagai *et al.*, 2001). Liposoluble vitamins such as vitamins A, D, E and K are absent (Li and Chen, 2003; Morita *et al.*, 2012; Nagai *et al.*, 2005; Ramadan and Al-Ghamdi, 2012;).

The vitamins content of RJ is subjected to seasonal changes as variation of the pollen of flowers collected by worker bees, as the mainly source of vitamins comes from the pollen(Biondi *et al*., 2003; Chen and Chen, 1995; Sabatini *et al.*, 2009).

2.5Minerals and other minor elements

Minerals and other elements are about 4% to 8% of RJ dry matter (Sabatini *et al*., 2009). The main elements are K, P, S, Na, Ca, Al, Mg, Zn, Fe, Cu and Mn, but there are also traces of Ni, Cr, Sn, W, Sb, Bi and Ti (Benfenati *et al.*, 1986; Li and Chen, 2003; Ramadan and Al-Ghamdi, 2012;Viuda-Martos*et al.*, 2008). The presence of minerals is related (and therefore variability) by the source of the feed, the production period, the environment and biological factors of bees (Benfenati *et al*., 1986; Garcia-Amoedo and de Almeida-Muradian, 2007; Nation and Robinson, 1971; Sabatini *et al*., 2009).

Moreover, RJ contains several minor components classified under various chemical classes, such as heterocyclic substances, biopterine and neopterine (Bogdanov, 2012). In RJ were also found low amounts of free nucleotides (adenosine, guanosine, cytidine, and iridine), phosphates, ATP, ADP, AMP, acetylcholine and gluconic, benzoic, malic, citric, and lactic acids (Bogdanov, 2012; Matsuka,1993; Sabatini *et al*., 2009). The functions of these components is still unclear, although their origin is assumed to arise from the nurse bee.

3. Mechanisms of Antimicrobial peptides action

Antimicrobial peptides (AMPs) are fundamental defence biomolecules that could protect the host from bacteria, viruses or fungi (Beutler, 2004; Gallo and Nizet, 2003;Zasloff, 2002).They have been preserved evolutionarily in their innate immune response, which represents the first line of defence in most living organisms (Hancock and Lehrer, 1998;Brogden*et al*., 2003).

AMPs are polypeptides of variable length containing from 10 to 50 amino acids, so relatively short, and they have a positive charge that goes from 2 to 9 (most commonly 4 or 6), due to an excess of basic residues of lysine, arginine and histidine (Ebenhan *et al*., 2014; Splith and Neundorf, 2011).These properties allow the interaction between AMPs and microbial surfaces (negatively charged), and to the cell membrane penetration by bilayer phospholipids head groups (Brogden *et al*., 2007; Pandey *et al.*, 2011).

The sequence of amino acids of the peptide has a significant role; in fact the presence of an amino acid or its substitution with another one, even with similar chemical properties, may change the effectiveness of peptide as its antimicrobial activity (Maróti *et al.*, 2011; Pandey *et al.*, 2011).

The interaction between the AMPs and the surface of the bacterial cell membrane seems to be strictly correlated to the electrostatic interactions of the AMPs sequence and the structure of the bacterial membrane surface. Furthermore, even the secondary structure of the peptide (α-helix or β-sheet) plays an important role primarily when the electrostatic interactions can not be established due to the distance between the charged groups (Scott *et al*., 1999; Yang *et al*., 2001; Zhao *et al*., 2001).

Several studies on the mechanism of action of AMPs have revealed different ways in which these substances exerted their effect (Bulet *et al.*, 1999). Although the antibacterial properties of many peptides present in RJ were demonstrated however their mode of actions were not been yet clarified in details.

The classic way which AMPs exert their action is by the ability to interact with cells membrane determining a permeabilization (Boman *et al*., 1994; Brogden, 2005 ;Huang *et al*., 2000).

There are three different models to describe possible AMPs mechanisms of action against bacteria: barrel-stave model, carpet-like model, and toroidal pore model (Li *et al.*, 2012).

The barrel-stave model contemplates the formation of pores in the hydrophobic core of the membrane created by a circular assembly of AMPs where their hydrophobic domains pointing toward the lipid chains of the membrane while the hydrophilics toward the interior of the pore (Li *et al.*, 2012; Maróti *et al.*, 2011;Shai *et al*., 2002).

In the carpet-like model the AMPs initially interact with the external surface of the membrane, subsequently the charged region of the peptide interacts with the anionic phospholipids forming a carpet, which extends on the surface of the target membrane. This mode of action cause a reducing of the lipid layer surface and a consequent membrane disruption with collapse of the lipid structure. The toroidal pore model also presents the formation of pores in the membrane like barrel-stave model, but in this case the phospholipids assumed a completely curvature as a double layer. In this process the lines of the double layer becomes a continuous structure, with the consequent formation on a pore. The toroidal pore model is an intermediate case between the two previously described models and in some cases it is difficult to establish a clear distinction. In the barrel-stave model and in the toroidal pore model the peptide causes a rearrangement of the polar heads of phospholipids by bundling the amphipathic helices and forming a transmembrane pore which the hydrophilic part of the peptide facing the lumen of the pore (Li *et al.*, 2012 ; Matsuzaki *et al.* 1996).Currently a fourth model was described, the aggregate channel model. Several studies indicate that permeabilization of the cell membrane alone may not be enough to kill bacteria (as predict in the other models). Like the carpet model there is no formation of pores in the cell membrane. After the formation of a binding between the peptide and the phospholipid head groups the peptide reaches the inner part of the cell without modified the membrane (a mechanism of transport through the lipid bilayer without the formation of a stable channel). Once inside, the peptide can interact with the targets (Pálffy *et al.*, 2009; Xiao *et al.*, 2015).Unfortunately, the type of aggregates that provide the insertion of the peptide inside the membrane is not well defined, so it is more difficult to predict molecular properties who can favour this mechanism (Herbig *et al*., 2005; Li *et al.*, 2012).

Besides the ability to interact with bacterial membranes, the AMPs could have other intracellular target (Ahn *et al*., 2006); in fact they can bind DNA, RNA and proteins and inhibit synthesis of different essential cell constituents as cell wall, DNA, RNA and proteins(Lan *et al*., 2010;Li *et al*., 2012). Moreover, AMPs can interfere with bacterial cytokinesis by cell filamentation by an unique mechanisms to translocation in side the cell in order to alter the cytoplasmic membrane septum formation (Brown and Hancock, 2006;Lan *et al*., 2010;Li *et al*., 2012).

Many constituent of RJ are ascribable to the antimicrobial peptides category such as MRJPs, Royalisin, Jelleines, Apismin, Royalactna, and apolipophorinIII-like. The natural origin of these composts could be a potential added value to different products as *in vitro* use. RJ, as well its by-products, could find a major role in the control of microorganisms growth both for their proven activities and for the low amounts needed.

4. Antibacterial activity

The presence of antimicrobial properties of RJ against Gram positive and Gram negative bacteria was scientifically showed for the first time in 1939 by McCleskey and Melampy.

Subsequent studies of Hinglais *et al.*(1955), Butenandt and Rembold (1957), Blum *et al.*(1959), Iizuka and Koyama (1964) and Muratova *et al.*(1967) reported the effects of RJ and 10-HDA against many bacteria, including *Escherichia coli* and *Micrococcus pyogenes*.

In 1990, Fujiwara *et al.* isolated and purified Royalisin from RJ.MIC (Minimum Inhibitory Concentration) evaluation of crude RJ showed that both Gram positive and Gram negative tested bacteria posses a low resistance of to this substance (Table 1).

Royalisin MIC evaluation reported a strong antibacterial activity against Gram positive bacteria, but not against Gram negative (Table 2). Bacterial strains belonging to *Bifidobacterium, Clostridium*, *Corynebacterium, Lactobacillus*, *Leuconostoc, Staphylococcus* and *Streptococcus* genera showed an inhibitory concentration of Royalisin comparable with the effective concentrations of several antibiotic classes.

The difference in antibacterial effectiveness between RJ and Royalisin could be explained by the presence of other compounds, such as 10-HDA, which were completely lost during this peptide splitting.

Further studies on antibacterial activity of Royalisin carried out by Bilikova *et al.* in 2001. These Authors investigated the specific action of Royalisin against Gram positive bacteria (Table 2).The aim of that study was to verify the action of the peptide against aetiological agent of American foulbrood, *Paenibacillus larvae* subsp. *larvae*. The results showed effective action of Royalisin against *Bacillus subtilis* and *Paenibacillus larvae* subsp. *larvae* while no inhibition was determined for *Micrococcus luteus* (*Sarcina lutea*).

Shen *et al.* in 2010 and 2012 isolated and purified recombinant Royalisins expressed by *Escherichia coli* after fusing in a vector the *Apis cerana cerana* cDNAs encoding for different Royalisin forms. These recombinant Royalisins showed higher antibacterial activity against Gram positive bacteria than Gram negative bacteria (MIC over 2000.0 µl/ml, Table 2); unfortunately no solid consideration could be formulated about the differences between the native and recombinant Royalisins for the different method of determination of the inhibitory activity.

In 2002, in Thailand, Ratanavalachai and Wongchai tested the antibacterial activities of crude RJ, and both the lipid and defatted extracts. Authors also evaluated different storage combination time/temperature in order to enhance RJ conservation. RJ freshly picked was stored at room temperature (25-27 °C), refrigerated temperature (2-4 °C) and deep frozen (-18 °C) for 12 hours, 24 hours and 3 days and subsequently was tested against several bacteria.

Results showed that conservation of RJ at frozen temperature did not affect antibacterial and bacteriostatic activities (Table 1). Instead, Authors observed a decrease in RJ antibacterial activity during storage time at all tested temperatures.

Fontana *et al.* in 2004 identified in RJ employing mass spectrometry four antimicrobial peptides that were called Jelleines.

The most relevant peptides with antimicrobial activity were Jelleines I and II, followed by Jelleine III, which has not demonstrated activity against all the microorganisms, and finally the Jelleine IV that has not given any evidence of activity (Table 3).
Few researches were carried out on antibacterial activities of Jelleines and their modified forms. Romanelli *et al*. (2011) showed that Jelleine I, II and III inhibit bacterial growth while the modifications of the structure in C and N terminals of these peptides caused a decreasing of activity (Table 3). Results of Capparelli *et al.* (2012) showed a wide range of activity against *Staphylococcus epidermidis* from 30 to 300 µg/ml for C-terminal modified peptides.

Brudzynski and Sjaarda (2015), in a recent study about the evaluation of honey glycoproteins against *Escherichia coli* and *Bacillus subtilis*, attributed the antibacterial activities to the presence of MRJP-1 and likely the presence of Jelleines.

In 2005Eshraghiinvestigated the different antibacterial properties of the crude RJ, the ether-non-soluble fraction and the ether-soluble fraction against different bacteria. The results showed a clear inhibitory effect of crude RJ; *Staphylococcus aureus* strain was the most sensible followed by *Streptomyces griseus* and *Escherichia coli*(Table 1).

The ether-soluble fraction of RJ showed a greater antibacterial action than the crude RJ, while the ether-non-soluble fraction, containing the Royalisin, was found to be less effective, even of crude RJ.

According to the experiment results, the antibacterial RJ action could be attributed to the ether-soluble fraction, i.e. the part containing lipids and fatty acids including 10-HDA, and not to the ether-non-soluble fraction, that includes Royalisin (inhibition zone of 10-HDA are reported in Table 4) (Eshraghi, 2005).

Recently, Boukraa (2008) and Boukraa *et al.* (2009) evaluated antibacterial effect of RJ against *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Escherichia coli.* Results showed that all tested bacterial strains were susceptible to RJ (Table 1).Antibacterial activity against *Pseudomonas aeruginosa* is probably related to the action of RJ demonstrated by Lerrer *et al.*(2007) that seems abrogate lectin-dependent infection-preceding by *Pseudomonas aeruginosa* dhesion.
RJ is also effective against some bacteria implicated in infection of skin wounds, as shown by the study conducted in Argentina on two different RJ samples in 2010 by Garcia *et al.* The MIC values of the two samples of RJ were reported in Table 1 as a range. Interesting results were reported as concern the inhibition and the bactericidal effects: the MIC values were around twenty times lower than MBC values. The observed differences in the values of MIC and MBC may be related to the RJ components associated to the geographical area or genetic variability between bee colonies.

In 2013 Garcia *et al.* evaluated the antibacterial activity of four Argentinean RJs, 10-HDA, ether--soluble fraction and fat-free RJ (Table 1 and Table 4).The results showed that the different samples of RJ tested had a significant antibacterial activity on almost all bacterial strains examined, with remarkable MIC values; both RJ and 10-HDA showed lower activity against Gram negative bacteria, as *Klebsiella pneumoniae, Escherichia coli* and *Pseudomonas aeruginosa* than against Gram positive bacteria.
In 2013, Moselhy *et al.* studied the antibacterial activity of Egyptian RJs (two samples collected in two different period, ie camphor and citrus seasons) and a Chinese RJ.

Authors reported that Gram positive bacteria (*Staphylococcus aureus* and *Bacillus subtilis*) were more sensitive to all three samples of RJ compared to Gram negative bacteria (*Pseudomonas aeruginosa* and *Escherichia coli*) (Table 1).

In addition, the samples of Egyptian RJ were found to have a more effective antibacterial action than Chinese RJ. The bactericidal or bacteriostatic action of RJ is closely linked to the geographical origin, the related botanical species and the genetic variability between colonies (Boukraa and Sulaiman, 2009; Garcia *et al.*, 2010; Garcia *et al*., 2013; Garcia-Amodeo and de Almeida-Muradian, 2007).

Another confirmation of the effectiveness of RJ against *Staphylococcus aureus* strains was recently

reported by an *in vivo* study carried out on rats by Gunaldi *et al.*(2014).

Rats with spinal implant inoculated with the bacteria and treated with RJ showed a decrease in severity of the infection if compared with the rats without RJ addition.

Bilikova *et al*. (2015) analysed Royalisin and Royalisin-D, a recombinant shortened form constructed in order to correlate the structure to the antimicrobial activity. Royalisin-D was structured as a reduced form of Royalisin that lacks of 11 amino acids at the C-terminal (Tseng *et al.,* 2011).

In addition to investigate the importance of the disulfide bonds in Royalisin, the two peptide were treated with dithiothreitol (DTT) as a reducing agent of the disulfide bonds. The action of each peptide, crude and treated with DTT, against each microorganism was evaluated with MIC and MBC (Table 2).

All bacteria were susceptible to the peptides, with the exception of *Escherichia coli*. Moreover, the activity of Royalisin and Royalisin-D were very similar to each other, while a significant and important difference was noted for the two peptides treated with DTT. In fact Royalisin and Royalisin-D treated with DTT showed a decreased inhibitory and bactericidal effects. These results highlight the importance of the disulfide bonds of Royalisin.

5. Conclusions

From available literature Royal Jelly and its derivate components, such as Royalisin, Jelleines and 10-hydroxy-2-decenoic acid (10-HDA), showed a high activity against Gram positive bacteria while their effectiveness decrease against Gram negative. Moreover, several studies carried out on Royal Jelly showed that this product is also effective against many multidrug resistant bacteria, such as MRSA (methicillin-resistant *Staphylococcus aureus*). This is particularly important since one of the major public health problems is currently represented right from the onset of an increasing number of antibiotic resistant bacteria. The indiscriminate use of antibiotics has led to the selection of resistant clones many for which it is often not provided an adequate therapy. Multidrug resistant bacteria management request an increasing attention to the antibacterial molecules/products used. For this reason researches in recent years has been directed toward the discovery of new antimicrobial substances, particularly natural substances such as plant extracts, essential oils and antimicrobial peptides isolated from many different animals. The interest in beehive products is also further enhanced by the fact that these products have always represented an important resource such as functional foods, which have not only the nutritional function, but also nutraceutical or rather to improve and promote human health due to the presence of molecules that prevent or fight various disease states.

On the basis of the results obtained by several studies about the antibacterial properties of Royal Jelly, it seems clear that this beehive product could be a potential subject of further investigation by the scientific world.

The new findings regarding its active components, their inner mechanisms of action and the possibility of isolation and purification of the pure substances, represent a starting point for the formulation of new products for therapeutic and pharmacological uses as an alternative to conventional antibiotics. Natural peptides, as Major Royal Jelly Proteins, could be taken into account as potential alternatives. The use of RJ could lead to the realization of nutraceutical products with a remarkable added value.

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Table 1. Antibacterial activities of Royal Jelly.

Bacterial strains		MIC	MBC	Method	Reference
Bacillus cereus		12.5 mg/ml		AI	Ratanavalachai and Wongchai, 2002
Bacillus subtilis	RCMBA 6005	7.8-500.0 µg/ml		AWD	Moselhy *et al.*, 2013
Bacteroides fragilis		nd		BM	Fujiwara *et al.*, 1990
Bacteroides vulgatus		nd		BM	Fujiwara *et al.*, 1990
Bifidobacterium adolescentis	ATCC 15703	10.0 µg/ml		BM	Fujiwara *et al.*, 1990
Bifidobacterium bifidum	ATCC 15696	10.0 µg/ml		BM	Fujiwara *et al.*, 1990
Bifidobacterium breve	ATCC 15700	10.0 µg/ml		BM	Fujiwara *et al.*, 1990
Bifidobacterium infantis	ATCC 15697	10.0 µg/ml		BM	Fujiwara *et al.*, 1990
Bifidobacterium longum	ATCC 15707	10.0 µg/ml		BM	Fujiwara *et al.*, 1990
Enteococcus faecium		50.0-70.0 w/w[3]		AWD	Garcia *et al.*, 2013
Enterococcus faecalis		40.0-100.0 w/w[3]		AWD	Garcia *et al.*, 2013
		40.0-80.0 w/w[3]		AWD	Garcia *et al.*, 2013
		50.0-90.0 w/w[3]		AWD	Garcia *et al.*, 2013
	ATCC 29212	60.0-80.0 w/w[3]		AWD	Garcia *et al.*, 2013
		3.7-7.6 mg/ml	125.0->250.0 mg/ml	BD	Garcia *et al.*, 2010
	ATCC 29212	5.0-13.7 mg/ml	>250.0 mg/ml	BD	Garcia *et al.*, 2010
Escherichia coli		13.5 mg/ml		AI	Ratanavalachai and Wongchai, 2002
		60.0-100.0 w/w[3]		AWD	Garcia *et al.*, 2013
	RCMBA 5003	500.0 µg/ml		AWD	Moselhy *et al.*, 2013
		2.0 v/v[1]		BD	Boukraa *et al.*, 2009
		7.0-7.1 mg/ml	>250.0 mg/ml	BD	Garcia *et al.*, 2010
	IID 861	10.0 µg/ml		BM	Fujiwara *et al.*, 1990
	ATCC 29532	12.0 mm[2]		DP	Eshraghi, 2005
Klebsiella pneumoniae		80.0-100.0 w/w[3]		AWD	Garcia *et al.*, 2013
		8.0-8.1 mg/ml	125.0-250.0 mg/ml	BD	Garcia *et al.*, 2010
	IFO-3321	nd		BM	Fujiwara *et al.*, 1990
Lactobacillus acidophilus	ATCC 314	10.0 µg/ml		BM	Fujiwara *et al.*, 1990
	ATCC 4356	10.0 µg/ml		BM	Fujiwara *et al.*, 1990
Lactobacillus helveticus subsp. *Jugurti*		10.0 µg/ml		BM	Fujiwara *et al.*, 1990
Micrococcus luteus	ATCC 9341	40.0-60.0 w/w[3]		AWD	Garcia *et al.*, 2013
	ATCC 9341	7.5-11.8 mg/ml	125.0 mg/ml	BD	Garcia *et al.*, 2010
Micrococcus luteus (Sarcina lutea)		0.3 mg/ml		AI	Ratanavalachai and Wongchai, 2002
Proteus vulgaris		15.5 mg/ml		AI	Ratanavalachai and Wongchai, 2002
Pseudomonas aeruginosa		15.5 mg/ml		AI	Ratanavalachai and Wongchai, 2002
	ATCC 27853	4.0 v/v[1]		AI	Boukraa, 2008
		70.0-100.0 w/w[3]		AWD	Garcia *et al.*, 2013
		60.0-100.0 w/w[3]		AWD	Garcia *et al.*, 2013
	RCMBA 1002	nd		AWD	Moselhy *et al.*, 2013
		3.3-14.4 mg/ml	63.0-250.0 mg/ml	BD	Garcia *et al.*, 2010
Salmonella infantis		10.0 µg/ml		BM	Fujiwara *et al.*, 1990
Salmonella typhi		14.5 mg/ml		AI	Ratanavalachai and Wongchai, 2002
Salmonella typhimurium		10.0 µg/ml		BM	Fujiwara *et al.*, 1990
Shigella flexneri		14.5 mg/ml		AI	Ratanavalachai and Wongchai, 2002
Staphylococcus aureus		12.5 mg/ml		AI	Ratanavalachai and Wongchai, 2002
	RCMBA 2004	15.6-500.0 µg/ml		AWD	Moselhy *et al.*, 2013
		1.7 v/v[1]		BD	Boukraa *et al.*, 2009
	ATCC 14776	15.0 mm[2]		DP	Eshraghi, 2005
Staphylococcus aureus MR 1		40.0-70.0 w/w[3]		AWD	Garcia *et al.*, 2013
		8.0-14.5 mg/ml	125.0 mg/ml	BD	Garcia *et al.* 2010
Staphylococcus aureus MR 2		30.0-70.0 w/w[3]		AWD	Garcia *et al.*, 2013
		8.0-12.5 mg/ml	125.0-250.0 mg/ml	BD	Garcia *et al.* 2010
Staphylococcus aureus MS 1	ATCC 25923	20.0-80.0 w/w[3]		AWD	Garcia *et al.*, 2013
	ATCC 25923	7.8-9.0 mg/ml	125.0->250.0 mg/ml	BD	Garcia *et al.* 2010
Staphylococcus aureus MS 2		40.0-80.0 w/w[3]		AWD	Garcia *et al.*, 2013

		3.4-8.8 mg/ml	125.0->250.0 mg/ml	BD	Garcia *et al.* 2010
Staphylococcus epidermidis		40.0-80.0 w/w[3]		AWD	Garcia *et al.*, 2013
		8.7-10.3 mg/ml	125.0 mg/ml	BD	Garcia *et al.*, 2010
Streptococcus agalactiae		50.0-100.0 w/w[3]		AWD	Garcia *et al.*, 2013
		70.0-90.0 w/w[3]		AWD	Garcia *et al.*, 2013
	ATCC 27956	50.0-90.0 w/w[3]		AWD	Garcia *et al.*, 2013
Streptococcus dysgalactiae		80.0-100.0 w/w[3]		AWD	Garcia *et al.*, 2013
		80.0-90.0 w/w[3]		AWD	Garcia *et al.*, 2013
	ATCC 27957	50.0-100.0 w/w[3]		AWD	Garcia *et al.*, 2013
Streptococcus uberis		60.0-70.0 w/w[3]		AWD	Garcia *et al.*, 2013
		5.8-14.5 mg/ml	250.0->250.0 mg/ml	BD	Garcia *et al.*, 2010
Streptomyces griseus	ATCC 11746	14.0 mm[2]		DP	Eshraghi, 2005

nd: value not determined.

AI: Agar Infusion; AWD: Agar Well Diffusion; BM: Broth Medium; BD: Broth Dilution; DP: Drop Plate; DT.

[1]: volume/volume of RJ on Muller Hinton agar medium.

[2]: RJ concentration 330 mg/ml.

[3]: weight/weight of RJ on water.

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Table 2. Antibacterial activities of Royalisin.

Bacterial strains		MIC	MBC	Method	Reference
Bacillus subtilis		5.4-108.0 μg/ml		DT	Bilikova *et al.*, 2001
	CMCC 63501	9.83 mm[1]		DT	Shen *et al.*, 2010
	CMCC 63501	10.53 mm[2]		DT	Shen *et al.*, 2010
	CMCC 63501	62.5 μg/ml[3]		MA	Shen *et al.*, 2012
Bacteroides fragilis		nd		BM	Fujiwara *et al.*, 1990
Bacteroides vulgatus		nd		BM	Fujiwara *et al.*, 1990
Bifidobacterium adolescentis	ATCC 15703	1.0 μM		BM	Fujiwara *et al.*, 1990
Bifidobacterium bifidum	ATCC 15696	1.0 μM		BM	Fujiwara *et al.*, 1990
Bifidobacterium breve	ATCC 15700	1.0 μM		BM	Fujiwara *et al.*, 1990
Bifidobacterium infantis	ATCC 15697	1.0 μM		BM	Fujiwara *et al.*, 1990
Bifidobacterium longum	ATCC 15707	1.0 μM		BM	Fujiwara *et al.*, 1990
Clostridium perfringens	ATCC 13124	1.0 μM		BM	Fujiwara *et al.*, 1990
Clostridium tetani	ATCC 19406	250.0 μg/ml[3]		MA	Shen *et al.*, 2012
Corynebacterium pyogenes		1.0 μM		BM	Fujiwara *et al.*, 1990
Escherichia coli		nd		DT	Bilikova *et al.*, 2001
		nd	nd	MA	Bilikova *et al.*, 2015
		nd[4]	nd[4]	MA	Bilikova *et al.*, 2015
		nd[5]	nd[5]	MA	Bilikova *et al.*, 2015
		nd[6]	nd[6]	MA	Bilikova *et al.*, 2015
	IID 861	nd		BM	Fujiwara *et al.*, 1990
	CGMCC1.1139	>2000.0 μg/ml[3]		MA	Shen *et al.*, 2012
Klebsiella pneumoniae	IFO-3321	nd		BM	Fujiwara *et al.*, 1990
Lactobacillus acidophilus	ATCC 314	1.0 μM		BM	Fujiwara *et al.*, 1990
	ATCC 4356	1.0 μM		BM	Fujiwara *et al.*, 1990
Lactobacillus bulgaricus	ATCC 11841	1.0 μM		BM	Fujiwara *et al.*, 1990
Lactobacillus helveticus subsp. *jugurti*		1.0 μM		BM	Fujiwara *et al.*, 1990
Lactobacillus lactis	ATCC 8000	1.0 μM		BM	Fujiwara *et al.*, 1990
Lactobacillus leichmannii	ATCC 7830	1.0 μM		BM	Fujiwara *et al.*, 1990
Leuconostoc cremoris	ATCC 19254	1.0 μM		BM	Fujiwara *et al.*, 1990
Micrococcus luteus (Sarcina lutea)		nd		DT	Bilikova *et al.*, 2001
	CMCC 28001	15.07 mm[1]		DT	Shen *et al.*, 2010
	CMCC 28001	16.70 mm[2]		DT	Shen *et al.*, 2010
	CMCC 28001	125.0 μg/ml[3]		MA	Shen *et al.*, 2012
Paenibacillus larvae subsp. *larvae*		6.0 μg/ml	15.0 μg/ml	MA	Bilikova *et al.*, 2015
		10.0 μg/ml[4]	nd[4]	MA	Bilikova *et al.*, 2015

		10.0 µg/ml[5]	20.0 µg/ml[5]	MA	Bilikova *et al.*, 2015
		50.0 µg/ml[6]	nd[6]	MA	Bilikova *et al.*, 2015
	ATCC 5084	5.4-108.0 µg/ml		DT	Bilikova *et al.*, 2001
	ATCC 5085	5.4-108.0 µg/ml		DT	Bilikova *et al.*, 2001
	ATCC 5086	5.4-108.0 µg/ml		DT	Bilikova *et al.*, 2001
Proteus vulgaris	CGMCC1.1527	>2000.0 µg/ml[3]		MA	Shen *et al.*, 2012
Pseudomonas aeruginosa		10.0 µg/ml	15.0 µg/ml	MA	Bilikova *et al.*, 2015
		nd[4]	nd[4]	MA	Bilikova *et al.*, 2015
		11.0 µg/ml[5]	17.0 µg/ml[5]	MA	Bilikova *et al.*, 2015
		nd[6]	nd[6]	MA	Bilikova *et al.*, 2015
Salmonella choleraesuis		9.0 µg/ml	11.0 µg/ml	MA	Bilikova *et al.*, 2015
		20.0 µg/ml[4]	nd[4]	MA	Bilikova *et al.*, 2015
		20.0 µg/ml[5]	18.0 µg/ml[5]	MA	Bilikova *et al.*, 2015
		nd[6]	nd[6]	MA	Bilikova *et al.*, 2015
Salmonella infantis		nd		BM	Fujiwara *et al.*, 1990
Salmonella typhimurium		nd		BM	Fujiwara *et al.*, 1990
	CGMCC1.1190	>2000.0 µg/ml[3]		MA	Shen *et al.*, 2012
Serratia marcescens		nd		DT	Bilikova *et al.*, 2001
Staphylococcus aureus		7.5 µg/ml	13.0 µg/ml	MA	Bilikova *et al.*, 2015
		20.0 µg/ml[4]	nd[4]	MA	Bilikova *et al.*, 2015
		9.5 µg/ml[5]	13.5 µg/ml[5]	MA	Bilikova *et al.*, 2015
		20.0 µg/ml[6]	nd[6]	MA	Bilikova *et al.*, 2015
	SC-D	1.0 µM		BM	Fujiwara *et al.*, 1990
	CMCC 26003	11.53 mm[1]		DT	Shen *et al.*, 2010
	CMCC 26003	10.50 mm[2]		DT	Shen *et al.*, 2010
	CMCC 26003	250.0 µg/ml[3]		MA	Shen *et al.*, 2012
Staphylococcus intermedius B		4.0 µg/ml	6.5 µg/ml	MA	Bilikova *et al.*, 2015
		nd[4]	nd[4]	MA	Bilikova *et al.*, 2015
		4.6 µg/ml[5]	7.0 µg/ml[5]	MA	Bilikova *et al.*, 2015
		nd[6]	nd[6]	MA	Bilikova *et al.*, 2015
Staphylococcus xylosus		10.5 µg/ml	12.0 µg/ml	MA	Bilikova *et al.*, 2015
		nd[4]	nd[4]	MA	Bilikova *et al.*, 2015
		18.0 µg/ml[5]	19.0 µg/ml[5]	MA	Bilikova *et al.*, 2015
		nd[6]	nd[6]	MA	Bilikova *et al.*, 2015
Streptococcus alactolyticus		9.0 µg/ml	11.0 µg/ml	MA	Bilikova *et al.*, 2015
		nd[4]	nd[4]	MA	Bilikova *et al.*, 2015
		12.0 µg/ml[5]	18.0 µg/ml[5]	MA	Bilikova *et al.*, 2015

		nd[6]	nd[6]	MA	Bilikova *et al.*, 2015
Streptococcus thermophilus	ATCC 19258	1.0 µM		BM	Fujiwara *et al.*, 1990
Vibrio parahaemolyticus		4.0 µg/ml	6.5 µg/ml	MA	Bilikova *et al.*, 2015
		8.0 µg/ml[4]	nd[4]	MA	Bilikova *et al.*, 2015
		4.6 µg/ml[5]	7.0 µg/ml[5]	MA	Bilikova *et al.*, 2015
		8.0 µg/ml[6]	nd[6]	MA	Bilikova *et al.*, 2015

nd: value not determined.

BM: Broth Medium; DT: Diffusion Test; MA: Microplate Assay.

[1]: 2 mg/ml of fusion protein from pre-pro-Acc-royalisin.

[2]: 2 mg/ml of fusion protein from mature Acc-royalisin.

[3]: Recombinant Acc-royalisin.

[4]: Royalisin treated with DTT.

[5]: Royalisin-D.

[6]: Royalisin-D treated with DTT.

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Table 3. The inhibitory activities of Jelleines against bacteria.

Bacterial strains		MIC[1]				Reference
		Jelleine I	Jelleine II	Jelleine III	Jelleine IV	
Bacillus cereus		nd	nd	nd	nd	Fontana *et al.*, 2004
Bacillus pumilis		nd	nd	nd	nd	Fontana *et al.*, 2004
Bacillus subtilis	CCT 2471	10.0	30.0	nd	nd	Fontana *et al.*, 2004
Bacillus thuringiensis		nd	nd	nd	nd	Fontana *et al.*, 2004
Enterobacter cloacae	ATCC 23355	10.0	15.0	nd	nd	Fontana *et al.*, 2004
Escherichia coli	CCT 1371	2.5	15.0	15.0	nd	Fontana *et al.*, 2004
Klebsiella pneumoniae	ATCC 13883	10.0	15.0	nd	nd	Fontana *et al.*, 2004
Listeria monocytogenes		≥200.0	200.0			Romanelli*et al.*, 2011
Proteus mirabilis		nd	nd	nd	nd	Fontana *et al.*, 2004
Pseudomonas aeruginosa	ATCC 27853	10.0	15.0	30.0	nd	Fontana *et al.*, 2004
*Salmonella enterica*Paratyphi		≥200.0	200.0			Romanelli*et al.*, 2011
Staphylococcus aureus	ATCC 6535	10.0	15.0	30.0	nd	Fontana *et al.*, 2004
		≥200.0	200.0			Romanelli*et al.*, 2011
Staphylococcus saprophyticus		15.0	10.0	30.0	nd	Fontana *et al.*, 2004

[1]: MIC values (µg/ml) obtained with Microplate Assay method;

nd: value not determined.

Table 4. The inhibitory activities of 10-hydroxy-2-decenoic acid against bacteria.

Bacterial strains		MIC	Method	Reference
Streptomyces griseus	ATCC 11746	29.0 mm[1]	DP	Eshraghi, 2005
Staphylococcus aureus	ATCC 14776	40.0 mm[1]	DP	Eshraghi, 2005
Staphylococcus aureus MS 1	ATCC 25923	1.9 mg/ml	AWD	Garcia *et al.*, 2013
Staphylococcus aureus MS 2		1.9 mg/ml	AWD	Garcia *et al.*, 2013
Staphylococcus aureus MR 1		1.9 mg/ml	AWD	Garcia *et al.*, 2013
Staphylococcus aureus MR 2		2.3 mg/ml	AWD	Garcia *et al.*, 2013
Escherichia coli	ATCC 29532	22.0 mm[1]	DP	Eshraghi, 2005
		nd	AWD	Garcia *et al.*, 2013
Enterococcus faecalis	ATCC 29212	2.3 mg/ml	AWD	Garcia *et al.*, 2013
		1.9 mg/ml	AWD	Garcia *et al.*, 2013
		1.9 mg/ml	AWD	Garcia *et al.*, 2013
		2.3 mg/ml	AWD	Garcia *et al.*, 2013
Enterococcus faecium		2.3 mg/ml	AWD	Garcia *et al.*, 2013
Streptococcus uberis		2.3 mg/ml	AWD	Garcia *et al.*, 2013
Streptococcus agalactiae	ATCC 27956	nd	AWD	Garcia *et al.*, 2013
		2.3 mg/ml	AWD	Garcia *et al.*, 2013
		0.9 mg/ml	AWD	Garcia *et al.*, 2013
Klebsiella pneumoniae		nd	AWD	Garcia *et al.*, 2013
Pseudomonas aeruginosa		nd	AWD	Garcia *et al.*, 2013

nd: value not determined.

DP: Drop Plate; AWD: Agar Well Diffusion.

[1]: ether-soluble fraction of RJ concentration 30 mg/ml.

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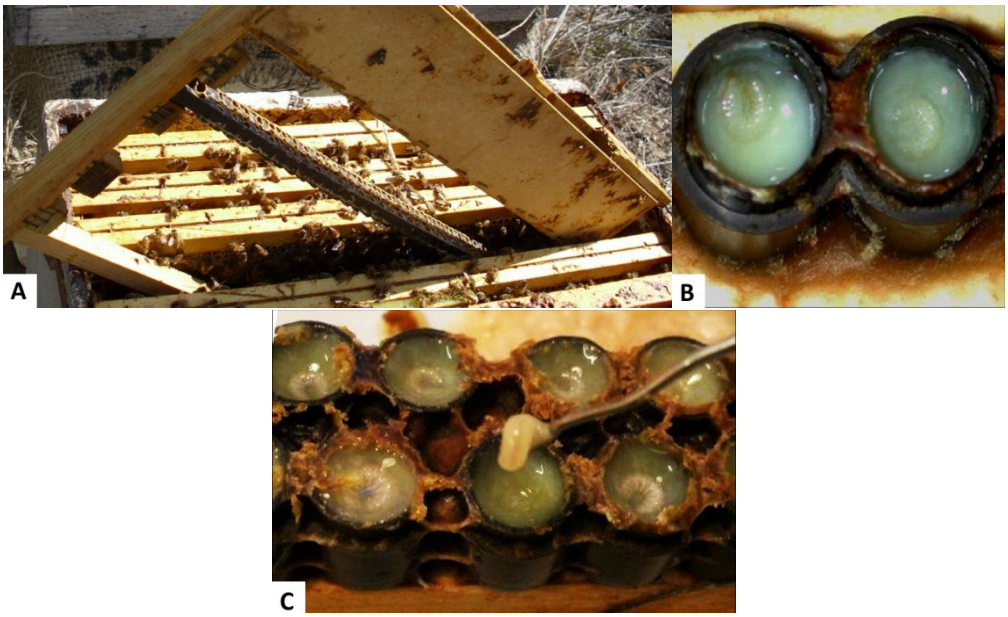
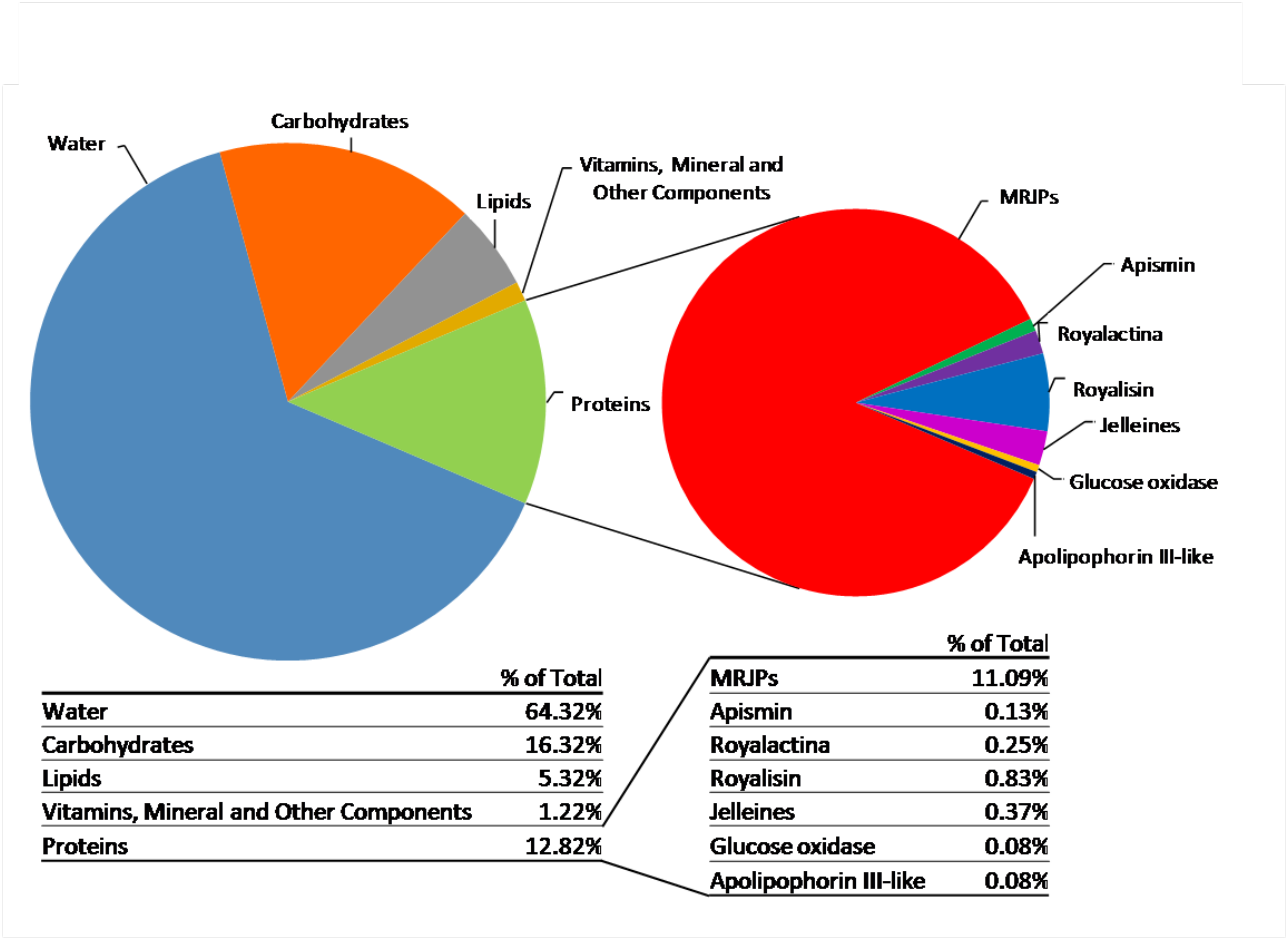
Figure 1. Hive and royal cells with Royal Jelly and queen bee larvae.



A. Hive for breeding queen bees and Royal Jelly production, constitued by only royal cells. **B.** Queen bee larvae during development in royal cells filled with Royal Jelly. **C.** Queen bee larvae removed by royal cells for the Royal Jelly collection.

Figure 2. Mean composition of Royal Jelly.



	% of Total
Water	64.32%
Carbohydrates	16.32%
Lipids	5.32%
Vitamins, Mineral and Other Components	1.22%
Proteins	12.82%

	% of Total
MRJPs	11.09%
Apismin	0.13%
Royalactina	0.25%
Royalisin	0.83%
Jelleines	0.37%
Glucose oxidase	0.08%
Apolipophorin III-like	0.08%