Antimicrobial Activity of Bee Bread Extracts Against Different Bacterial Strains

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Abstract

Bee bread is a product of the hive obtained from pollen collected by bees, to which they add honey, digestive enzymes and subsequently is stored in the combs. Increasing evidence suggests bee bread's potential therapeutic benefits, including antimicrobial properties. Bee bread is characterized by a bactericidal and bacteriostatic activity. The current study was carried out to test the antimicrobial activity of bee bread extracts, in various concentrations, against the bacterial strains: *Bacillus cereus, Staphylococcus aureus*, *Escherichia coli*, *Salmonella enterica* and *Pseudomonas aeruginosa*. The results of this study indicate that the first two dilutions of bee bread extract, respectively 33% and 16.66%, showed higher antimicrobial activity and the other dilutions had a lower, but visible activity depending on the pathogen on which they are tested. The best antimicrobial activity was manifested on the *Staphylococcus aureus* strain, where all dilutions had an inhibitory effect both at 8 hours and 12 hours.

Keywords: *antimicrobial effect, bacterial strains, bee bread extract*

INTRODUCTION

Modern researches confirm that the bee products are distinguished by unique content of biologically active substances, and they act as the biogenic stimulators and have ability to positively influence on the human organism that conditions expedience of their use in foods industry, apitherapy and pharmacological industry (Sobral *et al.*, 2017, Markiewicz-Zukowska *et al.*, 2013, Abouda *et al.*, 2011, Nagai *et al.*, 2004).

Bee bread is a fermented mixture of plant pollen, bee saliva and honey that worker bees use as food for all developmental stages in the hive. Pollen collected by bees is mixed with saliva, a small amount of honey and subsequently stored in the cells to the honeycombs where it undergoes a chemical change to form a product called bee bread (Zuluaga *et al.*, 2015).

Lately because of the development of microorganisms resistance to common antibiotics, it has become necessary to search for an alternative approach and it had been suggested that natural products are preferable to synthetic ones (Morais *et al.*, 2011).

Increasing evidence suggests bee bread's potential therapeutic benefits, including antioxidant (Leja *et al.*, 2007; Kroyer and Hegedus, 2001; Roldán *et al.*, 2001), and antimicrobial properties (Basim *et al.*, 2006; Carpes *et al.*, 2007; Morais *et al.*, 2011).

Bee bread has a positive effect on the immune system of healthy people and some authors have reported an antibiotic effect (Mutsaers *et al.*, 2005; Baltrušaitytė *et al.*, 2007).

The use of bee bread as a healthy food supplement and medicine is rising more and more due to its functional proprieties such as antioxidative ability and scavenging activities of reactive oxygen species and its benefits in liver diseases (Čeksterytė *et al.*, 2012).

Another study about bee bread antimicrobial effect it was the one made by Makarewiczet *et al.*, in 2012. They study the effect of propolis and bee bread extracts on microbiological stability of fresh fruit juices. The addition of bee bread extract in the amount of 8% or 12% to apple juice has shown, at the end of experiment, bactericidal effect because of the number of bacteria was significantly lower than at the first day of experiment. The addition of the aqueous bee bread extract to juices, in most cases, resulted in decrease of microorganism's growth. In the scientific literature there are no similar studies, there is only suggest that bee bread may have properties similar to propolis, due to the presence of lactic acid and biochemically active compounds, such as phenolic acids.

The purpose of this study was to evaluate the antimicrobial activity of bee bread against *Bacillus cereus, Staphylococcus aureus*, *Escherichia coli*, *Salmonella enterica* and *Pseudomonas aeruginosa*, pathogenic bacteria which frequently contaminate food and to determinate how different amount of the used extracts can influence the results of inhibition tests.

MATERIAL AND METHODS

Extract preparation

Different amounts of bee bread were milled, homogenized and individually extracted with 10 ml of water at room temperature for 24h. After sonication (15min) and filtration (Mărghitaş *et al.*, 2009), the resulting extracts were stored at 4 ˚C until antibacterial activity determination. The obtained extract was diluted with the purpose to see if the effect of bee bread was concentration dependent and to identify the minimal inhibitory concentration and 6 different concentrations were obtained, as follows1/3, 1/6, 1/12, 1/24, 1/48, 1/96 expressed as a percentage 33%, 16.66%, 8.33%, 4.16%, 2.08%, and 1.04%.

Bacterial strains and cultivation

The bacterial strains *Bacillus cereus, Staphylococcus aureus*, *Escherichia coli*, *Salmonella enterica* and *Pseudomonas aeruginosa* were obtained from the Department of Microbiology Bacteria Collection, Faculty of Animal Science and Biotechnology, University of Agricultural Science and Veterinary Medicine.

Escherichia coli, Salmonella enterica and *Pseudomonas aeruginosa* were used to test the effect of bee bread on gram negative bacteria and *Bacillus cereus, Staphylococcus aureus* were used to test the gram positive bacteria.

All bacteria were first cultivated at 37°C on agar plates from cultures stored at −80°C. A single colony was chosen to generate a streak plate that was stored at 4°C and served as inoculum for overnight in liquid media cultures. The nutrient broth used was made of: 10 g/L meat extract, 10 g/L peptone, 5 g/L sodium chloride. For agar plates, 10 g/L agar agar was added. After preparation the medium was autoclaved.

Bacterial growth assays

Bacterial growth assays were performed following the 96- well plate protocol of Erler *et al.*, (2014), which allows the monitoring of bacterial growth phase and aims at the determination of the lag phase length and of the slope during the logarithmic phase.

Briefly, for *Bacillus cereus, Staphylococcus aureus*, *Escherichia coli*, *Salmonella enterica* and *Pseudomonas aeruginosa* fresh overnight cultures in liquid media were used to inoculate 200 µl medium per well in a 96- well plate at an optical density (OD600 nm) of 0.001 cm^{-1}. The plates were incubated under medium shaking for 24 hours at 37°C in a BioTek Synergy 2 multichannel spectrophotometer (BioTek Instruments, USA) and the optical density, OD600 nm, was measured every 15 min.

This protocol implied the dilutions technique which were made in growth media followed by seeded with an equal microbial culture quantity. In parallel were conducted control samples in which the bee bread was not added, and where the microbial cultures must develop a growth curve according to specific cultural characteristics of the species.

Growth inhibition has been determined based on the slopes of the bee bread water extract bacterial growth curves in relation to media control growth curves.

RESULTS AND DISCUSSIONS

Physicochemical analysis of royal jelly water extracts

The amounts of sugars found in bee bread were regained in the water extracts with 16.22% fructose, 6. 57% glucose, 0.69 maltose, 0.61 turanose. No sucrose was found in bee bread

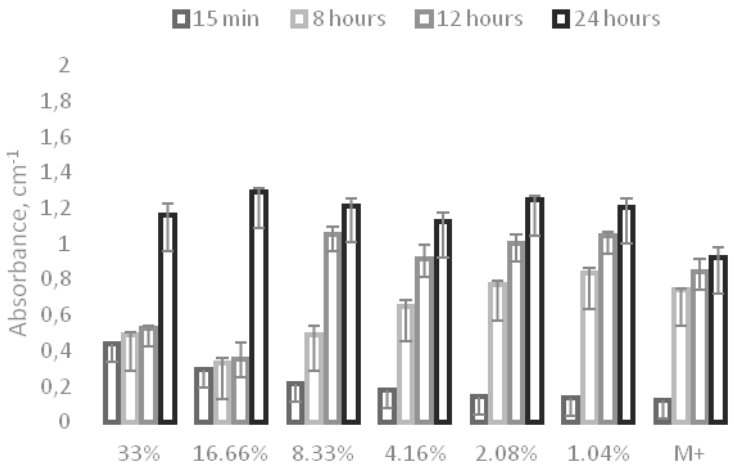

Figure 1. Effect of bee bread extracts against *Bacillus cereus* in comparison to control after 24 h of incubation (P > 0.05)

sample. The extract contained 19.10% total proteins. Total lipids content was 6.36%. The values obtained are comparable to those in the literature (Zuluaga *et al.*, 2015).

Effect of bee bread water extracts on bacterial growth

To determine the impact of bee bread on the growth of the different bacterial strains, growth curves were recorded in standard medium and supplemented with bee bread water extracts. The bacterial species showed remarkable different growth curves in their recommended media without bee bread. The extracts decelerated the growth of all four bacteria strains, by decreasing the slope and extending the log phase of the growth in a concentration- dependent manner. Growth inhibition in relation to the positive control was determined based on growth curve slopes during the log phases (Vezeteu *et al.*, 2017).

To see if the effect of bee bread was concentration dependent, and to identify the minimal inhibitory concentration serial dilution experiments with follows1/3, 1/6, 1/12, 1/24, 1/48, 1/96 expressed as a percentage 33%, 16.66%, 8.33%, 4.16%, 2.08%, 1.04%.

From our knowledge, the results of this study shown, for the first time, using spectrophotometric method, that bee bread decelerates the growth of *Bacillus cereus, Staphylococcus aureus, Escherichia coli, Salmonella enterica* and *Pseudomonas aeruginosa* some of the causative agents of food poisoning.

In the case of *Staphylococcus aureus*, 33% and 16.66% bee bread extract had a strong antimicrobial activity, followed by the other dilutions whose inhibitory effect was present, but lower. Negative control was represented by culture medium, while the positive control was the culture medium with *Staphylococcus aureus*, which actually represented the characteristic growth curves of the bacterial strain. By comparison with the positive control, we can express the inhibitory activity of bee bread extract. In the case of 33% bee bread extract, the optical density after 8 hours was 0.547cm^{-1} compared to 1.245cm^{-1} observed in the positive control sample. Compared to the characteristic growth curve of the *Staphylococcus aureus* where the density read at after 12 hours was 1.382 cm^{-1} the density of the tested extract was 0.624 cm^{-1} we can state that the extract has antimicrobial activity.

The optical density of 16.66 % bee bread extract, compared to the characteristic growth curve of the *Staphylococcus aureus* show that antimicrobial activity is also present. Dilutions 8.33 % and 4.16 % had inhibitory effect in both time points (8 and 12 hours), but showed a higher inhibition at 12 hours where the optical density for 8.33 % was 0.844 cm^{-1} and for 4.16 % 0.888 cm^{-1} beside to the control sample whose density observed was 1.382 cm^{-1}. A good result was also obtained in dilutions with a lower concentration 2.08 % and respectively 1.04 %. The extracts have an inhibitory effect at both 8 and 12 hours. *Staphylococcus aureus* was the only bacterial

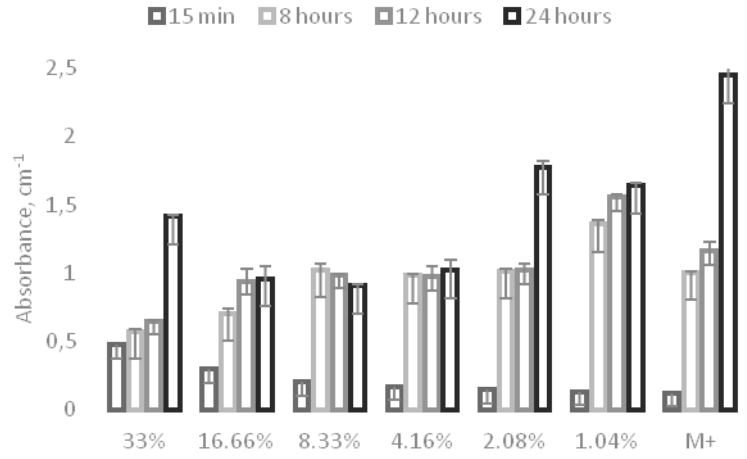

Figure 2. Effect of bee bread extracts against *Staphylococcus aureus* in comparison to control after 24 h of incubation (P > 0.05)

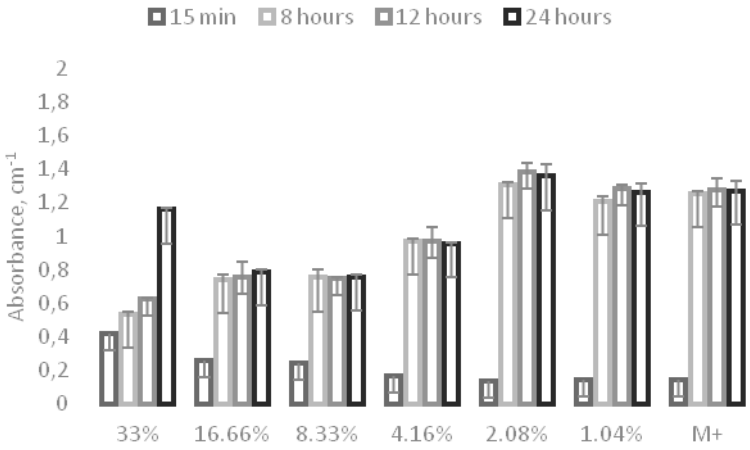

Figure 3. Effect of bee bread extracts against *Escherichia coli* in comparison to control after 24 h of incubation (P > 0.05)

strain on which all extract concentrations tested were effective. This density difference proves the inhibitory effect of the extract.

In case of *Bacillus cereus* the inhibitory activity of bee bread aqueous extract can be observed at concentrations between 4.16-33% in the first 12 hours. Under this concentration the inhibitory activity is absent. In the case of 33% bee bread extract, the inhibitory activity is visible, the growth curve being linear. Optical density at 8 hours is 0.570 cm^{-1} compared to 1.003cm^{-1} observed in the positive control and at 12 hours the optical density read was 0.648 cm^{-1} compared to 1.162 cm^{-1} observed in the control sample.

The inhibitory effect was also present in case of 16.66% dilution where the optical density was 0.706 cm^{-1} to 8 hours and 0.938 cm^{-1} to 12 hours. For the 8.33% and 4.16% bee bread extracts the optical density read after 8 and 12 hours are quite appropriate, the inhibitory effect being low. Concentration 2.08% and 1.04% did not have any inhibitory effect. The optical density read for 1.04% bee bread extract was 1.356 cm^{-1} at 8 hours compared to 1.003 cm^{-1} of the control sample. The optical density higher than the control could be explained by a stimulation of bacterial growth by the bee bread nutrients.

It was tested the inhibitory effect of the bee bread aqueous extract on the *Escherichia coli* strain. There has been a development of microbial culture regardless the dilution used. However, the growth rate is influenced by the bee bread concentration.

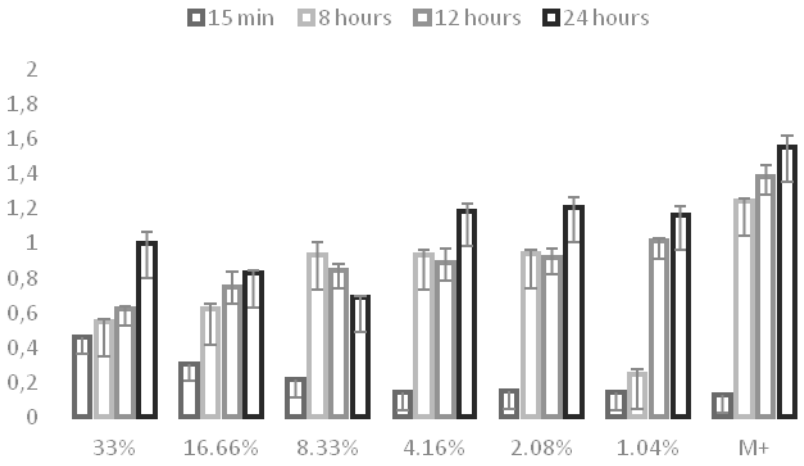

Figure 4. Effect of bee bread extracts against *Salmonella enteritis* in comparison to control after 24 h of incubation (P > 0.05)

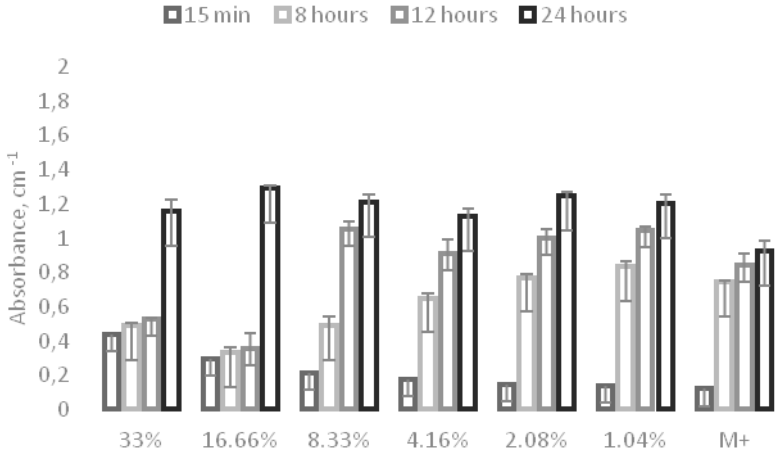

Figure 5. Effect of bee bread extracts against *Pseudomonas aeruginosa* in comparison to control after 24 h of incubation (P > 0.05)

In order to compare the effect of bee bread extract with the positive control, reference should be made to the optical density read at 8 respectively 12 hours. Thus, the inhibitory activity is observed at the maximum concentration used, namely 33%, the growth curve being linear. In this case the optical density at 8 hours is 0.531 cm^{-1}compared to 1.255 cm^{-1} observed in the positive control, and after 12 hours the observed absorbance was 0.625 cm^{-1} compared to 1.279 cm^{-1} observed in the control sample.

The inhibitory effect was also present when it was used 16.66% bee bread extract where the optical density was 0.738 cm^{-1} at 8 hours and 0.758 cm^{-1} at 12 hours. Extracts having a concentration lower than 8.33% bee bread do not exhibit inhibitory activity and may even stimulate the development of the bacterium at some point. For dilutions below 8.33%, the observed optical density values are closer to the positive control.

The 33% bee bread extract had inhibitory effect against *Salmonella enteritis* at 8 and 12 hours. 16.66% concentration inhibits bacterial growth only in the first 8 hours of incubation and the other dilutions do not have an inhibitory effect against this bacterial strain. By comparison with the characteristic growth curve of the *Salmonella enteritis* it was observed that after 12 hours this concentration even promote the development of the bacteria.

Concerning *Salmonella enteritis*, bee bread extracts did not have an inhibitory effect as good

as in the case of the other tested bacterial strains. Furthermore an increase in optical density was observed for lower concentrations which means that, bee bread even stimulate the growth of bacteria. We assume that this happened because the bacteria uses the bee bread extract as a nutritional support.

The extract with the highest concentrations of bee bread had an inhibitory effect against *Pseudomonas aeruginosa* bacterial strain. The characteristic growth curve of *Pseudomonas aeruginosa* after 8 hours had the value of optical density 0.742cm^{-1}. The absorbance read at 8 hours for 33% bee bread extract was 0.330 cm^{-1}and 0.487cm^{-1} for 16.66%. The optical density after 12 hours for the 33% concentration was 0.354cm^{-1} and 0.527cm^{-1} for the 16.66%. The absorbance for the control in this case was 0.842cm^{-1}. Dilutions 8.33% and 4.16% have a low inhibitory effect that occurs only within the first 8 hours. Dilutions 2.08% and 1.04% did not show any inhibitory effect.

The results obtained in this study ware similar with those reported by Abouda *et al.*, (2011) who studied the antimicrobial activity of samples of bee bread from different regions from Morocco on antibiotic resistant bacterial strains isolated from human pathology including *Escherichia coli, Staphylococcus aureus, Bacillus cereus* and *Pseudomonas aeruginosa.* In the study of Abouda *et al.*, (2011) the results revealed that most of strains were inhibited by the dilution ½ and ¼ and bee bread samples showed strong antimicrobial activities on the bacterial strains. Moreover, the Gram positive bacteria were more sensitive to bee bread than Gram negative bacteria (Markiewicz-Zukowska *et al.*, 2013).

Baltrušaitytė *et al.* studied in 2007 the antibacterial activity of bee bread samples against *Staphylococcus epidermidis* and *Staphylococcus aureus* and noticed difference in the diameter of the inhibition zone for all strains. In this study the extract of bee bread used were active in both tested cultures at 50 and 25 % fractions. In this experiment, the samples of bee bread were more effective than the honey bee samples.

Both studies only report the end- point measurements after an incubation of 24 hours, respectively (Abouda *et al.*, 2011, Baltrušaitytė *et al.*, 2007) not allowing for analyzing the temporal growth dynamics over time. Following the growth curves over a period of 24 hours, we show that the addition of bee bread extract indeed decelerates the growth of *Bacillus cereus, Staphylococcus aureus, Escherichia coli, Salmonella enterica* and *Pseudomonas aeruginosa* by decreasing the slopes of the growth curves but not the end- point measurements.

Using the spectrophotometric method to determine the impact of bee bread on the growth of the different bacterial strains, has enabled real-time monitoring of how the extract acts against bacterial strains. All tested bacterial species showed remarkable different growth curves.

This study indicate that the first two dilutions used, respectively 33% and 16.66%, showed good antimicrobial activity while the other dilutions had a lower, but visible activity, depending on the pathogen on which they were tested. It has been observed that the bee bread extract was able to inhibit the development of pathogenic strains such as *Staphylococcus aureus*, *Bacillus cereus*, *Escherichia coli*, *Salmonella enteritis* and *Pseudomonas aeruginosa.* The inhibitory effect was different, according to the concentration of bee bread used in the extract.

The best antimicrobial activity was manifested on the *Staphylococcus aureus* strain, where all dilutions had an inhibitory effect at both 8 hours and 12 hours after incubation. Based on these data, it can be appreciated that most of the bee bread extract dilutions showed differential antibacterial effect depending on the concentration of the bee bread. Another cause of these differences patterns of sensitivity are due to different compounds in pollen (Almedia-Muradian *et al.*, 2005). Each pollen type has its own specificity mainly linked to the floral species or cultivars and even for the same pallet of bee bread can vary widely (Nagai *et al.*, 2004).

CONCLUSION

In the present research work, it was concluded that the best antimicrobial activity was shown on the *Staphylococcus aureus* strain, where all dilutions had an inhibitory effect at both 8 hours and 12 hours. The Gram positive bacteria were more sensitive to bee bread than Gram negative bacteria. All bee bread extract dilutions presented a good antimicrobial activity, but some of them had a stronger effect than another that presented a lower activity. It was observed that the bee

bread was able to partial inhibit the developing of some pathogen strains such as: *Escherichia coli, Salmonella enteritis, Pseudomonas, Staphylococcus aureus* and *Bacillus cereus* and have a great potential in apitherapy use. It can be affirmed that the results obtained in this study are similar to those in the literature.

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