Egyptian Journal of Biological Pest Control

RESEARCH

Open Access

Double-coated microencapsulation of honeybee endogenous probiotics as a new strategy for the biocontrol of the American foulbrood disease

Fatma Mahmoud[1], Eman E. Essa[1*], Rasha M. A. Farag[2], Akila M. El Shafei[1], Ahmed S. Abou zeid[1], Hayam A. E. Sayed[3] and Shireen A. M. Ma'moun[1]

Abstract

Background: American foulbrood (AFB) is one of the potent and highly contagious bacterial diseases affecting honeybees of *Apis mellifera* and *A. cerana* species. *Paenibacillus larvae larvae* (*P. l. larvae*) is the causative agent of AFB. The present study evaluated a novel technique to control AFB disease, in vitro, depending on double-coated microencapsulated probiotics. Microencapsulation was performed for the preservation of five different locally isolated, honeybee endogenous lactic acid bacteria (LAB) (*Lactobacillus plantarum* MK780211, *L. plantarum* MK780215, *L. kunkeei* MK780216, *L. kunkeei* MK780218 and *Lactobacillus* sp. MK780212).

Results: Extrusion technique was used to encapsulate each strain separately in alginate beads coated with resistant starch (Hi-maize) and chitosan. Encapsulation efficiency was determined by testing the cell viability after encapsulation process, and it was ranging between 93.1 and 95.5%. The antimicrobial activity of both free and encapsulated LABs against *P. l. larvae* was evaluated by the agar well diffusion method. The encapsulation process decreased the activity of all the tested probiotics to some extent, except *Lactobacillus* sp. MK780212 which caused complete inhibition for *P. l. larvae* with good shelf-life expectancy time, for a month, compared to its free cells. The effect of whole capsule and its components was evaluated to ensure the activity of the tested probiotics.

Conclusion: The laboratory-controlled experiments revealed that the microencapsulation process with double coating can be used with some probiotics efficiently without affecting their activity.

Keywords: American foulbrood, *Paenibacillus larvae larvae*, Lactic acid bacteria, Microencapsulation

Background

American foulbrood (AFB) is a very dangerous, worldwide spread honeybee disease which affects the brood stage and worker bees act as infection reservoir (Erban et al. 2017). It is caused by the Gram-positive, rod-shaped and spore-forming bacterium *Paenibacillus larvae larvae* (*P. l. larvae*) (Genersch 2010). AFB is one of the bee diseases that listed in the Office International des Epizooties (OIE), where member countries and territories are obliged to report its occurrence (Erban et al. 2017).

Lactic acid bacteria are well known as beneficially valuable bacteria in human, animal and insect health (Vásquez et al. 2012). They are symbiotic bacteria that share in improving immunity and eliminating pathogens (Mitsuoka 1992). Previously, 13 species of endogenous honeybee-specific lactic acid bacteria (hbs-LAB) were isolated from the honey crop of honeybee

*Correspondence: eman.essa@sci.asu.edu.eg

[1] Department of Entomology, Faculty of Science, Ain Shams University, Abbassia, Cairo 11566, Egypt
Full list of author information is available at the end of the article

workers (Mahmoud et al. 2019). Lactic acid bacteria are well known for producing lactic acid during their metabolism within the honeybee crop (Olofsson and Vásquez 2008). They also produce many substances of antimicrobial properties such as hydrogen peroxide, organic acids, antimicrobial peptides and bacteriocins (De Vuyst and Vandamme 1994). Therefore, they preserve the healthy microbiota and maintain honeybee health. Also, they protect honeybee products from microorganisms and act as a safeguard for the foraging bees (Vásquez et al. 2012).

In 2010, it was reported that hbs-LAB inhibits and eliminates the growth of *P. l. larvae* spores (Forsgren et al. 2010). The strong antagonistic effect of LAB microbiota on the infectivity and pathogenicity of *P. l. larvae* has been identified as a new potential approach for AFB control, through the honeybee microbial defenses (Janashia and Alaux 2016). In addition to bio-biotics and antibiotics, there is also a shaking method to control AFB. These techniques were used to save bees and avoiding the burning of bee colonies (Del Hoyo et. al. 2001).

Maintenance of different types of hbs-LABs and associated active substances is a major challenge that faces field application at the colony level. Different field environmental conditions, different types of hbs-LABs and their reactions to surrounding sucrose medium are all obstacles to overcome (Douglas and Sanders 2008).

Encapsulation is commonly used in food preservation, and it acts as a barrier for liquid and solid ingredients against different environmental factors that may surround food and affect it such as light, oxygen and free radicals (Desai and Park 2005). Several studies assessed the encapsulation of LAB such as *Bifidobacterium lactis* and *Lactobacillus acidophilus* (Darukaradhya et al. 2013), *Lactobacillus paracasei* and *Lactococcus lactis* (Léonard et al. 2015), *Lactobacillus curvatus* (Barbosa et al. 2015) and *Lactobacillus plantarum* (Corbo et al. 2016), using different coating materials and taking in consideration bacterial viability, functionality and application.

In this study, double coating microencapsulation technique was applied to protect LAB for longer periods and to evaluate their viability and effectiveness after encapsulation. Laboratory bioassays against *P. l. larvae* were also estimated to indicate their efficiency for further field application.

Methods

Cultivation and preparation of lactic acid bacteria for microencapsulation

Five LAB strains of local isolates, extracted from honeybee worker guts, were used in this study. They were previously identified by 16S rRNA partial gene sequencing and deposited at NCBI GenBank under the accession numbers *Lactobacillus plantarum* MK780211, *L. plantarum* MK780215, *L. kunkeei* MK780216, *L. kunkeei* MK780218 and *Lactobacillus* sp. MK780212 (Mamoun et al. 2019). These isolates were stored in 11% skimmed milk for further applications (Stoianova and Arkad'eva 2000). Probiotic strains were activated through sub-culturing twice on freshly prepared MRS agar medium (Oxoid, Milan, Italy).

The activated probiotic cultures were cultured on MRS broth and incubated for 15 h at 37 °C under microaerophilic conditions using sodium bicarbonate (El-Gomhouria Company for Drugs, Egypt) and trichloroacetic acid (Oxford, India). Then, they were centrifuged under cooling conditions at 4670 × g for 15 min using cold centrifuge (Centurion Scientific Ltd K3 Series) and washed with NaCl solution (0.85%) (Bio. Chem. for laboratory fine chemicals—Egypt). After collecting the bacterial cells, they were suspended in saline to obtain a solution containing about 10 log CFU g^{-1} to be used for microencapsulation process. The concentration of microorganisms was adjusted by the bacterial growth curve (Silva et al. 2018).

Preparation of lactic acid bacteria capsules

Microencapsulation process was performed using the extrusion technique, developed by (Mahmoud et al. 2020), with some modifications, where the oriental wasp, *Vespa orientalis* (Linnaeus) was used as the source of chitosan.

Two different solutions were prepared:

The first solution

Contained 2% sodium alginate (ALG) (Fisher Chem. Alert TM)) and 2% Hi-maize (El Nasr Pharmaceutical Chemicals Company, Egypt) (Mahmoud et al. 2020),

The second solution

Chitosan used in the second solution was extracted locally in our laboratory from *Vespa orientalis* wasp's cuticles according to Rady et al. (2018). Calcium chloride ($CaCl_2$) of 0.2 M (El Nasr Pharmaceutical Chemicals Company, Egypt) was added to 0.4% chitosan. The pH was adjusted to 5.8 ± 0.2 with 1 M NaOH (Mahmoud et al. 2020).

Finally, the prepared solutions were autoclaved at 121 °C for 20 min.

Encapsulation process

For the encapsulation of LAB cultures, each strain was mixed with the first solution and then sprayed in the second solution by a medical syringe (3 cm). The particles were kept under stirring for 30 min in the second soln., and then, they were removed using a sterilized sieve (50 μm). The capsules were washed several times with

sterile distilled water to remove any debris from the manufacturing components. The moist microparticles were stored in sterile collectors (Mahmoud et al. 2020).

Capsule—size of capsules

The capsules were determined by using both the light microscope and a digital camera for image capture, and the scanning electron microscope. Sample preparation was carried out by immersion of the capsules in glutaraldehyde buffer (0.1 M) for 2 h at 4 °C (pH = 7.3), post-fixation by osmium tetroxide (0.1 M) for 1 h at 4 °C, followed by dehydrating the samples by 30, 50 and 70% ethyl alcohol consecutively for 2 min for each and remained in 100% ethyl alcohol for 30 min at 4 °C (Mahmoud et al. 2020). Finally, the samples were mounted on a piece of adhesive paper and gold coated using a vacuum coater (Sputter Coater, Japan).

Microencapsulation efficiency

The efficiency of the encapsulation process was judged through checking the viability of lactobacilli. It was assessed as described by Chávarri et al. (2010). After incubated under the appropriate conditions, the viable cell number was expressed as colony-forming unit per gram of microcapsule (CFU/gm.).

Encapsulation efficiency (EE) was determined according to Fareez et al. (2015):

$$EE = \mathrm{Log}_{10}N/\mathrm{Log}_{10}N_o \times 100$$

where N = Number of the bacterial cells loaded inside the microcapsules.

N_o = Number of the free bacterial cells added to the biopolymer mixture during the preparation of the microcapsules.

Capsule stability

The stability and viability of the double-coated microencapsulated LAB were assessed weekly over a month at freezing and refrigerator temperatures (Mahmoud et al. 2020). Two grams from each one of the double-coated LAB capsules were used in this experiment, where one gram stored at refrigerator temperature (4 ± 2 °C) and the other gram was stored at freezing temperature (− 18 ± 2 °C).

Encapsulated and free probiotics antimicrobial activity against P. l. larvae spores

Encapsulated and free probiotic cells were cultivated on MRS broth. Each strain was inoculated in a triplicate, and all the inoculated tubes were divided into three groups. These groups (1st, 2nd and 3rd) were incubated for 24, 48 and 72 h, respectively. All the groups were incubated at the appropriate conditions for the probiotic's growth. After incubation period of each group, the different cultures were centrifuged separately at 4 °C and 4000 rpm for 20 min, using cold centrifuge (Centurion Scientific Ltd K3 Series). The cell-free supernatant (CFS) was recovered and sterilized by filtration through syringe filter 0.22 µm, and then, the pH was adjusted to be around neutral (Barbosa et al. 2016).

The antimicrobial activity was evaluated by agar well-diffusion method according to Mallesha et al. (2010) with little modifications, where J-agar plates were inoculated with 0.5 McFarland suspension from spores of the pathogenic organism *P. l. larvae*. Then, a central well was made in each plate and filled with 150 µL of the neutralized CFS of each sample separately.

Antimicrobial activity of empty (bacterial free) capsules and its components against P. l. larvae spores

In separated plates, the effect of the capsule components (0.4% acetic acid and 0.4% chitosan dissolved in 0.4% acetic acid) on the pathogenic bacteria was checked through inoculation 150 µL of each component separately in the wells (Ansari et al. 2021). The plates incubated at upright position overnight at 37 °C. Also, the effect of the empty whole capsule on *P. l. larvae* was determined by growing the bacterial pathogen in the presence of the empty whole capsule in broth medium overnight and then the colony-forming units of the pathogen were determined and compared to the control experiment (overnight broth culture of *P. l. larvae*). The number of colony-forming units from every bacterial culture was carried out by plate counting to estimate the number of cells that were present based on their ability to give rise to the colonies (viability), where an inoculum of 0.1 mL from 1/10 diluted culture was spread over the surface of agar medium, using a sterile spreader, and after incubation at the appropriate growth conditions, the observed colonies were counted. Finally, the plates were used to calculate the bacterial number through using the formula:

Number of CFU = number of colonies per Petri /inoculum size* dilution of the culture (Harrigan and McCance 2014).

Statistical analysis

All the data are analyzed using IBM-SPSS-26.0 package. Mean and standard deviation (STD) were all calculated by one-way and repeated measurements ANOVA tests. The comparison between samples was done using Tukey test and significance in ANOVA ($P < 0.05$). All experiments were repeated 3 times.

Results

Morphology and size of capsules

The formed double-coated microcapsules were examined and characterized by both optical light microscope (Fig. 1) and SEM (Fig. 2). Both revealed that the obtained microcapsules had an irregular and spherical shapes with rough surface, with size ranged between 1530 µm for *Lactobacillus kunkeei* MK780216 and 1840 µm for *L. plantarum* MK780211. There were statistically significant differences ($P<0.05$) in the wet size of different strains (Fig. 3). ANOVA results that describe the effect of different types of LAB strains on the wet size of LAB microcapsules are listed in Table 1.

Encapsulation efficiency (EE)

The efficiency of the microencapsulation process was different from one bacterial strain to another; however, the values ranged between 95.5 and 93.1% (Fig. 4). The least cell viability loss was about 4.5% for *Lactobacillus sp.* strain MK780218, while the highest loss was about 6.9% for *Lactobacillus plantarum* MK780211. Statistical analysis among groups and within groups revealed that there was a significance difference between the LAB strains encapsulation efficiencies, *P* value < 0.0001. Table 2 shows ANOVA results, which describe the effect of different types of LAB strains on encapsulation efficiency of LAB microcapsules.

Capsule stability over refrigerated and freezing storage

Different encapsulated LAB proved to remain viable on storage either at refrigerating or at freezing temperatures, 4 ± 2 °C and -18 ± 2 °C, respectively. Refrigerated storage increased the viability of the LAB strains throughout the storage period. The encapsulation efficiency of all LAB strains increased significantly ($P<0.05$) under refrigerated storage (Fig. 5). During freezing storage, there were a decrease in the encapsulation efficiencies among LAB strains but with non-significant difference ($P>0.05$) between starting at initial time and bacterial count at the end of the fourth week for each strain (Fig. 6). ANOVA results of repeated measurement described the effect of LAB strains, period and the interaction of LAB strains with period on means of encapsulation efficiency of LAB microcapsules during a refrigerated storage at 4 ± 2 °C (Table 3) and during a freezing storage at -18 ± 2 °C (Table 4).

Probiotics antimicrobial activity against P. l. larvae spores

The antibacterial activity of both free and microencapsulated form, for all the tested probiotic strains, showed varying degrees of antimicrobial activity against *P. l. larvae* spores. The highest activity reported for both the free and the encapsulated forms of *Lactobacillus* sp. strain MK780212 was after 24 h incubation, where it caused a complete growth inhibition of the bacterial

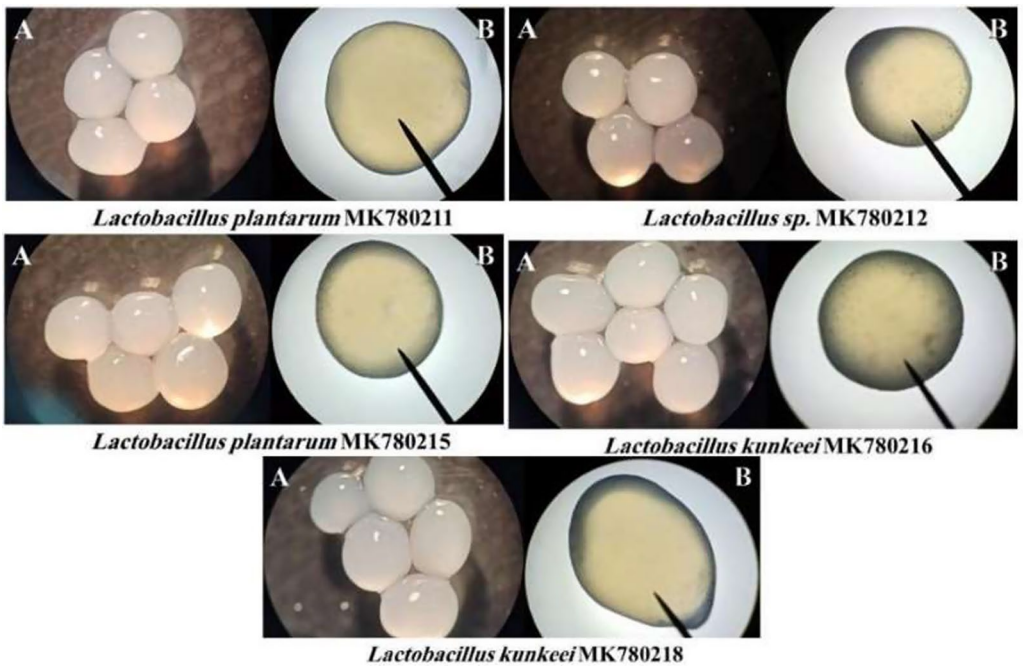

Fig. 1 Double-coated microcapsules loaded with the five LAB. **A** Capsules with magnification power 4×10^3 and **B** single capsules appeared with magnification of $40 \times 10.^3$

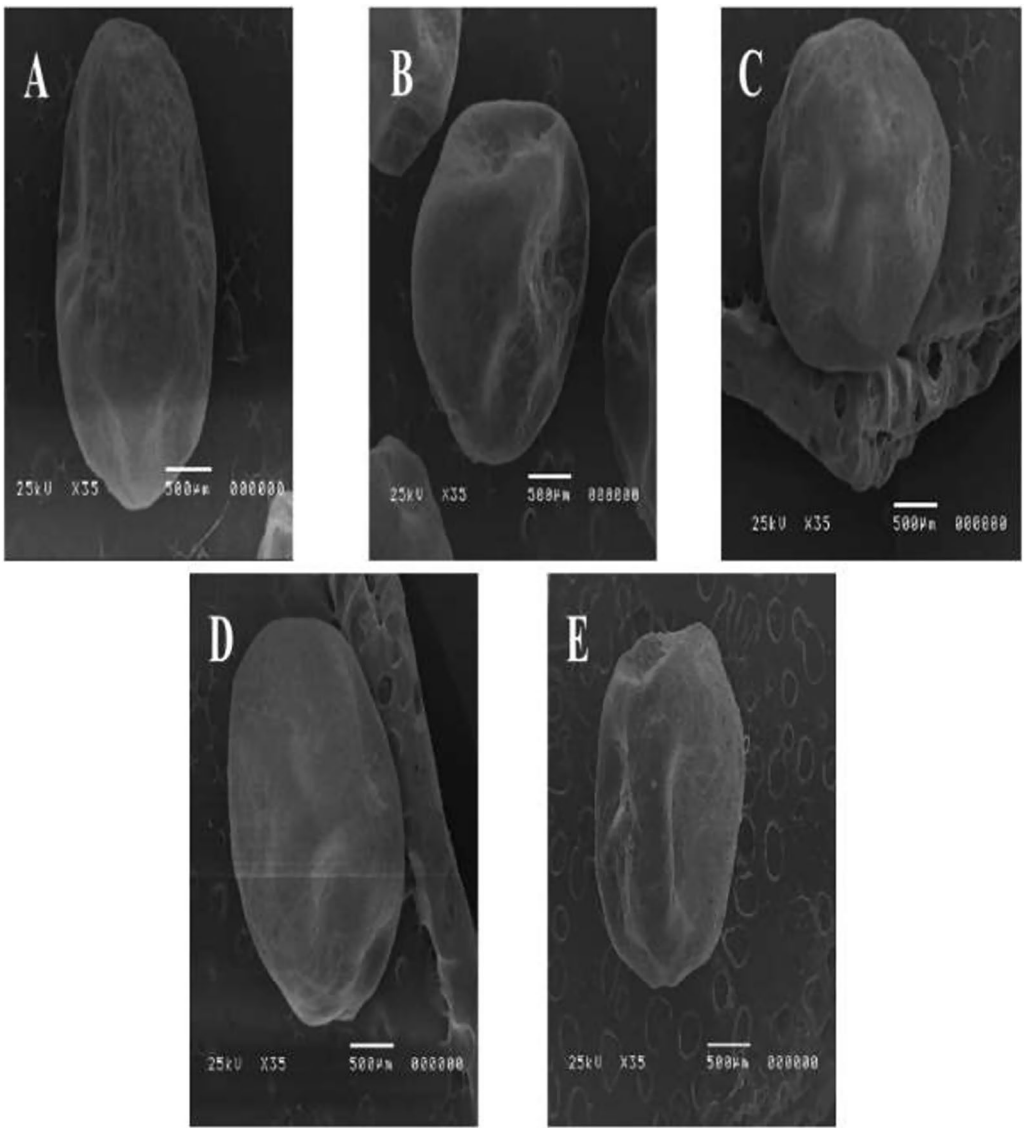

Fig. 2 Scanning electron micrograph of the double-coated microcapsules loaded with **A** *Lactobacillus plantarum* MK780211, **B** *L. plantarum* MK780215, **C** *L. kunkeei* MK780216, **D** *L. kunkeei* MK780218 and **E** *Lactobacillus* sp. MK780212

pathogen spores (Fig. 7: 2C, 2D), followed by *L. kunkeei* strain MK780218 (Fig. 7: 3E, 3F) inhibition zones and *L. kunkeei* strain MK780216 (Fig. 7: 4G, 4H). However, they showed a complete inhibition when tested in the free form after 24 and 48 h, respectively, and their encapsulated form decreased the *P. l. larvae* growth density, in comparison with the control plate (Fig. 7: 1A), making inhibition zones of 1.8 and 1.6 cm, respectively. *Lactobacillus plantarum* strain MK780211 (Fig. 7: 5I, 5 J), and *L. plantarum* strain MK780215 (Fig. 7: 6K, 6L), exhibited the lowest activity for both free and encapsulated cells after 72 h of incubation. *L. plantarum* strain MK780211 recorded 2.3 for free LAB and 2.1 for encapsulated form. However, *L. plantarum* strain MK780215 recorded 2.4 and 1.6 for free and encapsulated LAB. Upon comparing their results with the control plates, a less bacterial cell densities were noticed with the priority for the former strain.

The effect of each of the separated components of the capsule's material and the whole empty capsule showed a little effect on the growth of the *P. l. larvae* bacterial spores, where 0.4% acetic acid resulted in 1.4 cm inhibition zone. The wasp's chitosan dissolved in acetic acid showed 1.7 cm inhibition zone, while 2% sodium alginate with 2% starch polysaccharide polymers had no harmful effect on any bacterial cell (Table 5). For the assessment

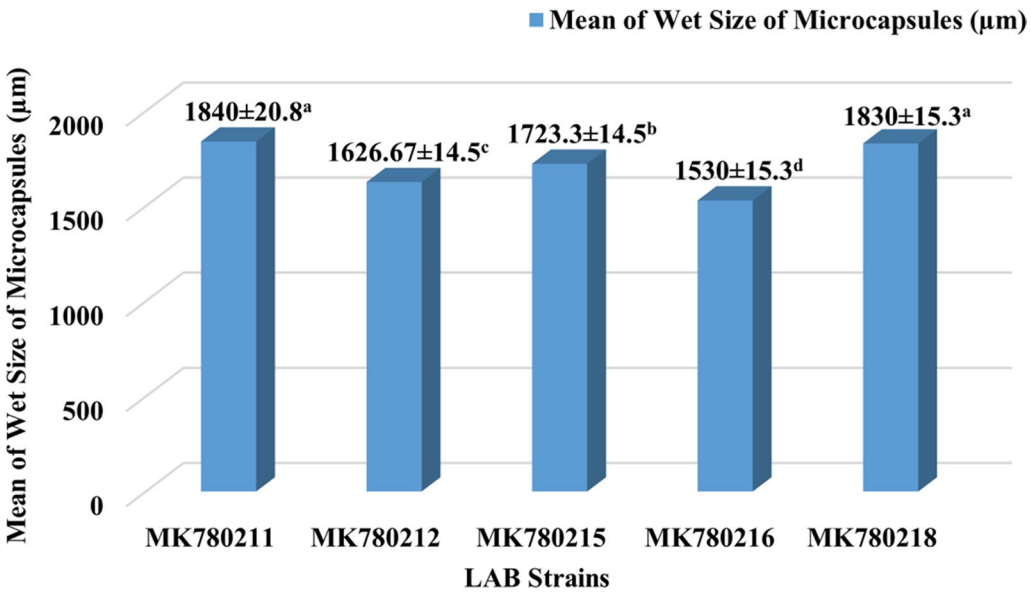

Fig. 3 Mean wet size (± STD) of the double-coated microcapsules. Uppercase letters are results of Tukey test. [a–d] Means with the same letter are not significantly different at sig. ($p \leq 0.05$)

Table 1 ANOVA table describes the effect of different types of LAB strains on wet size of LAB microcapsules

Source	DF	Sum of squares	Mean square	*F* value	Sig
LAB strains	4	212,466.667	53,116.667	66.95	0.0001
Error	10	7933.333	793.333		
Corrected total	14	220,400.000			

of the effect of the empty whole capsule, the pathogenic bacterial viability determined by comparing the CFU count of an overnight culture of the *P. l. larvae* grown in the presence of the whole empty capsules (62×10^4) with the CFU count of an overnight culture of the *P. l. larvae* alone (67×10^4). There was about 7.5% decrease in the pathogenic bacterial count (Table 6).

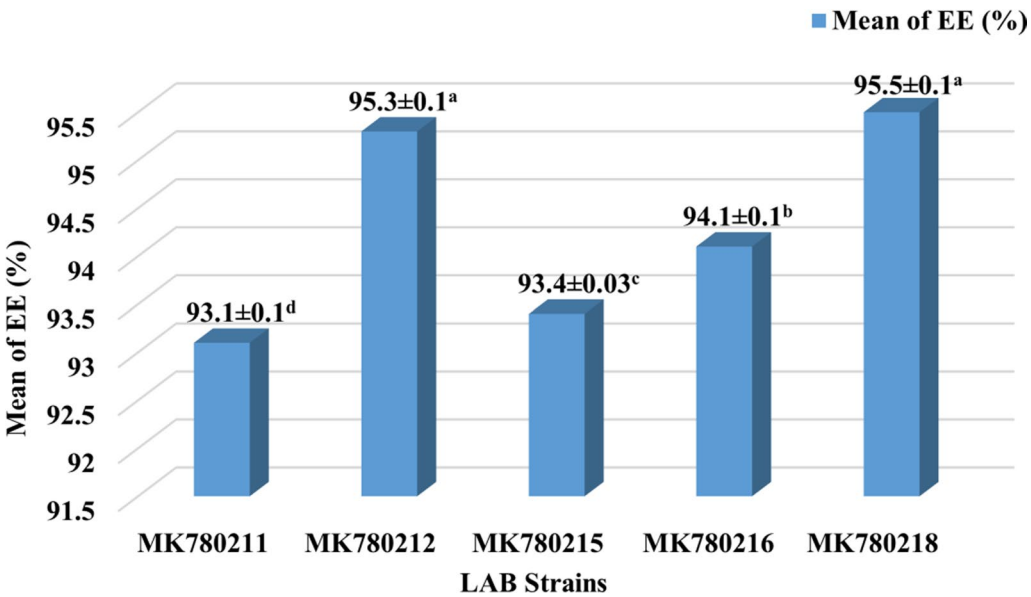

Fig. 4 The relation between bacterial strain types and mean encapsulation efficiency (± STD). Uppercase letters are results of Tukey test. [a–d] Means with the same letter are not significantly different at sig. ($p \leq 0.05$)

Table 2 ANOVA table describes the effect of different types of LAB strain on encapsulation efficiency of LAB microcapsules

Source	DF	Sum of squares	Mean square	*F* value	Sig
LAB strains	4	13.983	3.496	169.15	0.0001
Error	10	0.207	0.021		
Corrected total	14	14.189			

Discussion

Several encapsulating materials have been studied and tested for their ability to improve the viability of many probiotic strains. Alginate is one of the coating materials that is commonly selected for microencapsulation in most studies. It is characterized by simple manipulation, being compatible with majority of encapsulation methods, cheap and safe (Cook et al. 2012). Unfortunately, alginate microcapsules are sensitive to low pH, so biofilm-forming materials, as chitosan, are used in over coating alginate beads to make it stable at acidic environment as that of the bees' stomach (Chávarri et al. 2010).

The number of bacterial cells that entrapped inside the capsule and known as the encapsulation efficiency is considered as a very important criteria for determining the efficiency of the encapsulation process (Burgain et al. 2014). There was a statistically significance difference in the encapsulation efficiency among the tested strains. The strain type explains about 99% of the causes of those differences in the encapsulation efficiency between different tested LABS. The encapsulation efficiency for different strains ranged between 93.1 and 95.5%, thus falling within the range as reported by Mahmoud et al. (2020), and higher than that recorded by Tee et al. (2014) who reported encapsulation efficiency of about 92.26%.

Fascinatingly, on studying the encapsulated cells survivability during the refrigerated storage for a month, the count of the encapsulated five LAB showed statistically significant increase over the storage period with effect size 33%. This suggests that the refrigerator temperature contributes about 33% from the factors, which caused that increase in number. That may be due to LAB can grow at low rate of refrigerating temperature utilizing starch as a nutritional source, this notation was not implemented by any other relevant study. Upon freezing conditions, there was a statistically neglectable decrease in the cell count, this may be due to the different components of the capsules, which protect the bacterial cells from the formation of ice crystals inside and thus reduced the cell injury and cell loss, and this notation was recorded by Wang et al. (2015) through concerning the presence of milk protein with LAB during storage. The same effect may be occurred here where the starch which adsorbed on the cell surface led to a partial efflux of water from the cell, thus inhibiting the ice crystal formation inside the cells.

All the encapsulating materials that were used in this study were effective in enhancing the viability of the five LAB during the refrigerated storage at 4 ± 2 °C up to one

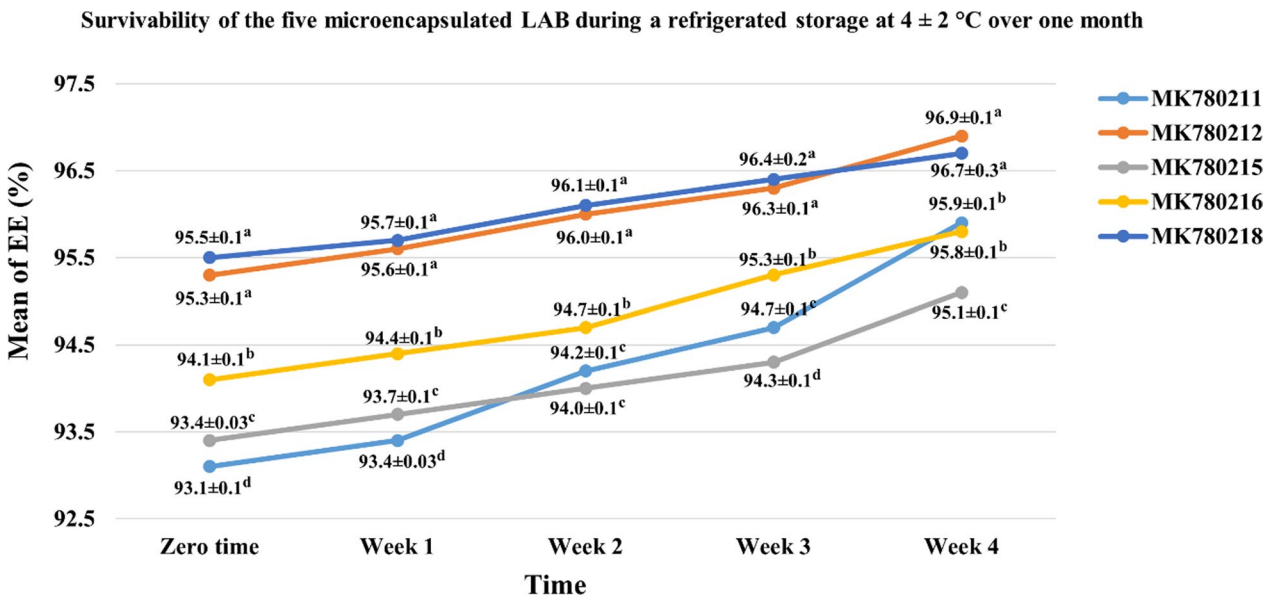

Fig. 5 Survivability of the five microencapsulated LAB during a refrigerated storage at 4 ± 2 °C. Data are represented as weekly bacterial cell counts during one month for each strain. Data were expressed as mean (± STD). Uppercase letters are results of Tukey test. $^{a-d}$ Means with the same letter are not significantly different at sig. ($p \leq 0.05$)

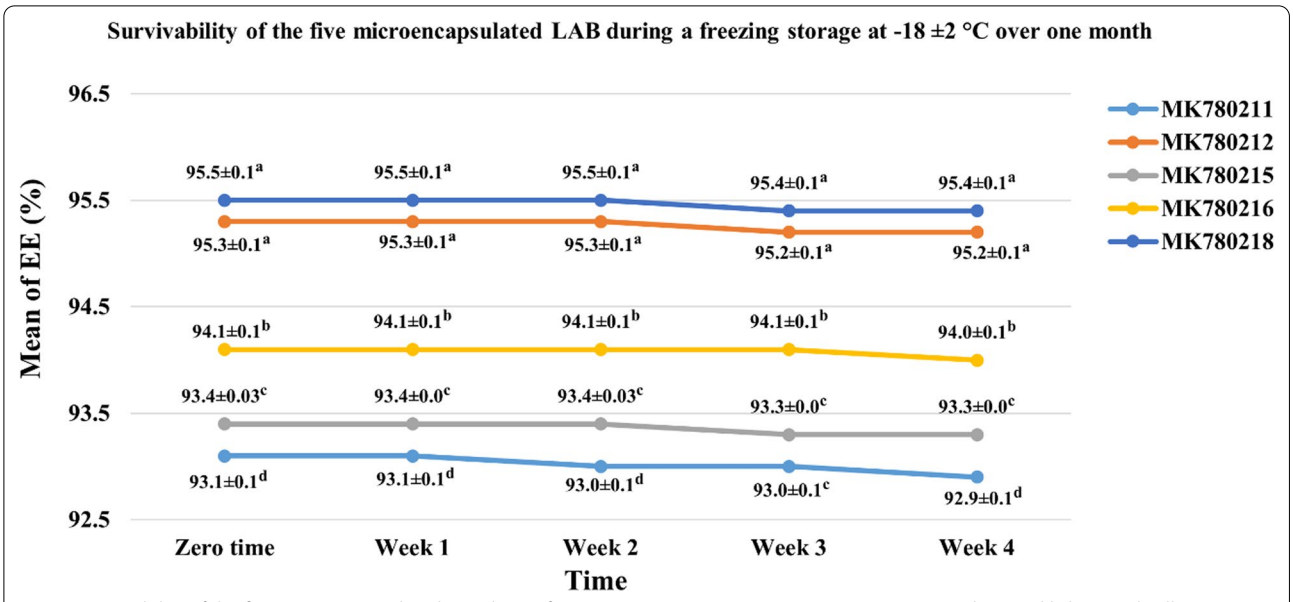

Fig. 6 Survivability of the five microencapsulated LAB during freezing storage at -18 ± 2 °C. Data are represented as weekly bacterial cell counts during one month for each strain. Data were expressed as mean (± STD). Uppercase letters are results of Tukey test. $^{a-d}$ Means with the same letter are not significantly different at sig. ($p \leq 0.05$)

Table 3 ANOVA table of repeated measurement describes the effect of LAB strains, period and the interaction of LAB strains with period on means of encapsulation efficiency of LAB microcapsules during a refrigerated storage at 4 ± 2 °C

Source	DF	Sum of squares	Mean square	*F* value	Sig
LAB strains	4	52.7075	13.177	312.74	0.0001
Period	4	29.443	7.361	235.17	0.0001
LAB strains *period	16	2.993	0.187	5.98	0.0001
Error (LAB strains)	10	0.421	0.042		
Error	40	1.252	0.031		
Corrected total	74	86.817			

Table 4 ANOVA table of repeated measurement describes the effect of LAB strains, period and the interaction of LAB strains with period on means of encapsulation efficiency of LAB microcapsules during a freezing storage at − 18 ± 2 °C

Source	DF	Sum of squares	Mean square	*F* value	Sig
LAB strains	4	70.779	17.695	153.07	0.0001
Period	4	0.190	0.048	50.93	0.0001
LAB strains *period	16	0.021	0.001	1.37	0.2033
Error (LAB strains)	10	1.156	0.116		
Error	40	0.037	0.001		
Corrected total	74	72.183			

month. This may be due to the fact that alginate–chitosan was the most efficient materials used in encapsulation when comparing them to the other tested materials.

In order to declare the possible antimicrobial effect of the whole capsule (free from LAB) upon the pathogenic bacterial spores, and to evaluate the effect of the encapsulated LAB honestly after neutralizing any other possible effects, the CFU of an overnight culture of *P. l. larvae* with the LAB free capsules was determined. About 7.5% decrease in the pathogenic bacterial count ensures that there was an antimicrobial effect for the empty capsules. The final pH of the broth culture was markedly decreased indicating the liberation of the acidic component into the growth medium, which means that the decrease in the pathogenic spores may be due to both chitosan and the acetic acid. We had to compensate the effect of the acid upon testing the LAB microcapsules by neutralizing the CFS, but we cannot delete the effect of the chitosan.

The tested probiotics normally produce lactic acid, so upon testing both the free and the encapsulated samples we have to cancel the effect of various acids, which liberated from the capsule and those produced by LAB via adjusting the pH of the CFS to be around

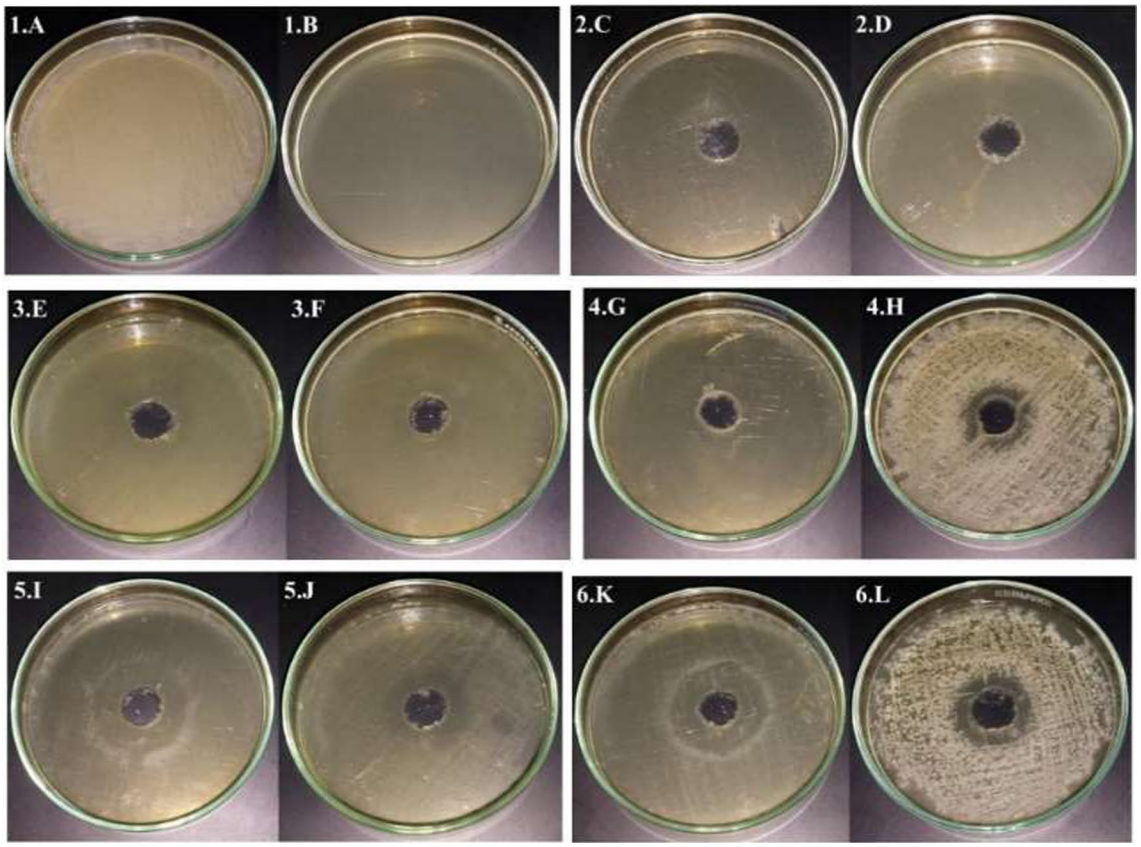

Fig. 7 The highest inhibition zones formed by LAB over different incubation periods, 1. Control 1A: -ve Control, and 1B: + ve Control. 2. *Lactobacillus* sp. MK780212 (After 24 h of incubation) 2C: Free LAB and 2D: encapsulated, 3. *Lactobacillus kunkeei* MK780218 (After 24 h incubation) 3E: Free LAB and 3F: encapsulated, 4. *Lactobacillus kunkeei* MK780216 (After 48 h of incubation) 4G: Free LAB and 4H: encapsulated, 5. *Lactobacillus plantarum* MK780211 (After 72 h of incubation) 5I: Free LAB and 5 J: encapsulated, 6. *Lactobacillus plantarum* MK780215 (After 72 h of incubation) 6 K: Free LAB and 6L: encapsulated

Table 5 Effect of the capsule components on the area of inhibition zone of *P. l. larvae*

Inhibition substances	Inhibition zone (cm)
0.4% acetic acid	1.4
0.4% chitosan + 0.4% acetic acid	1.7
2% sodium alginate + 2% starch	0

Table 6 Effect of whole capsule on *P. l. larvae* viability

	No. of CFU	% of viable cells
P. l. larvae	≈ 670,000	100
P. l. larvae incubated with empty (bacterial free) Capsule	≈ 620,000	92.5

neutral using 4 N NaOH solution, and thus, the produced antimicrobial effect will be due to the production of bacteriocins and antioxidants and not due to the acid accumulation.

Lactobacillus plantarum strain MK780211 and *L. plantarum* strain MK780215 gave the lowest activity for both free and encapsulated forms and needed 72 h of incubation to produce their best activities, which differed from the results of Iorizzo et al. (2020) who reported the antimicrobial activity of *L. plantarum* after 48 h incubation.

Conclusion

The microencapsulation process of LAB strains in alginate beads coated with starch (Hi maize) and chitosan raised the survival of the probiotics, as the encapsulation efficiency increased upon refrigeration storage. Microencapsulation process did not affect the antimicrobial activities of *Lactobacillus kunkeei* MK780212 and *L. plantarum* MK780211

and positively increased their efficiencies. However, based on our findings, the encapsulation could be promising in different food and treatment applications in general and treatment of honeybee diseases in particular. Giving a hope for further hive application of a natural promising commercially stable therapeutic products, those beneficial microbes should be used to inoculate the bee gut and colonies to ensure health and diet. Building a community of bacteria such as *Lactobacillus* sp. inside the bee gut is a very important process, and LAB not only helps in food digestion but also produces some beneficial materials (vitamins) and immunomodulator substances (antibiotics) that eliminate pathogenic microorganisms. This new technique may be an initial promising step for the application of probiotic commercial products.

Future studies

The survivability of the double-coated microcapsules will be determined over extended periods, especially at room temperature for economic production and application on the honey bee colony level. Encapsulation of probiotics also insures the protection and good viability of LAB cultures, for future field application of a promising commercially stable therapeutic products.

Abbreviations

AFB: American foulbrood; LAB: Lactic acid bacteria; hbs-: Honeybees specific; OTC: Oxytetracycline; TCR: Tetracycline-resistant; OTCR: Oxytetracycline-resistant; SEM: Scanning electron microscopy; ANOVA: Analysis of variance; MRS agar: De Man, Rogosa and Sharpe agar.

Acknowledgements

Authors are grateful to Prof. Dr. Mohamed. S. Salama, the founder and director of the Molecular Biology & Microbiology Unit at the Entomology Department, Faculty of Science, Ain Shams University, for providing the necessary laboratory facilities for this research.

Author contributions

AES, ASA, SAMM, RMAF and EEE conceived and designed the experiments, contributed to reagents and materials and revised the manuscript. FM, EEE, HAES, SAMM and RMAF performed the experiments, analyzed and interpreted the data, contributed to reagents, materials and analysis tools and wrote the paper. All authors read and approved the final manuscript.

Funding

Not applicable.

Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Declarations

Ethics approval and consent to participate

This research was approved by the Research Ethics Committee of Faculty of Science, Ain Shams University, Cairo, Egypt (Approval code: ASU-SCI/ENTO/2022/10/1).

Consent for publication

Not applicable.

Competing interests

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: The authors declare the possible existence of financial competing interests derived from the introduced patent held by Academy of scientific research, Egyptian patent office regarding the described technology especially after the success of field study. (Data have not been published yet.)

Author details

[1]Department of Entomology, Faculty of Science, Ain Shams University, Abbassia, Cairo 11566, Egypt. [2]Honeybee Research Department, Agriculture Research Centre, Plant Protection Research Institute, Dokki, Giza 12311, Egypt. [3]Department of Microbiology, Faculty of Science, Ain Shams University, Abbassia, Cairo 11566, Egypt.

Received: 1 August 2022 Accepted: 29 November 2022
Published online: 06 December 2022

References

Ansari J, Kushwaha SP, Ansari VA, Singh K, Hasan SM (2021) Agar well diffusion: a prominent method for in vitro screening of antimicrobials. Int J Bot Stud 6(5):836–839

Barbosa MS, Todorov SD, Jurkiewicz CH, Franco BD (2015) Bacteriocin production by *Lactobacillus curvatus* MBSa2 entrapped in calcium alginate during ripening of salami for control of Listeria monocytogenes. Food Control 47:147–153. https://doi.org/10.1016/j.foodcont.2014.07.005

Barbosa MS, Todorov SD, Ivanova IV, Belguesmia Y, Choiset Y, Rabesona H, Chobert JM, Haertle T, Franco BDGM (2016) Characterization of a two-peptide plantaricin produced by *Lactobacillus plantarum* MBSa4 isolated from Brazilian salami. Food Contr 60:103–112. https://doi.org/10.1016/j.foodcont.2015.07.029

Burgain J, Scher J, Lebeer S, Vanderleyden J, Cailliez-Grimal C, Corgneau M, Francius G, Gaiani C (2014) Significance of bacterial surface molecules interactions with milk proteins to enhance microencapsulation of *Lactobacillus rhamnosus* GG. Food Hydrocoll 41:60–70. https://doi.org/10.1016/j.foodhyd.2014.03.029

Chávarri M, Marañón I, Ares R, Ibáñez FC, Marzo F, del Carmen VM (2010) Microencapsulation of a probiotic and prebiotic in alginate-chitosan capsules improves survival in simulated gastro-intestinal conditions. Int J Food Microbiol 142(1–2):185–189. https://doi.org/10.1016/j.ijfoodmicro.2010.06.022

Cook MT, Tzortzis G, Charalampopoulos D, Khutoryanskiy VV (2012) Microencapsulation of probiotics for gastrointestinal delivery. J Control Release 162(1):56–67. https://doi.org/10.1016/j.jconrel.2012.06.003

Corbo MR, Bevilacqua A, Speranza B, Di Maggio B, Gallo M, Sinigaglia M (2016) Use of alginate beads as carriers for lactic acid bacteria in a structured system and preliminary validation in a meat product. Meat Sci 111:198–203. https://doi.org/10.1016/j.meatsci.2015.10.005

Darukaradhya J, Phillips M, Kailasapathy K (2013) Effect of encapsulation on the survival of probiotic bacteria in the presence of starter and non-starter lactic acid bacteria in Cheddar cheese over a 6-month ripening period. Int J Ferment Foods 2(1):63–76

Del Hoyo ML, Basualdo M, Lorenzo A, Palacio MA, Rodriguez EM, Bedascarrasbure E (2001) Effect of shaking honey bee colonies affected by American foulbrood on *Paenibacillus larvae larvae* spore loads. J Apic Res 40(2):65–69. https://doi.org/10.1080/00218839.2001.11101053

Desai KGH, Park HJ (2005) Recent developments in microencapsulation of food ingredients. Dry Technol 23(7):1361–1394. https://doi.org/10.1081/DRT-200063478

Douglas LC, Sanders ME (2008) Probiotics and prebiotics in dietetics practice. J Am Diet Assoc 108:510–521. https://doi.org/10.1016/j.jada.2007.12.009

Erban T, Ledvinka O, Kamler M, Nesvorna M, Hortova B, Tyl J, Titera D, Markovic M, Hubert J (2017) Honeybee (*Apis mellifera*) associated bacterial community affected by American foulbrood: detection of *Paenibacillus larvae* via microbiome analysis. Sci Rep 7(1):1–10. https://doi.org/10.1038/s41598-017-05076-8

Fareez IM, Lim SM, Mishra RK, Ramasamy K (2015) Chitosan coated alginate-xanthan gum bead enhanced pH and thermotolerance of *Lactobacillus*

plantarum LAB12. Int J Biol Macromol 72:1419–1428. https://doi.org/10.1016/j.ijbiomac.2014.10.054
Forsgren E, Olofsson TC, Vásquez A, Fries I (2010) Novel lactic acid bacteria inhibiting *Paenibacillus larvae* in honeybee larvae. Apidologie 41:99–108. https://doi.org/10.1051/apido/2009065
Genersch E (2010) American foulbrood in honeybees and its causative agent, *Paenibacillus larvae*. J Invertebr Pathol 103:S10–S19. https://doi.org/10.1016/j.jip.2009.06.015
Harrigan WF, McCance ME (2014) Laboratory methods in microbiology. Academic press
Iorizzo M, Testa B, Lombardi SJ, Ganassi S, Ianiro M, Letizia F, Succi M, Tremonte P, Vergalito F, Cozzolino A, Sorrentino E, Coppola R, Petrarca S, Mancini M, De Cristofaro A (2020) Antimicrobial activity against *Paenibacillus larvae* and functional properties of *Lactiplantibacillus plantarum* strains: potential benefits for honeybee health. Antibiotics 9(8):442. https://doi.org/10.3390/antibiotics9080442
Janashia I, Alaux CC (2016) Specific immune stimulation by endogenous bacteria in honeybees (Hymenoptera: Apidae). J Econ Entomol 109:1474–1477. https://doi.org/10.1093/jee/tow065
Léonard L, Beji O, Arnould C, Noirot E, Bonnotte A, Gharsallaoui A, Degraeve P, Lherminier J, Saurel R, Oulahal N (2015) Preservation of viability and anti-Listeria activity of lactic acid bacteria, *Lactococcus lactis* and *Lactobacillus paracasei*, entrapped in gelling matrices of alginate or alginate/caseinate. Food Control 47:7–19. https://doi.org/10.1016/j.foodcont.2014.06.020
Mahmoud F et al (2019) Field application of honeybees' endogenous lactic acid bacteria for the control of American foulbrood disease. Egypt Acad J Biolog Sci 11(1):19–82
Mahmoud M, Abdallah NA, El-Shafei K, Tawfik NF, El-Sayed HS (2020) Survivability of alginate-microencapsulated *Lactobacillus plantarum* during storage, simulated food processing and gastrointestinal conditions. Heliyon 6(3):e03541. https://doi.org/10.1016/j.heliyon.2020.e03541
Mallesha Shylaja R, Selvakumar D, Jagannath JH (2010) Isolation and identification of lactic acid bacteria from raw and fermented products and their antibacterial activity. Recent Res Sci Technol 2(6):42–46
Mamoun SAM, Mahmoud F, Farag RMA, Elshafae AM, Abou Zeid AS (2019) Inhibitory Effects of Lactic Acid Bacteria Isolated from the Honeybee (*Apis Mellifera*) collected from Egypt, Against *Paenibacillus Larvae Larvae*. J Commun Med Health Res 1(3):117
Mitsuoka T (1992) The human gastrointestinal tract. In: Wood BJB (ed) The lactic acid bacteria in health and disease 1. Elsevier, London, pp 69–114. https://doi.org/10.1007/978-1-4615-3522-5_4
Olofsson TC, Vásquez A (2008) Detection and Identification of a novel lactic acid bacterial flora within the honey stomach of the honeybee *Apis mellifera*. Curr Microbiol 57:356–363. https://doi.org/10.1007/s00284-008-9202-0
Rady MH, Essa EE et al (2018) Characterization and solubilization of chitosan from the oriental hornet (*Vespa Orientalis*). J Egypt Soc Parasitol 48(3):669–676
Silva APRD, Longhi DA, Dalcanton F, Aragão GMFD (2018) Modelling the growth of lactic acid bacteria at different temperatures. Braz Arch Biol Technol. https://doi.org/10.1590/1678-4324-2018160159
Stoianova LG, Arkad'eva ZA (2000) Comparison of methods of storing lactic acid bacteria. Mikrobiologiia 69(1):98–104
Tee WF, Nazaruddin R, Tan YN, Ayob MK (2014) Effects of encapsulation on the viability of potential probiotic *Lactobacillus plantarum* exposed to high acidity condition and presence of bile salts. Food Sci Technol Int 20(6):399–404
Vásquez A, Forsgren E, Fries I, Paxton RJ, Flaberg E, Szekely L, Olofsson TC (2012) Symbionts as major modulators of insect health: lactic acid bacteria and honeybees. PLoS ONE 7(3):e33188. https://doi.org/10.1371/journal.pone.0033188
De Vuyst L, Vandamme EJ (1994) Bacteriocins of lactic acid bacteria. Blackie academic and professional, Chapman and Hall, printed in Great Britain by the Alden Press, Oxford. ISBN 0751401749. https://doi.org/10.1007/978-1-4615-2668-1.
Wang SY, Ho YF, Chen YP, Chen MJ (2015) Effects of a novel encapsulating technique on the temperature tolerance and anti-colitis activity of the probiotic bacterium *Lactobacillus kefiranofaciens* M1. Food Microbiol 46:494–500. https://doi.org/10.1016/j.fm.2014.09.015

Publisher's Note