Enhancement of probiotic bioactivity by some prebiotics to produce bio-fermented milk

Amnah A. H. Rayes

Biology Department - Faculty of Applied Science, Umm Al-Qura University Kingdom of Saudi Arabia
ahlel7aei@gmail.com; rayes1025@hotmail.com

Abstract: Fermented milk Samples were prepared by adding 3% (w/v) of honey I, II or Inulin to cow's milk, and 2% starter fermented milk. The culture consisted of *Lactobacillus delbreuckii* subspp *bulgaricus* and *Streptococcu thermophilus* 1:1 plus 5% *(Bifidobacterium bifidum* (*B. bifidum) or 2% Lactobacillus rhamnosus* or2 % *Lactobacillus reuteri).* Lactic acid bacterial count, acetaldehyde, acidity as lactic acid values and organoleptic evaluation when fresh and during storage were determined. Counts of LAB and probiotic strains reached their maximum on the 5th days of storage in different samples. Honey II had highest effect on the growth and viability of probiotic strains. All fermented milk samples with prebiotic substances had a high scores compared to controls. All treatments with *B. bifidum* had higher organoleptic scores. Non-significant differences between effects of honey I, II or inulin on organoleptic scores.
[Amnah A. H. Rayes. **Enhancement of probiotic bioactivity by some prebiotics to produce bio-fermented milk**. *Life Sci J* 2012;9(3):2246-2253] (ISSN:1097-8135). http://www.lifesciencesite.com. 322

Key words: Function food, Probiotic, prebiotic, honey, inulin.

1-Introduction

Probiotics are technically defined as live microbial food ingredients that have a beneficial effect on human health. Some of the important beneficial effects are antimicrobial activity, immune system modulation, antimutagenic activity, colonization resistance activity, maintenance of micro-ecology of bowel, stimulation of *Bifidobacteria,* deactivation of carcinogens etc. Commercially available probiotic strains belong to genera *Lactobacilli*, *Bifidobacterium, Streptococcus, Bacillus, Bacteriodes, Pediococcus, Leuconostoc, Propionibacteruim* **(Douglas & Sanders, 2008; Meile, et al., 2008; and Tharmaraj, et al., 2004),** *Saccharomyces cerevisia* and *Aspergillus oryzae* **(Verma & Singh,1995)**

Dairy products are the most common carrier that have been used as probiotic food products **(Lourens-Hattingh and Viljoen, 2001; TianHong and XiangChen, 2004).**

Some Lactobacillus spp. and som *Bifidobacterium spp*. are considered sensitive in dairy products, but this sensitivity was different in different strains (**Nighswonger *et al.*, 1996; Gilliland *et al.*, 2002)**. However, it has found that Bifidobacteria have been reported to show weak growth in milk and require an anaerobic environment and the addition of bifidogenic factors (as prebiotic) to achieve the desired levels of growth **(Kailasapathy and Chin, 2000; Lourens-Hattingh and Viljoen, 2001; Matto *et al.*, 2006b; Donkor *et al.*, 2007).**

Prebiotics are nondigestible food ingredients that beneficially affect the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in colon that can improve the host health **(Nimess, 1999).**

When prebiotics are used in combination with probiotics or live bacteria, the resultant has synergistic effects, referred to as "synbiotic". This is because in addition to the action of probiotics that promote the growth of existing strains of beneficial bacteria in the colon, prebiotics such as inulin and oligofructose also act to improve the survival, implantation and growth of newly added probiotics strains.

Inulin is a mixture of polymers consisting mainly of fructose unit; its partial enzymatic hydrolysis yields oligofructose (**Amiri et al., 2010**). A lot of scientific studies *in vivo* have shown that FOS and Inulin have bifidogenic effect on host; When consumed at a dose of 5g/day for oligofructose and ≤ 8 g/day for inulin, they significantly modify the composition of the intestinal (faecal) flora, selectively increasing the numbers of Bifidobacteria and reducing the harmful bacteria (**Gibson and Wang, 1994b; Reddy *et al.* 1997; Roberfroid *et al.* 1998; Rao. 1999; Tuohy *et al.* 2000; Menne *et al.* 2000, Mehanna, et al., 2003a)**

As a prebiotic, honey contains carbohydrates called oligosaccharides, which may improve gastrointestinal health by stimulating the growth of good bacteria in the colon. Honey has been shown to enhance growth, activity of *Bifidobacteria* in fermented dairy food **(Mehanna et al., 2003b; Sanze *et al*, 2005)**

The consumption of probiotic bacteria within food products is the most popular way to re-establish the gastrointestinal microflora balance. (**Adhikari *et al.*, 2003).**

The aim of this research is enhancement of probiotic bioactivity by some prebiotics to produce bio-fermented milk; this is because in addition to the

action of probiotics that promote the growth of existing strains of beneficial bacteria in the colon, also it acts to improve the survival, implantation and growth of newly added probiotics strains. Thus this study showed which one of prebiotic substances when added to milk maintains a viable, large population during refrigerated storage, not less than 10^6 in order to meet the requirement of a "probiotic" food (**Rybka and Kailasapathy, 1995; Dave and Shah, 1997; Lourens-Hattingh and Viljoen, 2001; Adhikari *et al.*, 2003)**

2. Materials And Methods

Bacterial strains:

Streptococcus thermophilus, Lactobacillus delbreuckii subsp. Bulgaricus and *Bifidobacterium bifidum* were obtained from Chr. Hansen's Lab., Denmark. Strain of *Lactobacillus reuteri, B-14171and Lactobacillus rhamnosus B-445* were provided by Northein Regienal Research Lab., Illinois, USA, (NRRL).

Prebiotics:

Inulin: was obtained from Canada market. Chemical composition of it was 91(±2.0) %

Honey: Two kinds from row Honey were obtained from Kingdom of Saudi Arabian markets**.**

Milk: Fresh whole cow's milk was obtained from Kingdom of Saudi Arabian markets**.**

Expermintal procedure

Preparation of fermented milk with different additives:

Samples were prepared by adding 3 percent (w/v) of honey I, II or Inulin to cow's milk as described by **Mehanna (2003a,b).** A control of cow's milk without any additives was also prepared. All samples were heated to 85° C for 30 min., cooled to 42° C and each sample was divided into three portions. The first portion was inoculated with 2% starter fermented milk + 5% *Bifidobacterium bifidum*, the second portion was inoculated with 2% starter fermented milk + 2% *Lactobacillus reuteri,* where as the third one was inoculated with 2% starter yogurt + 2% *Lactobacillus rhamnosus*. The inoculated milk samples were incubated at 42° C for 3 hours. The samples were stored at refrigerator temperature (5° C), three replicate were made from each treatment.

Analytical procedures:

Growth of strains in batch cultures:

Hundred ml of sterilized respective media for growth of each strains were prepared in Erlenmeyer flasks (250 ml in volume). The flasks were inoculated with 1 ml standerd inoculum of respective strain and shaked (rotary shaker incubator with 160 rpm for 24 hrs. at 37° C). Samples (5 ml) were taken from the growing cultures periodically under aseptic condition to determine the bacterial growth rate by measuring the O.D. at 650 nm using a UV-VIS spectrophotometer (model 8452, Hewlett-Packard).

Growth rate and generation time were calculated from exponential phase using the following equation according to **Shin et al., (2000).**

$\mu = L n x – L n x o / t – t o$

$d t = Ln2 / \mu$

Where : h = growth rate (hr-1)

xo = growth density at zero time

x = growth density after time

dt = generation time (hr)

Microbiological Analysis

Bifidobacterium bifidum was determined according to **Blanchette et al. (1996)** using modified MRS agar (Oxoid) supplemented with 0.05% L. cysteine-HCL (Merck, Germany). Plates were incubated at 37°C for 48 h. under anaerobic conditions (BBL Gas Pak, Becton Dickinson, Cockeysville MA, USA).

3.3.4. *Lactobacillus rhamnosus* was counted on LC agar (**Ramakanth and Nagendera, 1998).** Plates were incubated anaerobically at 27°e for 72- 96h.

Lactobacillus reuteri was enumerated on MRS-arabinose agar. MRS basal medium was prepared without dextrose, and 10 ml of membrane-filtered sterile solution of 10%L-arabinose was added per 90 ml of basal medium (10% final concentration) just before pouring the agar medium. Plates were incubated anaerobically at 37°e for 48 h.

Coliforms were enumerated according to **Harrigan and McCance (1996)** using Violet Red Bile agar medium. The plates were incubated at 37°C for 24 h.

Moulds and yeasts were determined according to Standard Methods for Examination of Dairy Products **(APHA, 1994).**

Chemical analysis:

All fermented milk samples were examined for titratable acidity (T.A%) according to International Dairy Federation **IDF (1991),** acetaldehyde content was determined as described by Lees and Jago (1969).

Sensory Evaluation

Fermented milk samples were organoleptically scored when fresh and throughout the storage period by 25 volunteer panelists according to the International Dairy Federation IDF(1997) as follow: acceptability, flavour, appearance and texture.

3. Results and Dissection

Fig (1) (A, B, C) show the effect of prebiotics on specific growth of all probiotic strains. This effect was dependent on the strains and kind of prebiotic in the growth medium.

All prebiotics substances were effective in enhancing growth rate of all probiotic strains without significant differences between them compared with control.

Table (1) shows the doubling time of each probiotic strain in the presence of prebiotics substances. Doubling time was used as a measure of

the efficacy of prebiotic substance in modulating growth rate. Growth promotion of probiotic strains by prebiotics were dependent on kind of prebiotic as evidenced by decreased doubling time with all prebiotics substances. The data indicated that for all probiotic strains provided the shortest doubling times compared with control. We can notice that honey II had the highest effect on the doubling time for all strains.

These results are in line with those found by **Dubey & Mistry (1998) and Mehanna et. al. (2003a,b)** .who found that generation times were affected by honey and inulin.

These results are consistent with previous reports on the ability of FOS (fractoligosaccharied) – honey contain FOS and other oligosaccharides beside another sweeteners - to stimulate the proliferation of Bifidobacteria relative to other intestinal microflora in vitro culture models simulating the colon **(Gibson and Wang 1994a).**

Activity of *B. bifidum La. rhamnosus, and Lac. reuteri* greatly enhanced when these organisms were grown in the presence of honey or inulin especially with honey II as evidenced by acetaldehyde and acidity (as lactic acid) production (Fig. 2) this result agree with **Amiri et al., 2010**. The effect of acetaldehyde production was more pronounced. Also, we noticed that the acetaldehyde was higher in the strains of probiotic *B. bifidum*. These results agree with **Baranowska (2006), Salama (2002), Rasic & Kurmann (1978) and (Abo-Donia et al., (1992**). They reported that, adding of *bifidobacterium* to lactic ferment starter highly activated the production of the acetaldehyde. This may be due to threonine which present in honey (**Janiszewska et al., 2012)**. Threonine had enhancement effect to the production of acetaldehyde (**Baranowska, 2006).**

Table (1) Effect of prebiotics on doubling time of probiotic bacteria

Species	Doubling time (min)			
	0	3% honey I	3% honey II	Inulin
B. bifidum	289	154	132	143
La. rhamnosus	264	169	159	164
Lac. reuteri,	245	201	185	195

Doubling time (dt) = Ln 2/μ (specific growth rate); μ = Ln x_2-Ln x_1/ t_2-t_1.
Doubling time (dt) = Ln 2/μ (specific growth rate); μ = Ln x2-Ln x1/ t2-t1.

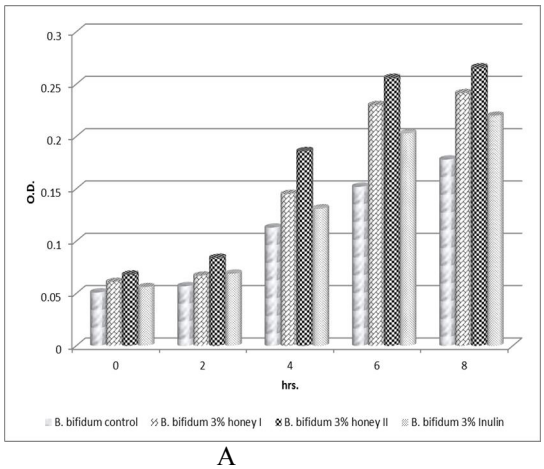
Data presented in Fig (3) shows that the acidity values were gradually increased along the storage period in fermented milk made without or with honey or inulin of different types of probiotic strains. One the other hand, fermented milk made with *B. bifidum* was higher in acidity values as compared with other strains.

The behavior of *Bifidobacterium bifidum* during manufacturing and refrigerated storage period of fermented milk made without or with honey I, II or inulin as shown in Fig. (4). It could be observed that the numbers of *B. bifidum* were increased in all treatments reached maximum in fermented milk made with honey II, honey I and inulin after 5 days of storage respectively. This could be due to the fact that during the manufacture process bacterial starter increase in number and continue to multiply for about five days, whilst lactose is available. From data given in Fig (4) we can noticed that the decrease in population of *B. bifidum* in fermented milk with prebiotics substances was lower than that fermented milk made without prebiotics after 10 days of refrigerated storage period. This may be due to the effect of honey as prebiotic or as stimulate the growth of *B. bifidum*. These results agree with **(Chick et al., 2001 and Mehanna et al., 2003b),** reported that the honey has variety of oligosaccharides with low DP (degrees of polymerization) it may be the favored substrate as Bifidobacteria support (bifidogenic factor).

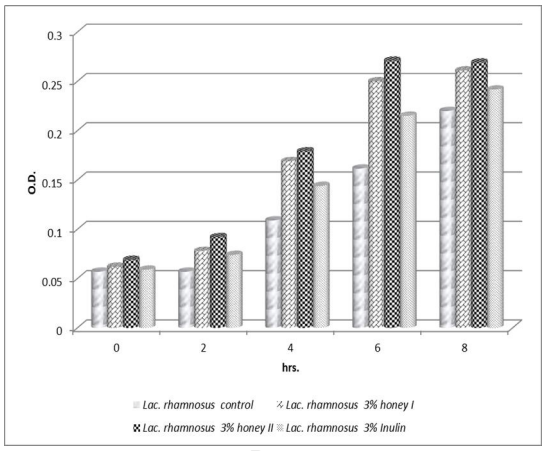
The data given in Fig (5) indicated that the viability of *La. rhamnosus* and *Lac. reuteri* increased in numbers till 5 days of refrigerated storage then dramatically decreased after 10 days of storage period in fermented milk without prebiotics, while slightly decrease in numbers was observed in *La. rhamnosus* at the end of refrigerated storage period in fermented milk made with 3% honey I, II or inulin. Also, these results could be due to the effect of honey or inulin as a prebiotic which stimulates the growth of *La. rhamnosus* and *Lac. reuteri.*

Data present in Fig (7) showed that fermented milk with *B. bifidum* and 3% honey II had highest acceptability scored followed by fermented milk with *B. bifidum* and 3% honey I, or fermented milk with *B. bifidum* and 3% inulin respectively. All samples which contain probiotic bacteria and prebiotics had non-significant effect between acceptability scored but had significant effect between them and control.

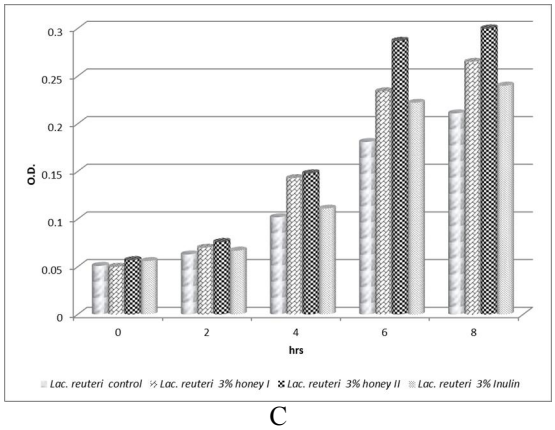
Mould & yeasts and coliform bacteria were also examined in all treatments and were not detected until the end of storage period. This could be attributed to an inhibition effect of probiotic strains against yeasts & moulds and coliform bacteria.



A



B



C

Fig (1): effect of prebiotic substences on specific growth rat of *Bifidobacterium bifidum (A); Lactobacillus rhamnosus (B) and Lactobacillus reuteri (C)*

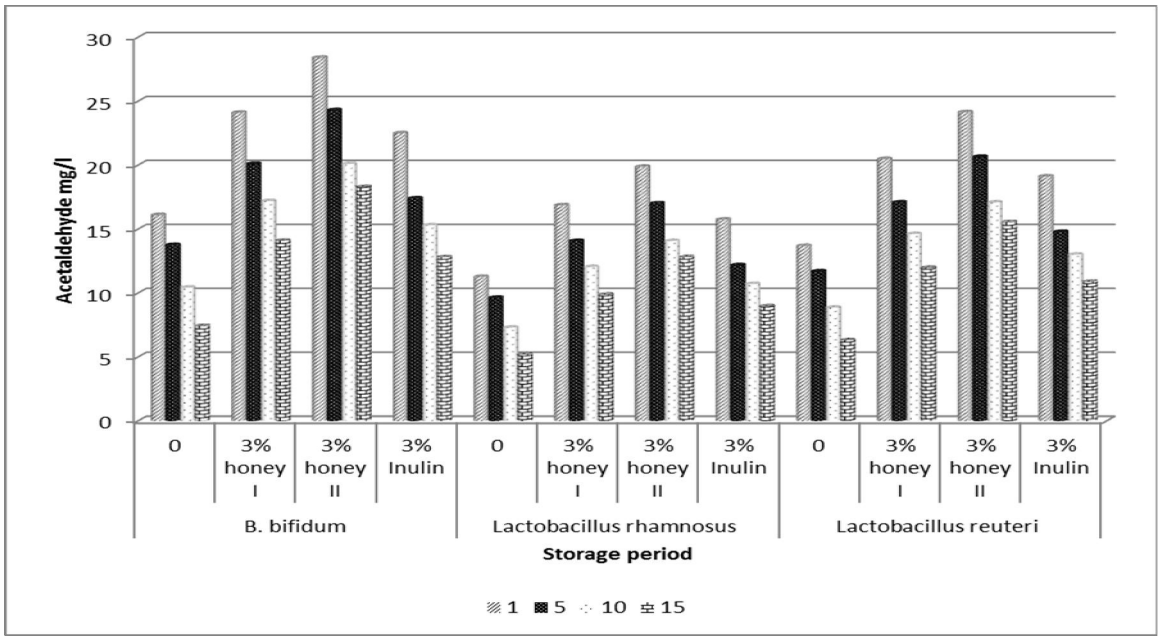


Fig (2): Effect of probiotic strains and prebiotics on the productionof acetaldehyde during storage period.

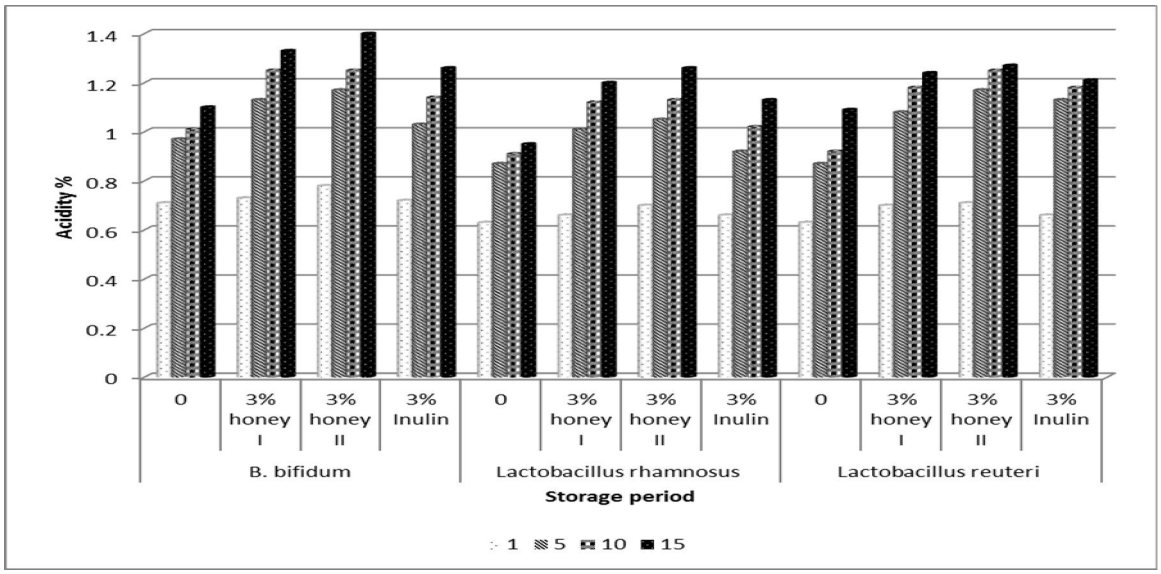


Fig (3): effect of probiotic strains and prebiotics on the production of acidity during storage period.

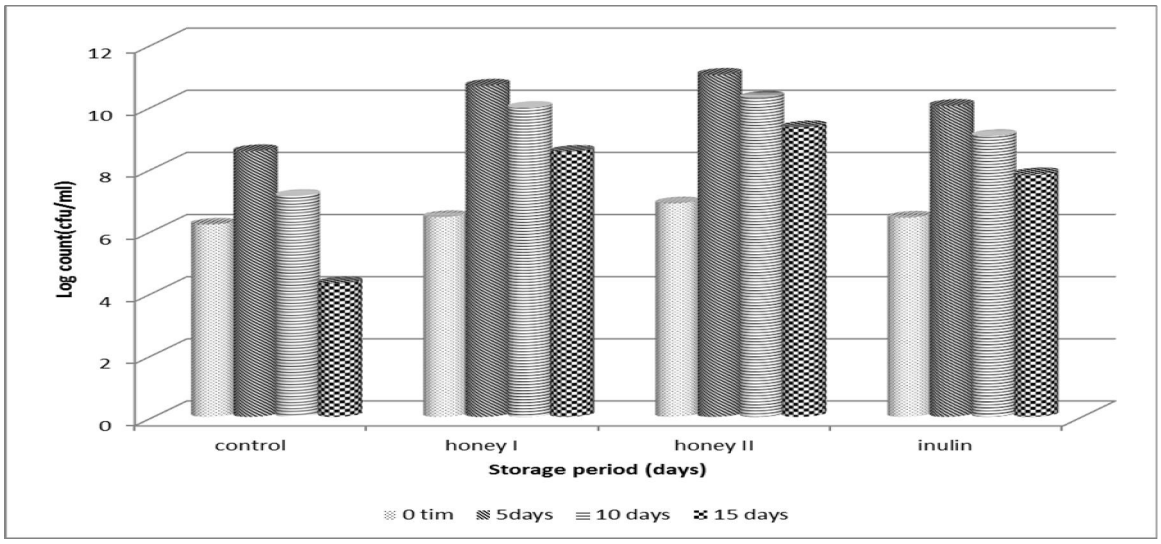


Fig (4): Log count of *B.bifidum* in fermented milk during storage period

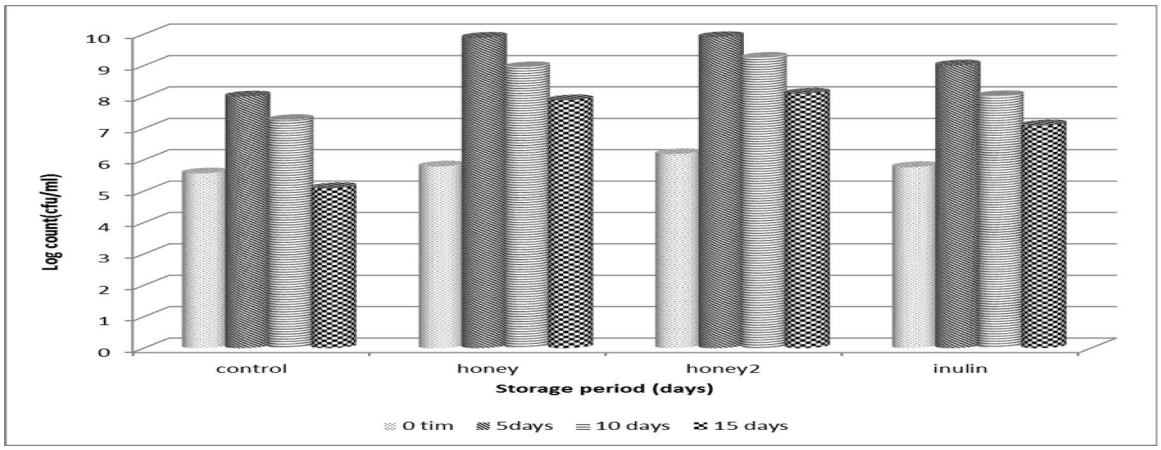


Fig (5): Log count of *Lac. reuteri* in fermented milk during storage period

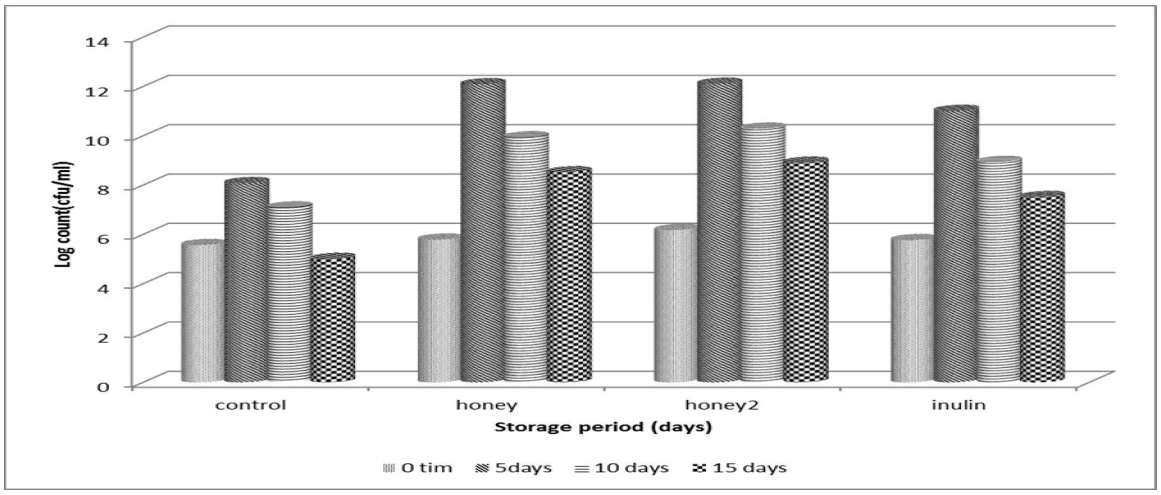


Fig (6): Log count of *Lac. rhamnosus in* fermented milk during storage period

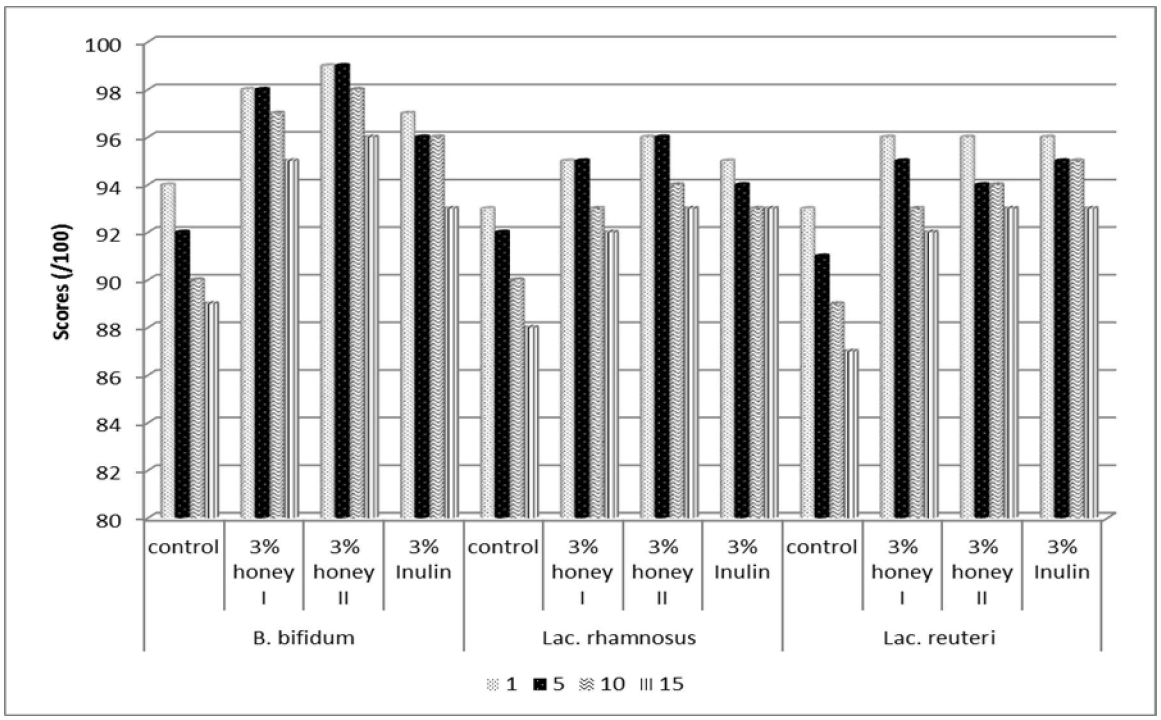


Fig (7): Organoleptic scores of fermented milk manufactured with prebiotics and different of probiotic strains during storage at refrigerator temperature.

4. Conclusions

From all these results, it can be concluded that the probiotic bacteria were enhanced by adding honey or inulin as a healing agent, but honey was more effect than inulin. These results can help us to prove that the consumption of probiotic bacteria and prebiotics within food products is the most popular way to re-establish the gastrointestinal microflora balance.

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8/8/2012