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.

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.

INTRODUCTION

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.

Microorganisms

Shigella flexneri, Salmonella typhi, and enteropathogenic E. coli provided by Juyuj Children Hospital were identified by biochemical tests. The strains were transferred weekly to Luria-Bertani and selenite media and incubated for 24 h at 37°C. They were stored at 4°C in 20% glycerol medium (Ruiz et al., 2018). 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 (10^6 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 of the reagent 1N Folin-Ciocalteau reagent (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 (PALJU), 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).
(CCA) was performed to establish the relationship between the botanical origin, 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 | 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±0.27   | 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  |

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 Ríos and 8.66 mg / L in pollen from Nequén. 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 Ríos showed a greater inhibitory effect than antibiotics.

### Table 2 Pollen grains against pathogens strains.

| Origin                  | Total phenols mg / L | 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     | -       |
| Nequén                  | 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 samples were monofloral. The predominant pollen types were from Arceaeae (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 praeceps, Cleratia, Fraxinus, Gleditsia amorphoides, Helianthus annus, Peltophilia dawburni and Ziziphus mistol species. Other pollen types related to inhibition were: a) Prosopsis, in samples with lower inhibition from Jujuy (Palpalá Organic and Santa Bárbara) and Entre Ríos; b) Astereae 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 (Palpala) 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, Lauracea and Ilex in traces. Ilex was also present in the Misiones sample. Other pollen types associated with inhibitory capacity are Salix, Solanaceae and Myrtaceae, 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 Arceaeae, 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 Lamiaeae and Boraginaceae exclusively, while than in Chaco sample were from Aspidosperma quebracho blanco, Helianthus annus, Acacia, Cercidium praeceps, Peltopherum dawburni, Gleditsia amorphoides, Fraxinus, Clematis, Ziziphus mistol. The pollen types in the Corrientes sample were Laueuch, Echium and Zanthoxylum, Eucalyptus, Prosopsis, Baccharis, Astereae, Euphorbiaceae and one type identified as Monocoltodorella.

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, Serjantia, Gomphrena, Sebastiana, Taxaracum 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 Arceaeae, 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.

### Table 3 Antibiotics

| Antibiotics                | Inhibition halos (mm) |
|---------------------------|----------------------|
|                          | S. typhi | S. flexneri | E. coli |
| Erythromycin (60µg)       | 9        | 3           | -       |
| Vancomycin (30µg)         | 21       | 21          | -       |
| Tetracyclin (30µg)        | 15       | 36          | -       |
| Rifampicin (15µg)         | 13       | 20          | -       |
| Cephalothin (30µg)        | -        | -           | -       |
| Penicillin (10U)          | -        | 16          | -       |
| Neomycin (30µg)           | 10       | 9           | 16      |
| Gentamicin (10µg)         | 9        | 10          | 14      |
| Kanamycin (120µg)         | -        | -           | 22      |
| Streptomycin (300µg)      | 14       | -           | 25      |

### Botanical origin 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, Anacardiacae, Boraginaceae, Myrtaceae and Sapindaceae two, and the remaining families only one. Two
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 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-Recalec et al., 2019). Shown inhibition halos are higher than those proposed in other studies indicating that honeys from Argentina have a greater inhibitory effect (Fraturová-Srhamková et al., 2013). Other studies have shown, the action of the compounds of honey, in bacteria, they act on the cell wall (murin/pihtopidoglycan) 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 and permeation of 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 action of the hydrogen peroxide. This would generate free radicals which break DNA and oxidize the groups of proteins and lipids, causing damage of the bacterial cells (Jantake and Tragoolpua, 2015). This work coincides with Fraturová-Srhamková (Fraturová-Srhamková et al., 2013), and would demonstrate that the effect of pollen is due to the concentration of flavonoids and phenolic acids present in different plants associated to regions. The high resistance values obtained by Adesfioye (Adesfioye 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 (Adesfioye 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 pollen. The effect shown by pollens could be attributed to the phenols concentration.

Acknowledgements: The authors appreciate the financial support of SECTER, from the National University of Juyn. Tejerina Marcos Raúl has a postdoctoral studies fellowship from Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET-Argentina).

REFERENCES

Adesfioye, M.A., Okoh, A.I. (2016). Identification and antimicrobial resistance prevalence of pathogenic Escherichia coli strains from treated wastewater effluents in Eastern Cape, South Africa. Microbiology Open; 5(1): 143–151. https://doi.org/10.1002/mbo3.319

Bauer, A.W., Kirby, W.M.M., Sherris, J.C., Turck, M. (1966). Antibiotic susceptibility testing by a standardized single disk method. Am J Clin Pathol; 45: 493–496. https://doi.org/10.1016/s0002-9173(66)80078-6

Brudzynski, K., Szaarda, C. (2015). Honey. Glycoproteins Containing Antimicrobial Peptides, Jeelins of the Major Royal Jelly Protein 1, Are Responsible for the Cell Walllytic and Bacterial Activities of Honey. PLoS ONE; 10(4): e0120238. https://doi.org/10.1371/journal.pone.0120238

Cabrera, C., Montenegro, G. (2013). Pathogen control using a natural Chilean bee pollen extract of known botanical origin. Cienc. Inv. Agr.; 40 (1):223-230. https://doi.org/10.4067/S0718-16202013000100020

De Toro, M., Seral C, Rojo-Bezares, B., Torres, C., Castillo, F.J., Sáenz, Y. (2014). Antibiotic resistance and virulence factors in clinical Salmonella enterica isolates. Enferm. Infecc. Microbiol. Clin.; 32(1):1-10. https://doi.org/10.1016/j.eimc.2013.03.002

Della Gaspera, A.M.I., Caffé, M.P., Vinas, M.R., Hebe, A., Barrios, S.S., Viora, R.J.A. (2015). Brote de shigelosis en la ciudad de Luján, Argentina. Rev. Argent. Microbiol.; 26(2): 1-6. https://doi.org/10.1016/j.ram.2015.02.003

Di Rienzo, J.A., Casanoves, F., Balzanni, M.G., González, L., Tablada, M., Robledo, C.W., (2008). Grupo Infostat, versión 2008, FCA, Univ. Nacional de Córdoba, Argentina. http://www.infostat.com.ar

Erdman, G. (1960). The acetylcolin method, a revised description. Svensk. Bot. Tidskr.; 54: 561-564. https://doi.org/10.1080/00373136009429446

Ewnetu, Y., Wossenseged, L., Birhane, N. (2013) Antibacterial effects of Apis mellifera and stingless bee honeys on susceptible and resistant strains of Escherichia coli, Staphylococcus aureus and Klebsiella pneumoniae in Gondar, northwest Ethiopia. BMC Complementary and Alternative Medicine; 13: 269. https://doi.org/10.1186/1472-6831-13-269

Fraturová-Srhamková, K., Nőzökì, J., Kačánirová, M., Máriaááyová, M., Rovná, K., Stríčik, M. (2013). Antioxidant and antimicrobial properties of monofloral bee pollen. Journal of Environmental Science and Health, Part B: Pesticides, Food Contaminants, and Agricultural Wastes; 48(2): 133-138. https://doi.org/10.1080/03601234.2013.727664

Jantake, K., Tragoolpua, Y. (2015). Activities of different types of Thai honey on pathogenic bacteria causing skin diseases, tyrosinase enzyme and generating free radicals. Biological Research; 48(1). https://doi.org/10.1186/1687-6287-48-4

Kwakman, P.H., Te Velde, A.A., Boer, L., Speijer, D., Vandenbroucke-Grauls, C.M., Zaat, S.A. (2010). How honey kills bacteria. Faseb J.; 24(7): 2576 – 2582. https://doi.org/10.1096/fj.10-150789

Lalak, A., Wasyl, D., Zaja, M., Skarzynska, M., Hoszowski, A., Saincik, I., Wonzniakowski, G., Szulowski, K. (2016). Mechanisms of cephalosporin resistance in indicator Escherichia coli isolated from food animals. Vet. Microbiol.; 194; 69-73. https://doi.org/10.1016/j.vetmic.2016.01.023

Leps, J. and Smilauer, P. (2003). Multivariate Analysis of Ecological Data Using CANOCO. Cambridge University Press, Cambridge. https://doi.org/10.1017/cbo9780511615146.005

Libonatti, C., Soledad, V., & Marina, B. (2014). Antibacterial activity of honey: A review of honey around the world. Journal of Microbiology and Antimicrobials, 6(3), 51-56. https://doi.org/10.8977/n14ma203600308

Louveaux J., Maurizio A., Vorwol G. (1970). Methods of melissopalynology by International Commission of Bee Botany of IUBS. Bee World; 59: 139-157. https://doi.org/10.1006/micro.2015.02.003

Mandal, M.D., & Mandal, S. (2011). Honey: its medicinal property and antibacterial activity. Asian Pacific Journal of tropical biomedicine, 1(2): 154–160. https://doi.org/10.1016/s2221-1691(11)60016-6

Montero-Recalec, M., Morroco-Núñez, M.J., Áviles-Esquível, D., Carrasco-Cando, A., & Erazo-Gutierrez, R., (2019). Eficacia antimicrobiana del aceite esencial de eucalipto (Eucalyptus spp) sobre cepas de Escherichia coli y Staphylococcus aureus subsp. aureus. Revue of Investigaciones Veterinarias del Perú; 30(2): 932-938. https://doi.org/10.15381/revipe.v30i2.16099

Rzepecka-Stojko, A., Stojko, J., Kurek-Górecka, A., Górecki, M., Kabala-Dzik, A., Kubina, R., Mozdzier, A., Buszman, E. (2015). Polyphenols from bee pollen: structure, absorption, metabolism and biological activity. Molecules 2015; 20; 21732-21749. https://doi.org/10.3390/molecules20121900

Saurabh, A., Sárkar, P., Sahai, D.R., Patra, A., Ramamurthy, T., Prasanta, K. (2015). Intracellular and membrane-damaging activities of methyl gallic isolated from Terminalia chebula against multidrug-resistant Shigella spp. J Med Microbiol.; 64 (8): 901-909. https://doi.org/10.1099/im.0.000107

Töpfer G. 2018. Folin-Ciocalteu-Methode. In: Gressner A., Arndt T. (eds) Lexikon der Medizinischen Laboratoriumsdiagnostik. Springer Reference. Medizin. Springer, Berlin, Heidelberg. https://doi.org/10.107798-3-662-49054-9.1157-1