Effect of Microwave Treatment on Microbial Contamination of Honeys and on Their Physicochemical and Thermal Properties

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In recent years, microwave heating has become a common method for pasteurization and sterilization of food. Honey is a sweet substance produced by worker honeybees from nectar of flowers. The major microbial contaminants include moulds and yeasts, as well as the spore-forming bacteria, being their counts indicative of honey’s commercial quality and safety. Paenibacillus larvae is also of interest since it causes American foulbrood (AFB) in honeybee larvae. The main quality factors that are used in the honey international trade are moisture, hydroxymethylfurfural content (HMF), and enzymatic indices. Moreover, honey exhibits several thermal events, the most important being the glass transition temperature (Tg). The aim of this work was to evaluate microwave effect (800 watts during 45 and 90 seconds) on microbial content in particular over P. larvae spores retained in honey, and on physicochemical and thermal properties. Microwave promoted a decrease of microbial count with time of exposure, including P. larvae. Moisture content diminished after treatment, while Tg increased linearly, and acidity decremented in the majority of cases. Honeys darkened and HMF exceeded the permissible value. Diastase and glucose-oxidase enzymes were totally inactivated by microwave treatment.

INTRODUCTION

In industrial food processing, microwave energy has been considered a faster process of pasteurization and sterilization of food than conventional methods. Consequently, microwave radiation is regarded as an alternative method for killing bacteria because of its effectiveness, commercial availability, and lower cost compared with other technologies [Celandroni et al., 2004]. However, the effect of microwave on bacteria depends on food composition and type of microorganism [Castro et al., 1997].

Honey is a sweet product elaborated by worker honeybees from nectar and secretion of flowers. It is collected and transformed by combining with specific substances, then stored in honeycombs until maturation. Chemical composition involves a complex mixture of fructose (average 38.4 g/100 g), glucose (average 30.3 g/100 g), sucrose (average 1.3 g/100 g), other carbohydrates (around 12 g/100 g), minerals (average 0.169 g/100 g) and proteins (enzymes and amino acids) (average 169 mg/100 g), with a moisture content of 17.2 g/100 g [White et al., 1963]. It also contains organic acids such as gluconic (0.43 g/100 g), and in a minor amount, acetic, citric, lactic, succinic and formic acids, aromatic substances, pigments, wax and pollen grains [Desalegn, 2013]. Honey has own flavor and aroma, and its color varies from colorless to dark amber. Color has been frequently attributed to presence of polyphenols, tannins, among others [Acquarone, 2004].

Due to its natural properties the number and variety of microorganisms present in honey is expected to be low. Low water activity (aw: 0.56 – 0.62), low pH (3.5 – 4.5), high organic acids content, presence of hydrogen peroxide, phytochemicals and/or antioxidants [Estrada et al., 2005; Coll Cardenas et al., 2008] inhibit microorganisms growing. Handling and manipulation post harvest are the main sources of contamination, usually by yeasts (e.g. Saccharomyces, Schizosaccharomyces and Torula) and spore-forming bacteria (e.g. Aerobic Bacillus and anaerobic Clostridium spores). Spores and small
fragments of moulds may appear [Frazier & Westhoff, 1978; Kedzia et al., 1996]. Among these, microorganisms that cause honeybee diseases are of special interest. *Paenibacillus larvae*, a spore-forming bacterium, is one of the major pathogens of *Apis mellifera* responsible for an infection known as American foulbrood (AFB) [Alippr et al., 2002; Lauro et al., 2003]. Also, the species *Paenibacillus alvei* and *Melissoceoccus ptylon* in association may cause European foulbrood disease (EFB), which is less important [Coll Cardenas et al., 2008]. The Argentine Food Code (AFC) requires total absence of coliforms and *Salmonella-Shigella*, and less than 10 CFU/g of moulds and yeasts. Other countries include a limit of 10^3 CFU/g for aerobic mesophilic bacteria [Peréz et al., 2009].

According to AFC [1969], honey must meet the following requirements: maximum moisture of 18 g/100 g, an acidity of 40 meq/kg, a minimum of 8 diastase units (Gotho scale) and a maximum of 40 mg/kg of hydroxymethylfurfural (HMF).

Moisture is the major factor that influences the keeping quality or storability of honey especially concerning the risk of spoilage due to fermentation [White, 1975]. The control of moisture is relevant to prevent microbial growth. Acidity is due to the presence of organic acids that contribute not only to honey flavour, but also to stability against microbial spoilage [Coll Cardenas et al., 2008].

Several enzymes are present in honey, such as invertase, diastase, glucose-oxidase, catalase and acid phosphatase. Invertase, glucose-oxidase and catalase are derived mostly from the honeybees. Their activities depend on the intensity of the nectar flow and the amount of nectar processed by the honeybees [Bachmann, 2007]. Enzyme activities are usually measured to evaluate heating or storage effects. The activity of diastase enzyme decreases with time of storage and heating [Kowalski et al., 2012]. Glucose-oxidase is more sensitive to temperature than diastase, so that its determination constitutes a good complement in thermally-treated honey’s studies [Montenegro et al., 2006].

HMF is formed through the acid catalyzed dehydration of hexoses (glucose and fructose) as well as from the decomposition of 3-deoxysorose in Maillard reaction [Belitz et al., 2004]. Its content in fresh honey is very low, and it depends on the floral origin and the chemical properties of honey [Singh & Bath, 1998; Fallico et al., 2004; Zappala et al., 2005]. Its concentration increases during storage in relation to pH and time [Bath & Singh, 1999], but also due to honey heating [Bath & Singh, 1999; Bartakova et al., 2011]. The level of HMF in food depends on the equilibrium between formation from precursors and destruction by oxidation [Morales et al., 1997].

Honey is a supersaturated solution composed mainly of sugars and water and can be thermally characterized by its glass transition temperature (Tg). This is the temperature at which an amorphous material changes from rubbery to glassy state upon cooling. As Tg is very sensitive to water content as well as to chemical composition [Bhandari & Howes, 1999], it is a very useful tool to detect adulteration [Cordella et al., 2003].

The consequences of microwave treatment over honey properties, such as moisture content, acidity [Dranca & Oroian, 2013], HMF and enzyme activities [Kowalski et al., 2012], as well as over mould and yeasts [Hebbar et al., 2003] and bacteria spores have already been reported. Microwave radiation cannot be thought as simple heating from the inside; the E-field would produce effects on the biological molecules affecting spore structure, different from those attributable only to heat [Celandroni et al., 2004]. Gamma radiation method was used to control this disease, in order to inactivate *P. larvae*, although significant effect on chemical parameters and enzyme activities of honey were observed [Baggio et al., 2005].

At present, the effect of microwave radiation on *P. larvae* has not been studied yet. So, the aim of this study was to evaluate the microwave impact on microbial content, in particular over *P. larvae* spores, and on physicochemical and thermal properties.

**MATERIALS AND METHODS**

**Samples**

Eight honeys were obtained from different geographic sites of Argentina. Five from Corrientes province (27°8’16” S; 58°50’25” W), one from Entre Ríos province (32°2’51.72” S; 60°16’51.6” W) and the other two from Buenos Aires province (37°52’59” S; 57°36’09” W). They were kept in hermetic containers and stored at 20–27°C and 50% relative humidity [AOAC, 2000a]. Superficial layer was removed and honey was homogenized with sterile scoop. Microbial content and physicochemical and thermal properties of honeys were determined.

**Microwave thermal treatment**

Microwave heating studies were conducted in a micro-convective oven with turntable attachment (Sharp R-3A55, maximum power of 800 watts; Thailand). Experiments were carried out at high microwave power of 800 watts, and for heating periods of 45 and 90 seconds [Kohn’s, 1998]. Microbial content and physicochemical and thermal properties of honeys were anew determined. The samples temperature was measured at the end of each heating period (Mean temperature at 45 seconds: 83°C; mean temperature at 90 seconds: 108°C).

**Analysis of microbial content**

Aerobic mesophilic bacteria, moulds and yeast and sulphite-reductive *Clostridium* were counted. Also, *P. larvae* was determined. Untreated honeys were used as a control.

In microbial counts of aerobic mesophilic bacteria and moulds and yeast, ten grams of each sample were homogenized into 90 mL of sterile phosphate-buffered dilution water (0.25 mol/L KH₂PO₄ adjusted to pH 7.2 with NaOH). Decimal dilutions were made into the buffer. Aerobic mesophilic bacteria were counted onto standard plate count agar (PCA) incubated at 37°C for 24–48 h [ICMSF, 1983]. Moulds and yeasts were counted onto standard yeast extract–glucose–chloramphenicol (YGC) agar incubated at 22–25°C for 3–5 days [ICMSF, 1983]. Microbial counts were expressed as colony-forming units per gram of honey (CFU/g).

Sulphite-reductive *Clostridium* count was determined with horizontal method in anaerobes on Agar Sulphite Iron (TSC)
with virgin cape to avoid contact between oxygen and micro-organism. Plates were incubated in anaerobe jar at 37°C for 24–48 h [ISO 15213, 2003].

In order to quantify viable spore abundance of *P. larvae*, five grams of honey were homogenized with 5 mL of sterile water [Alippi, 1995]. Serial dilutions (1:2 and 1:10) of the honey solution were prepared and their aliquots (100 μL) were plated on MYPGP agar [Dingman & Stahly, 1983], supplemented with 9 μg/L nalidixic acid to inhibit *Paenibacillus alvei* growth, and incubated at 37°C in micro-aerophila (5–10 g/100 g CO₂). The plates were checked after 2 and 4 days to count the number of *P. larvae*-like colony forming units (CFU) for each dilution, which permitted to calculate the number of viable spores per honey gram. Colonies were identified by catalase test [Heyndrickx et al., 1996], Gram staining [Beveridge, 2001] and phenotypic characterization [Alippi, 1992].

All microbial tests were performed in triplicate.

**Analysis of physicochemical properties**

Moisture content, acidity, color, diastase, glucose-oxidase and HMF were determined for all the samples. Untreated honeys were used as a control.

Determination of moisture content was based on the refractometric method [AOAC, 2000b]. The refractive index of samples was measured with a refractometer (Abbe; Science Instrumentation, Argentina) and results were converted with the Chataway Table. Free acidity was determined by titrimetric method with a pH-meter (HANNA Instruments, HI 2211 pH, pH/ORP meter; Rumania, Europe) according to AOAC [2000c] technical, readings were corrected to meq of acid/kg of honey. Measurements of color are based on a visual comparison with a colored glass reference. It was measured in the Pfund colorimeter (HANNA Instruments, C22f digital, The United States) [Tabera et al., 2002].

Diastase catalyzes the transformation of starch to maltose. It was determined quantitatively by official method in AOAC [2003] and qualitatively according to Bianchi [1990]; both methods are based in diastase action on starch solution, using iodine-iodized solution to visualize the hydrolysis. In qualitative determination diastase was analysed together with glucose-oxidase. Glucose-oxidase activity determination is based in action of glucose-oxidize on glucose, to produce gluconic acid and hydrogen peroxides [Bianchi, 1990].

The HMF was measured by spectrophotometric method suggested by White [1979] using Carrez solutions. The absorbance of the solutions at 284 and 336 nm was determined using a spectrophotometer (Helios Unicam Gamma, England). The quantitative value of HMF was determined using the proposed formula for the method reported by IHC [1999].

**Analysis of thermal properties**

Glass transition temperature (Tg) was determined for all the samples. Untreated honeys were used as a control. Tg was analyzed by differential scanning calorimetry (DSC) using a Pyris 1, Perkin Elmer (Massachusetts, USA). Samples were previously homogenized with a mechanical device (IEC CC31R Multispeed Centrifuge Thermo Scientific, United States) for 20 min, then placed in aluminum pans and heated at a rate of 10°C/min from -65°C to 25°C under N₂ atmosphere. Tg was determined as the onset of the step decrease in heat flow plot [Cordella et al., 2003].

**Statistical analysis**

It was performed with RStudio software, and data were considered as binomial distribution and inflated zero. Widespread Linear Model was used for analyzed differences between microbial content and treatment time. Pearson’s correlation was used to analyze chemical data. Pearson’s linear correlation coefficient (r) was calculated to the relationship between moisture content and Tg, and between HMF and color. For all analyses, α=0.05 was the level of statistical significance.

**RESULTS AND DISCUSSION**

**Microbial content**

Honey may be expected to contain a small number and a limited variety of microorganisms. Treated honeys presented a lower count of aerobic mesophilic bacteria, moulds and yeast and *P. larvae* than virgin honeys (Table 1). Sulphite-reducing *Clostridium* was not observed.

In this study, aerobic mesophilic bacterial count was lower than those obtained by Iurlina & Fritz [2005]. Microwave reduced significantly (p<2 × 10⁻¹⁰) count in response to the exposure time. Untreated honeys presented higher microbial variety in relation to treated ones. White colonies with radial or spiral forms and yellow colonies were observed. All presented positive-Gram coloration with basilar or coco-basilar structure. Wakita et al. [2001] and Iurlina & Fritz [2005] reported colonies of *Bacillus* with concentric ring and irregular border. Mould and yeast counts ranged between 0 to 766 UFC/g in virgin honeys, whereas, Iurlina & Fritz [2005] observed a lower values within a narrower range (164–191 UFC/g) for honeys of southeast region of Buenos Aires province, Argentina. Microwave treatment resulted in a total reduction of these counts after 90 seconds of exposure (p=0.015). Similar results were achieved by Hebbar et al. [2003].

*P. larvae* has the ability to generate extremely resilient and long-lived spores [Shimanuki, 1997]. They can be transmitted by beekeepers unwittingly, shifting honeycombs between colonies, or by the honeybees themselves through robbing of colonies weakened by AFB [Fries & Camazine, 2001; de Graaf et al., 2001]. It is widely recognized that honey will retain spores of *P. larvae* [Sturtevant, 1936; Hansen & Rasmussen, 1986], and that inter-colony transmission of spores in contaminated honey does occur [Fries & Camazine, 2001; de Graaf et al., 2001]. In this study, *P. larvae* count was on average 37 CFU/g, data according to Iurlina & Fritz [2005]. The microwave treatment reduced the count of *P. larvae*, but this decrease was not statistically significant, a different result from gamma radiation method was observed [Baggio et al., 2005].

**Physicochemical and thermal properties**

The moisture content in honeys depends on several factors, such as harvesting season, degree of maturity and climatic factors. The values determined for virgin honeys are
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higher than others reported for Argentine honeys [Acquarone, 2004; Iurlina & Fritz, 2005; Silvano et al., 2014] and Spanish ones [Bentabol et al., 2014]. But, these values were similar to those reported by Karabagias et al. [2014] for unifloral Greek honeys. Meanwhile, the values were close to or even higher than those permitted by international food codes as Codex Alimentarius standard for honey 12–1981 (≤20 g/100 g) and the AFC (≤18 g/100 g). After microwave treatment, moisture content diminished (Table 2), as it was expectable because of the sample temperature rise during the exposure to microwave radiation.

Glass transition temperatures (Tg) of honeys from different botanical and geographic origins have already been reported [Cordella et al., 2003; Lazaridou et al., 2004; Costa et al., 2011] but there is no available information about Argentine honeys. From the results it can be seen that Tg shifted to higher values after treatment, which is consistent with the reduction in moisture content. It is well known that water acts as a plasticizer lowering the Tg [Bhandari & Howes, 1999; Ahmed et al., 2007]. Moreover, a linear relationship between Tg and moisture content was found (r = -0.94), which is in agreement with other authors [Cordella et al., 2003].

Acidity is produced in honey, principally, by the action of the enzyme glucose-oxidase on glucose. The values of most virgin honeys analyzed were below the limit permitted by the Argentine Food Code (40 meq/Kg) and were similar to these described by Acquarone [2004]. Bentabol et al. [2014] reported similar results for this parameter, these values did

| Sample | Aerobic mesophilic bacteria (CFU / g of honey) | Moulds and yeasts (CFU / g of honey) | P. larvae (CFU / g of honey) |
|--------|-----------------------------------------------|------------------------------------|-------------------------------|
|        | Unt. | 45'' | 90'' | Unt. | 45'' | 90'' | Unt. | 45'' | 90'' | Unt. | 45'' | 90'' |
| 1      | 87   | 23   | 15   | 19   | ZC   | ZC   | 133  | 5    | ZC   |
| 2      | 45   | 40   | 20   | 766  | 10   | ZC   | 5    | ZC   | ZC   |
| 3      | 140  | 62   | 25   | 269  | ZC   | ZC   | ZC   | ZC   | ZC   |
| 4      | 188  | 115  | 63   | 293  | ZC   | ZC   | 59   | 5    | ZC   |
| 5      | 124  | 59   | 5    | 35   | 10   | ZC   | ZC   | ZC   | ZC   |
| 6      | 85   | 28   | 10   | 45   | ZC   | ZC   | 50   | ZC   | ZC   |
| 7      | 24   | 5    | ZC   | ZC   | ZC   | ZC   | 49   | ZC   | ZC   |
| 8      | 30   | 19   | 4    | ZC   | ZC   | ZC   | ZC   | ZC   | ZC   |

Mean ± SD 90.3 ± 57.7° 43.8 ± 34.7° 17.8 ± 20.1° 178.4 ± 265.7° 2.5 ± 4.6° 0° 37 ± 46.5° 1.3 ± 2.3° 0°

Unt. (untreated), 45'' (treated honey 45 seconds at 800 watts), 90'' (treated honey 90 seconds at 800 watts). ZC. Zero Count. p significance value (widespread lineal model with binomial distribution and inflated zero). The same letter indicates no significant difference at the 95% confidence level.

| Sample | Moisture (g/100 g) | Tg (°C) | Acidity (meq/kg) |
|--------|--------------------|---------|-----------------|
|        | Unt. | 45'' | 90'' | Unt. | 45'' | 90'' | Unt. | 45'' | 90'' |
| 1      | 19.6 | 19   | 18.8 | -52.9 | -51.7 | -47.8 | 29   | 33   | 44   |
| 2      | 19.6 | 17.6 | 16.6 | -50.4 | -46.3 | -44.2 | 42   | 35   | 35   |
| 3      | 21.4 | 19.8 | 16.2 | -55.3 | -53.3 | -46.3 | 46   | 34   | 43   |
| 4      | 20.2 | 18.6 | 17.6 | -53.4 | -52.2 | -47.3 | 36   | 41   | 30   |
| 5      | 19   | 17.2 | 16.6 | -52.5 | -48.8 | -47.9 | 39   | 30   | 35   |
| 6      | 19.4 | 18.4 | 17.6 | -51.1 | -48.5 | -44.9 | 36   | 31   | 32   |
| 7      | 21.8 | 20.8 | 18.4 | -56.9 | -56.2 | -53.3 | 36   | 27   | 27   |
| 8      | 17.6 | 17.4 | 16.8 | -47.1 | -47.9 | -45.3 | 32   | 29   | 32   |

Mean ± SD 19.8 ± 1.3 18.6 ± 1.2 17.3 ± 0.9 -52.5 ± 3 -50.6 ± 3.3 -47.1 ± 2.8 – – –

r -0.9 -0.9 -0.6 – – – – – –

Unt. (untreated), 45'' (treated honey 45 seconds at 800 watts). 90'' (treated honey 90 seconds at 800 watts). Pearson correlation coefficient (r) between moisture and Tg.
not exceed the maximum established in the European Legislation for free acidity (50 meq/Kg). Microwave caused, in most cases, reduced acidity probably due to volatile acids evaporation. This was in disagreement with Dranca & Oroian [2013].

The color of honey has not been legislated, but can be considered as another quality criterion. In this study a little range of color was observed. It is recognized that the color varied according to the melliferous areas, our results agreed with data previously obtained by Aloise [2010] for the same geographic zone. Similar range was observed in Greek unifloral honey [Karabagias et al., 2014].

One of the effects of thermal treatments of honey is the acceleration of Maillard reaction [Ibarz et al., 2000; Wong & Stanton, 1989]. These reactions would be associated to the non-enzymatic chemical changes of browning, leading to the formation of a variety of brown pigments, and formation of intermediate products as the HMF. In honey, Maillard reactions are the sugars condense with free amino acids producing a variety of brown pigments. The difference in browning rate of honey could be explained by differences in its amino acid and reducing sugar contents. Other factors that would influence the kinetics of Maillard browning could be the type and thermal stability of amino acids and reducing sugars which participate in the reaction [Turkmen et al., 2006].

In this study, a darkening of honeys was observed and HMF amount incremented rapidly (r=0.3) (Table 3). The trends in the variation of HMF content of the samples clearly depicted the sensitivity of honey to the period of heating and temperature. The same was observed by Kowalski et al. [2012], the increase caused by microwaves of the HMF is not gradual and its formation did not take place in the individual honeys in the same way, depending on floral origin. On the other hand, sample eight showed high concentration of HMF before treatment, associated to, probably, adultera-

### Table 3. Color changes and hydroxymethylfurfural content in the analysed honeys.

| Sample | Color (mm Pfund) | HMF (mg/kg) |
|--------|-----------------|-------------|
|        | Unt. 45'' 90''  | Unt. 45'' 90'' |
| 1      | 55 73 73 25 55 101 |
| 2      | 66 89 86 0 42 51   |
| 3      | 68 125 90 0 8 49   |
| 4      | 71 117 90 5 13 80  |
| 5      | 70 90 92 4 21 67   |
| 6      | 51 68 66 0 37 84   |
| 7      | 53 68 63 30 19 50  |
| 8      | 73 81 107 108 87 110 |
| Mean ± SD | 63.4 ± 9 | 88.9 ± 21.6 | 83.4 ± 14.9 | 22 ± 36.9 | 35.3 ± 26.2 | 74 ± 23.7 |
| r      | 0.25 0.1 0.26 – – – |

Unt. (untreated), 45'' (treated honey 45 seconds at 800 watts), 90'' (treated honey 90 seconds at 800 watts). Pearson correlation coefficient (r) between color and HMF.

### Table 4. Diastase index, diastase activity and glucose-oxidase activity in the analysed honeys.

| Samples | Diastase index (DU) | Diastase activity | Glucose-oxidase activity |
|---------|-------------------|------------------|-------------------------|
|         | Unt. 45'' 90''    | Unt. 45'' 90''   | Unt. 45'' 90''          |
| 1       | 22 0 UM*          | + – –            | + – –                   |
| 2       | 11 0 UM*          | + – –            | + – –                   |
| 3       | 31 0 UM*          | + – –            | + – –                   |
| 4       | 18 2 UM*          | + – –            | + – –                   |
| 5       | 15 2 UM*          | + – –            | + – –                   |
| 6       | 5 0 UM*           | + – –            | + – –                   |
| 7       | 9 0 UM*           | + – –            | + – –                   |
| 8       | 7 0 UM*           | + – –            | + – –                   |

Unt. (untreated), 45'' (treated honey 45 seconds at 800 watts), 90'' (treated honey 90 seconds at 800 watts), UM*: unmeasured, + (activity present), – (activity absent).
tion or previous overheating. Excessive amount of HMF has been observed as evidence of overheating. Also, HMF is toxic and cancerogenic [Michail et al., 2007].

Diastase index values of virgin honeys were in agreement with reported values of Argentine honeys [Aquarone, 2004]. In Greek unifloral honeys, the range of diastase index values was a little more wide [Karabagias et al., 2014]. It this thought that the amount of enzyme activity is influenced by nectar thickness and sugar content and by the fact how many times it is transferred from honeybee to honeybee before nectar is turned into honey [Bonvhei et al., 2000].

The parameter of diastase activity is regulated by international codes, while there are no requirements for the activity of glucose-oxidase. Diastase index dropped below the acceptable value (Table 4), meanwhile, Hebbbar et al. [2003] obtained similar results. Kowalski et al. [2012] showed that a short microwave treatment (0–2 min) with a low power level (63 watts) did not influence the honey quality estimated by means of diastase number.

In qualitative determination of both enzymes it was not observed activity neither of diastase (blue color) nor glucose-oxidase (colorless) in treated honeys (Table 4). Glucose-oxidase data were in agreement with the study by Kretavictus et al. [2010], where the authors observed a decrease of its activity after exposure to a conventional thermal treatment.

CONCLUSIONS

Microwave treatment was found very effective in reducing aerobic mesophilic bacteria, moulds, yeasts and P. larvae too. This was the first register about the effect of microwave on P. larvae.

Concerning to physicochemical and thermal properties variation, the treatment led to several undesirable consequences such as darkening, increase of HMF content and enzymes inactivation. Despite this, from this study it can be concluded that microwave treatment could be successfully applied by properly selecting power and exposure time to control benefits and disadvantages.

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