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BEE-GATHERED POLLEN LOADS SUSPENSION: PRELIMINARY ASSESSMENT OF INTERACTION WITH MICROBIAL GROWTH FOR A POTENTIAL EMPLOYMENT AS A NATURAL FOOD ADDITIVE

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ABSTRACT

Pollen collected by honey bees is currently considered a health food with several nutritional and therapeutic properties; it contains essential aminoacids, several vitamins, bioflavonoids and other remarkable molecules such as phenolic compounds, phytosterols and phytochemicals. While numerous studies are available about the antimicrobial effect of some beehive products (honey, propolis), few researches were carried out on the potential bee pollen antibacterial activity. The aim of our investigation was to evaluate the effect on *in vitro* bacterial growth of the addition of multifloral bee-gathered pollen loads suspensions (1%, 2%, 4% v/v) to standard growth media. The employed pollen employed was gathered from bees in Lucca Province (Tuscany, Italy). *Staphylococcus aureus* ATCC 6538, *Enterococcus faecalis* ATCC VanBV583, *Pseudomonas aeruginosa* ATCC 27853, *Escherichia coli* ATCC 25922, *Lactobacillus casei* ATCC 334 and *Lactococcus lactis* subsp *lactis* ATCC 19435 were selected for the study. All concentrations determined approximately 1 log (CFU/mL) decrease in *S. aureus* and *E. faecalis* growth. *P. aeruginosa*, *E. coli* and *L. lactis* showed an almost unaffected growth rate. *L. casei* revealed instead a significant increase of growth rate in presence of added bee pollen. To the best of our knowledge, this is the first report on antimicrobial activity of an Italian bee pollen. Moreover, this is the first survey concerning the effect of bee-gathered pollen loads suspension on lactic acid bacteria growth. Our data seem to be promising for a potential use of bee pollen loads suspension as natural additive.

Keywords: Bee-gathered pollen loads, antimicrobial activity, *Staphylococcus aureus*, *Enterococcus faecalis*, Lactic acid bacteria

INTRODUCTION

Bee pollen is the pollen gathered from flowering plants by honeybees and brought back to their hive after enzymatic elaboration and addition with peculiar substances produced by the bees themselves. Pollen appears as a fine, powder-like material that has been packed by worker honeybees into granules (**Basim *et al.*, 2006**), together with honey or nectar.
Bee pollen contains all the essential aminoacids in remarkable amounts, which are five to seven times higher than those found in traditional high protein foods. Bee pollen contains also vitamins A, D, E, K, C and bioflavonoids, as well as the complete B-complex, especially pantothenic acid (B5) and niacin. Moreover, phenolic compounds are detectable in bee pollen, together with considerable quantities of phytosterols and phytochemicals (**Balch and Balch, 1990; Broadhurst, 1999; Carpes, 2008**).
Relevant beneficial effects for human health are associated with dietary intake of phytochemicals, such as phenolic compounds, since they can reduce the risk of degenerative diseases. This is due to the decrease of oxidative stress and inhibition of macro-molecular oxidation (**Silva *et al.*, 2004**). In addition to their reported anticarcinogenic properties, phenolic compounds have been shown to possess free radical-scavenging and metal chelating activities (**Middleton, 1998**).
Bee-pollen has been successfully used for the treatment of prostatitis and for oral desensitization of allergic children (**Campos *et al.*, 1997; Mizrahi and Lensky, 1997**), moreover the employment of bee pollen as a natural supplementation has been proposed for human and animal diets (**Morais *et al.*, 2011; Hleba *et al.*, 2013**).
Reports about bee pollen antimicrobial activity are steadily increasing. The most sensitive microrganisms seem to be Gram positive bacteria, particularly *Staphylococcus aureus*, as shown by **Carpes *et al.* (2007), Fatrcová-Šramková *et al.* (2012)** and **Pascoal *et al.* (2014).** Some Authors have also described an inhibitory activity against Gram negative bacteria, such as *Escherichia coli* and *Pseudomonas aeruginosa*, as well as against yeasts and fungi (**Carpes *et al.*, 2007; Kacániova *et al.*, 2012**). Antibacterial activity is not only ascribed to the content of phenolic compounds, but also to their nature (**Morais *et al.*, 2011).** Thus, different patterns in pollen loads antimicrobial activity could be due to different compositions in phenolic compounds of the pollen itself **(Kacániova *et al.*, 2012**).
Examples of the successful employment of beehive products, such as honey and propolis, as meat or beverage additives are already present in literature (**Nagai *et al.* 2006; Sagdic *et al.* 2007; Rabaa *et al.* 2013**). Particularly, bee pollen has been recently employed for beer and cookies production **(Solgajová *et al.*, 2014a; Solgajová *et al.*, 2014b**). However, some doubts have been raised on bee pollen digestibility. Maceration of pollen for several hours in water or other liquids has been already proposed in order to improve digestibility and ensure the effective intake of nutritional and nutraceutical bee pollen compounds (**Campos *et al.*, 2010**). Several methods for the extraction of the antimicrobial bee pollen compounds have been described (aqueous, methanol or ethanol extraction), each of them leading to different results; moreover they could be a potential problem in food processing. Unaltered bee pollen loads could be instead promptly employed as a food additive and at the same time could represent an additional source of nutritional and nutraceutical compounds.
The aim of the present work was to investigate the antimicrobial activity of bee-gathered pollen loads suspensions, without treating them neither physically nor chemically to extract the active molecules.

MATERIAL AND METHODS

Bee-Gathered Pollen Loads Suspensions

Dried, organic and bee-gathered pollen loads were obtained directly from Italian beekeepers and employed for all experiments. Pollen was gathered by honey bees in Lucca Province (Tuscany, Italy) in year 2012. Pollen loads were sterilized by gamma-irradiation at a dose of 25 kGy, stored refrigerated until use. Palynological analysis showed a multifloral composition. Eight families were found in pollen loads samples. *Castanea* taxon was the most representative,

followed by *Eucalyptus*, *Compositae T* (*Forma Taraxacum*), *Cucumis*, *Compositae S* (*Forma Carduus*), *Trifolium pratense gr*, *Rubus* and *Hedera*. Enumeration of total mesophilic bacterial amount was performed as sterility control before each trial. For this purpose Plate Count Agar (PCA) (Oxoid, Milan, Italy) was employed. Plates were incubated at 30 °C for 72 h in aerobic conditions.

Bacterial Strains

The type strains *Staphylococcus aureus* ATCC 6538, *Enterococcus faecalis* ATCC Van B V583, *Pseudomonas aeruginosa* ATCC 27853, *Escherichia coli* ATCC 25922, *Lactobacillus casei* ATCC 334 and *Lactococcus lactis* subsp *lactis* ATCC 19435 were employed for the tests. The strains were obtained from the American Type Culture Collection (Rockville, MD) and maintained stored at -80 °C in growth media added with 20% v/v glycerol.

All the strains, except lactic acid bacteria, were revitalized in Brain Hearth Infusion (BHI) broth (Oxoid, Milan, Italy) at 37 °C for 24 h in aerobic conditions. Lactic acid bacteria strains were revitalized in Man Rogosa Sharp (MRS) broth (Oxoid, Milan, Italy) at 37 °C for 24 h in aerobic conditions.

Strains were then grown on selective agar media at different optimal culture conditions. The employed media were Baird Parker (37 °C, 24 h) for *S. aureus*, Kanamycin Aesculin Azide Agar (42 °C, 24 h) for *E. faecalis*, Pseudomonas Agar Base (30 °C, 24 h) for *P. aeruginosa*, Tryptone Bile X-Glucuronide (42 °C, 24 h) for *E. coli*, MRS (37 °C, 48 h) for *L. casei*, M17 Agar (30 °C, 24 h) for *L. lactis* subsp. *lactis*. All media were purchased from Oxoid, Milan, Italy.

For each strain, bacterial suspensions with a turbidity equivalent to McFarland standard 3 (corresponding approximately to $9x10^8$ CFU/mL) were prepared in sterile saline solution.

Preparation Of Bee-Gathered Pollen Loads Suspensions

An homogeneous pollen suspension was prepared adding 3 g of bee pollen loads into 4 mL of sterile saline solution and mixing the preparation with a stomacher.

The pollen loads suspension was added to BHI broth together with 1 mL of standardized bacterial inoculum. For each strain a pollen concentration of 1%, 2% and 4% v/v was tested in a final volume of 10 mL. A control test consisting of strains incubated in BHI or MRS broth, without addition of bee pollen was also evaluated. The strains were then incubated at optimal growth conditions.

Enumeration Of Bacterial Cells

After incubation the samples were serially diluted to enumerate bacterial cells on selective agar media. Bacterial growth rate in presence of different percentages of bee pollen was evaluated and compared to bacterial growth rate of control samples. Each experiment was carried out in triplicate.

Statistical Analysis

Statistical analysis was performed using the R v. 3.0.2 software (R Foundation for Statistical Computing, Vienna, Austria). For each bacterial strain the statistical significance of differences in growth rate in presence of different percentages of organic bee pollen was tested with a one-way ANOVA test followed by Tukey HSD *post-hoc* comparisons. Results were considered significant if associated with a p value lower than 0.05.

RESULTS AND DISCUSSION

Table 1 shows the obtained results. We observed a significant difference in *S. aureus* ATCC 6538 growth in absence and in presence of bee pollen suspensions. All the tested concentrations (1%, 2%, 4% v/v) determined approximately 1 log (CFU/mL) decrease in *S. aureus* ATCC 6538 growth. The same trend could be observed for *E. faecalis* ATCC Van B V583. This effective inhibitory activity was not detected against Gram negative strains. *P. aeruginosa* ATCC 27853 and *E. coli* ATCC 25922 showed an almost unaffected growth rate, with an increase or decrease of bacterial enumerations in presence of different pollen concentrations. However, changes detected in *P. aeruginosa* and *E. coli* enumerations were always lower than 1 log (CFU/ml). In particular, *P. aeruginosa* growth seems to be promoted in presence of 4% bee pollen, but not with 1% and 2%; while *E. coli* showed an increased growth in presence of 1% bee pollen and a reduced growth with 4% bee pollen. As concerns lactic acid bacteria, we observed a different trend in *L. casei* ATCC 334 and *L. lactis* subsp. *lactis* ATCC 19435 growth rates: while *L. lactis* growth was slightly reduced by the addition of organic bee pollen in the medium, especially in presence of 4%, *L. casei* revealed a significant increase of growth rate in presence of bee pollen, regardless of the added concentration.

Our results are in accordance with data reported by **Abouda *et al.* (2011), Morais *et al.* (2011), Fatrcová-Šramková *et al.* (2013)** and **Pascoal *et al.* (2014).** These Authors observed a higher antimicrobial effect of beehive products on Gram positive bacteria, especially against *S. aureus*, than on Gram negative ones. As suggested by **Pascoal *et al.* (2014)** this may be due to the presence of the additional outer layer membrane, impermeable to most molecules, that consists of phospholipids, proteins and lipopolysaccharides. Nevertheless, unlike *S. aureus*, few studies were carried out on *E. faecalis* pollen susceptibility (**Erkmen and Özcan, 2008**), while numerous studies are available on the antimicrobial effect of propolis, honey and royal jelly against *Enterococcus* spp. as oral pathogen (**Cooper *et al*., 2002; Boukraâ and Sulaiman, 2009; Moncla *et al*., 2012**).

As concerns Gram negative bacteria, our results on *E. coli* and *P. aeruginosa* agree with those reported from **Abouda *et al.* (2011)** and **Morais *et al.* (2011)**. For both microrganisms, a slighter bee pollen effect was observed. However, while *Enterobacteriaceae* have been frequently detected in bee pollen and consequently a specific antimicrobial activity was not expected, *Pseudomonadaceae* as well as other Gram negative rods have been rarely found either in honey bees or beehive products (**Gilliam and Morton, 1974; Gilliam and Valentine, 1974; Vanneste *et al*., 2011**).

Regarding lactic acid bacteria, the observed 1 log (CFU/mL) increase in *L. casei* growth rate in presence of different bee pollen concentrations could be due to the ability of this strain to metabolize carbohydrate substrates carried by pollen. It is know indeed that lactic acid bacteria, especially *Lactobacillus* spp., are present in natural bee pollen and play an important role together with the yeasts in the conversion and preservation of pollen (**Gilliam, 1997; Vasquez and Olofsson, 2009**).

Table 1 Strains growth rate (log CFU/mL mean values±standard deviation) in presence of different percentages of organic bee pollen (1%, 2%, 4% v/v) and without addition of organic bee pollen (control)

Strains	Control	1%	2%	4%
S. aureus ATCC 6538	9.39 ± 0.08^{a}	8.53 ± 0.11^{b}	8.49 ± 0.14^{b}	8.57 ± 0.19^{b}
E. faecalis ATCC Van B V583	9.24 ± 0.10^{a}	8.82 ± 0.10^{b}	8.74 ± 0.10^{b}	8.49 ± 0.09^{c}
P. aeruginosa ATCC 27853	8.25 ± 0.12^{a}	8.23 ± 0.19^{a}	$8.53 \pm 0.11^{a,b}$	8.77 ± 0.21^{b}
E. coli ATCC 25922	9.18 ± 0.13^{a}	9.57 ± 0.11^{b}	$9.39 \pm 0.12^{a,b}$	8.78 ± 0.17^{c}
L. casei ATCC 334	8.67 ± 0.10^{a}	9.52 ± 0.11^{b}	9.66 ± 0.15^{b}	9.76 ± 0.18^{b}
L. lactis lactis ATCC 19435	8.22 ± 0.15^{a}	$8.15 \pm 0.16^{a,b}$	$7.77 \pm 0.13^{a,b}$	7.71 ± 0.24^{b}

Legend: different letters in the same row show statistically significant differences

CONCLUSION

The present work aimed to evaluate the antimicrobial potential of dried bee-gathered pollen loads suspensions. Pathogen, spoilage and useful bacteria were chosen for the trials. Our findings highlight a significant inhibitory effect against Gram positive bacteria, such as *E. faecalis* and *S. aureus*, while a slighter antimicrobial activity was detected for Gram negative bacteria (*P. aeruginosa* and *E. coli*). As concerns lactic acid bacteria, *Lc. lactis* subsp. *lactis* and *Lb. casei*, which were here for the first time evaluated, a different trend was observed. *Lc. lactis* was negatively affected by the addition of 4% bee-pollen in the synthetic medium, while *Lb. casei* always showed an improved growth rate.

Our findings could suggest the employment of pollen loads suspension as a food additive with the advantage to reduce the proliferation of spoilage or pathogen bacteria, without inhibiting the growth of useful ones. Particularly, since no negative effects against lactic acid bacteria were detected in presence of 1% and 2% bee pollen, it would be interesting to evaluate the impact of the addition of bee pollen in dairy products, both in terms of safety and organoleptic characteristics. At the same time the addition of pollen would enrich dairy products with nutraceutical compounds. Further studies would be also required in order to evaluate the antimicrobial activity of bee-pollen load suspensions on wild bacterial strains growth rate, since they could likely be more resistant.

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