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Biological properties and activities of major royal jelly proteins and their derived peptides

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ABSTRACT

Royal jelly (RJ) is a complex beehive product that is important for larval development and queen nutrition in the hive and may also have beneficial effects on human health, according to *in vitro* and *in vivo* studies. The main proteins in RJ belong to the Major royal jelly proteins family (MRJPs), representing up to 90% of the total proteins. This narrative review aims to compile the results of studies on MRJPs and their derived peptides to understand their biological effects better, their most important activities being antioxidant, antimicrobial, anti-tumor, hypotensive, hypolipidemic, cell growth promoting, wound healing, anti-aging, neuroprotective, anti-inflammatory and immune-modulatory.

1. Introduction

Honey bee (*Apis* spp.) products are used by humans for millennia. This is undoubtedly recorded in numerous mesolithic rock paintings showing humans collecting honey from nests across the native range of honey bees (Dams & Dams, 1977; Mathpal, 2015; Pager, 2015). The earliest evidence for humans deliberately using honey bee products (in this case wax) was dated to around 38,000 BCE (D'Errico et al., 2012) and true beekeeping was first found in ancient Egypt (2,450 BCE) in a relief showing honey processing and honey storing (Borchardt, 1900; Kuény, 2015) [The interested reader on the history of beekeeping is referred to the excellent books by Gene Kritsky and Eva Crane (Crane, 1999; Kritsky, 2005)]. While wax and honey have obviously been known to mankind for centuries, a third honey bee product, food jelly, has only been first mentioned in the 18th century (Swammerdam, 1737). Food jelly is an acidic secretion (pH 4.0) of the hypopharyngeal and mandibular glands of young worker honey bees (Hoffmann, 1960; Kratky, 1931; Schiemenz, 1883) that is fed to the developing larvae. Special attention has always been on food jelly fed to queen larvae, aptly named royal jelly (RJ) (Huber, 1792), due to two reasons: first and foremost as it has the potential to turn a growing larva into a queen and second as it is available in larger quantities (100–200 mg per queen cell) than food jelly fed to worker or drone larvae (approximately 1 and 10 mg per cell, respectively) (von Planta, 1888). Whereas RJ is produced by all *Apis* species (Koeniger et al., 2011), research on RJ is almost exclusively limited to the Western honey bee *Apis mellifera*. Thus, if not mentioned otherwise, all research summarized here is based on *A. mellifera* RJ.

The investigation of RJ as potentially being beneficial for human health started in the 1930s with the question whether RJ has an antibacterial effect against certain human pathogenic bacteria (McCleskey & Melampy, 1939). Even though research on RJ started comparatively late, RJ has nowadays a considerable commercial value as it is utilized in the pharmaceutical, cosmetic and food industry (Sabatini et al., 2009) with China being the largest producer (3,500 tons in 2010) and exporter (220 tons in 2014, 39 Mio. USD export value) in the world (Cao et al., 2016). As of today, various studies suggest that RJ has functional activities such as antioxidant, antimicrobial, anti-inflammatory, immunomodulatory, wound healing, cell proliferation stimulation, anticancer, anti-aging, anti-allergic, etc. (Ahmad et al., 2020; Collazo et al., 2021; Ramanathan et al., 2018). Those activities are associated with several bioactive components found in RJ. We here review one of

Abbreviations: RJ, royal jelly; MRJP, major royal jelly proteins.

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these groups of bioactive components - major royal jelly proteins (MRJPs) which make up 10 – 14 % (*w/w*) of RJ (Furusawa et al., 2008; Hanes & Šimuth, 1992; Rembold, 1983; Schmitzová et al., 1998; von Planta, 1888).

The genome of *A. mellifera* encodes nine MRJPs, namely MRJP1 to MRJP9 (Table 1) (Weinstock et al., 2006; Helbing et al., 2017) with amino acid sequence identities between 47 and 74 % (Buttstedt et al., 2014). All nine MRJPs have been identified via mass spectrometry in RJ (Schönleben et al., 2007; Zhang et al., 2014) with MRJP1 being the most abundant (31–66 % of total RJ proteins), followed by MRJP3, MRJP2 and MRJP5 (Bíliková & Šimúth, 2010; Schmitzová et al., 1998; Šimúth, 2001). Furthermore, honey bees do actively add MRJPs during honey production and ripening (Lewkowski et al., 2019). Thus, MRJPs can not only be found in RJ but also in honey (Chua et al., 2013). All MRJPs have 1–8 predicted *N*-glycosylation sites (Table 1) (Buttstedt et al., 2014) and for MRJP1–4, MRJP6, MRJP7 and MRJP9 glycosylation has been experimentally confirmed (Kimura et al., 1996, 2010; Okamoto et al., 2003; Zhang et al., 2014). MRJP1 and MRJP2 have been shown to be differentially glycosylated (Bilikova et al., 2009; Kimura et al., 2010) and this differential glycosylation does for MRJP2 influence the antibacterial activity of the protein (Bilikova et al., 2009). This is partially explained by agglutinating activity for which the glycosylation is essential (see Chapter 3) (Brudzynski et al., 2015; Brudzynski & Sjaarda, 2015). Furthermore, glycosylation might effect IgE binding of MRJP1 and 2 (see Chapter 10) (Hayashi et al., 2011). These are so far the only known cases in which a MRJP glycosylation influences the activity.

MRJP1 function has been shown to dependent on the oligomeric state. The monomeric form is a 55 kDa protein also named royalactin (Buttstedt et al., 2016; Kamakura et al., 2001) while the oligomeric form of MRJP1, also known as apisin (Kimura et al., 2003), is an association of MRJP1 monomers with apisimin and 24-methylenecholesterol (Mandacaru et al., 2017; Tian et al., 2018). This oligomeric form of MRJP1 builds a fibrillary network that confers the needed viscosity to RJ (Buttstedt et al., 2018; Kurth et al., 2019). In addition, peptides derived via enzymatic cleavage from MRJP1, called jelleines have an antibacterial effect against a variety of bacteria (Fontana et al., 2004). Besides for MRJP1, the only other MRJP for which a function in honey bees has been described is MRJP3. MRJP3 binds and stabilizes RNA and is thought to share this RNA among individuals in the diet (Maori et al., 2019).

Whereas the functions of MRJPs for honey bees, except for the listed examples, are largely unclear, various biological activities (Fig. 1) of MRJPs and their derived peptides that might benefit humans have been demonstrated by cell culture, animal, and *in silico* studies (Table 2). In this review, we examine and summarize those studies.

2. Antioxidant activities

A few studies indicated that RJ has antioxidant activity (Jamnik et al., 2007; Pavel et al., 2014). Furthermore, it was shown that the antioxidant activity derived from its proteins (MRJPs) and peptides (Guo et al., 2009; Nagai & Inoue, 2004). For example, it was reported that the peptides obtained from RJ hydrolyzed with proteases had a strong antioxidant effect against lipid peroxidation (Guo et al., 2009). Furthermore, a number of 29 antioxidative peptides were isolated from RJ hydrolysate and their hydroxyl radicals and hydrogen-peroxide scavenging activities were tested. 12 peptides with 2–4 residues had the highest activity, while 3 dipeptides, namely Lys-Tyr, Arg-Tyr, and Tyr-Tyr, had strong scavenging activity derived from donating the hydrogen atom of the phenolic hydroxyl group (Guo et al., 2009).

Moreover, a study on *Drosophila melanogaster* fed with diets containing MRJPs demonstrated the up-regulation of superoxide dismutase and lifespan extension which was associated with MRJPs acting as antioxidants in intracellular cytoplasmic compartments (Xin et al., 2016).

Antioxidant assays on recombinant MRJP1–7 showed that they decreased the activity of a key mediator of apoptosis, namely caspase-3, and that they diminished the oxidative stress-induced apoptosis leading to enhanced viability of H_2O_2-exposed NIH 3 T3 cells. In addition, MRJPs have DPPH radical-scavenging activity and can protect DNA against oxidative damage (Park et al., 2020). In addition, recombinant MRJP2 from *Apis cerana* (AcMRJP2) protected cells by reducing the caspase-3 levels and oxidative stress-induced cell apoptosis leading to an increase of cell viability. MRJP2 was also found to have an antioxidant effect on mammalian and insect cells. Furthermore, AcMRJP2 protected the DNA against reactive oxygen species (Park et al., 2020).

Intending to understand the contribution of proteins from honey to its antioxidant activity, a team of researchers isolated total honey proteins and found that honey has a potent antioxidant pentapeptide, namely TSNTF. This peptide was the dominant peptide in the most effective antioxidant fractions derived from total honey proteins, which exhibited strong superoxide-scavenging and DPPH reducing activity. In addition, the TSNTF peptide had a protective effect for human HCT-116 colon cells challenged with hydrogen peroxide and diethyl maleate in terms of cell viability and antioxidant defense. The pentapeptide TSNTF derives from MRJP1 and corresponds to the residues 208–212 of MRJP1 (Ibrahim et al., 2021).

3. Antimicrobial activity

Recombinant MRJPs 2–5 and MRJP7 obtained from baculovirus-infected insect cells had antibacterial activity, while MRJP1 and MRJP6 had almost no antibacterial activity against the gram-negative bacterium *Escherichia coli*. Furthermore, the study showed that the antibacterial activity of recombinant MRJPs 2–5 and MRJP7 is due to

Table 1
Major royal jelly proteins (MRJPs) properties.

Protein	Number of amino acids	Experimental MW (kDa)*	Theoretical MW (kDa)	pI	Predicted phosphorylation sites	Predicted *N*-glycosylation sites
MRJP1	413	55	46.86	5.03	S: 13/T: 2/Y: 09	3
MRJP2	435	50–55	49.15	6.65	S: 05/T: 4/Y: 06	2
MRJP3	524	60–70	59.49	6.50	S: 09/T: 2/Y: 09	1
MRJP4	444	60	50.67	5.74	S: 14/T: 4/Y: 08	8
MRJP5	578	77–87	68.13	5.95	S: 16/T: 8/Y: 11	4
MRJP6	417		47.58	6.01	S: 09/T: 2/Y: 10	5
MRJP7	426		48.66	4.85	S: 11/T: 9/Y: 09	3
MRJP8	400		45.06	5.81	S: 04/T: 2/Y: 05	6
MRJP9	403		46.27	8.62	S: 06/T: 2/Y: 09	3

The sequence data from UniProt Knowledgebase (https://www.uniprot.org/) was used to determine the theoretical MW (molecular weight) and pI by using Expasy ProtParam (https://web.expasy.org/protparam/), while the phosphorylation and glycosylation sites were predicted using NetPhos (Blom, Gammeltoft & Brunak, 1999) and NetNGlyc (https://www.cbs.dtu.dk/services/NetNGlyc/); * values taken from scientific literature: MRJP3 and 5 (Schmitzová et al., 1998); MRJP4 (Zhang et al., 2019). The experimental MW differs from the theoretical MW due to post-translational modifications (phosphorylation and glycosylation) and repetitive regions in MRJP2, 3 and 5.

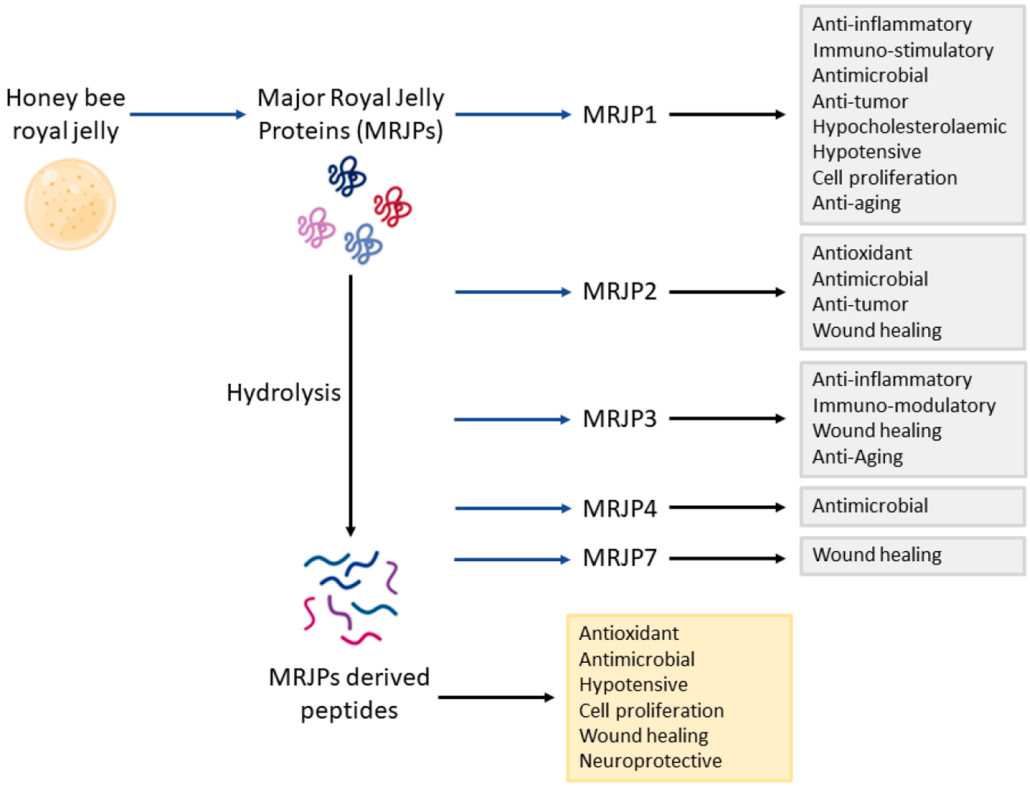

Fig. 1. Biological activities of major royal jelly proteins (MRJPs) and their derived peptides.

their capacity to bind to the walls of the bacterial cells (Park et al., 2020).

In accordance, it was found that glycosylated proteins, named glps, which were isolated from honey, had growth inhibitory and bactericidal effects (Brudzynski et al., 2015). The glps were found to have two characteristics: they could specifically bind and determine the agglutination of bacterial cells, and they possessed non-specific membrane permeabilization of bacterial cells. Light and Scanning Electron Microscopy showed that the glps induced changes in the bacterial cell shape in a concentration- and time-dependent manner. Glps had sequence identity with MRJP1, which also harbors the antimicrobial peptides named jelleins. Based on experiments with glycosylated and de-glycosylated proteins, the authors concluded that the high-mannose structures of the glycosylations added to the proteins could explain the lectin-like effect of MRJP1, whilst jelleins found in the MRJP1 structure could explain the membrane permeabilization of bacterial cells (Brudzynski & Sjaarda, 2015). If the antibacterial effect of MRJP1 is indeed at least partially linked to the glycosylation, this might explain why in the aforementioned study using recombinant proteins expressed in a heterologous system no antibacterial activity was found for MRJP1.

Still, the antimicrobial activity of MRJP1 may not be confirmed with certainty. Several studies report the antibacterial effect (Brudzynski et al., 2015; Brudzynski & Sjaarda, 2015), while other studies do not (Bucekova & Majtan, 2016; Feng et al., 2015). A recent study tested the antibacterial effect of MRJP1 on *Enterococcus faecalis*, *Bacillus pumilus*, *E. coli*, and *Pseudomonas fluorescens*, and showed that it significantly inhibits the growth of bacteria at a concentration of 60 μg/mL (Vezeteu et al., 2017). Bucekova and Majtan (2016) tested the effect of MRJP1 only until a concentration of 47.5 μg/mL which might explain the discrepancy.

In addition, it was shown that the glycosylated MRJP1 isolated from honey has antibacterial effect against multi-drug resistant bacteria: vancomycin-resistant Enterococci and methicillin-resistant *Staphylococcus aureus* starting from a protein concentration of 5.4 μg/mL (Brudzynski et al., 2015).

Moreover, several studies demonstrated the wide range of antimicrobial activity of MRJP2 and MRJP4 against both Gram-positive and Gram-negative bacteria, fungi, and yeasts. The proteins behave as antimicrobial peptide (AMP)-like proteins because they are able to attach to the cell wall and damage its structure (Bilikova et al., 2009; Kim & Jin, 2019; Park et al., 2019; Park et al., 2020).

N-glycosylated MRJP2 isolated from RJ inhibits the growth of the Gram-positive *Paenibacillus larvae*, whereas the deglycosylated form could not. The antibacterial effect was due to the cell wall biosynthesis perturbation, the cell membrane permeability increase, aerobic respiration inhibition, cell division limitation, and cell death induction (Feng et al., 2021).

Furthermore, jelleins, the peptides derived from MRJP1, exhibit antimicrobial effect against yeast, Gram-positive and Gram-negative bacteria (Fratini et al., 2016) by affecting the bacterial membranes (Dos Santos Cabrera et al., 2014). Jelleine-1 and jelleine-2 at low concentrations (2.5–30 μg/mL) inhibited the growth of *S. aureus*, *Staphylococcus saprophyticus*, *Bacilus subtilis*, *E. coli*, *Enterbacter cloacae*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa* and the yeast *Candida albicans* at a concentration of 10^6 colony forming units (CFU)/mL after 18–24 h of incubation (Fontana et al., 2004). Jelleine-1 had antibacterial activity at a concentration of more than 200 μg/mL against *S. aureus* A170, *Listeria monocytogenes* and *Salmonella typhimurium* at the concentration of approximately 10^6 CFU/mL, while jelleine-2 and jelleine-3 were found to have no activity even at a concentration of 200 μg/mL (Romanelli et al., 2011).

Initially, the antimicrobial peptide jelleine-1 with a short sequence of PFKLSLHL (~1 kDa) was isolated from the RJ of *Apis mellifera*. There is only one residue difference between the sequences of jelleines. Still, this difference has a significant impact on their antimicrobial activities due to the lysine, arginine, and histidine residues in their sequences. Thus, the antibacterial peptides have net positive charge which enables the interaction with anionic phospholipids present on the bacterial cell membranes leading to their disintegration (Splith & Neundorf, 2011).

In order to improve the antimicrobial activity, novel analogs of

Table 2
Summary of Major royal jelly proteins (MRJPs) and their derived peptides' biological activities.

Biological Activity	Proteins/ Peptides	References
Antioxidant	Proteins isolated from RJ*, MRJP-mix and MRJP-derived peptides	Guo et al., 2009; Nagai & Inoue, 2004; Xin et al., 2016
	Recombinant MRJP1–7	Park et al., 2020
Antimicrobial	Recombinant MRJP1-7	Park et al., 2020
	MRJP1 and its derived jelleins	Brudzynski et al., 2015; Brudzynski & Sjaarda, 2015
	MRJP1	Vezeteu et al., 2017; Bucekova and Majtan 2016
	MRJP2 and MRJP4	Bilikova et al., 2009; Kim & Jin, 2019; Park et al., 2019; Park et al., 2020
	MRJP2	Feng et al., 2021
	Jelleins	Fratini et al., 2016; Dos Santos Cabrera et al., 2014; Fontana et al., 2004; Romanelli et al., 2011
	Analogs of jelleine-1	Zhou et al., 2021
	Halogenated derivatives of jelleine-1	Jia et al., 2019
	Synthesized phosphorylated jelleines	Han et al., 2014
Anti-tumor	MRJP2 and its isoform X1	Abu-Serie & Habashy, 2019
Hypotensive and Hypolipidemic	MRJP1, MRJP2, and MRJP3	Kashima et al., 2014
	MRJP1	Fan et al., 2016
	Peptides derived from MRJP1 after gastrointestinal digestion	Matsui et al., 2002
	RJ proteins, including royalisin and degradation products of MRJP1 and MRJP3	Sato et al., 2021
	The peptide "EALPHVPIFDR" derived from MRJP1	Tahir et al., 2020
Cell proliferation, Growth-promoting and Wound healing	MRJP-Mix	Chen et al., 2016; Jiang et al., 2018; Park et al., 2020
	Oligomeric MRJP1	Kimura et al., 2003; Moriyama et al., 2015; Tamura et al., 2009
	Monomeric MRJP1	Kimura et al., 1996; Watanabe et al., 1996; Wan et al., 2018
	MRJP2, MRJP3, and MRJP7	Lin et al., 2019
	The carboxyl-terminal penta-peptide repeats (TPRs) of MRJP3	Minegaki et al., 2020
Anti-aging	MRJP-Mix	Xin et al., 2016; Jiang et al., 2018
	Monomeric MRJP1	Detienne et al., 2014
Neuroprotective	Crude royal jelly peptides (RJPs), obtained by digesting RJ proteins	Zhang et al., 2019
	MRJP-Mix	Chen et al., 2017
Reproductive and Fertility	MRJP-Mix	Xin et al., 2016; Liu et al., 2020
Anti-inflammatory and Immune-Modulatory	MRJP1 and MRJP2	Majtan et al., 2006; Šimúth et al., 2004; Majtan et al., 2010; Bilal & Azim, 2018; Rosmilah et al., 2008; Thien et al., 1996; Hayashi et al., 2011
	MRJP3	Okamoto et al., 2003; Kohno et al., 2004

If not stated otherwise, the proteins were isolated from RJ and do thus contain all post-translational modifications, such as glycosylation, which are made by the honey bees. Recombinant MRJPs might contain no or different post-translational modifications. * - consist of about 90% of MRJPs.

jelleine-1 were designed, and tested in terms of antimicrobial effects. Amino acids substitution enhanced the activity of jelleine-1 at concentrations between 1 μM and 256 μM against *E. coli, P. aeruginosa, E. sakazakii, S. aureus, B. subtilis* and *Staphylococcus epidermidis* at the concentration of 10^5 CFU/mL. Among all the analogs, the one enriched in arginine and leucine had the most potent activity against Gram-negative and Gram-positive bacteria *in vivo* and *in vitro*. This was due to the cationity of the amino acids, which leads to electrostatic interaction with the bacterial membrane which is negatively charged. The increased arginine content in the sequence of jelleine-1 promoted antimicrobial activity significantly by 2 to 4-fold (Zhou et al., 2021).

Another study examined the antibacterial activity of halogenated derivatives of jelleine-1 at concentrations ranging from 1 μM to 256 μM against *S. aureus*, *B. subtilis*, *S. epidermidis*, *E. coli*, *P. aeruginosa*, *Klebsiella influenza*, and *Cronobacter sakazakii* at 10^5 to 10^6 CFU/mL. Antimicrobial activity and antibiofilm activity were improved 1- to 8-fold after halogenation. In addition, the proteolytic stability was improved 10- to 100-fold by halogenation. Chlorine-jelleine-I (Cl-J-I), bromine-jelleine-I (Br-J-I), and iodine-jelleine-I (I-J-I) were more effective compared to fluorine-jelleine-I (F-J-I)(Jia et al., 2019). This effect could be associated with the shift in the binding affinity to the bacterial wall after halogenation.

Furthermore, protein phosphorylation affects the antibacterial activity of jelleines for the concentration gradient of 0.625 to 320 μg/mL. Native jelleine-2 (TPFKLSLHL) inhibited the growth of all tested bacteria: *S. aureus*, *B. subtilis*, *E. coli*, *P. aeruginosa*, and *P. larvae*. In contrast, the two forms of synthesized phosphorylated jelleine-2, jelleine-2 (pT) and jelleine-2 (pS), had a different antibiotic spectrum. Jelleine-2 (pS) had no effect on *S. aureus* and *P. aeruginosa*, while jelleine-2 (pT) with a concentration of 160 μg/mL inhibited just the growth of *E. coli* at a concentration of approximately 10^6 CFU/mL. Thus, the antimicrobial activity of jelleine-2 diminished after phosphorylation (Han et al., 2014).

Exosome-like extracellular vesicles (EVs) found in honey from *Apis mellifera* have antibacterial and antibiofilm effects against oral streptococci. The molecular characterization of the EVs contained MRJP1, defensin-1, and jellein-3 as intravesicular cargo. Therefore, the authors concluded that honey-derived EVs could represent innovative approaches for preventing dental caries (Leiva-Sabadini et al., 2021).

In conclusion, MRJPs and jelleines have antimicrobial activity. This is explained by the interaction with bacterial cell membranes of the positively charged amino acids (lysine, arginine, and histidine) and hydrophobic residues (Ahmad et al., 2020; Fratini et al., 2016). The antibacterial activity depends on the type of glycosylation, phosphorylation, and halogenation (Brudzynski et al., 2015; Han et al., 2014; Jia et al., 2019). Therefore, MRJPs and jelleines have potential as innovative antibacterial and antifungal agents.

4. Anti-tumor activities

MRJP2 and its isoform X1 manifest anti-tumor activity and protect against CCl4-induced hepatotoxicity in hepatocytes isolated from the liver of male Albino rats. They do so by stimulating caspase-dependent apoptosis, scavenging intracellular free radicals, suppressing TNF-α, as well as by mixed lineage kinase domain-like protein activation (Abu-Serie & Habashy, 2019). Thus, their mode of action should be characterized in-depth in future animal and human studies.

5. Hypotensive and hypolipidemic activity

By using a cholic acid-conjugated column, MRJP1, MRJP2, and MRJP3 from RJ were identified as bile acid-binding proteins (Kashima et al., 2014). MRJP1 had *in vitro* taurocholate-binding activity, while it significantly lowered the micellar solubility of cholesterol. *In vivo*, liver bile acids were significantly elevated, and cholesterol 7a-hydroxylase (CYP7A1) mRNA and protein involved in cholesterol metabolism

increased when feeding rats with MRJP1. In addition, CYP7A1 mRNA and protein levels were significantly increased in hepatocytes (human HepG2 cells) treated with MRJP1 tryptic hydrolysate. MRJP1 demonstrated the most potent hypocholesterolaemic effect among the three tested MRJPs because it was found to interact with bile acids, enhance the excretion of fecal bile acids, increase fecal excretion of cholesterol, and increase the hepatic cholesterol catabolism. The difference in the bile acid-binding capacity could be explained by the difference in the degree of hydrophobicity between MRJPs since the hydrophobic environment influences the binding of bile acids to proteins (Kashima et al., 2014). MRJP1 inhibits cholesterol absorption in the jejunum, leading to an altered concentration of blood lipids in rats fed with a test diet containing 600 mg/kg/day of MRJP1. Furthermore, MRJP1 also stops the reabsorption of bile acids.

Heterologous MRJP1 expression in mouse vascular smooth muscle cells (VSMCs) significantly lowered the contraction and migration of the cells by inhibiting muscle filament activities, while VSMCs proliferation was hindered by reducing the energy supply. Thus, MRJP1 has a potential hypotensive effect through its action on VSMCs, which regulate blood pressure (Fan et al., 2016).

RJ hydrolyzed with protease N and the resulting peptides (Ile-Tyr, Val-Tyr, and Ile-Val-Tyr) were able to inhibit angiotensin-converting enzyme (ACE) activity and had anti-hypertensive effects by decreasing systolic blood pressure in a dose-dependent manner after 28-days oral treatment in spontaneously hypertensive rats. Therefore, the peptides resulting from RJ hydrolysis may be useful for ameliorating blood pressure in patients with hypertension (Tokunaga et al., 2004).

Peptides derived from MRJP1 after gastrointestinal digestion had strong ACE inhibitory activity in spontaneously hypertensive rats (Matsui et al., 2002). In addition, RJ proteins, including royalisin and degradation products of MRJP1 and MRJP3 inhibited macrophage proliferation in atherosclerotic plaque in a concentration-dependent manner (Sato et al., 2021). The degradation products of MRJP1 and MRJP3 did bind LDL and oxidized LDL, a component of atherosclerotic lesions. Furthermore, an *in silico* experiment on MRJP1 aimed to identify the ACE inhibitory peptides, and the peptide "EALPHVPIFDR" exhibited strong binding affinity and high anti-hypertensive activity (Tahir et al., 2020). Therefore, the degradation products of MRJP1 and MRJP3 could lead to the regression of atherosclerotic plaque by lowering plaque inflammation. Further studies of these molecules may lead to the discovery of novel anti-atherosclerotic agents (Sato et al., 2021).

6. Cell proliferation, growth-promoting and wound healing activities

MRJPs extracted from RJ display growth-promoting activity in several cell lines, including human lymphoid and myeloid cell lines (Moriyama et al., 2015; Watanabe et al., 1996; Watanabe et al., 1998), rat liver primary cultured cell (Kimura et al., 1996), human monocytes (Kimura et al., 2003; Kimura et al., 1996), Tn-5B1-4 insect cells (Salazar-Olivo & Paz-González, 2005; Shen et al., 2010), human embryonic lung fibroblast cells (Jiang et al., 2018), human keratinocytes (Lin et al., 2019; Majtan et al., 2010), rat small intestine epithelial cell lines (Moriyama et al., 2015), murine fibroblast cell lines (Park et al., 2020), stem cells (Wan et al., 2018) and monkey kidney epithelial cell lines (Minegaki et al., 2020).

In addition, a study found that MRJPs isolated from RJ could induce proliferation of human cell lines and could partially replace fetal bovine serum (FBS) in the cultivation of cells (Chen et al., 2016).

For example, the human embryonic lung fibroblast (HFL-I) cell line treated with a MRJP mixture extracted from RJ showed greater proliferation, minimum senescence, and elongated telomeres. The molecular mechanism was associated with superoxide dismutase-1 (SOD1) upregulation and mammalian target of rapamycin (mTOR), catenin beta like-1, and tumor protein p53 downregulation (Jiang et al., 2018). Recombinant MRJPs 1–7 increased murine fibroblast NIH-3 T3 cell line viability by protecting them against oxidative stress-induced cell apoptosis (Park et al., 2020).

Oligomeric MRJP1 stimulates human monocyte (U-937 and HB4C5) proliferation (Kimura et al., 2003), while monomeric MRJP1 sustained high viability for rat liver primary cultured cells but did not stimulate the human monocytes proliferation (Kimura et al., 1996). The oligomeric form of MRJP1 has cell proliferative effects on Jurkat, and IEC-6 cells, and its proliferative activity is resistant to heat treatment (Moriyama et al., 2015; Tamura et al., 2009). In addition, monomeric MRJP1 stimulated the growth of the following human lymphoid cell lines: U-937, THP-1, U-M, HB4C5, HF10B4 (Watanabe et al., 1996). The crude protein extract obtained by ammonium sulfate precipitation from RJ stimulated Tn-5B1-4 insect cells growth (Salazar-Olivo & Paz-González, 2005), while in a more recent study MRJP1 was found to have proliferative activity on Tn-5B1-4 insect cells (Shen et al., 2010). Elevated growth level and proliferation of cells in response to MRJP1 treatment was also detected in human keratinocytes (Majtan et al., 2010) and human myeloid cell lines, U-937 and THP-1(Watanabe et al., 1998).

The monomeric form of MRJP1 is able to activate a pluripotency gene network that enables self-renewal in mouse embryonic stem cells (mESC). This is an important functional role of MRJP1 in terms of cell state and fate regulation and its effects are yet to be further elucidated by future studies (Wan et al., 2018).

A protein fraction containing MRJP2, MRJP3, and MRJP7 has the potential to promote wound healing by inducing human epidermal keratinocyte cells proliferation and migration (Lin et al., 2019). Another study showed wound healing activity for the carboxyl-terminal pentapeptide repeats (TPRs) of MRJP3. The TPRs consist of basic residues which induce the growth of THP-1 and monkey kidney epithelial cell line (Vero) growth and wound healing activity in the case of Vero cells (Minegaki et al., 2020).

The wound healing process is complex and is related to inflammation, cell proliferation, differentiation, and migration, involving many intracellular and extracellular components, like cytokines, growth factors, ATP, etc (Breitkreutz et al., 2009). Therefore, future studies should investigate the regulatory effects of MRJPs involved in this wound healing activity. Understanding the biological functions of MRJPs in terms of cell proliferation and growth could open the way for these proteins to be integrated in tissue regeneration and wound closure interventions.

7. Anti-aging effect

The longevity of *Drosophila melanogaster* was increased by MRJPs, especially by MRJP1 and MRJP3, via the promotion of the epidermal growth factor receptor (EGFR)-mediated signaling pathway (Xin et al., 2016). Analysis by microarray data and gene ontology revealed that the diet supplemented with MRJPs determined the upregulation of S6K, MAPK, and EGFR in the EGFR-mediated signaling pathway. In addition, MRJPs increased the antioxidant SOD1 gene expression and decreased the levels of malonaldehyde, a marker of oxidative stress (Xin et al., 2016).

Moreover, a more recent *in vitro* study showed that the human embryonic lung fibroblast (HFL-I) cell line treated with MRJPs had greater proliferation, minimum senescence, and elongated telomeres (Jiang et al., 2018).

Likewise, monomeric MRJP1 had the same effects on the lifespan of *Caenorhabditis elegans*, by promoting the epidermal growth factor (EGF) and EGFR signaling pathways (Detienne et al., 2014). Therefore, the anti-aging function of MRJPs was associated with antioxidant function and enhanced EGFR signaling pathway, known for its role in promoting cell division and cellular differentiation (Rongo, 2011).

However, at least the lifespan prolonging effect was not maintained after protease treatment of the samples indicating that full-length MRJP1 is necessary for the effect. While it has been shown that full-length MRJP1-3 can somewhat withstand *in vitro* gastric digestion

(12.0–84.5 % of the full-length proteins still detectable after 1 h of pepsin digestion), MRJP1 and 3 were rapidly digested within 10 min by trypsin and α-chymotrypsin (60 min for MRJP2), questioning that MRJP1 would be able to withstand the human digestive system for any full-length effect (Mureşan et al., 2018).

8. Neuroprotective activity

Crude royal jelly peptides (RJPs), obtained by digesting RJ proteins, reduced at a concentration of 1 to 9 μg/mL the production of external beta-amyloid 40 (Aβ1-40) and beta-amyloid 42 (Aβ1-42) peptides involved in Alzheimer's disease (AD) as a consequence of beta-site amyloid precursor protein cleaving enzyme 1 (BACE1) down-regulation in N2a/APP695 neuroblastoma cells. Thus, RJPs could have the potential for ameliorating AD-related amyloid β-peptide (Aβ) pathology (Zhang et al., 2019).

When the diet of aged rats was supplemented with MRJPs, the brain metabolism was improved by an enhanced glucose and phosphoenolpyruvic acid levels compared to the control group (Chen et al., 2017). Metabolomics analysis of urine revealed that the MRJPs-treated aged rats had similar metabolites as the young rats, especially increased levels of nicotinic acid mononucleotide (NaMN), a precursor of NAD+, and xanthosine, which sustains the nucleic acid metabolism and could support DNA repair in aged rats. Furthermore, supplementation with MRJPs stimulated the production of neuroprotective molecules in aged rats, mainly of cysteic acid, revealing the association of the cysteine-taurine metabolism pathways in the memory enhancement. This effect could be related to the fact that the MRJPs are proteins rich in the amino acid cystine, which further can be converted through metabolism into cysteine and cysteic acid (Chen et al., 2017).

Whether these results derive from a single protein, the combination of MRJPs or their metabolites is not clear yet, therefore future studies should address these findings leading to practical applications for the prevention of cognitive decline in humans.

9. Effects on reproduction and fertility

The supplementation with MRJPs at 1.25 %, 2.50 % or 5.00 % (*w/w*) of the traditional corn-yeast diet increased fecundity in *Drosophila melanogaster,* and the diet supplemented with 2.50 % MRJPs was the the optimal dose (Xin et al., 2016).

MRJPs supplementation increased the onset of puberty and sustained the follicular development in immature female mice (FM). The reproductive function of MRJPs was connected with an elevated estrogenic activity, an antioxidant potential of the reproductive system, the upregulation of estrogen receptor beta gene (ER beta) expression, hormone secretion and ovary development in FM (Liu et al., 2020).

10. Anti-inflammatory and immune-modulatory activities

Monomeric MRJP1 and MRJP2 stimulate mouse macrophages to secrete tumor necrosis factor (TNF)-α (Majtan et al., 2006; Šimúth et al., 2004). An *in vitro* experiment on human skin cells showed that 25 μg/mL of monomeric MRJP1 increased TNF-α mRNA expression (Majtan et al., 2010). The authors of the study argue that monomeric MRJP1 could be a novel agent for treatment of skin wounds. Also, MRJP1 extracted from honey was found to have an immuno-stimulatory effect by elevating TNF-α production in mice peritoneal macrophages (Bilal & Azim, 2018).

Moreover, the proteins MRJP1 and MRJP2 can cause allergic reactions. Some studies determined that these proteins interact with immunoglobulin E (IgE) of sera from patients exhibiting RJ allergy (Rosmilah et al., 2008; Thien et al., 1996). Furthermore, IgE binding is depending on the glycosylation degree of the MRJP1 (Hayashi et al., 2011).

MRJP3 can manifest strong immuno-modulatory effects by inhibiting IgE and immunoglobulin G1 (IgG1) levels *in vivo* when using an allergic mouse model and was proposed as a useful agent with anti-allergic action and reduced antigenicity (Okamoto et al., 2003). In addition, another study showed that MRJP3 has anti-inflammatory activity because of suppressing the pro-inflammatory cytokine secretion in mice (Kohno et al., 2004). Another study confirmed these results (Qu et al., 2008).

These studies and their results indicate that MRJP1, MRJP2 and MRJP3 have immunoregulatory function *in vivo*.

11. Conclusions

RJ is an attractive beehive product in terms of nutritional and health beneficial effects. Besides its nutritional role, its biological activities are involved in physiological mechanisms like anti-inflamatory and immuno-modulatory, antioxidant, antimicrobial, anti-tumor, cell proliferation, neuroprotective, reproductive, and anti-aging (Table 2). RJ's biological effects are multi-factorial due to the complex pattern of bioactive compounds it contains. Diverse studies demonstrated that MRJPs are the dominant proteins found in RJ. This review article aims to draw attention to the array of current knowledge on RJ, its MRJPs, and their derived peptides which have a great potential for applications as nutraceuticals and emphasizes the demand for further research on RJ and its protein components in order to unravel the mechanisms by which they influence the physiological pathways in cells, animals as well as in humans. For instance, the growth-factor-like activity of MRJPs and their derived peptides should be screened on more cell lines, and the mechanisms corresponding to this activity should be further investigated. Thus, characterizing MRJPs and their derived peptides should be an objective of future studies.

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Ethics statement

On behalf of, and having obtained permission from all the authors, I declare that: the material has not been published in whole or in part elsewhere; the paper is not currently being considered for publication elsewhere; all authors have been personally and actively involved in substantive work leading to the report, and will hold themselves jointly and individually responsible for its content.

CRediT authorship contribution statement

Carmen Ioana Mureşan: Conceptualization, Methodology, Investigation, Writing – original draft. **Daniel Severus Dezmirean:** Supervision, Writing – review & editing, Visualization, Investigation. **Bianca Dana Marc:** Writing – review & editing. **Ramona Suharoschi:** Supervision, Writing – review & editing. **Oana Lelia Pop:** Investigation, Writing – original draft, Writing – review & editing. **Anja Buttstedt:** Investigation, Writing – original draft, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

No data was used for the research described in the article.

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