International Journal of
Food Science & Technology
www.ifst.org

International Journal of Food Science and Technology 2010, **45**, 520–529

Original article

Characterisation of Argentinean honeys and evaluation of its inhibitory action on *Escherichia coli* growth

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(Received 10 August 2009; Accepted in revised form 26 November 2009)

Summary The aim of this work was to evaluate the physicochemical, sensory and *Escherichia coli* growth inhibitory characteristics of honey of different botanical sources from two geographic origin of Argentina. Honeys were obtained from apiaries located in two zones. The floral identification of honeys allowed to clustered them as monofloral, mixed and polyfloral honeys. The study of the physicochemical parameters such as colour, free acidity, pH and moisture showed that the last one reflected significant differences between honeys. These differences were markedly reflected in the average values of moisture content for each zone, being 18.96% and 14.29% to centre and east zone, respectively. In general, honeys evaluated presented an inhibitory effect on the *E. coli* growth at different periods of time (bacteriostatic action). Only, two of the samples would show a bactericide action against *E. coli* at 48 h of incubation. Honeys with higher non-hydrogen peroxide activity, were collected from a same geographic place at the same season of year, showing a relationship between the antimicrobial activity and the geographic origin, which could be associated with the typical flora of the place.

Keywords Antimicrobial activity, *Escherichia coli,* honey, physicochemical properties.

Introduction

Honey is the natural sweet substance produced by *Apis mellifera* bees from the nectar of plants, which the bees collect and transform by combining with specific substances of their own. Honey consists of a solution that contains fructose (38.5%) and glucose (31.3%) as the predominant monosacharides, maltose (7.2%), sucrose (1.5%) and oligosaccharides (4.2%), acids organics and their esters, minerals, volatile compounds, proteins, amino acids and pollen (Beretta *et al*., 2005). In general, moisture content of honey varies between 12% and 23%, with a range of water activity from 0.562 to 0.62 and values of pH between 3.2 and 4.5 (Molan, 1992).

In Argentina honey production constitutes a productive economic activity, yielding per comb between 50 and 100 kg of honey. In general, Argentinean honey present good acceptance at international level for its quality and purity. Buenos Aires province focus the major number of beehives, the major quantity of small producers and the most representative production that is destined to both internal consumption and international market. The quality of honey is mainly determined by its sensory, chemical, physical and microbiological characteristics.

Physical and chemical properties of honey vary with botanical and geographical origin (Bath & Singh, 1999; Cano *et al*., 2001). Colour of honey is ranged from water white to dark, being related to both the content of pigments such as carotenoids and flavonoids (many of which have antioxidant and antibacterial properties) and the content of minerals. Light coloured honeys are produced generally in prairie zones, which in our country are mainly placed in the wet pampa region. Because of this, colour evaluation is important, since light coloured honeys are preferred by the majority of consumers and they are better valuated in the market (Andrada, *et al*., 2000). Acidity and pH of honey are mainly due to the content of the gluconolactone/gluconic acid present as result of the enzymatic action of glucose-oxidase (Molan, 1992). Honey moisture content is a quality criterion that determines its shelf-life during storage because of resistance to spoilage by yeast fermentation. Differences on moisture content also depend on the harvest season and the degree of maturity reached in the hive (Corbella & Cozzolino, 2006). Although, there are some methods that have been

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doi:10.1111/j.1365-2621.2009.02159.x

optimised for characterise honeys as its floral and geographical sources (Ruoff *et al*., 2006; Woodcok *et al*., 2007), normally the identification of the botanical origin of honeys is made through microscopical analysis of pollen (Louveaux *et al*., 1978; Tellería, 2001).

There are different studies about the antimicrobial activity of honeys came from different origins and their action against bacteria, moulds and yeasts (Allen *et al*., 1991; Molan, 1992; Fangio *et al*., 2007). Antimicrobial activity of honey against Gram positive and negative bacteria, has been demonstrated (Garedew *et al*., 2004), although, the nature and mechanism of action of honeys have not been still totally elucidated. Osmolarity, acidity, hydrogen peroxide and non-peroxide compounds are considered the main factors associated to the antimicrobial activity of honeys (Weston & Brocklebank, 1999). Moreover, the hydrogen peroxide is recognised as the principal antimicrobial factor in diluted honeys. In addition, the presence of non-peroxide factors such as lysozyme, phenolic acids and flavonoids may also contribute to antimicrobial properties of honey.

The aim of this work was to evaluate the physicochemical, sensory and *Escherichia coli* growth inhibitory characteristics of honey of different botanical sources from two geographic origin of Argentina.

Materials and methods

Area of study

The area of study belongs to the austral district to the pampeana phytogeographic region (Cabrera, 1976). The pampeana phytogeographic region is the most suitable territory for the agriculture and the ranching. Natural vegetation has been altered by the agricultural activity, with culturing of cereals, foragers and oleaginous. Also there are plantations of *Eucalyptus*, and other woody species that surround the facilities of the stays or use as protection to the cattle. Some of these honey-producing plants are: eucalyptus, clover, pine, alfalfa, sunflower, fruit trees, blueberries, pastures, wheat, corn, soya. Honey samples were obtained from apiaries located in two zones: centre zone and east zone, both of them lay in the southeast region of Buenos Aires province, Argentina (Table 1).

Honey samples

Thirty honey samples were collected during the 2005–2007 flowering seasons from apiaries placed in different geographical districts, being ten samples from centre zone and twenty-three samples from east zone. Extraction of honeys from combs was done by centrifugation without heating. The samples were collected in sterile containers and stored at 10 °C until their analyses.

Table 1 Geographical origin of honey samples

Zone of production	Identification of honey	Geographical districts	Location
Centre	H1, H3	Ayacucho	37°15′ S–58°46′ W
	H2, H4–H8	Balcarce	37°52′ S–58° 15′ W
	H9–H10	Tres Arroyos	38° 22′ S–60° 16′ W
East	H11	Mar del Plata	37° 59′ S–57° 19′ W
	H12	Mar Chiquita	37° 40′ S–58° 46′ W
	H13–H33	Miramar	38° 16′ S–57° 50′ W

Pollen analysis

In order to perform the floral identification of the honeys, the procedures established by the International Commission of Bee Botany were carried out (Louveaux *et al*., 1978). The frequency classes for the pollen types were determined according to SAGPyA Res. 274/95 classification.

Sensory and physicochemical characterisation of honeys

Sensory characteristics as aroma, flavour and texture as viscosity/granularity, were assessed. The honey samples (30–40 g) were poured into transparent glass coded jars at room temperature. Thirty untrained panellists from the Mar del Plata University (Argentina), graduate and undergraduate students, performed the evaluation. The assessors were instructed to examine the sample by smelling it with two long sniffs, swirling it in their mouths for a few seconds and then swallowing it (Gonnet & Vache, 1973). Texture attributes were divided in two categories: viscosity (liquid honey) and granularity (crystalline honey). Viscosity was considered as the fluidity capacity. This property could vary with the water content of the samples (Gonnet & Vache, 1973). Granularity is an attribute relating to the perception of the size and shape of particles in crystalline honey (Galán-Soldevilla *et al*., 2005).

The colour of honey was measured by spectrophotometric method (Bath & Singh, 1999). Solutions containing 5 g of honey in 20 mL of distilled water was filtered and its absorbance was measured at 420 nm using an UV–Vis Scanning Spectrophotometer Shimadzu (UV–2101PC).

Acidity content was determined in agreement to the technique proposed by Bianchi (1984). A solution containing 10 g of honey in 75 mL of CO_2-free distilled water was titrated with a standard sodium hydroxide solution approximately 0.1 N. pH measurements were made using honey samples diluted at various concentrations, 1%, 5%, 10%, 25%, 50% and 75% (w/v), in CO_2-free distilled water, being the pH value of full-strength honey (100%) obtained by extrapolation (Iurlina & Fritz, 2005). A HANNA pH-meter (model HI 9321) was used for these measurements.

Moisture content was determined through the refractive index of honey using an Abbe refractometer at 20 °C, being the values recorded converted to moisture content (% w/w) according to Chataway's table (Bianchi, 1984). For honeys naturally liquid and transparent the measurement was directly made. Solid or crystalline honeys were previously heated in a water bath at 40 °C for melting.

Qualitative determination of both diastase and glucose-oxidase activity was made according to the method suggested by Bianchi (1984). A solution containing 1 g of honey referred to dry residue dissolved in a buffer phosphate solution (monosodium phosphate $NaPO_4H_2{\cdot}H_2O$, 0.2 M and disodium phosphate, Na_2PO_4H, 0.2 M, pH 6.5) was incubated in a water bath at 37 °C for 1 h. A solution of potassium iodide (KI) 3% (w/v), an aliquot of honey solution and starch solution 0.1% (w/v) were placed in the tube where glucose-oxidase activity was detected. On the other way, starch solution 0.05% (w/v) and an aliquot of honey solution were deposited in the tube where diastase activity was investigated. Both tubes were incubated in a water bath at 37 °C during 10 min and then cooled. A drop of iodine working solution was added to the diastase tube. Hydrogen peroxide (H_2O_2) and gluconic acid are produced by the enzyme glucose-oxidase. KI is oxidised to iodine (I_2) by H_2O_2 that in presence of starch produces a blue colour. Starch is hydrolysed by diastase activity, because of that the solution remains colourless with the addition of I_2. Glucose oxidase tube becomes blue, while diastase tube remains colourless in honey with normal glucose-oxidase and diastase activities. Any variant of colour indicated a scanty or void enzymatic activity.

Assay for inhibitory activity of honey on *Escherichia coli* growth

Pure cultures of *Escherichia coli* ATCC 25922, incubated in brain heart broth at 35 °C for 18 h, were standardised in Butterfield's phosphate-buffered dilution water 0.25 M KH_2PO_4, pH 7.2 (Butterfield, 1932) until a turbidity equivalent to 0.5 of McFarland scale, which would correspond to a bacterial concentration of 1.5×10^8 cfu mL^{-1}. Then, this suspension was diluted in Butterfield's dilution water to achieve an inoculum of 1.5×10^4 cfu mL^{-1} In order to avoid antagonistic or symbiotic interaction due to the presence of *Bacillus* spp., *Paenibacillus larvae* and any other bacteria, samples were filtered by sterile millipore filters (GSWP025, 0.22 μm of pore, MF-Millipore). Tubes with 4 mL of honey solution 50% (w/v) in Muller-Hinton broth was inoculated with 100 μL of the bacterial suspension (1.5×10^4 cfu mL^{-1}) and incubated at 35 °C during the following times of contact: 0, 1.5, 3, 6, 9, 24 and 48 h. For every tested time, *E. coli* was counted onto standard plate count agar (PCA) incubated at 35 °C for 24 h. A control was made without honey to evaluate the curve of growth of *E. coli*.

Evaluation of total and non-hydrogen peroxide antibacterial activity of honeys

Honeys were also screened for total antibacterial activity against *E. coli* (ATCC 25922) according to the agar well diffusion method (Allen *et al*., 1991). Cultures (100 μL) grown in trypticase soy broth (TSB) at 37 °C for 18 h were added to nutrient agar (150 mL) and immediately poured onto plates. The plates were stored at 4 °C for 24 h before being used, and wells (8 mm diameter) were cut in the agar. Solutions containing 50% (w/v) of honey were made in sterile distilled water. A 100 μL aliquot of each sample was added to each well. Cultures were incubated at 37 °C for 18 h. Antibacterial activity was assessed by measuring the size of the zones of inhibition surrounding wells (Fangio *et al*., 2007). In order to evaluate the non-hydrogen peroxide antimicrobial activity, honeys were previously treated with bovine liver catalase (450 units mg^{-1}) (C-10) (Sigma, St Louis, MO, USA). Then the antimicrobial activity was tested following the method above mentioned.

Statistical analysis

Analysis of variance was used to examine the differences between physicochemical data, and the values found for every honey to the different times of contact. The level of significance selected for the probability was of 0.05 (Zar, 1984). Principal component analysis (PCA) was performed on the physicochemical data (Corbella & Cozzolino, 2006). All the analyses were achieving using SPSS 15.0 to Windows.

Results

Pollen analysis

In accordance with the Argentinean legislation (SAGPyA Res 274/95), honeys were classified as monofloral of *Eucalyptus* spp., when the pollen content of these species is equal or higher than 70%. Honeys were considered as monofloral of *Lotus* spp. when pollen of that plant was present at least in 20%. In addition, when *Eucalyptus* spp. pollen was dominant (> 45%) but lower than 70%, and clovers pollen, which includes *Melilotus*, *Lotus*, *Trifolium* and *Medicago* represented the secondary pollen class (45–15%) honeys were classified as mixed. Table 2 shows the frequency classes of pollen types in the honeys studied.

Monofloral honeys corresponded to *Lotus* spp., t. Fabaceae and *Eucalyptus* spp. (Table 2). *Lotus* spp. honeys had an important contribution of *Lotus* spp. pollen representing around 47% of the total pollen. *Eucalyptus* spp. species were present in monofloral honeys (80–85%) and mixed honeys (50–65%). Mixed honeys had an important contribution of *Eucalyptus*

Table 2 Honey types and frequency classes of pollen identified in the samples

Type of honey	Family	Pollen type *D* > 45%	Pollen type *S* = 15–45%	Honey
Monofloral	Fabaceae	*Lotus* spp.		H1
	Myrtaceae		*Eucalyptus* spp.	
	Myrtaceae	*Eucalyptus* spp.		H2, 5, 6, 11, 12
	Fabaceae		*Lotus* spp.	
	Fabaceae	t. Fabaceae		H13, 31
	Myrtaceae		*Eucalyptus* spp.	
	Myrtaceae	*Eucalyptus* spp.		
	Fabaceae		t. Fabaceae	H7, 8, 16, 19–26, 29, 32
	Myrtaceae	*Eucalyptus* spp.	–	H17
Mixed	Myrtaceae	*Eucalyptus* spp.		H4, 15, 18, 27,
	Fabaceae		t. Fabaceae	28, 30, 33
	Myrtaceae	*Eucalyptus* spp.		
	Fabaceae		*Lotus* spp.	H3, 14, H10
Polyfloral	Fabaceae		t. Fabaceae	H9
	Myrtaceae		*Eucalyptus spp.*	
	Asteraceae		*Helianthus annuus*	

spp. pollen with *Lotus* or other Fabaceae as secondary pollen (*Melilotus* spp., *Trifolium* spp., *Robinia pseudoacacia*). Myrtaceae and t. Fabaceae were the most representative families. Polyfloral honeys mainly included a pollen contribution from t. Fabaceae, *Eucaliptus* spp. and *Helianthus annuus*.

Sensory and physicochemical characterisation of honeys

Table 3 shows the sensory characteristics and the physicochemical parameters of samples. All the evaluated honeys corresponded to partial or totally crystallised honeys, observing variations in the size of the crystals (small and big). All honey samples also presented typical flavour and aroma. None sample presented visible signs of fermentation.

The sensorial analysis showed that the colour of honeys is light or slightly dark. However, colour measures showed significant differences on its intensity, although, the colour mean values for honeys came from both zones were around 0.45 ± 0.08.

Values of free acidity ranged between 16.62 and 28.7 meq NaOH kg^{-1} of honey with an average of 20.24 ± 3.53 meq NaOH kg^{-1} of honey. Average values of free acidity did not show significant differences between both geographical zones, being for zone centre and to zone east 20.06 and 20.32 meq NaOH kg^{-1} of honey, respectively. On the other hand values of pH ranged from 3.25 to 3.72 with an average value of 3.44 ± 0.11.

Moisture content for all honeys varied between 13.04% and 20.48% with an average value of 15.71% ± 2.6%, showing the values measured significant differences between them. Indeed, these differences were markedly reflected in the average values for each zone, being 18.96% and 14.29% to centre and east zone, respectively.

Diastase and glucose-oxidase activity was detected in all the samples which indicated that the honeys were not submitted to thermal treatment before their study.

Assay for inhibitory activity of honey on *E. coli* growth

Figure 1 shows *E. coli* mean counts of honeys from both zones obtained from cultures performed in presence of 50% (w/v) of honey for different times of contact. In general, all the honeys evaluated presented an inhibitory effect on the *E. coli* growth in contrast to the control cultures (bacterial suspension in absence of honeys) for the different times.

Average values of counts for all centre honeys showed similar growth curves during the first hours of incubation, with a minimum at 6 h. After 48 h of incubation of the samples, the counts showed a significant increases ($P < 0.05$).

In general, east honeys showed a progressive decrease on the growth which reached the maximum at 48 h of incubation. Only, two of the samples (H16 and H17) showed a complete reduction of the growth (bactericidal effect) at 48 h of incubation (data not showed).

Consequently, different degrees of antibacterial actions were observed, although a significant decrease ($P < 0.05$) of bacteria counts compared to the time zero and to the culture control in the different times of incubation was observed for all honey samples.

Evaluation of total and non-hydrogen peroxide antibacterial activity of honeys

In order to know the nature of the antimicrobial activity (hydrogen peroxide or non-hydrogen peroxide), the sensitivity of *E. coli* against the honey samples studied was screened. Table 4 shows the diameter values of

Table 3 Sensory properties, colour, free acidity and moisture content of honeys

	Sensory properties		Physicochemical parameters		
Honey samples	Sensory analysis	Colour (Abs. 420 nm)	Free acidity (meq NaOH kg^{-1} of honey)	pH	Moisture content (%)
H1	Slight aroma, light to medium colour, granular (small crystals)	0.45 ± 0.002^{a}	$17.50 \pm 0.14^{a,b}$	$3.42 \pm 0.005^{a,b,c}$	$18.2 \pm 0.17^{a,b}$
H2	Medium to strong aroma, medium to dark colour, granular	0.51 ± 0.001^{e}	$18.65 \pm 0.84^{f,g}$	$3.44 \pm 0.01^{a,b,c}$	20.4 ± 0.2^{c}
H3	Medium aroma, light to medium colour, granular	0.49 ± 0.001^{g}	$17.85 \pm 0.53^{a,b,c}$	$3.42 \pm 0.005^{a,b,c}$	18 ± 0.2^{d}
H4	Weak to medium aroma, light to medium colour, granular	0.37 ± 0.001^{b}	$17.84 \pm 0.53^{a,b,c}$	$3.41 \pm 0.005^{a,b,c}$	20.24 ± 0.08^{c}
H5	Medium to strong aroma, medium to dark colour, granular (small crystals)	0.63 ± 0.002^{k}	18.2 ± 0.66^{c}	3.60 ± 0.9^{k}	$18.2 \pm 0.11^{a,b}$
H6	Medium to strong aroma, medium to dark colour, granular	0.61 ± 0.005^{k}	28 ± 0.61^{l}	$3.40 \pm 0.2^{b,c}$	$18.33 \pm 0.11^{a,b}$
H7	Medium to strong aroma, medium colour, granular (small crystals)	0.61 ± 0.01^{k}	28.7 ± 1^{l}	3.60 ± 0.7^{k}	20.06 ± 0.08^{n}
H8	Medium to strong aroma, medium colour, granular (small crystals)	$0.43 \pm 0.002^{d,j}$	$17.81 \pm 0.55^{a,b}$	$3.44 \pm 0.005^{a,b,c}$	19.46 ± 0.11^{o}
H9	Weak aroma, light colour, creamy, thick	$0.41 \pm 0.01^{c,d}$	$17.5 \pm 0.14^{a,b}$	3.72 ± 0.02^{d}	$18.04 \pm 0.95^{a,d}$
H10	Medium aroma, light to medium colour, granular	$0.43 \pm 0.002^{d,j}$	$17.5 \pm 0.14^{a,b}$	3.63 ± 0.02^{k}	18.8 ± 0.2^{e}
H11	Medium to strong aroma, medium to dark colour, granular (small crystals)	0.52 ± 0.002^{e}	$20.83 \pm 0.28^{d,e}$	$3.44 \pm 0.01^{a,b,c}$	$18.52 \pm 0.44^{b,e}$
H12	Medium to strong aroma, medium to dark colour, granular (small crystals)	0.51 ± 0.003^{e}	$17.78 \pm 0.09^{a,b}$	$3.32 \pm 0.005^{e,f,g,h}$	20.48 ± 0.24^{c}
H13	Weak aroma, light to medium colour, granular	$0.38 \pm 0.005^{b,f}$	$19.83 \pm 0.57^{f,g}$	$3.43 \pm 0.005^{a,b,c}$	14.2 ± 0.33^{f}
H14	Medium aroma, light to medium colour, granular, thick	0.47 ± 0.015^{g}	16.62 ± 0.19^{a}	$3.41 \pm 0.005^{a,b,c}$	13.12 ± 0.24^{g}
H15	Medium aroma, light colour, creamy	$0.38 \pm 0.02^{b,f}$	$20.17 \pm 0.56^{d,g}$	3.72 ± 0.01^{d}	13.04 ± 0.08^{g}
H16	Medium to strong aroma, medium colour, granular (small crystals)	$0.33 \pm 0.004^{h,i}$	$18.55 \pm 0.08^{f,g}$	$3.4 \pm 0.01^{b,c}$	$13.44 \pm 0.19^{h,i}$
H17	Medium to strong aroma, medium to dark colour, slightly granular	0.34 ± 0.005^{i}	$19.51 \pm 0.03^{f,g,d}$	3.25 ± 0.015^{e}	$13.36 \pm 0.08^{g,h}$
H18	Weak to medium aroma, light to medium colour, granular	$0.44 \pm 0.001^{a,j}$	$17.89 \pm 0.48^{a,b,c}$	$3.43 \pm 0.01^{a,b,c}$	$13.66 \pm 0.19^{i,l,m}$
H19	Medium to strong aroma, medium colour, granular (small crystals)	0.51 ± 0.001^{e}	$18.5 \pm 0.08^{f,g}$	$3.44 \pm 0.005^{a,b,c}$	$13.86 \pm 0.95^{j,k,l,m}$
H20	Medium aroma, medium colour, granular	0.42 ± 0.002^{d}	$17.58 \pm 0.14^{a,b}$	$3.32 \pm 0.03^{e,f,g,h}$	13.13 ± 0.11^{g}
H21	Medium to strong aroma, medium colour, granular (small crystals)	0.49 ± 0.002^{g}	$18.55 \pm 0.08^{f,g}$	$3.53 \pm 0.01^{i,j}$	$13.53 \pm 0.11^{b,e}$
H22	Strong aroma, medium colour, granular	0.49 ± 0.002^{g}	$22.13 \pm 0.53^{e,h}$	$3.29 \pm 0.06^{e,f}$	$14.06 \pm 0.11^{j,k,m}$
H23	Medium aroma, medium colour, granular (small crystals)	0.40 ± 0.003	16.62 ± 0.19^{a}	3.27 ± 0.01^{e}	13.13 ± 0.08^{g}
H24	Medium to strong aroma, medium colour, granular	0.42 ± 0.002^{d}	$18.84 \pm 0.55^{c,f,g}$	$3.45 \pm 0.01^{b,c,i}$	$14.06 \pm 0.11^{j,k,m}$
H25	Medium to strong aroma, medium colour, granular	$0.40 \pm 0.001^{c,f}$	$22.39 \pm 0.13^{h,i}$	$3.40 \pm 0.01^{b,c}$	$13.86 \pm 0.19^{j,k,l,m}$
H26	Medium to strong aroma, medium colour, granular (small crystals)	0.32 ± 0.001^{h}	14.86 ± 0.51^{j}	$3.39 \pm 0.1^{g,h,a,b,c}$	14.53 ± 0.17^{f}
H27	Weak to medium aroma, light to medium colour, granular	0.34 ± 0.00^{i}	$22.21 \pm 0.58^{e,h}$	$3.40 \pm 0.005^{b,c}$	14.2 ± 0.2^{f}
H28	Medium aroma, light colour	0.53 ± 0.002^{e}	$23.23 \pm 0–59^{h,i}$	$3.39 \pm 0.01^{g,h,a,b,c}$	14.26 ± 0.33^{f}
H29	Medium to strong aroma, medium to dark colour, granular	0.63 ± 0.001^{k}	26.65 ± 1^{k}	$3.36 \pm 0.01^{f,g,h,a}$	$14.13 \pm 0.95^{f,j,k}$
H30	Medium aroma, light colour	$0.40 \pm 0.002^{c,f}$	25.97 ± 0.58^{k}	$3.32 \pm 0.02^{e,f,g,h}$	$13.66 \pm 0.2^{i,l,m}$
H31	Weak aroma, light to medium colour, granular	0.42 ± 0.002^{d}	25.96 ± 0.59^{k}	3.54 ± 0.01^{j}	$14.13 \pm 0.11^{f,j,k}$
H32	Medium to strong aroma, medium colour, granular	0.37 ± 0.002^{b}	$18.79 \pm 0.29^{f,g}$	$3.47 \pm 0.02^{c,i,j}$	13.73 ± 0.17^{j}
H33	Medium aroma, light colour, creamy	$0.40 \pm 0.001^{c,f}$	23.91 ± 0.59^{i}	$3.45 \pm 0.005^{b,c,i}$	14.53 ± 0.19^{f}

Values of the same parameter (vertical columns) with different superscripts differ significantly ($P < 0.05$).

inhibition of *E. coli* growth in presence of honey solution 50% (w/v). The antibacterial activity was classified as: no sensitive, for diameters lower than 8 mm; sensitive, for diameters from 8 to 14 mm; very sensitive, for diameters from 15 to 19 mm; extremely sensitive, for diameters higher than 20 mm. Antimicrobial activity of non-hydrogen peroxide compounds was mainly detected for samples came from Miramar, which included monofloral and mixed honeys. The results obtained revealed that the antibacterial activity was

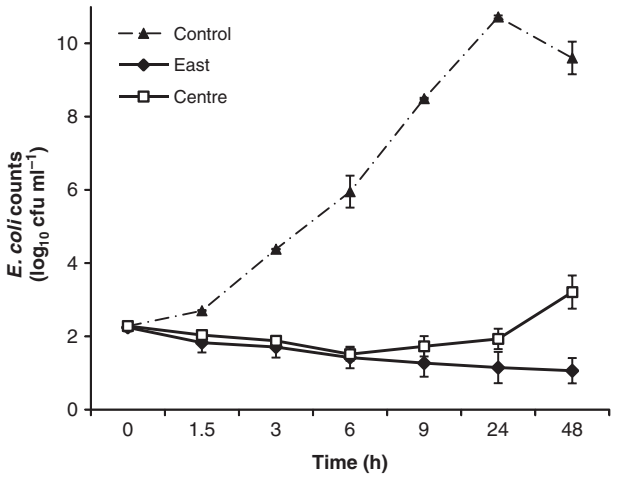

Figure 1 Log growth of *E. coli* (expressed as $\log_{10}$ cfu mL^{-1}) after the exposure to honey solution (50%) over hours 0 to 48. East and centre correspond to mean values of honeys from both zones. The control was made without honey to evaluate the curve of growth of *E. coli*.

mainly due to the enzymatic formation of hydrogen peroxide, although most of the samples also showed an important non-hydrogen peroxide activity, being the bacterium in some cases, extremely sensitive (diameters higher than 20 mm).

Discussion

Pollen analyses

Monofloral honeys of *Eucalyptus* spp. were mainly recognised, being this pollen also identified as dominant in mixed honeys. This could be explained by the distribution of *Eucalyptus* spp. in the different geographical regions where the samples were taken. *Eucalyptus* spp. is cultivated as a forest and ornamental tree in these regions. Unifloral honeys of *Lotus* spp. were also found. *Lotus* (Lotus tenuis Waldst et Kit) is a forage legume broadly accepted and used by ranchers in the Flooding Pampas because its nutritional value, productivity, natural re-sowing and tolerance to grazing and soil flooding and alkalinity conditions (Cambareri *et al*., 2007). Malacalza *et al*. (2005) reported the predominance of monofloral honeys of *Eucalyptus* spp. and *Lotus* spp. in the province of Buenos Aires. On other hand, monofloral honeys of *Eucalyptus* spp. were also reported by Basualdo *et al*. (2006), while *Lotus* spp. was always found as secondary or minority pollen. Mixed and multifloral honeys were associated with the presence of *Eucalyptus* spp., *Lotus* spp., *Helianthus annuus* and t. Fabaceae which agreed with the results showed by Basualdo *et al*. (2006).

Table 4 Total and non-hydrogen peroxide antibacterial activity of honeys against *E. coli* by agar well-diffusion method

	Antibacterial activity				
Honey sample	Total 50%	Non-hydrogen peroxide 50%		Total 50%	Non-hydrogen peroxide 50%
H1	$32 \pm 1.5^{a,b,c,d}$	14 ± 0.3^{a}	H18	30 ± 0.5^{d}	$21 \pm 1^{e,f}$
H2	$32 \pm 0.9^{a,b,c,d}$	$11 \pm 1.1^{b,c}$	H19	$36 \pm 0.5^{e,f,h}$	$25 \pm 1^{j,h}$
H3	$31 \pm 0.58^{a,c,d}$	15 ± 0^{a}	H20	$35 \pm 1^{b,e,f,h}$	$29 \pm 1,1^{i}$
H4	$32 \pm 0.9^{a,b,c,d}$	0 ± 0^{d}	H21	$34 \pm 0.5^{a,b,c,e,f}$	$19 \pm 1.5^{f,k}$
H5	$31 \pm 0.5^{a,c,d}$	14 ± 1.1^{a}	H22	$34 \pm 2^{a,b,c,e,f,}$	$19 \pm 0.5^{f,k}$
H6	$31 \pm 1.1^{a,c,d}$	$11 \pm 1.1^{b,c}$	H23	$33 \pm 0.5^{a,b,c,d,e}$	20 ± 0.5^{f}
H7	$31 \pm 1.1^{a,c,d}$	$11 \pm 1.7^{b,c}$	H24	$36 \pm 0^{e,f,h}$	$19 \pm 0.5^{f,k}$
H8	$31 \pm 1.1^{a,c,d}$	9 ± 1.1^{b}	H25	$33 \pm 0.5^{a,b,c,d,e}$	$24 \pm 0.5^{j,h}$
H9	$34 \pm 2^{a,b,c,e,f}$	$13 \pm 0.5^{a,c}$	H26	$33 \pm 1^{a,b,c,d,e}$	$23 \pm 1.1^{e,j}$
H10	30 ± 0^{d}	$12 \pm 1^{a,c}$	H27	30 ± 1.5^{d}	$21 \pm 1.1^{e,f}$
H11	$31 \pm 1^{a,c,d}$	$10 \pm 0.4^{b,c}$	H28	$31 \pm 0.5^{a,c,d\ a,c,d}$	$19 \pm 0.5^{f,k}$
H12	$31 \pm 0.5^{a,c,d}$	$11 \pm 0.1^{b,c}$	H29	$36 \pm 2.5^{e,f,h}$	$23 \pm 1.1^{e,j}$
H13	$31 \pm 0.7^{a,c,d}$	22 ± 0.6^{e}	H30	$35 \pm 2.3^{b,e,f,h}$	$24 \pm 0.5^{j,h}$
H14	25 ± 1.3^{g}	20 ± 0^{f}	H31	38 ± 1.1^{i}	$23 \pm 1.1^{e,j}$
H15	23 ± 1.1^{g}	20 ± 0.5^{f}	H32	22 ± 2.3^{g}	18 ± 1^{k}
H16	40 ± 2^{i}	35 ± 0.8^{g}	H33	24 ± 0.5^{g}	17 ± 0.5^{k}
H17	30 ± 0.8^{d}	$27 \pm 1^{h,i}$			

Ref: The activity is shown as the diameter (mm) of the clear zone obtained. Data expressed as mean ± standard deviations.
Values of the same parameter (vertical columns) with different superscripts ($^{a, b, c, d, e, f}$ and ^{g}) differ significantly ($P < 0.05$).

Sensory and physicochemical characterisation

The sensory properties such as colour, taste and texture of honey vary according to the geographical and seasonal conditions as well as the floral source (Anupama *et al*., 2003). Viscosity is one of the important properties of honey and depends on water and sugar quantities (Matsuda & Sabato, 2004). In general, the sensory properties are a complex function of physicochemical parameters, which are depending on the botanic and geographic origin (Acquarone *et al*., 2007).

Honey colour is associated to minerals content and to the presence of pigments such as carotenoids and flavonoids (Beretta *et al*., 2005). In our study, monofloral honeys showed darker colour than mixed and polyfloral honeys. *Eucalyptus* spp. monofloral honeys showed an average value of absorbance a 420 nm between 0.480 ± 0.009, which are lower than absorbance values reported by Bath & Singh (1999) for *Eucalyptus lanceolatus* (0.683 ± 0.01). The honey free acidity is attributed to a diversity of organic acids, mainly gluconic acid (70–90%), and others acids such as malic acid, maleic acid, citric acid, succinic acid and fumaric acid which are present in a minor proportion (Suárez-Luque *et al*., 2002). The acidity values obtained were similar to those reported by Cunsolo *et al*. (2003) whose values ranged from 10 to 29.9 meq NaOH kg^{-1} for the 92% of honey samples. Respects to pH, values were between 3.25 and 3.72, being these results in agreement with those reported by Malacalza *et al*. (2005). On the other hand, as the results obtained in the present work, these authors informed not significant differences in pH values between honeys of *Eucalyptus* spp., *Lotus* spp. and *Helianthus* spp. Our results showed no significant differences between the pH average values of monofloral, mixed and polyfloral honeys.

Moisture content of honey is a parameter that depends on the climatic conditions, season of the year and degree of maturity of every sample of honey. Moisture content has an influence on honey colour, viscosity, flavour, density and refractive index, being one of the most important physical chemical parameters for the analysis of conservation and stability of honeys (Cano *et al*., 2001). The mean values of moisture determined for honeys came from the southeast region of Buenos Aires province were ranged between 16% and 17.9%. In our study, most of the honeys showed moisture values lower than 18%, which is the maximum allowed by local regulations (Código Alimentario Argentino (CAA), 1997 and all of the values were below 21%, which is the maximum fixed by Codex Standards (1981/2001) European Economic Community Honey Commission. These results are also coincident with those reported by Acquarone *et al*. (2007). Besides, moisture content inferior to 18% and 20% is important in stability of the product during its storage.

All honeys presented free acidity values below 40 meq NaOH kg^{-1}, being this value the maximum allowed by local regulations (CAA) and international regulations.

Silva *et al*. (2009) reported for *Eucalyptus* spp. honeys from Portugal moisture content varying from 14.30% to 19.20%, being the mean value 16.73. Free acidity and pH ranged between 10.50 to 29.70 and 3.45 to 4.30, being mean values 19.96 and 3.82 respectively. Serrano *et al*. (2004) for *Eucalyptus* spp. honeys came from Spain have obtained values ranged between 14.90 to 18.60 for moisture content, 19.20 to 41.51 for free acidity, and 3.72 to 4.64 for pH, being mean values 16.64, 26.94 and 4.10 for each parameter respectively. In heterofloral honeys moisture varied from 18.59% to 19.25%. The comparative analysis between our results and those obtained for *Eucalyptus* spp. honeys from other geographical regions showed significant differences for pH measures and free acidity. Meanwhile, for moisture content these monofloral samples did not showed significant differences.

PCA is a mathematical procedure for resolving sets of data into orthogonal components whose linear combinations approximate the original data to any desired degree of accuracy, in such way the data presented in those axes (principal components) are uncorrelated with which other (Naes *et al*., 2002). The principal component analysis (PCA) (Table 5) explained 43% of the total variability for first factor and was predominantly a function of colour and moisture content, whilst the second PC explained 26% of the variance is made up of the variable free acidity and pH. The number of significant principal components was selected on the basis of the Kaiser criterion with eigenvalue higher than one (Kaiser, 1960). According to this criterion, only the first two components were retained because subsequent eigenvalues were less than one. Consequently, reduced dimension ability of the descriptor space is two. Figure 2 shows the score plots of the first two principal components (PC1 and PC2) for the discrimination of honey samples according to the separation for zones. From the PCA analysis is shown that colour and moisture content are the most important variables that explained this discrimination of the samples studied.

Taking into account the limited region where the studied zones are placed the present results lead us to be

Table 5 Component matrix for physicochemical parameters

Variable	Principal component	
	1	2
Moisture content	0.684	−0.467
colour	0.854	0.159
pH	0.499	0.684
Free acidity	0.525	0.772

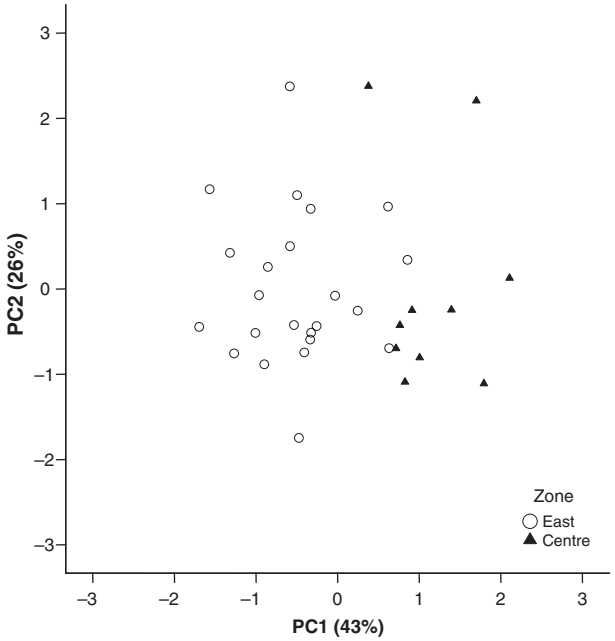

Figure 2 Principal component score plots of the separation of honey samples of Zone East and Centre.

cautious about extrapolating them to other regions or conditions. Further studies are needed in order to establish in general the relationship between the floral or the geographical origins and the honey antimicrobial activities.

Assay for inhibitory activity of honey on *E. coli* growth

In previous research the sensitivity of different microorganisms in presence honey has been studied showing differences of antimicrobial activity in agreed with their geographical and floral origin (Willix *et al*., 1992). Then, significant different values of sensitivity of *E. coli* strains in presence of honey with antibacterial properties have been published. These variations have been observed in concentrations of honeys that produce complete and partial inhibitions of growth (Molan, 1992).

The antimicrobial properties of the honey are due to different factors. However, in diluted honeys, hydrogen peroxide and phytochemical compounds would be considered the main substances responsible to the antimicrobial activity. Garedew *et al*. (2004) studied the action of the honey by a micro calorimetric method on different microorganisms, between them *E. coli*, demonstrating that immediately after the treatment with high concentrations of honey, the production of metabolic heat of the microorganisms (measured by a micro calorimeter) decrease in considerable form. The minimal concentration of honey that produced a drop in the curve of heat flow was considered to be MIC's value. When the factor of dilution of the honey was doubling (1:10 to 1:20), the activity of honey was passing of bactericidal to bacteriostatic. These authors considered that the bacteriostatic activity is due to the fact that after an initial drop of the heat production, an increase in the bacterial counts was registered, which was kept in stationary phase within the 10 h.

In the present work the effects of the inclusion of honey solution were evident. The Fig. 1 shows an important decrease of growth for all samples as well as extending inhibition in some samples. Some honeys showed an antimicrobial activity during few hours, showing a significantly increase of the counts of *E. coli* at longest times. This behaviour might be explained considering that after treatment with honey a proportion of the cells died, whereas others came in a viable but nonculturable state or were not affected. Next, with the course of the time they adapted to the way driving to an increase in the bacterial counts. Consequently the effect achieved by the honey might be associated to a reversible inhibition of the growth of the bacteria. On the other hand, Miramar honeys supported the antimicrobial activity to the different times of contact, being detected for two of the samples a bactericidal action (more than 99.9% of death compared to the initial microbial suspension).

Evaluation of total and non-hydrogen peroxide antibacterial activity of honeys

Agar well-diffusion method showed that Miramar honeys have the highest non-hydrogen peroxide antimicrobial activities among all the samples studied. Moreover, these honeys were the only samples with bactericide action against *E. coli* at long times of contact.

All samples showed non-hydrogen peroxide activity. However, the superior antibacterial activity of samples H16 and H17 could be associated to the presence of particular phytochemical compounds of the nectar, which would give a highest activity against *E. coli*. Most of the honeys studied in the present work with major antimicrobial efficiency came from the same geographical zone (Miramar). However, differences interpreted as caused by geographical origin may be indirect effects of the botanical origin (Ruoff *et al*., 2006). Nectar contributions of the accompanying flora in monofloral honeys could change with the geographical region where the honey is produced.

Conclusion

In presence of concentrations of 50% (p/v), the honeys obtained from the Southeast of Buenos Aires province had an inhibitory effect on the *Escherichia coli* growth. In general the antimicrobial properties could be associated

with the release of hydrogen peroxide, while some honeys showed an additional phytochemical effect which could be related to the geographical origin. The assessment of growth curve as a function of the contact times allowed to verify an antimicrobial action in short times (not higher than 24 h), being this important for optimising the potential use of this agent in therapeutic treatments.

Acknowledgments

We gratefully acknowledge the financial supports of the Universidad Nacional de Mar del Plata (Project N° EXA 415/08) and the CIC Buenos Aires province grant (Res. N° 673/07)

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