Honey as a natural preservative of milk

N S A Krushna, A Kowsalya, S Radha & R B Narayanan*
Centre for Biotechnology, Anna University, Chennai 600 025, India

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The anti-bacterial property and preservative nature of honey has been studied by evaluating the role of hydrogen peroxide in these properties, against bacterial strains isolated and identified from pasteurized milk samples. The anti-bacterial property of honey examined by agar incorporation assay and turbidometry, indicated a concentration dependent inhibition of bacterial growth in all catalase negative strains in comparison with catalase positive strains, highlighting a probable role of hydrogen peroxide. Samples of commercial milk stored at 4°C in presence of honey were shown to inhibit opportunistic bacterial growth better compared to samples stored without honey. Due to the bactericidal property of hydrogen peroxide and its preservative nature, honey which is chiefly a combination of various sugars and hydrogen peroxide, can be used a preservative of milk samples.

Keywords: Antibacterial activity, Honey, Hydrogen peroxide, Preservative

Milk and other dairy related products comprise a major chunk of food products for their nutritive values. However, the rich source of proteins and vitamins in them turns out to be a very good growth medium for several pathogenic microorganisms. Members of Klebsiella, Bacillus, Pseudomonas, and Staphylococcus are some of the most commonly encountered bacterial species in contaminated milk. The preservation of milk is a major bottleneck especially where, refrigeration facilities are a limitation or in places where the ambient temperatures exceed 30°C. Preservation of food products aims in maintaining quality, physico-chemical properties and functionality by providing safe products having low spoilage potential during their shelf life. On the basis of the inhibitory activity on food-borne pathogens, it was suggested that antioxidants were the significant contributors in non-peroxide mediated bacteriostatic/bactericidal activity of honey.

Hydrogen peroxide was found to prevent the souring of raw milk and was used for preservation of milk in cheese making. It is a potent anti-microbial agent that acts in a concentration dependent fashion, and is influenced by environmental factors such as pH and temperature. The destruction of approximately 85% of the total bacterial flora of the milk may be attained through the addition of 0.2% edible hydrogen peroxide solution (30-35% hydrogen peroxide) at 48.9°C (120°F) for 10-15 min prior to the inactivation of hydrogen peroxide with catalase.

Numerous studies on the inhibitory activity of honey on clinically significant bacteria have been reviewed. The anti-microbial activity of honey can be grouped into peroxide component and non-peroxide components. The major anti-bacterial factor in honey is hydrogen peroxide, which is formed due to the oxidation of glucose and other monosaccharides by glucose oxidase present in the hypopharyngeal glands of the honey bees. The non-peroxide factors contributing to the anti-bacterial property of honey are the components like lysozyme, phenolic acids, flavonoids and a number of aromatic acids, of which syringic acid and phenyllactic acid are most abundant. Other phenolic components having antioxidant property are known to inhibit the growth of a wide range of gram-negative and gram-positive bacteria.

In the present study, an attempt has been made to study the anti-bacterial property of honey in relation to hydrogen peroxide and its potential applicability as a natural preservative of milk samples.

Materials and Methods

Chemicals — Microbiological media and/or individual components of agar and broths of Luria-Bertani and Nutrient agar were from HiMedia Laboratories, Mumbai, India. Common chemicals,
salts and sugars unless mentioned were from HiMedia Laboratories, Mumbai, India or Sisco Research Laboratories, Mumbai, India.

*Milk and honey* — Four commercially available pasteurized milk samples were obtained and refrigerated at 4°C until further use. Samples stored from 1 to 4 days were serially diluted and the isolated pure cultures were used for further analyses.

Honey was obtained commercially and dilutions of honey (Table 1) were made in sterile water with or without heating at 100°C for 10 min in water bath. The honey solutions were freshly prepared for each assay to make sure that the hydrogen peroxide that is accumulated upon the dilution of honey remains viable. This is very important considering the fact that the present study has been carried out to assess the role of hydrogen peroxide in the antibacterial activity of honey and thus its preservative nature. Artificial honey [80% (w/v) sugar] was prepared as described. This was used as a control as it reflects the sugar composition in most of the honeys. Sterile water served as a negative control for milk and honey samples.

To evaluate the potential of honey in preserving milk, different dilutions of honey were added to the diluted milk samples and checked for spoilage of milk.

Isolation of bacterial cultures — To enumerate and isolate the bacteria in the milk samples, 1ml of refrigerated milk samples were serially diluted (10^{-1} to 10^{-7}) in sterile water and 200μl of samples were plated onto nutrient agar plates and incubated at 37°C for 24 hr. The randomly picked colonies were re-inoculated in 15 ml Luria-Bertini broth and incubated for 14-16 hr at 37°C. The culture thus obtained from individual colonies, identified from milk were characterized, using IMViC test, starch hydrolysis test, catalase test, TSI agar test, Carbohydrate fermentation test and motility was examined by hanging drop method as per standard protocols.

Disc diffusion assay for inhibitory activity — Sterile filter discs (10 mm, diameter) were immersed in 5μl diluted honey solutions (Table 1), and air-dried. The cells were harvested from the cultures grown to mid log phase and the pallet was suspended in 3 ml fresh LB medium to remove any traces of dead cells or their products. From this culture, 200 μl corresponding to 1×10^7 CFU/ml was plated on LB plates. The discs containing honey of different concentrations were placed on the culture plates, and incubated at 37°C for 24 hr. The diameter of zones of inhibition were recorded. All the species considered for the present study were used and the assay was done in triplicates.

**Honey inhibits growth of both catalase positive and negative bacteria** — Cultures of bacteria isolated from milk were grown to mid log phase with a density of 1×10^7 CFU/ml and 5% of this pre inoculam was inoculated into 15 ml nutrient broth and each flask was supplemented with honey of different concentrations (100, 200, 300, 400 and 500mg/ml). The cultures were incubated at 37°C for 24 hr and the effect of honey on the growth of catalase positive and catalase negative was assessed by monitoring the change in the absorbance at 550 nm using a photoelectric colorimeter (Systronics). Sterile water, instead of honey was used as a negative control and the assay was done in triplicate with all catalase negative and catalase positive bacteria identified from milk.

**Honey as a preservative** — Studies on the preservative use of honey were done by monitoring the bacterial growth in 500 ml of milk samples that were stored with 100 μl of 500 mg/ml solution of honey added, at 4°C for 3-6 days and it was compared with a milk sample stored with out honey. The bacterial growth was quantitated by measuring the absorbance of 50 ml nutrient broth (550 nm) inoculated with 100 μl milk sample supplemented with honey and a similar volume of the same milk sample with out honey at 24 hr intervals from 3-6 days. In the present experiment the absorbance was taken after incubating the cultures for 10 hr in order to make sure that the cultures do not grow beyond the exponential phase.

**Results**

Spoilage of milk during shelf-life — Four locally available and widely used commercial milk samples stored at 4°C over a period of 4 days were evaluated
for contaminating bacterial species by serially diluting
the samples and plating them on nutrient agar plates.
Mean of the number of bacterial colonies obtained
with all the four milk samples at various dilutions
from day 1 to 4 is shown in Table 2. This indicates an
increase in the growth of bacterial species with time.
Sterile water (used for dilutions) used as negative
control, did not yield any bacterial colonies,
indicating milk as the source of microbial
contamination. In order to test the samples for proper
pasteurization, milk samples were tested for
phosphatase activity by phosphatase test11, an
indicator for effective pasteurization. All the milk
samples tested, were negative for phosphatase
activity.

Isolation and characterization of bacteria — The
bacterial colonies were randomly picked and five
selected colonies from each milk sample and dilutions
were isolated for pure cultures. The pure cultures
were characterized based on the morphological
characteristics, biochemical and microbiological tests.
The most common bacterial species were found to be
Bacillus sp., Staphylococcus sp., Pseudomonas sp.,
and Klebsiella sps. However, in the context of the
present study, the organisms were broadly classified
into catalase positive and catalase negative based on
the results of catalase test, where the bacterial species
identified from milk were monitored for their ability
to produce oxygen bubbles when hydrogen peroxide
was added to the slide containing the bacteria.

Disc diffusion assay — The growth of the bacterial
cultures in presence of discs containing various
concentrations of honey was measured by the zones
of inhibition. The presence and diameter of the zones
of inhibition were found to depend more on the
bacterial species and the concentration of honey.
Mean diameters of the zone of inhibition obtained
with all the bacterial species identified from milk in
presence of different concentrations of honey are
presented in Table 3. Honey was found to inhibit
catalase negative cultures, while catalase positive
cultures were not inhibited to the same extent. These
results suggest that the anti-bacterial activity of honey
on catalase negative bacteria could be due to
hydrogen peroxide. In order to confirm that this anti-
bacterial activity is due to an active component in
honey, artificial honey (equivalent to honey) was used
at various concentrations. It was found that artificial
honey had no inhibitory effect on the growth of either
catalase positive or negative bacteria. Further, the

| Table 2 — Number of bacterial colonies in milk samples
| [Values are mean ± SD] |
|----------------------|---------------------|----------------|----------------|
| Dilution  | Day-1  | Day-2  | Day-3  | Day-4  |
| 10⁻¹   | 16±6   | 38±11  | 159±10 | 207±17 |
| 10⁻²   | 3      | 4±3    | 95±7   | 132±19 |
| 10⁻³   | 2±1    | 5±2    | 63±12  | 86±12  |
| 10⁻⁴   | 1      | 1      | 41±13  | 53±16  |
| 10⁻⁵   | 0      | 0      | 19±7   | 30±12  |
| 10⁻⁶   | 0      | 0      | 11±3   | 16±8   |
| Water  | 0      | 0      | 0      | 0      |

| Table 3 — Inhibitory action of honey on catalase negative and catalase positive bacteria
| [Values are mean ± SD] |
|-----------------------------|-----------------------------|-----------------------------|
| Concentration (mg/ml) of honey used  | Diameter (mm) of zone of inhibition with catalase negative bacteria | Diameter (mm) of zone of inhibition with catalase positive bacteria |
| 5                             | 10.3±4                      | ---                        |
| 10                            | 10.6±4                      | ---                        |
| 15                            | 12.6±4                      | ---                        |
| 20                            | 15.6±5                      | ---                        |
| 25                            | 18.6±6                      | ---                        |

(---)Indicates the absence of zone of inhibition

anti-bacterial activity of honey was not observed
when honey was subjected to 100°C for 10 min
(data not shown). Sterile water used as negative
controls did have any inhibitory effect on the growth
of the bacteria.

Honey inhibits growth of both catalase positive and negative bacteria — In order to evaluate whether the
addition of honey to the bacterial cultures has any
effect on their growth, honey was added at various
concentrations to the isolated pure cultures and the
turbidity was measured after incubating at 37°C over
a period of 24 hr. The inhibition of growth increased
in a concentration dependent manner at 24 hr
incubation in catalase negative cultures containing
honey, with a percentage inhibition averaging around
50-60% (Fig.1a). Sterile water when used instead of
honey showed a drastic increase in turbidity in
comparison to cultures with honey.

A similar study done with catalase positive
bacteria, with the same concentrations of honey, did
not reveal a similar extent of inhibition of growth, that
was observed with catalase negative bacteria in
comparison with water. The decrease in this case was
only around 4-35% (Fig. 1b). Nevertheless, an
increase in the percentage inhibition with the increase
in the concentration of honey was observed even in
this case, as with catalase negative bacteria
highlighting the bactericidal nature of honey even in
conditions where the hydrogen peroxide in the honey is enzymatically broken down.

**Honey as a preservative** — In an attempt to evaluate the possible use of honey as a natural preservative for milk, milk samples stored at 4°C from 3 to 6 days in the presence or absence of honey were assayed for their bacterial content and growth. The addition of honey at a final concentration of 50mg/ml had a considerable inhibitory effect (50-55% inhibition) on the growth of bacteria in comparison to milk samples devoid of honey (Fig. 2 and Table 4).

**Discussion**

Milk from various sources has been used as food since pre-historic times. Although much of it is consumed with minimal processing, highly processed milk products and components find usage in several foods. Due to the presence of several nutrients, care should be taken while drawing and in the proper storage of milk, to avoid contamination with pathogenic organisms. The preservation of milk in the developing countries presents a problem in that, the ambient temperatures tend to be high with limited refrigeration facilities. The results from the present study suggest that very low levels of microbial contamination in milk samples that escape detection by phosphatase detection, could infact result in the spoilage of milk samples during shelf life. This poses an additional problem especially in conditions, where the cold chain is a limiting factor.

The lactoperoxidase (LP) system is naturally occurring antimicrobial system in raw milk, which is active against both gram-positive and gram-negative microbes to varying extents. The system requires hydrogen peroxide and thiocyanate for optimal activity and is thus primarily active against microorganisms producing H₂O₂. Many attempts have been made to use the lactoperoxidase system in the preservation of raw milk and to manufacture products from such preserved milk. The preservative action of the LP system in bovine milk has been well-established, and it was reported that activation of the system depends on the concentration of the two reactants, thiocyanate (SCN⁻) and hydrogen peroxide H₂O₂. In particular, the LP system has the ability to catalyze the oxidation of thiocyanate by hydrogen peroxide with the production of the antibacterial hypothiocyanite (OSCN⁻) and other intermediates. These compounds, which can be further oxidized to end products that are harmless to humans, have the ability to reduce bacterial growth by damaging the cell membranes and inhibiting the activity of many cytoplasmic enzymes. Hydrogen peroxide is not known to occur naturally in milk, but is produced in...
milk via microbial metabolism. Therefore only organisms which produce hydrogen peroxide are generally inhibited by lactoperoxidase system. Non-hydrogen peroxide producing organisms are generally less sensitive to lactoperoxidase system, but if a source of hydrogen peroxide is added some, including susceptible catalase positive strains are inhibited or killed.

Lactoperoxidase is heat sensitive but retains the majority of its activity in milk, which has been pasteurized at 72°C/15 sec. However, as the temperature is increased, the LP activity decreases rapidly until it cannot be detected following treatment at approximately 80°C. It can therefore be postulated that if the LP system remains active in a pasteurized milk sample, it is able to exert some effect on the flora therein.

Therefore the preservation of milk samples with hydrogen peroxide involves the activation of lactoperoxidase system in milk by hydrogen peroxide resulting in effective antibacterial activity aiding in preservation. But FAO/WHO strongly discourages the chemical preservation of milk, except the application of H₂O₂, which again needs to be completely destroyed before consumption either by heat treatment or by catalase. However, the increasing negative awareness regarding the usage of chemical preservatives, and effectiveness of the naturally occurring anti-microbial molecules has targeted research at finding alternative approaches for quality, preservative-free, safe processed foods with extended shelf life. The therapeutic applicability of natural products in the fields of Ayurvedha and Sidha medicine has drawn significant interest in the global scientific fraternity. Honey has been one such product that has the ability to promote wound healing which is mediated by the anti-bacterial property of hydrogen peroxide. Honey has been an effective remedy in the clearing of bacterial infections in ulcers, abscesses etc., and in dyspepsia. A study with the effect of honey on bacteria isolated from wounds confirming the bactericidal property and thus its medicinal value has been done.

The antimicrobial activity of honeys against several bacterial species known to cause spoilage of food has been found to be variable and the anti bacterial activity of honeys on several food borne pathogens has been proved. But there are no evidences showing honey as a preservative due its bactericidal activity. In the present study, to address the susceptibility of contaminating microorganisms to hydrogen peroxide in honey, the isolated bacteria have been classified as catalase positive or negative.

Milk upon storage is safe to consume till 24-72 hr as evidenced by a lesser number of bacteria on LB plates, plated with 1 to 3 day old milk samples. An increase in the bacterial numbers in milk samples after 3 days of storage, demonstrates the degree of bacterial contamination of stored milk samples. Several studies in the past have used disc diffusion assays to determine the antimicrobial activity of honey.

In the present study, various dilutions of honey were analysed, with the idea of evaluating the least concentrations of honey that inhibits at least 50% growth. The inhibition was found to be concentration dependent and more specific on catalase negative bacteria compared to catalase positive bacteria. The results of turbidity assays (550 nm) further prove the previous findings on the role of hydrogen peroxide in antibacterial property of honey, where an increase in the percentage inhibition, with the increase in the concentration of honey was observed. The catalase positive bacteria under the same experimental conditions did not show an appreciable decrease in growth with an increase in the concentration of honey like the catalase negative bacteria, but a concentration dependent reduction in the percentage inhibition was found even when the total hydrogen peroxide of honey was presumed to be broken down by catalase produced by the bacteria. Catalase positive bacteria were found to be more resistant to low concentrations of honey than catalase negative ones were, but were susceptible to higher concentrations of honey. This reduction in the absorbance, with an increase in the concentration of honey used even in the case of catalase positive bacteria may be attributed to the residual non-peroxide antibacterial activity observed in honey. Previous studies along with present findings indicate, “far greater amounts of catalase were required to destroy hydrogen peroxide than indicated by the total amount of hydrogen peroxide produced.” Moreover it has also been demonstrated that catalase is not effective at destroying physiological levels of hydrogen peroxide, which may have accounted for the current observations with catalase positive bacteria. In this context, identical concentrations of honey were not considered for disc inhibition assay and turbidity assay because of the fact that the concentrations used in disc inhibition assay showed a very minimal inhibition in turbidity.
 assay with catalase positive and catalase negative bacteria (data not shown)

Several authors 26-33 are of the opinion that sugar content of the honey is exclusively responsible for the anti bacterial effect. To rule out the role of fructose, as the main component involved in the antibacterial activity in the present study, a minimum inhibitory concentration assay was done using 80% (w/v) fructose syrup (artificial honey) instead of honey. No inhibition was observed with any of the concentrations used, though the concentrations were similar to honey used in all the previous experiments, thus confirming the role of hydrogen peroxide in the present study. The turbidity assay (550 nm) carried out with LB broth inoculated with milk samples stored for different time periods (3, 4, 5 & 6 days) in the presence of honey, showed around 50-55% decrease in turbidity compared to the samples that were preserved with out honey. Thus the findings of the present study, evaluating the role of both peroxide and the non-peroxide components of honey, in the anti bacterial activity confirmed its role as a safe and an effective preservative of milk samples and it is presumed that honey provides hydrogen peroxide as a substrate for the lactoperoxidase of milk thus, generating the antibacterial activity aiding in effective preservation.

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