ANTIBACTERIAL EFFECT OF ARGENTINIAN POLLENS AND HONEYS

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ABSTRACT

Honey and pollen are considered functional foods, due to their multiple properties since they have a great diversity of active principles according to their botanical origin. The aim of this work was to analyze the antimicrobial effect of pollens and honeys from different origins of Argentina against the antibiotic resistant *Shigella flexneri,* enteropathogenic *Escherichia coli* and *Salmonella typhi*. Honey samples showed a significant effect over the inhibition of them, being higher in *Salmonella*. Pollen inhibited *Shigella* and *Salmonella* and showed no effect on *Escherichia*. The concentration of total phenols was higher in pollen than in honey. The highest value observed in honey was 7.48 mg/L in a sample from Entre Ríos, and it was 8.66 mg/L in pollen from Neuquén.

Keywords: *Shigella flexneri,* enteropathogenic *Escherichia coli, Salmonella typhi,* honey, pollen, phenols

INTRODUCTION

Honey and pollen bee are products stored by the hive and they are considered as functional food (**Libonatti *et al*., 2014**). Its production in Argentina has increased in recent years. Several compounds contribute significantly to the antibacterial properties of honey, such as hydrogen peroxide, methylglyoxal, melanoidins, oxidative stress and hydroxyl radicals, through a multimodal action mechanism (**Kwakman *et al* 2010**). **Brudzynski and Sjaarda (2015)** demonstrated in honey a glycoprotein that inhibits the growth of *Escherichia coli* and *Bacillus subtilis* through cell lysis and/or spheroplast formation.

The pollen collected by bees is known as antibacterial, antifungal and immunomodulatory. It is composed mainly of polysaccharides, simple sugars, lipids, amino acids, proteins and other side compounds, such as flavonoids, vitamins, minerals and pigments such as carotenoid. Its chemical compositions, depends on its floral origin (**Cabrera and Montenegro, 2013**).

Natural compounds against antibiotic resistant microorganisms are sought because the bacteria that accumulate multiple antibiotic resistances are able to spread in different geographical regions (**Rosenblatt-Farrell, 2009**).

The species of *Salmonella* and *Shigella* are one of the major causes of diarrhea and dysentery worldwide, responsible for significant morbidity and mortality in developing countries (**Della Gaspera *et al*., 2015**). Although a variety of antibiotics have been effective in treating shigellosis and salmonellosis, the options are increasingly limited due to drug resistance (**Saurabh *et al*., 2015**).

Enteropathogenic *E. coli* remains the most common cause of infant morbidity. The diarrheagenic strains can be transmitted through the fecal-oral route by ingesting food or water contaminated (**Adefisoye and Okoh, 2016**). An increase in resistance has been observed in the last six decades after the introduction of new antibiotics (**De Toro *et al*., 2014**)

The aim of this study was to analyze the antimicrobial effect of pollens and honeys from different origins of Argentina against enteropathogenic *Escherichia coli, Salmonella typhi,* and *Shigella flexneri,* which are antibiotic resistant. Also, know the phenol concentrations in honeys and pollen and their relationship with the botanical origin.

MATERIAL AND METHODS

Samples

Honey samples were obtained from the provinces of Misiones, Chaco, Entre Rios, Corrientes and Jujuy and pollen from the provinces of Buenos Aires, Santa Fe, Entre Rios, Neuquén, Tucumán, Misiones, Santiago del Estero and Jujuy. Samples were collected in sterile containers and kept at room temperature.

Microorganisms

Shigella flexneri, Salmonella typhi, and enteropathogenic *E. coli* provided by Jujuy Children Hospital were identified by biochemical tests. The strains were transferred weekly to Luria-Bertani or selenite media and incubated for 24h at 37°C). They were stored at 4° C in 20% glycerol medium (**Ruíz *et al*., 2018**).

The antibiograms were performed by the disk-plate technique (**Bauer, Kirby *et al*., 1966**). The antibiotics were erythromycin, vancomycin, tetracycline, rifampicin, cephalotin, penicillin, neomycin, gentamicin, kanamycin, and streptomycin.

The resistance tests were made using 200 μL of fresh culture (10^5 cfu / mL) onto Mueller Hinton agar, against 20 μL of honey or pollen suspension (1: 1) in sterile water. They were incubated at 37 ° C up to stationary growth phase. The inhibition halos were measured after 24 hours in millimeters.

Determination of phenols

One mL honey or 1 g of pollen was dissolved in 50 mL of distilled water and 0.5 mL of the solution was added to 0.75 mL Folin-Ciocalteau reagent 1N (**Töpfer, 2018**). It was settled for about 5 minutes and 0.75 mL of sodium carbonate 20% was added. It was shaken and allowed to settle for 2 h and reading at 760 nm. Measurements were performed by duplicate.

Botanical origin of honeys and pollen

Qualitative analysis of the honey sample was carried out following the method described by **Louveaux *et al*. (1970)** using the acetolysis method (**Erdtman, 1960**). The pollen samples were disintegrated and homogenized with mortar, taking 0.5 g of the total for further acetolysis treatment (**Erdtman, 1960**). Pollen sediment was mounted into glycerin and sealed with paraffin. Observations were made with a light microscope. To determine the frequency classes, 600 pollen grains were counted (**Louveaux *et al*., 1970**). Pollen types were identified according the palynotheca of the Facultad de Ciencias Agrarias, UNJu (PALJUA), and palynological atlas. The classes are: predominant pollen 'D' (> 45%), secondary pollen 'S' (16–45%), important minor pollen 'M' (3–15%), trace pollen 'T' (< 3%) (**Louveaux *et al*., 1970**).

Analysis of results

Statistical analysis of the results will be performed by ANOVA test and Tukey comparison with 0.05 probability of committing Type I error, using a InfoStat statistical package (**Di Rienzo *et al*., 2008**). A canonical correspondence analysis

(CCA) was performed to establish the relationship between the botanical origen, concentration of phenol and measuring inhibition halos using CANOCO 4.5 software package (*Leps* and *Smilauer,* 2003).

RESULTS

All honey samples showed a significant effect (p=0.04) in the inhibition of *S. flexneri, E. coli,* and *S. typhi* strains, being higher in the latter (Table 1). Pollen inhibited *Shigella* and *Salmonella*, but not the *Escherichia* strain (Table 2).

Table 1 Honeys against pathogen strains.

Origin	Total phenols mg / L	Inhibition halos (mm)		
		S. typhi	*S. flexneri*	*E. coli*
Entre Ríos	6.38	37±1.41	40.75±1.75	13.25±2.75
Misiones	3.35	44.5±0.71	41±9.27	36.5±2.12
Jujuy (San Salvador)	5.28	30.5±0.71	27.5±2.12	25±1.41
Chaco	4.37	45	36.25±5.06	34.25±4,11
Jujuy (Palpala)	5.4	41.5±4.95	23	34±3.46
Corrientes	4.72	39.5±0.71	49.5±0.71	34±4.62
Jujuy(Palpala Orgánica)	6.42	39.5±0.58	28.5±3.54	34±8.13
Jujuy (Tilquiza)	6.12	41.75±0.5	27±1.41	28.8±2.3
Jujuy (Santa Bárbara)	5.87	32.5±0.58	32.75±1.71	32±0.83

Table 2 Pollen grains against pathogens strains.

Origin	Total Phenols mg / L	Inhibition halos (mm)		
		S. typhi	*S. flexneri*	*E. coli*
Buenos Aires	4.58	31.5±2.12	28±1	-
Santa Fe	4.34	35.5±4.95	25.75±2.87	-
Entre Ríos	7.48	40	31±2.83	-
Neuquén	8.66	37±1.41	26.75±1.26	-
Tucumán	5.78	32.25±0.96	16±5.66	-
Misiones	5.98	29±1.41	24.25±2.87	-
Santiago del Estero	4.61	26	12.5±0.71	-
Jujuy (San Salvador)	8.32	27.75±1.5	18.75±0.96	-

The concentration of total phenols was different according to the regions, being higher in pollen than in honey, showing significant difference between them (p=0.02) (Tables 1 and 2). The highest values observed were 7.48 mg / L in honey from Entre Rios and 8.66 mg / L in pollen from Neuquén. *E. coli* was the most influenced by phenols content of honey.

Antibiotics tested were found to have lower inhibitory effect in relation to the samples of honey and pollen (p=0.001) (Table 3). Honeys from Corrientes and Entre Rios showed a greater inhibitory effect than antibiotics.

Table 3 Antibiograms

Antibiotics	Inhibition halos (mm)		
	S. typhi	*S. flexneri*	*E. coli*
Erythromycin (60μg)	9	23	-
Vancomycin (30μg)	21	21	-
Tetracyclin (30μg)	15	36	-
Rifampicin (15μg)	13	20	-
Cephalotin (30μg)	-	-	-
Penicillin (10U)	-	16	-
Neomycin (30μg)	10	9	16
Gentamicin (10μg)	9	10	14
Kanamycin (120μg)	-	-	22
Streptomycin (300μg)	14	-	25

Botanical origen of honeys

From the 9 honey samples analyzed, 56 pollen types were identified belonging to 34 botanical families. *Fabaceae* presented eight pollen types, *Asteraceae* seven, *Euphorbiaceae* and *Rhamnaceae* three, *Anacardiaceae, Boraginaceae, Myrtaceae* and *Sapindaceae* two, and the remaining families only one. Two

samples were monofloral. The predominant pollen types were from *Arecaceae* (Entre Ríos) and *Schinus* (Jujuy, Tilquiza) (Fig. 1).
The results obtained with the Monte Carlo Test that perform an exploratory analysis are presented, which show a trend of the association of the variables analyzed.
The samples with the highest *S. typhi* inhibition halo were those from Chaco and Misiones, which had a lower content of phenols. The pollen traces from Chaco honey sample belonged to: *Acacia, Aspidosperma quebracho-blanco, Cercidium praecox, Clematis, Fraxinus, Gleditsia amorphoides, Helianthus annus, Peltophorum dubium* and *Ziziphus mistol* species.
Other pollen types related to inhibition were: a) *Prosopis*, in samples with low inhibition from Jujuy (Palpalá Organic and Santa Bárbara) and Entre Ríos; b) Asteraceae in all the samples studied reaching the secondary category (Misiones); c) *Eucalyptus*, found in minor frequency class and secondary, in the most inhibited samples Chaco, Misiones, Jujuy (Tilquiza), Jujuy (Palpalá) and Corrientes. *Salix* was registered in trace also in samples of greater inhibition but in Corrientes sample was found in less importance.
The sample with the highest inhibition of *S. flexneri* halo was that from Corrientes characterized by pollens of: a) *Echium* and *Euphorbiaceae* recorded as minor importance; b) *Zanthoxylum, Lauraceae* and *Ilex* in traces. *Ilex* was also present in the Misiones sample. Other pollen types associated with inhibitory capacity are *Salix*, Solanaceae and Myrtaceae, those that are also present in samples with less inhibition capacity corresponding to Misiones, Chaco, Jujuy (Santa Bárbara), Jujuy (Tilquiza) and Jujuy (Palpalá). The Entre Ríos sample showed an inhibitory capacity related to Arecaceae, registered as dominant.
The highest inhibition of *E. coli* it was observed that the samples from Misiones, Chaco and Corrientes with the lowest content of phenols. The pollen types in Misiones sample were from *Lamiaceae* and *Boraginaceae* exclusively, while than in Chaco sample were from *Aspidosperma quebracho blanco, Helianthus annuus, Acacia, Cercidium praecox, Peltophorum dubium, Gleditsia amorphoides, Fraxinus, Clematis, Ziziphus mistol*. The pollen types in the Corrientes sample were *Lauraceae*, *Echium* and *Zanthoxylum*, *Eucalyptus*, *Prosopis*, *Baccharis, Asteraceae, Euphorbiaceae* and one type identified as *Monocotyledonea*.
Samples from Jujuy (Palpalá, Palpalá Organic, and Santa Bárbara) had similar inhibitory capacity, but different from the three previous samples, because they had a higher content of phenols. The pollen types of these samples were: *Schinus, Schinopsis* type, *Senecio, Serjania, Gomphrena, Sebastiania, Taraxacum officinale*, *Solanaceae*, *Myrtaceae, Apiaceae, Poaceae, Bignoniaceae,* and *Verbenaceae*.
The samples from Entre Ríos, Jujuy (Tilquiza) and Jujuy (San Salvador) are those with the least inhibition but with a high content of phenols. In the three analyzes, phenols contents are related to some pollen types such as *Arecaceae, Schinus, Schinopsis* type, *Senecio, Fabaceae, Rapistrum rugosum, Eupatorium, Veronica, Bignoniaceae* and *Rosaceae*. The first three were found as dominant or secondary pollen, while the others as less importance or in trace.

Botanical origen of pollens

From the eight pollen samples analysed, 35 types belonging to 20 botanical families, one subfamily, one class and one undetermined, were identified. Among these families, *Fabaceae* and *Asteraceae* presented five and eight types respectively, *Myrtaceae* and *Poaceae* two, and the others one pollen type each. According to their frequency class, in three samples were predominant *Rapistrum rugosum* (Jujuy, Entre Ríos y Misiones), in one *Myrtaceae* type (Neuquén) and in the other *Asteraceae* (Tucumán) (Fig. 2).
The samples with the highest inhibition of *S. typhi* were those from Entre Ríos and Neuquén, which are characterized by presenting the highest phenolic content values, followed in order of importance by Santa Fe, Tucumán, Buenos Aires, Misiones, Jujuy and Santiago of Estero. The pollen types were: *Caesalpinoideae*, exclusive to Entre Ríos; *Cardus,* in Entre Ríos, Misiones and Buenos Aires samples, and *Myrtaceae* in Neuquén sample. The lasts three pollen types were a high content of phenols together with *Taraxacum officinale* and *Rapistrum rugosum*. The *S. typhi* inhibition by Santa Fe sample (with the lowest phenols content) was associated with *Mutisieae*, but in Tucumán sample to *Ziziphus mistol*. *Helianthus annus*, Amaranthaceae-Chenopodiaceae, Fabaceae and Asteraceae, were present in Santa Fe, Buenos Aires and Tucumán samples.
The greatest inhibition *S. flexneri* was observed with the Entre Ríos sample, following by that of Buenos Aires. Pollen types that are important in the inhibition of this bacterium, were the same for *Salmonella.*
The pollen types related to the highest phenols content were: *Myrtaceae*, in the sample of Neuquén; *Taraxacum officinale* and *Rapistrum rugosum* in Neuquén, Jujuy, Entre Ríos, Misiones, Buenos Aires and Santa Fe; *Zea mays* in Jujuy, Entre Ríos, Misiones.
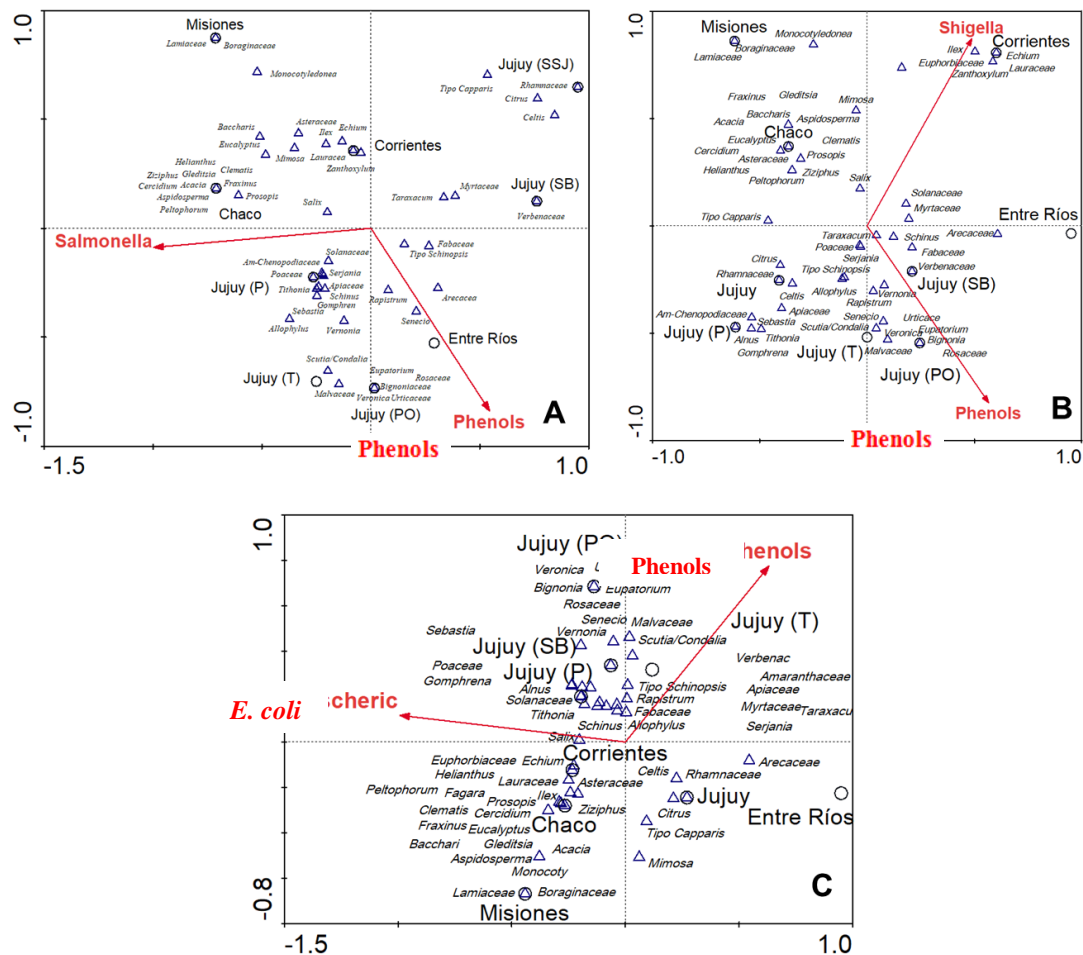
Figure 1 Samples of honey. CCA plot showing the relationship between the content of phenols and the inhibition of bacteria: A. *Salmonella*; B. *Shigella*; C. *Escherichia coli.*
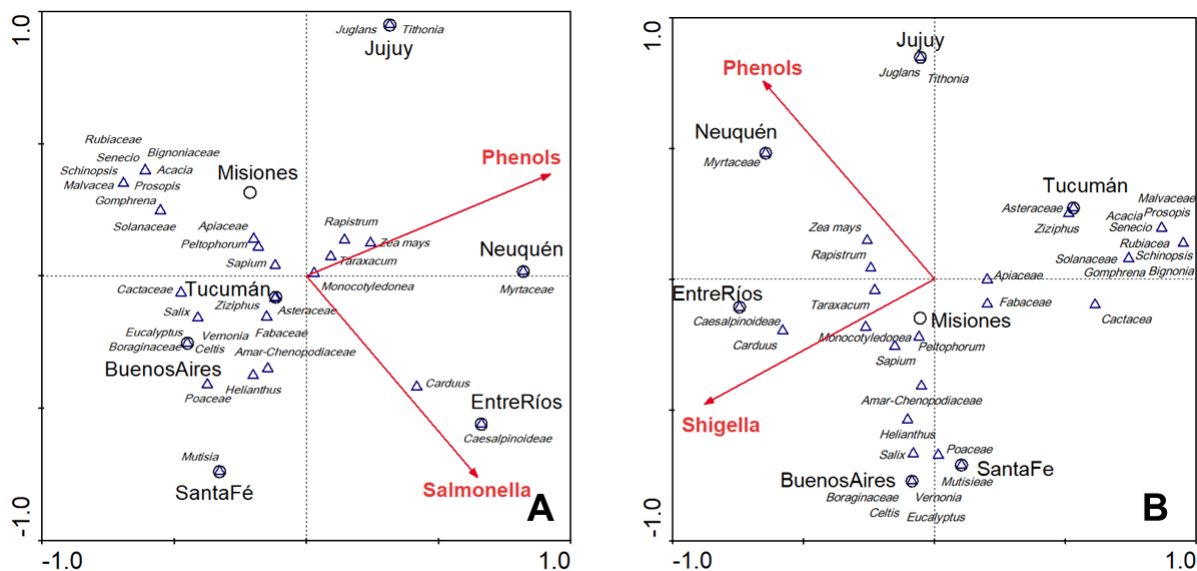

Figure 2 Samples of pollen. CCA plot showing the relationship between the content of phenols and the inhibition of bacteria: A. *Salmonella*; B. *Shigella.*

DISCUSSION

The antimicrobial activity of honey depends on several factors which function singularly or synergistically, related to hydrogen of peroxide, phenolic compounds, low pH, osmotic pressure and other phytochemicals contents (**Mandal and Mandal, 2011**). Honey has the ability to generate hydrogen peroxide related to antimicrobial activity. The production of hydrogen peroxide is due to the transformation of glucose acting as substrate for glucose oxidase enzyme present in honey, which concentration depends on the floral origin (**Jantakee and Tragoolpua, 2015**), which is related to the results of this study where differences in the inhibitory capacity of the different provinces were shown due to different floral origins, since in this work it resulted in lower phenolic contents than pollens.

The greatest inhibition halos were produced by honey from Corrientes and pollen from Misiones indicating that *Apis mellifera* L. uses different floral sources for the production of honey, which explains the difference in their antibacterial

effect, that at the same time its effect could be due to the pollen types found as *Eucalyptus* and *Laurel* (**Ewnetu *et al*., 2013; Montero-Recalde *et al.,* 2019**). Shown inhibition halos are higher than those proposed in other studies indicating that honeys from Argentina have a greater inhibitory effect (**Fatrcová-Šramková *et al*., 2013**). Other studies have shown, the action of the compounds of honey, in bacteria, they act on the cell wall (murein/peptidoglycan) in *E. coli* inducing perturbations in its integrity, many of these effects are due to active anti-bacterial proteins, including the glycoproteins resistant to diseases found in plants, this would allow them to recognize and bind bacterial cells, affecting their growth, survival and other mechanisms (**Brudzynski *et al*., 2015**).

Polyphenols are able to inhibit microorganisms and antimicrobial activity is dependent on their chemical structure and environmental conditions. The effect of flavonoids and phenolic acids present in pollen cause interruption of bacterial metabolism. The mechanism consists of the formation of complexes with the cell wall by exposure on the surface of adhesins and polypeptides, and/or cell membrane enzymes, which leads to interruption of the integrity of the cell wall, block of ion channels, and inhibition of the flow of electrons from the transport chain by scanning electron (**Rzepecka-Stojko *et al*., 2015**).

Therefore, antibacterial activity of the different varieties of honey is due to the activity of the hydrogen peroxide. This would generate free hydroxyl radicals which break DNA and oxidize thiol groups of proteins and lipids, causing damage of the bacterial cells (**Jantakee and Tragoolpua, 2015**).

This work coincides with Fatrcová-Sramkova (**Fatrcová-Šramková *et al*., 2013**), and would demonstrate that the effect of pollen is due to the concentration of phenols in the sample and not the effect of honey that might be related to proteins similar to those found in plants that are resistant to diseases (**Brudzynski and Sjaarda, 2015**) and/or the concentration of the enzyme glucose oxidase from different plants associated to regions.

The high resistance values obtained by Adefisoye (**Adefisoye and Okoh, 2016**) may suggest exposure to bacterial antibiotics isolated, this could be due to improper use of antibiotics among the population, and can lead to increase the development of multi-drug resistance. They have identified genes that confer resistance mainly to tetracycline and ampicillin (**Adefisoye and Okoh, 2016**, **Lalak *et al*., 2016**).

CONCLUSION

In this study it was found the antimicrobial effect of pollen and honey against *S. flexneri*, *E. coli* and *S. typhi* enteropathogenic, were superior to those shown by commercial antibiotics. Honeys showed greater inhibitory effect than pollens. The effect shown by pollens could be attributed to the phenols concentration.

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