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Effect of a Polyfloral Honey Supplement on the Growth and Viability of Lactic and Probiotic Flora in Fermented Milk

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ABSTRACT

Background: The study involved evaluating the growth and viability of probiotic lactic acid bacteria strains in milk spiked with polyfloral honey.

Methods: Beforehand, the selected honey was characterized by analysis of its physicochemical properties, including water content, pH, electrical conductivity and HMF. Sugar content was also assessed by NMR. The growth of a probiotic strain of Lactobacillus plantarum seeded in milk enriched with honey at different concentrations was evaluated. Four types of fermented milk were prepared ; one natural without honey, one with honey and two containing honey with a mixture of lactic and probiotic flora.

Result: Physicochemical analysis of the polyfloral honey revealed a water content of 18.6%, a pH of 4.0 and an appreciable HMF level of 13.51 mg/kg. The addition of honey to milk stimulated the growth of Lactobacillus plantarum, particularly at a honey concentration of 5%. The highest viability rate (72.41%) was observed in honey-fermented milk after 15 days of storage. These results confirm the beneficial effect of polyfloral honey on lactic acid bacteria in terms of growth and viability, underlining its biotechnological interest in improving the quality of fermented milks.

Key words: Fermented milks, Growth, Lactic acid bacteria, Polyfloral honey, Probiotics, Viability.

INTRODUCTION

Produced by bees from nectar and/or honeydew, honey has been, for millennia in the Western world, the only abundant source of sweet substances available (Bonsignore *et al.,* 2024). Indeed, carbohydrates are the major components of this natural product, mainly made up of monosaccharides represented by glucose (31%) and fructose (38%). Other carbohydrates have also been identified in honey, but in small quantities, notably disaccharides and oligosaccharides. The physicochemical and biological properties of honey can vary according to its floral origin. Floral sources contribute to honey's specific composition of sugars, amino acids and phenolic compounds, which in turn influence its antimicrobial, anti-inflammatory and antioxidant properties (Bonsignore *et al.,* 2024 ; Sousa *et al.,* 2016 ; Wang *et al.,* 2021). In nature, bees forage on a wide variety of plants, resulting in polyfloral honey, or limit their selection to a single plant, resulting in monofloral honey (Mouhoubi-Taffinine *et al.,* 2018).

For several years now, considerable interest has focused on the health benefits of lactic acid bacteria, also known as "probiotics", for food applications, particularly in dairy products (fermented milks, cheeses, etc.), which have been chosen as the preferred carriers for this type of microorganism (Soni *et al.,* 2021). The majority of probiotic bacteria belong to the genera Bifidobacterium and Lactobacillus (Adriani *et al.,* 2024). Probiotics must be active, abundant and viable in the finished product by the minimum durability date. To support and maintain this viability, as well as in vivo growth, substances commonly known as "prebiotics" are often added to milk or dairy products, the majority of prebiotics being oligosaccharides (Rosa *et al.,* 2021).

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Honey is traditionally used in the dairy industry, particularly in fermented dairy products such as yoghurt, mainly to improve flavor. However, some studies have reported that the growth and survival of lactic acid bacteria in milk and the gastrointestinal tract are stimulated by the presence of bee honey (Kowalska *et al.,* 2024). Its highly varied composition, particularly in carbohydrates, suggests the potential beneficial effects of incorporating this natural sweetener into dairy products.

The aim of this study was to investigate the influence of polyfloral honey at different concentrations on the growth and viability of lactic acid bacteria strains in a skimmed milk medium during and after fermentation.

MATERIALS AND METHODS

Location and sampling

The experimental study was carried out at the Research Laboratory of Sciences and Techniques of Animal Production ″LSTPA″ of Hassi-Mameche, Faculty of Natural and Life Sciences, Abdelhamid Ibn Badis University of Mostaganem, Algeria from October 20, 2023 to August 30, 2024.

Samples of the honey

Polyfloral honey (250 g) from the Sidi-Ali forest in Mostaganem, northwest Algeria, collected during a summer harvest in September 2023, was used for this study. The sample was stored in plastic containers at 4°C, protected from light.

Lactic acid bacteria strains

A probiotic strain belonging to the *Lactobacillus plantarum* species, isolated from honey at the LSTPA research laboratory and two thermophilic commercial ferments, namely a monoculture of the *Streptococcus thermophilus* strain and a mixed culture of the two strains *Streptococcus thermophilus* and *Lactobacillus bulgaricus*, from Danish producer CHR HANSEN, were used for this study.

Revivification of lactic acid bacteria

Strains were activated from their frozen forms (stored in glycerol at -80°C) by culturing 1000 µl of them in a tube containing 5 ml of De Man, Rogosa and Sharpe (MRS) broth for; then incubated anaerobically at 37°C for 48 h.

Physico-chemical analysis of honey

Physicochemical parameters were determined according to the harmonized methods of the International Honey Commission (IHC, 2009). The water content of the honey was assessed by measuring the refractive index, which can be used to deduce water content using the Wedmore (1955) table. A drop of liquefied honey was placed on the refractometer, the refractive index was read and corrected for a standard temperature of 20°C by adding a correction factor of 0.00023/°C. The pH of the honey solution, diluted 10% (w/v) in distilled water, was measured using a LOVIBOND SD 400 Oxi pH meter. The electrical conductivity (EC) of the honey sample was measured using an ADWA AD3000 EC/TDS standard calibration conductivity meter. The honey sample was diluted with distilled water to a concentration of 20% (w/v) dry matter. The conductivity value was measured directly by the cell after immersion in the solution and the results were expressed in micro-Siemens per centimeter (µS/cm). HMF content, a marker of honey quality, was determined by White's spectrophotometric method. A honey solution (5 g in 25 ml distilled water) was prepared with Carrez reagents, made up to 50 ml and filtered. Absorbance measurements at 284 and 336 nm were carried out using a JENWAY 7305 UV visible spectrophotometer, with a reference solution containing 0.2% sodium bisulfite. For analysis of sugar composition, we used nuclear magnetic resonance (NMR), using a BRUKER NMR spectrometer to measure in %, two monosaccharides (glucose and fructose), three disaccharides (sucrose, maltose and turanose) and one trisaccharide (melezitose).

Evaluation of honey addition on *Lactobacillus plantarum* strain growth

Three variants of skimmed milk with 12.5% total dry extract, enriched with 1, 5 and 10% honey, were evaluated for the use of the *Lactobacillus plantarum* strain as a lactic leaven. A honey-free reference medium served as a control. Briefly, the strain was cultured in MRS broth at 37°C for 24 hours. Then, 3% of this microbial culture was inoculated into sterile reconstituted skimmed milk with different concentrations of honey and incubated at 37°C. *Lactobacillus plantarum* growth was assessed by counting colonies after 24 and 48 hours incubation on MRS agar under anaerobic conditions at 37°C, expressed as colony-forming units per milliliter (CFU/ml).

Observation

The milks used (at 12.5% total dry extract) for the preparation of lactic ferment and fermented milks were reconstituted from skimmed milk powder (from EPI Ingredients France) solubilized in treated water and pasteurized at 90°C for 15 seconds.

Fermented milk production

Three types of sourdough were prepared for the production of various variants of fermented milk enriched with honey. We developed these sourdoughs by fermenting 500 ml of skimmed milk with the different cultures at 37°C until the target acidity of 80° Dornic was reached.

Leaven A consisted of a mixed culture of *Streptococcus thermophilus* and *Lactobacillus bulgaricus* at a concentration of 3%. Leaven B consisted of a mixed culture of *Streptococcus thermophilus* (2%) and *Lactobacillus plantarum* (1%). Leaven C consisted of a 3% *Lactobacillus plantarum* culture. Milk enriched with 5% honey was distributed in aseptic jars. Each jar of honey-enriched milk was inoculated with one of the leavens, while the control group consisted of honey-free milk inoculated with a mixture of *Streptococcus thermophilus* and *Lactobacillus bulgaricus*. Four varieties of fermented milk were produced: milk fermented with 3%

of the mixed culture with honey (Product P1), milk fermented with a sourdough composed of 3% *Lactobacillus plantarum* (Product P2), milk fermented with honey with a sourdough composed of 2% *Streptococcus thermophilus* and 1% *Lactobacillus plantarum* (Product P3) and natural milk fermented without honey (Product P4).

Determining the viability of lactic acid bacteria after fermentation

The viability of lactic acid bacteria in the various fermented milks was assessed by measuring the number of viable lactic acid bacteria, from day 1 of milk fermentation through to day 15, the cut-off date for storage at 4°C. Dilutions were prepared from various fermented milk samples for enumeration of lactic acid bacteria on Man, Rogosa and Sharpe (MRS) agar from Biokar, France. Dilutions (0.1 ml) were inoculated using the MRS spreading plate. Inoculated Petri dishes were incubated at 37°C for 72 h under anaerobic conditions, obtained by deoxygenating the incubation enclosure.

Statistical analysis

The study of the similarity of the means of the results of the influence of the studied supplementation of natural honey in milk on the growth and viability of lactic acid bacteria was made possible by the use of a statistical test in SYSTAT MYSTAT 13 SOFTWARE. The level of statistical significance is estimated at $P<0.05$.

RESULTS AND DISCUSSION

Physico-chemical analysis

The results obtained for the physico-chemical analysis of our polyfloral honey sample are shown in Table (1).

The water content of the honey sample analyzed was 18.6%. This parameter is essential for assessing honey quality, maturity and shelf life (Islam *et al.,* 2021). Excessive moisture can lead to fermentation, loss of flavor and quality and accelerated crystallization (Mouhoubi- Taffinine *et al.,* 2018). Regulatory standards stipulate that honey should not exceed 20% moisture, hence our sample's compliance with Codex Alimentarius (2001) standards. Factors influencing moisture content are climatic conditions, extraction and storage practices and the beekeeper's management methods, as well as the harvesting season (Bonsignore *et al.,* 2024).

Honey is generally acidic due to the presence of organic acids such as gluconic acid, acetic acid and citric acid and pH serves as an indicator of its floral origin (Sousa *et al.,* 2016). According to Codex Alimentarius standards, the pH of nectar-derived honey is between 3.5 and 4.5, while honeydew-derived honey is generally between 5.0 and 5.5. The pH of our sample was 4.0, indicating that it was a nectar honey.

The electrical conductivity (EC) of the honey sample analyzed was 0.500 mS/cm, indicating good quality and compliance with Codex Alimentarius standards for nectar honeys, which recommend values <0.8 mS/cm. The conductivity level reflects the mineral content, which influences the ionic composition of honey (IHC, 2009). Factors such as the presence of pollen and organic acids can also affect conductivity, reinforcing the importance of this parameter in honey classification (Suto *et al.,* 2020).

Results for hydroxymethylfurfural (HMF) content, a marker of honey quality, show a value of 13.51 mg/kg for the sample used. This level complies with honey quality standards, which set a threshold of 40 mg/kg (Codex Alimentarius, 2001). It is important to note that HMF concentration can increase in the event of excessive heat treatment or prolonged storage (Mouhoubi *et al.,* 2018). These results indicate that appropriate practices were followed during honey extraction and storage.

The results for sugar content are shown in Table (2). Total reducing sugars indicate a crucial aspect of honey quality. The observed predominance of fructose and glucose is in line with the findings of Susilowati et Azkia (2022), who stress the importance of sugar composition in determining the physical and chemical properties of honey. Reducing sugars (Glucose G+ Fructose F) were found to conform to standard values, generally suggesting a total reducing sugar content of at least 60% for honey. The observed F/G ratio of 1.68 indicates a potential tendency towards crystallization, since a higher fructose content relative to glucose can influence honey stability and texture (Bonsignore *et al.,* 2024).

The sucrose content of the honey sample analyzed was 1.2%. This value is below the maximum limit of 5% set by Codex Alimentarius (2001), indicating that the honey has not been adulterated and meets authenticity criteria. In our study, the maltose concentration was 3%. High maltose concentrations, above 50 mg/g, may indicate potential adulteration, as some beekeepers use maltose as an additive to enhance sweetness (Islam *et al.,* 2021). Turanose and gentiobiose were not detected in the sample, although these disaccharides are commonly analyzed and frequently found in honeydew (Mouhoubi-Taffinine *et al.,* 2018).

Oligosaccharides, which are more complex sugars with more than two rings, are usually formed by the interaction of bee enzymes and are often associated with honeydew or indicate adulteration (Islam *et al.,* 2021). In

Table 1: Average results with standard deviation from physico-chemical analysis of polyfloral honey sample.

Parameters	pH	Moisture (%)	EC (mS/cm)	HMF
honey Sample	4±0.01	18.4±0.01	0.55±0.01	13.51±0.05

EC : Electrical conductivity ; HMF : Hydroxy-methyl-furfural.

Average results from 05 repetitions.

our results, no trisaccharides were detected with the exception of raffinose. The presence of oligosaccharides, such as raffinose, albeit in low concentrations, may contribute to the biological properties of honey, including its antioxidant activity and potential prebiotic effects (Susilowati *et al.,* 2022). These sugar compositions may also reflect honey's floral sources and processing methods, influencing both its sensory attributes and its health benefits (Susilowati *et al.,* 2022; Wang *et al.,* 2021).

Assessment of honey addition on Lactobacillus plantarum strain growth

On Fig 1, in the absence of honey, the *Lactobacillus plantarum* strain prepared in sourdough showed slow growth, with biomass rising from 3×10^4 to 10^5 CFU/ml after 24 hours and reaching 2.2×10^7 CFU/ml after 48 hours. However, the addition of honey favored bacterial growth. After 24 hours, the highest proliferation was observed in milk supplemented with 5% honey (8.9×10^7 CFU/ml), followed by 10% honey (2.8×10^7 CFU/ml) and 1% honey (2.1×10^7 CFU/ml). At the end of fermentation, sourdough biomass reached 2.46×10^8 CFU/ml with 5% honey, compared with 2.18×10^8 CFU/ml with 10% honey and 8.7×10^7 CFU/ml with 1% honey.

Table 2: Analysis of the average reducing sugar content of polyfloral honey.

Total reducing sugars	% content
Glucose + Fructose (G+F)	63.6
Fructose/ Glucose (F/G)	1.68
Fructose	39.8
Glucose	23.6
Sucrose	1.2
Turanose	1.4
Maltose	3
Melezitose	N.D
Maltotriose	N.D
Gentiobiose	N.D
Raffinose	0.8
Mannose	N.D

Average results from 05 repetitions, ND: Not defined.

These results are consistent with several studies that have demonstrated the prebiotic potential of honey to stimulate the growth of probiotic lactic acid bacteria, in particular *Lactobacillus plantarum* (Bhola *et al.,* 2023 ; Machado *et al.,* 2017). Honey is an essential energy source due to its high glucose, fructose and fructo-oligosaccharide (FOS) content. In addition, its low pH and organic acids, such as acetic and citric acids, create a favorable environment for *Lactobacillus* while inhibiting the proliferation of pathogenic microorganisms (Bonsignore *et al.,* 2024).

Interestingly, milk supplemented with 5% honey showed the highest bacterial growth, suggesting an optimal balance between nutrient availability and osmotic effects. A higher concentration (10%) did not promote growth, possibly indicating an inhibitory effect linked to increased sugar concentration or osmolarity. This result aligns with previous research highlighting the importance of optimal honey concentration for probiotic stimulation (Mouhoubi *et al.,* 2018).

Although milk makes a good culture medium, its limited nitrogen and carbon sources can restrict bacterial growth (Bonnet *et al.,* 2019). The addition of honey improves the nutritional quality of the medium, thus favoring the development of probiotics. In addition, its enzymatic production of hydrogen peroxide enhances the ability of *Lactobacillus plantarum* to compete with undesirable microorganisms (Sari *et al.,* 2020).

The prebiotic effects of honey are largely attributed to its oligosaccharide composition, which varies according to its botanical and geographical origin (IHC, 2009). These oligosaccharides selectively promote the growth of beneficial bacteria such as *Bifidobacterium* and *Lactobacillus*, while suppressing potentially harmful species such as Bacteroides and Clostridia (Kowalska *et al.,* 2024).

Fermented milk production

Based on preliminary tests, milk with 5% polyfloral honey added was selected for the production of fermented milk.

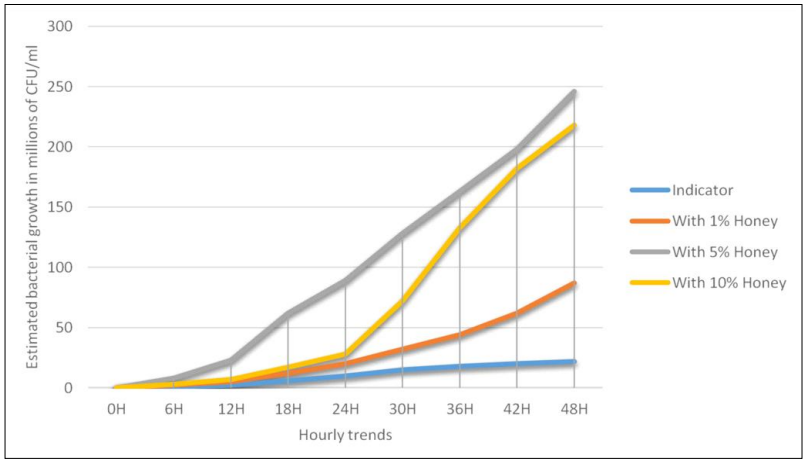

Fig 1: Assessment of *Lactobacillus plantarum* strain growth in prepared sourdough.

The final products had a firm texture, with no change in appearance except for a slight change in color, probably due to the natural pigments and chemical reactions induced by the honey during fermentation. Replacing *Lactobacillus bulgaricus* with *Lactobacillus plantarum* did not affect the final appearance, indicating that the biotechnological profile of this strain is suitable for lactic fermentation. However, fermentation with *Lactobacillus plantarum* alone resulted in a brittle and less cohesive texture, underlining the need for co-culture with other lactic acid bacteria. These results align with those of Machado *et al*. (2017). who reported that the addition of bee honey to fermented goat's milk influenced acidity over time without compromising sensory acceptability. In addition, the presence of flavonoids, phenolic compounds and organic acids in honey has been shown to interact with milk proteins, potentially affecting viscosity, creaminess and stability (Sousa *et al.,* 2016).

Viability of lactic acid bacteria in post-fermented milk

Total lactic acid flora was higher in milk fermented with honey than in natural milk without honey. In Fig 2, the highest bacterial count was observed in milk fermented with honey (1.74×10^6 CFU/ml), followed by mixed culture of fermented milk with honey (1.66×10^6 CFU/ml) and monoculture of *Lactobacillus plantarum* with honey (1.62×10^6 CFU/ml). In contrast, natural fermented milk without honey showed a lower bacterial load (1.55×10^6 CFU/ml) on the first day of storage. These results indicate that honey promotes bacterial viability, in line with previous studies demonstrating its protective effect on probiotic lactic acid bacteria cultures (Landry *et al.,* 2018).

After a fortnight's storage, bacterial populations decreased in all samples produced, but survival rates varied. P3, milk fermented with honey maintained the highest viability (72.41% with 1.26×10^6 CFU/ml), followed by P2, milk fermented with *L. plantarum* (69.14% with 1.12×10^6 CFU/ml), P1 milk fermented with mixed culture (49.40% with 0.82×10^6 CFU/ml) and P4, natural fermented milk without honey (32.90% with 0.51×10^6 CFU/ml). Similar trends were observed by Machado *et al*. (2017), where honey enhanced the viability of *Lactobacillus acidophilus* in a yogurt-like fermented milk, maintaining counts above 10^6 CFU/g until the 28th of its production.

In fact, to promote health benefits for consumers, a 100 g serving of dairy product must contain at least 10^6 to 10^7 CFU/g or mL of viable probiotic bacteria on the best-before date (IDF, 2020).

Microbial survival is influenced by nutrients, inhibitory compounds, fermentation time and storage conditions (Adriani *et al.,* 2024). Previous studies have shown that honey can stabilize probiotic bacteria in dairy products. For example, Caldeira *et al*. (2018) reported that incorporating 5-10% Africanized bee honey and 10-15% Jata bee honey into bioyogurt maintained viable probiotic bacteria for 35 days under refrigeration. The protective effect of honey depends on its floral origin, as variations in oligosaccharides and polyphenols influence its prebiotic properties (Wang *et al.,* 2021). Sugars in honey, such as sucrose, lactose, glucose and fructose, not only contribute to sweetness, but also serve as energy sources for microbial metabolism, promoting bacterial growth and the stability of fermented dairy products (Sari *et al.,* 2020; Wang *et al.,* 2021).

Statistical analysis

Statistical analysis using Systat Mystat 13 software to assess the growth kinetics of lactic acid and probiotic bacteria produced significant $P<0.05$ values. The formulation of product 3, fermented milk with 5% honey using a sourdough composed of 2% *Streptococcus thermophilus* and 1% *Lactobacillus plantarum*, was best suited to lactic fermentation and the production of a typical fermented milk. The viability of the bacterial cells was consistent with technological application and bioconservation of the product with DLC and consumer satisfaction with rheological qualities typical of local fermented milks (Dahou *et al.,* 2024). On the other hand, the post-fermentation activity for storage at 4°C, the incorporation of honey as a 5% prebiotic and the mixture of *Lactobacillus plantarum* with *Streptococcus thermophilus* enabled lactic acid synthesis to continue even after 15 days of production.

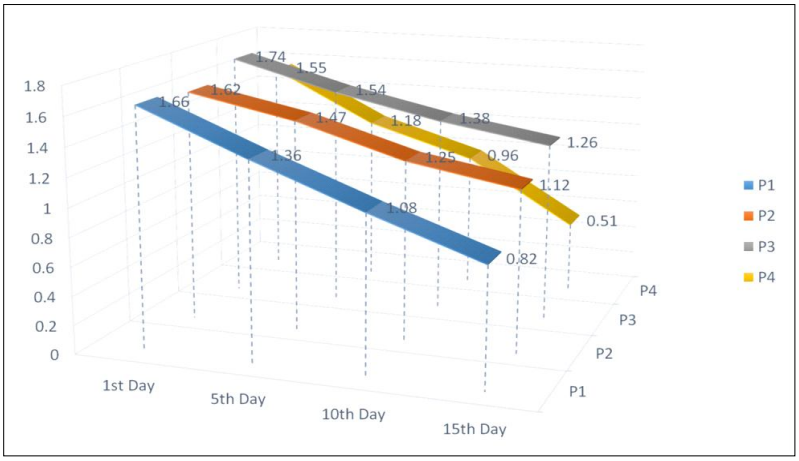

Fig 2: Viability of lactic acid bacteria on post-fermented milks.

With this culture combination, the number of viable bacterial cells was significantly improved by the presence of honey in the fermented milk and far exceeded ($P<0.05$) the level required by international legislation (IDF, 2020) with a count > to 10^6 CFU/ml.

This improvement compared to the control product P4 (without honey) is a significant difference with a rate of 39.51% giving a cell viability of 72.41% for P3 compared to 32.90% for P4. These results confirm the protective effect of polyfloral honey, on the one hand on growth and on the other hand on the viability of lactic ferments during lactic fermentation and storage of the fermented milks produced.

CONCLUSION

The incorporation of honey in the manufacture of fermented dairy products is of considerable interest for the growth of lactic acid bacteria and probiotics and on the other hand enables their survival to ensure the controlled post-acidification necessary to preserve fermented dairy products against biochemical alterations in storage. These results suggest that polyfloral honey has significant functional potential for fermented dairy products, indicating its potential use in the food industry to improve both the technological abilities, bio-protective activities, probiotic properties and rheological qualities of finished products.

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Disclaimers

The views and conclusions expressed in this article are solely those of the authors and do not necessarily represent the views of their affiliated institutions. The authors are responsible for the accuracy and completeness of the information provided, but do not accept any liability for any direct or indirect losses resulting from the use of this content.

Conflict of interest

The authors declare no conflict of interest.

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