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ORIGINAL ARTICLE

Honey and royal jelly, like human milk, abrogate lectin-dependent infection-preceding *Pseudomonas aeruginosa* adhesion

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Pseudomonas aeruginosa **antibiotic resistance has led to the search of natural compounds, which would competitively block its fucose > fructose/mannose-binding lectin (PA-IIL) that mediates its biofilm formation and adhesion to animal cells. Such compounds were found in human milk (HM) and avian egg whites. The present research has revealed that honey and royal jelly (RJ), which are assigned to protect beehive progeny and are applied for human infection therapy, match HM in PA-IIL blocking. The function of their fructose (higher in honey) and mannosylated glycoproteins (higher in RJ) as powerful decoys in PA-IIL neutralization is of ecological/biological importance and implementability for the antibacterial adhesion therapeutic strategy.**
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Introduction

Pseudomonas aeruginosa, a widely spread saprophytic soil and water-borne bacterium, contributes to the nitrogen and carbon cycles in nature by decomposing plant, microbial and animal debris. For this goal, it is endowed with diverse adhesive proteins and a rich arsenal of extracellular toxins and digestive hydrolytic enzymes (Nicas and Iglewski, 1985). The adhesins include the lectins PA-IL (*P. aeruginosa* first, galactophilic lectin, LecA) and PA-IIL (*P. aeruginosa* second, fucose > fructose/mannose-binding lectin, LecB) (Gilboa-Garber, 1982), that mediate auto (own type) as well as hetero cell-to-cell interactions for biofilm formation (Gilboa-Garber *et al.*, 1997; Tielker *et al.*, 2005; Diggle *et al.*, 2006) and adherence to target cells/organisms (Gilboa-Garber, 1996; Gilboa-Garber *et al.*, 1997). Those two *P. aeruginosa* lectins are considered as virulence factors (VIFs) together with the toxins and hydrolytic enzymes since their expression is coregulated (Gilboa-Garber *et al.*, 1997) and they contribute to the activities of the other VIFs by homing to the target cells. Owing to its ubiquitous distribution in nature and in hospitals and to its multiple, wide-spectrum adhesins, *P. aeruginosa* occasionally adheres to living organisms. Healthy adult animals generally resist its infections, but immuno-deficient, cystic fibrosis or burn patients, as well as wounded or immature animals (newborns or newly hatched vertebrates or insect larvae) are very sensitive to them. This bacterium may aggressively attack them, as if they are destined to decomposition, leading to severe morbidity and mortality. In mammals this situation is prevented by protection of immature newborns by maternal milks (Lesman-Movshovich *et al.*, 2003; Ruiz-Palacios *et al.*, 2003; Newburg *et al.*, 2005). Similarly, avian embryos are protected by avian egg whites (Lerrer and Gilboa-Garber, 2001). In addition to rich nutrients, these maternal secretions contain immunoreactive components, bactericidal factors and saccharides in free form or carried on macromolecules, mainly glycoproteins (gps), which act as decoys, competitively blocking the bacterial lectin-mediated adhesion to the target cells.

In the case of the beehive, owing to the fascinating social behavior of the honey bees (*Apis melifera*), the maternal contribution of feeding and protection of the new progeny is substituted by parental brood care. For up to 3 days, the larvae are supplied with royal jelly (RJ) that is secreted by young worker bees (nurse bees) in the hive. Thereafter, only larvae

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designated to become queens receive RJ, while a mixture of honey, pollen and water is fed to larvae selected to become workers (Drapeau *et al.*, 2006).

Honey is produced by worker honeybees from floral nectars, followed by water evaporation in the honeycomb. It is rich in carbohydrates (approximately 79%, including around 38% fructose (Fru), 31% glucose (Glc), 1% sucrose and 9% other sugars), but its protein content is only around 0.7% (Moritz and Southwick, 1992; Qiu *et al.*, 1999; Simuth *et al.*, 2004).

RJ, which is produced by young nurse bees from a honey and flower pollen mixture with secretions from their cephalic salivary glands (Moritz and Southwick, 1992), differs from honey in its higher water content (60–70%), 10–16% carbohydrates (some of honey origin) and much higher protein levels (around 14%), most of them gps (Kimura *et al.*, 2000, 2002; Scarselli *et al.*, 2005), and 3–7% lipids as well as richer content of other vital components (Takenaka, 1982; Schmitzova *et al.*, 1998; Albert *et al.*, 1999; Stocker *et al.*, 2005). The most prominent 'major' RJ proteins, 'MRJPs' 1–5, ranging from 49 up to 87 kDa and also named apalbumins (Albert and Klaudiny, 2004), constitute around 90% of the total RJ proteins (Schmitzova *et al.*, 1998; Sano *et al.*, 2004; Santos *et al.*, 2005; Scarselli *et al.*, 2005). Apalbumin 1 was reported to constitute 48% of the RJ proteins and also to be present in honey (Scarselli *et al.*, 2005). The genes of those proteins were recently identified (Schmitzova *et al.*, 1998; Drapeau *et al.*, 2006) with the unveiling of the honeybee complete genome, and they are suggested to be multifunctional, performing diverse nutritional, physiological and developmental roles and affecting various tissues, including the brain (Drapeau *et al.*, 2006).

Beehive products have long been used for various therapeutic purposes. Over the last decade, there is a renewed usage of both honey and RJ for treatment of infections due to the emergence of antibiotic-resistant bacteria. There have been many case reports and clinical trials proving their effectiveness against such bacteria, including the opportunistic human pathogen *P. aeruginosa* (Cooper *et al.*, 2002; French *et al.*, 2005; Molan, 2006). The honey antibacterial effects were mainly attributed to a combination of high osmolarity and bacteriocidal/static activities, including hydrogen peroxide, phenolic compounds and antioxidants (Taormina *et al.*, 2001; Cooper *et al.*, 2002) as well as cytokine induction (Tonks *et al.*, 2003; Simuth *et al.*, 2004; Majtan *et al.*, 2006). In parallel, there are searches for modern anti-bacterial adhesion strategies aimed at abrogating microbial (including *P. aeruginosa*) adhesion to host cells, preceding infection establishment (Gilboa-Garber, 1996; Sharon, 2006). One of these strategies is based on the usage of soluble saccharides and conjugated glycans that mimic those of the host cell receptors and function as decoys for competitive hampering of the pathogen-binding to the cell-membrane receptors. This mechanism operates widely in nature – as best exemplified by human milk (HM) saccharides that protect newborns against infections (Ruiz-Palacios *et al.*, 2003; Morrow *et al.*, 2005; Newburg *et al.*, 2005).

Our group has used HM to block *P. aeruginosa* adhesion to human cells via its fucose (Fuc)-binding lectin, PA-IIL (Lesman-Movshovich *et al.*, 2003; Lesman-Movshovich and Gilboa-Garber, 2003), which also binds Fru and mannose (Man) (Gilboa-Garber *et al.*, 1997; Mitchell *et al.*, 2002). The groups of Imberty and Wimmerova endorsed our results by elucidating the 3-dimensional crystallographic and thermodynamic interactions of that lectin with the fucosylated lewis a (Le^a) receptor (which mediates *P. aeruginosa* binding to the lungs of cystic fibrosis patients) and with the competing milk saccharides (Mitchell *et al.*, 2002; Perret *et al.*, 2005).

In the present study, we have used the same *P. aeruginosa* PA-IIL model to analyze honey and RJ anti-adhesion potential, comparing their PA-IIL-blocking efficiencies with those of human and cow's milk. The PA-IIL interactions with the honey, RJ, and milk were also compared with those of two additional lectins: Con A (Concanavalin A; Moothoo and Naismith, 1998) (from the plant *Canavalia ensiformis*) that shares with PA-IIL Fru and Man (without Fuc) sensitivity but also reacts with Glc derivatives and UEA-I (from the plant *Ulex europaeus*) (Konami *et al.*, 1991) that is Fuc-specific. The interactions of these lectins with the low and high molecular weight glycotopes were concomitantly assayed by hemagglutination inhibition test using human erythrocytes and by western blotting using peroxidase-labeled lectins.

Materials and methods

The lectins

PA-IIL was prepared in our laboratory from *P. aeruginosa* ATCC 33347, as described previously (Gilboa-Garber, 1982). Con A and UEA-I were purchased from Sigma–Aldrich (St Louis, MO, USA). Peroxidase labeling of these lectins was attained using glutaraldehyde coupling, as described previously (Lesman-Movshovich and Gilboa-Garber, 2003).

Honey and RJ preparations

Three honey samples from commercial sources, labeled 'wild flower honey', 'Eucalyptus honey' and 'Field flower honey', and an additional undefined commercial one were used. One RJ was obtained from Benjamin Avrahami and the second from the beehives of Kfar Habad, Israel. All honey and RJ samples were kept at 5°C and used both without and following overnight dialysis (2 ml inside the dialysis membrane of 10 kDa limit),

against 1 l of 0.85% NaCl solution (with three changes of the saline) at 5°C.

Milks
HMs were obtained from healthy O-type (secretor positive, Se) mothers (volunteers) (Lesman-Movshovich *et al.*, 2003). Each sample was centrifuged (10 000 × *g*) for 10 min and the intermediate skim phase was carefully collected. Cow's milk was purchased from a food market.

Lectin hemagglutinating activity and its inhibition by the beehive products and milks
Lectin solution (20 μg/ml) was serially 2-fold-diluted in 50 μl saline volumes and mixed with an equal volume of 5% (v/v) suspension of 0.1% papain-treated O-type human erythrocytes, as described previously (Gilboa-Garber, 1982; Lesman-Movshovich *et al.*, 2003). After 30 min, the tubes were centrifuged for 30 s (1000 × *g*) and hemagglutination intensity was examined. The highest dilution leading to agglutination of all the erythrocytes in one mass was determined. The lectin concentration chosen for the inhibition test was twice the minimal one that still gave agglutination in one mass.

Hemagglutination inhibition was assayed by adding 50 μl of the chosen lectin concentration to each tube in a series of 2-fold dilutions in saline of the examined sample (milks were initially diluted 2 ×, honey 6 × and RJ 8 ×, to avoid viscosity) in 50 μl volume. After 30 min interaction, the erythrocytes were added and the hemagglutination was examined as above. The hemagglutination inhibition intensity was represented by the number of tubes without hemagglutination preceding its reappearance (Lesman-Movshovich and Gilboa-Garber, 2003). The four different commercial honey samples and the two RJs were each examined at least in triplicates. The results were analyzed by Student *t*-test.

Western blot analyses
Fifteen microliters of the honey (from wild flowers) 6-fold dilution (around 0.12% protein) and RJ (from Kfar Habad) 200-fold dilution (around 0.07% protein) were used for this test so that both their discrete bands and the intensities of their interactions with the lectins would be represented (western blots with 3-fold honey and 50-fold RJ dilutions, were used with the peroxidase-labeled UEA-I. When those were used with Con A and PA-IIL they were found to be overloaded.) The milks were treated as described previously (Lesman-Movshovich *et al.*, 2003). Non-diluted HM and 2-fold-diluted cow milk (both around 1.5% protein) were used. These samples were mixed with sample buffer 1:1, boiled, and applied to the wells in 10% SDS–PAGE (at 140 V) in Mini Protean Cell 3 Electrophoresis (Bio-Rad Laboratories, Inc., Hercules, CA, USA) according to Laemmli (1970) and as described previously (Lesman-Movshovich and Gilboa-Garber, 2003). Following SDS–PAGE, the proteins were transferred to nitrocellulose (0.45 μm; Bio-Rad) membrane at 4°C for 2 h (85 mA/40–50 V), using the Minitrans-Blot Module (Bio-Rad). The membranes were incubated overnight in blocking buffer (0.01 M PBS, pH 7.2, containing 3% BSA and 0.05% Tween 20), exposed to the peroxidase-labeled lectins (about 1 μg/ml) in blocking buffer (with 0.1% Tween 20) at room temperature for 2 h, and then washed thoroughly. The peroxidase reaction was visualized using enhanced chemiluminescence (Amersham International PLC, Buckinghamshire, UK) and recorded on photographic films.

Controls with the labeled lectins in the presence of 0.3 M of the relevant blocking sugar (Man for Con A and PA-IIL, Fuc for UEA-I) in their reaction mixtures were parallely used for ruling out non-specific (sugar-independent) lectin binding.

Results and discussion

Examination of honeys from the four unrelated sources using Con A, UEA-I and PA-IIL demonstrated an apparent variability of the lectin specificities, but significantly high uniformity among the diverse honeys themselves. The strongest, almost equal inhibition of Con A and PA-IIL by the four undialyzed honey samples, without any inhibition of UEA-I (Figure 1), has indicated that despite being from different commercial sources, they all contain most similar, very high-concentrations of inhibiting saccharides, not including Fuc. The fact that most of the honey-inhibiting activities towards Con A and PA-IIL were removed by dialysis indicates that they are mainly associated with low molecular weight components. These results are in accord with both the reported honey composition (Qiu *et al.*, 1999) and the Con A and PA-IIL shared affinity to Fru (Gilboa-Garber *et al.*, 1997; Moothoo and Naismith, 1998), which constitutes almost 40% of the honey mass (Moritz and Southwick, 1992; Qiu *et al.*, 1999; Simuth *et al.*, 2004). The addition of Fru at the above, original (40%) concentration to the dialyzed honey sample restored the PA-IIL hemagglutination inhibition, while addition of both Fru (40%) and Glc (30%) restored Con A hemagglutination inhibition. The added Glc did not affect PA-IIL activity, which in contrast to Con A, is not inhibited by glucose. The finding that, following dialysis, the honey only weakly inhibited PA-IIL while still nicely inhibiting Con A suggests that the honey gps bind Con A more than PA-IIL.

The preferential interactions of these honey gps with Con A, as compared to PA-IIL, were confirmed well in the respective western blots (Figure 2), showing that both Con A and PA-IIL bind to certain

honey gps, most strongly to the 55 kDa MRJP, apalbumin-1 equivalent, but Con A tagged them more profoundly than PA-IIL and also reacted with additional gps. In the presence of 2-fold higher honey concentration (giving overloaded pattern), Con A stained more gps in the range of 35–105 kDa, while PA-IIL-stained discrete gps in the range of 50–105 kDa.

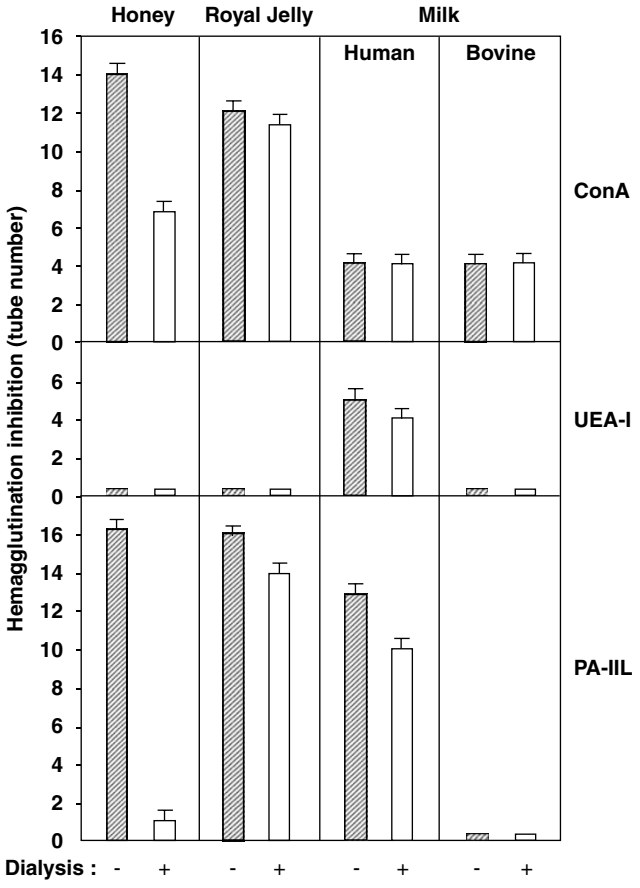

Figure 1 Inhibition of the hemagglutinating activities of Con A, UEA-I, and PA-IIL by four honey and two royal jelly (RJ) samples compared to human (HM) and bovine (BM) milks, without (filled bars) and following (empty bars) dialysis. Each sample of the honeys and RJs are analyzed at least three times. The data in the figure represent their means and s.e.m. bars.

As opposed to Con A and PA-IIL, UEA–I was neither inhibited by any of the honeys (Figure 1) nor did it tag any of their components using the 2 × honey concentration that was overloaded with Con A and PA-IIL (Figure 2). The negative UEA-I results indicate that there is no α1-2 fucosylated gps in the honeys, confirming again the conclusion that the honey-induced inhibition of PA-IIL is not due to fucose (to which this bacterial lectin is most sensitive).

On the basis of the western blotting data, it is assumed that the low inhibition of PA-IIL by honey gps is mainly associated with the 55 kDa apalbumin, followed by a weak interaction with around 75 kDa and scanty interaction with 105 kDa gps. Con A tagging of additional gps between 36 and 105 kDa, observed in the more loaded western blots might be due to their terminal N-acetylglucosamine (GlcNAc) residues and internal Man residues, to which only Con A, but not PA-IIL, binds (Moothoo and Naismith, 1998).

Inhibitions of Con A and PA-IIL hemagglutinating activities by the two undialyzed RJ samples were almost equal to those of the honeys and also indicated uniformity of the two RJ preparations that were obtained from different sources (Figure 1). However, as opposed to the honeys, following dialysis a considerable Con A and PA-IIL inhibiting activity was retained. The reason for the different effects of dialysis on the honey and RJ Con A and PA-IIL-inhibiting activities (Figure 1) is the much higher RJ gps concentration (14%) combined with much lower free saccharide concentration as opposed to low gp concentration (0.7%) and high free saccharide levels (79%) of honey, as described in the

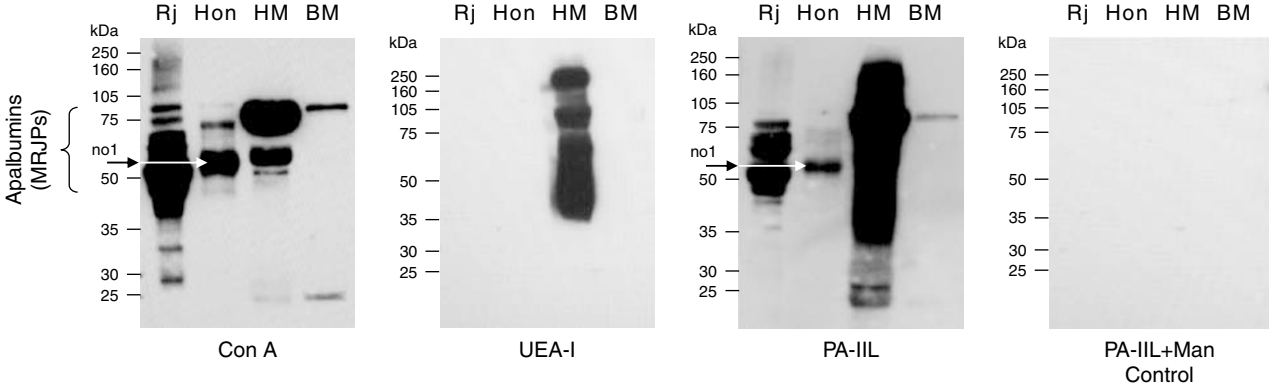

Figure 2 Western blots of RJ, (from Kfar Habad), honey (Hon, from 'wild flowers'), human milk (HM, from O, Se-positive volunteers) and cow's (bovine) milk (BM, commercial) stained by peroxidase-labeled Con A, UEA-I, and PA-IIL, showing the RJ MRJPs distribution and indicating (by an arrow) apalbumin-1 location in both RJ and honey. The details of the test are described in the Materials and methods, and detailed information on the tagged RJ gps might be found in Kimura *et al.* (2000), Schmitzova *et al.* (1998), Qiu *et al.* (1999), Sano *et al.* (2004), and Scarselli (2005).

Introduction. This interpretation is confirmed in the western blot results showing the relatively prosperous interactions of the two lectins with the RJ gps (Figure 2). As seen in that figure, Con A again reacted not only with stronger avidity but also with a much wider gps spectrum than PA-IIL, which tagged at least 11 bands in the range of 20–105 kDa in the more highly loaded blot. Taking into account the dilutions used for the western blotting (RJ × 200 vs honey × 6 and HM almost without dilution), the results indicate enormous affinity of PA-IIL as well as Con A to the RJ gps. Under the overloading conditions, UEA-I did not react with any RJ component. This finding is also in consensus with Kimura's results, indicating the absence of Fuc (Kimura *et al.*, 2000).

Con A interactions with RJ gps was described by Kimura *et al.* (2000), who characterized the structures of RJ N-glycans and showed that typical high-mannose-type structure ($Man_{9\text{-}4}$ $GlcNAc_2$) (Figure 3) accounts for about 72% of them, followed by a biantennary structure ($GlcNAc_2$ Man_3 $GlcNAc_2$) (about 8%), and a hybrid-type structure ($GlcNAc_{1\text{-}}$ $Man_4GlcNAc_2$) (about 3%), all of them reacting with Con A.

PA-IIL-selective strong binding to the MRJPs is attributable to their documented high mannosylation (Figure 3) (Kimura *et al.*, 2000). It was already shown by us to display similar very high affinity to highly mannosylated quail egg white gps (Lerrer and Gilboa-Garber, 2001). High PA-IIL affinity to the 72% highly mannosylated gps (Kimura *et al.*, 2000) may explain its very strong inhibition by RJ, resembling that of Con A, which also binds to them and to around 11% additional gps that bear the terminal GlcNAc. PA-IIL, in contrast to Con A, differentiates between the highly mannosylated and the GlcNAc-bearing gp groups.

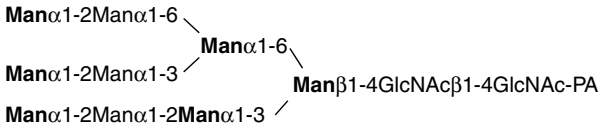

Figure 3 RJ's major highly mannosylated glycoprotein glycan structure, according to Kimura *et al.* (2000).

Finally, the critical comparison of Con A, UAE-I, and PA-IIL interactions with honey and RJ to those with bovine (BM) and human (HM) milks, knowing that the latter provides the best and widest natural anti-adhesion protection to human newborns (Newburg *et al.*, 2005; Ruiz-Palacios *et al.*, 2003), including against *P. aeruginosa* (Lesman-Movshovich *et al.*, 2003; Lesman-Movshovich and Gilboa-Garber, 2003) shows different patterns of the three lectin interactions in both hemagglutination inhibition (Figure 1) and western blotting (Figure 2). As seen in Figure 1, in contrast to Con A's strong inhibition by the beehive products, its inhibition by HM was much weaker than those of PA-IIL and UEA-I, being equal to its inhibition by the cow's milk (BM), which did not inhibit PA-IIL and UEA-I. The main difference between PA-IIL and UEA-I is that the latter, which is specific for human α 1–2 Fuc-bearing H type 2 human blood group antigen (Konami *et al.*, 1991), was selectively inhibited by HM from Se-positive individuals (Lesman-Movshovich *et al.*, 2003). Figure 2 also shows that PA-IIL interactions with HM are much stronger and involve more gps than those of Con A, with a significantly wider gp spectrum extensively overlapping the combination of Con A and UEA-I. The high PA-IIL affinities to them is due to the fucosylation of the HM gps, which do not attract Con A, whereas its affinities to the RJ gps (which are at a 20-fold higher concentration than in the honey) is owing to their mannosylation that also attracts Con A.

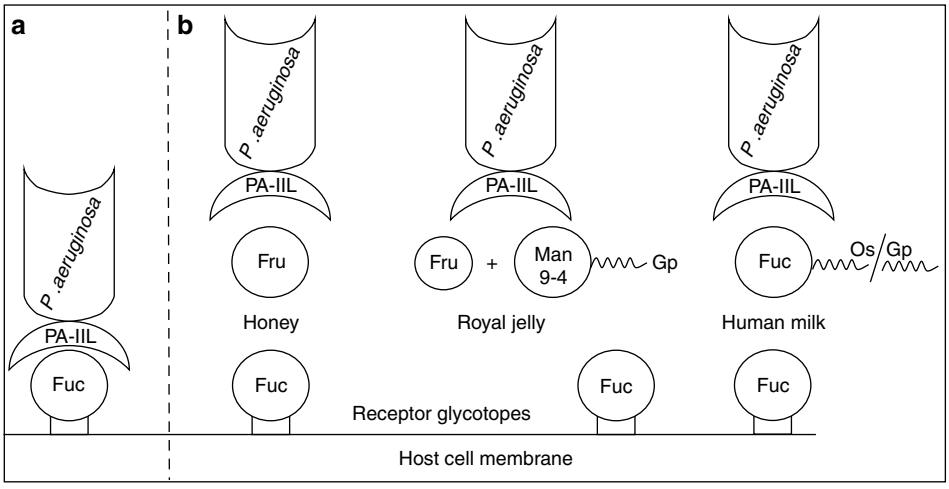

Figure 4 Schematic simplified model of PA-IIL blocking with honey Fru, RJ highly mannosylated (9–4 residues) gps and Fru as well as human milk (HM) fucose-bearing oligosaccharides and gps. Attraction of this lectin to the cell surface fucosylated receptors (**a**) blocking by honey, RJ and HM saccharides functioning as decoys that mimic them (**b**).

Taken together, using Fuc > Fru/Man-binding *P. aeruginosa* lectin, PA-IIL, as a probe, has shown that both honey and RJ provide excellent HM-like protection against PA-IIL-mediated *P. aeruginosa* adhesion. Con A, which shares Fru and Man avidity with PA-IIL, also displays a very high sensitivity to the honey and RJ saccharides, but not to those of HM. UEA-I, which shares Fuc affinity and sensitivity to HM with PA-IIL, does not interact at all with the honey and RJ glycans. Hence, the honey PA-IIL-blocking effect is mainly attributable to its high Fru content (which is removable by dialysis) together with low mannosylated gp contribution, while the RJ powerful PA-IIL inhibition is probably mainly associated with highly mannosylated MRJPs (retained after dialysis) together with lower Fru contribution. The adhesion-blocking competitive cross-reactivity of the honey, RJ, and HM-active components, hampering the lectin attraction by the animal cell surface glycans, is schematically represented in Figure 4.

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