# In vitro assessment of gastrointestinal viability of potentially probiotic Lactobacilli

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**Abstract:** The objectives of this study were to assess the potential of four probiotic lactobacillus strains, *Lactobacillus bulgaricus*, *Lactobacillus johnsonii* B-2178, *Lactobacillus gasseri* B-14168 and *Lactobacillus salivarius* B-1950 in human upper gastrointestinal tract in vitro and evaluate the effect of milk proteins addition on viability of these strains in simulated gastric juices and in yoghurt during storage for 15 days at 4°C. The viability of lactobacilli strains in simulated gastric transit conditions (pH 2.0, pH 3.0 and pH 4.0) gastric juices with or without milk proteins singly or in combination with starch was tested. All the treatments were determined with three replicates. The simulated gastric transit tolerance of *L. johnsonii ,L. gasseri* and *L. salivarius* strains was pH-dependent and correspondingly showed lower viability at pH 2.0 after 180 min compared with pH 3.0 and pH 4.0. The addition of milk proteins singly or in combination with starch enhanced the survival of probiotic lactobacilli strains in simulated gastric juices different tested pH values. Results showed that addition of milk proteins in combination with starch improved the viability of *L.johnsonii* B-2178, *L. gasseri* B-14168 and *L. salivarius* B-1950 in yoghurt during storage. Sensory evaluation showed that yoghurt fortified with milk proteins plus starch recorded the highest score for and overall acceptability than the other treatments. However, yoghurt manufactured with *L. johnsonii* and *L. gasseri* and fortified with sodium caseinate plus starch showed the highest organoleptic score. It is suggested that the yoghurt of acceptable quality and high total probiotic bacterial count during storage can be made from milk supplemented with 0.5% (w/v) starch plus 0.5% (w/v) sodium caseinate. [Journal of American Science. 2010;6(11):357-367. (ISSN: 1545-1003).

**Keywords:** Probiotics, Gastric tolerance, *L. johnsonii, L. gasseri,L. salivarius*

## 1. Introduction

In the last decades consumer demands in the field of food production has changed considerably.Consumers more and more believe that foods contribute directly to their health (Qiang *et al*,2009). Today foods are not intended to only satisfy hunger and to provide necessary nutrients for humans but to prevent nutrition- related diseases and improve physical and mental well-being of consumers (Siro *et al*, 2008). In this regard, functional foods play an outstanding role.The increasing demand on such foods can explained by the increasing cost of healthcare, the steady increase in life expectancy, and desire of old people for improved quality of their later years(Kotilainen *et al*, 2006). In addition, functional dairy products offer requirements, benefits to health that are strengthened by the addition of probiotics as well as by certain types of soluble fibers known as prebiotics.

Probiotics for human consumption, generally either lactobacilli or bifidobacteria, are of increasing interest due to the growing evidence of health benefits associated with their use. Probiotic bacteria that are delivered through food systems have to firstly survive during the transit through the upper gastrointestinal tract, and then persist in the gut to provide beneficial effects for the host (Huang and Adams 2004). In order to be used as potential probiotics, lactobacillus strains need to be screened for their capacity of transit tolerance to the upper gastrointestinal tract conditions.

The low pH of the stomach and the antimicrobial action of pepsin are known to provide an effective barrier against entry of bacteria into the intestinal tract (Holzapfel *et al*, 1998). The pH of the stomach could be as low pH 1.5 or as high as pH 6.0 or above after food intake (Jonhson, 1977), but generally ranges from pH 2.5 to pH 3.5 (Holzapfel *et al*, 1998). There are no agreed rules for the screening of acid tolerance of potential probiotic strains. A range of pH values, from pH 1.0 to pH 5.0 has been used to screen in vitro acid tolerance of *Lactobacillus* and *Bifidobacterium* (Chung *et al*, 1999 and Zarate *et al*, 2000).

Food and food ingredients have been shown to protect probiotic bacteria from acid conditions and enhance gastric survival. Milk has been reported to increase the viability of acid sensitive *Lactobacillus* and *Bifidobacterium* strains during simulated gastric tract transit (Huang and Adams, 2004). The protective effect may be due to the increase of gut pH after milk addition. Maize starch granules at pH 3.5 have also been found to increase the viability of the more sensitive Bifidobacterium strains (Wang *et al*, 1999). Currently, orally ingested probiotic bacteria for humans are mainly prepared in conjunction with dairy products (Huang and Adams, 2004).

Ice cream, yoghurt and cheese have been found to be carriers of probiotic organisms (Madureira *et al*, 2005); another potential food vector is whey cheese- on which, unfortunately very little (if any) research has been performed to date. Whey is the aqueous portion of milk that obtained following acid-or rennet –driven coagulation (i.e. precipitation of caseins) in cheesemaking and is still disposed of in significant overall volumes, especially by small dairy industries, to public sewage systems (Pintado *et al*, 2001).

Yoghurts fortified with casein based ingredients (SMP, Na-caseinate or Ca-caseinate) showed an increase in firmness (or viscosity) and a reduction in syneresis compared with unfortified yoghurt (Amatayakul *et al*,2006).On the other hand, there were no consistent trends between the physical characteristics of yoghurts and the addition of whey protein-based ingredients. Therefore, the objectives of this study were to test the viability of four strains of lactobacilli in simulated gastric transit conditions (PH 2.0, pH 3.0 and pH 4.0 gastric juices). In addition, the effects of milk proteins in combination with starch on viability of probiotic lactobacilli in simulated gastric juices and in yoghurt during storage were determined.

## 2. Material and Methods

Bacterial Strains:

Three *Lactobacillus* strains were obtained from Northern Regional Research Lab., Illinois,USA (NRRL).These organisms are *Lactobacillus johnsonii* B-2178, *Lactobacillus gasseri* B-14168 and *Lactobacillus salivarius* B-1950. All strains had previously been shown to possess properties required of probiotic microorganisms including bile salt tolerance, tolerance to low pH values and antagonistic activity (Amin *et al*, 2002). Additionally, *Streptococcus thermophilus* and *Lactobacillus bulgaricus* were provided from Dairy Microbiology Lab., National Research Centre, Dokki, Cairo, Egypt.

Preparation of simulated gastric juice:

Simulated gastric juice was prepared fresh daily as described by Huang and Adams (2004). It prepared by suspending pepsin (obtained from Sigma, BDH Chemicals Ltd., Poole, England) (1:1000, ICN) in sterile saline (0.5%w/v) to a final concentration of $3gl^{-1}$ and adjusting the pH to 2.0,3.0 and 4.0 with concentrated HCl or sterile 0.1mol $l^{-1}$ NaOH using a pH meter (Model 8417N, Hanna Instrument, Singapore).

Effect of different components in simulated gastric juice on viability of lactobacilli:

Sodium cseinate (Listowel,Co. Kerry, Ireland) whey protein (El-Masserin Milk products Co., Egypt) and Hi-maize starch (National Starch, Melbourne, Victoria, Australia) were used in this investigation.The solutions of these components were prepared fresh daily by suspending each singly in sterile saline (0.5%w/v) at a concentration of $1gl^{-1}$.

In order to analyze the effects of various components of simulated gastric juice on viability of lactobacilli, an aliquot (0.2ml) of each washed cell suspension was transferred to a 2.0 ml capacity screw–cap tube and then mixed with 0.3ml of NaCl (0.5%w/v) and 1.0ml of simulated gastric (pH 2.0, pH 3.0 or pH 4.0) and this treatment served as a control. Solutions of various components were replaced the sterile saline addition and added as follows:

1- Treatment I: Sodium caseinate at 1% concentration.
2- Treatment II: Whey protein at 1% concentration.
3-Treatment III: Sodium caseinate and starch (each, at 0.5% concentration).
4- Treatment (IV): Whey protein and starch (each, at 0.5% concentration).

These mixtures were then vortexed at maximum sitting for 10s and incubated at 37°C. When screening gastric transit tolerance, aliquots of 0.1 ml were removed after 0, 60,120 and 180 min for determination the viability of lactobacilli. Viability was assessed in three repeat experiments.

Viability of probiotics in yoghurt supplemented with prebiotics Yoghurt was manufactured in triplicate according to ( Donkor et al, 2007) from standardized fresh cow`s milk (3% fat). Milk was divided into three main portions. The first portion was applied as a control (C), without addition of prebiotics. The other two yoghurt base was supplemented with 0.5% (w/v) starch (I) and then whey protein (TI) or sodium caseinate (TII)(each,0.5 w/v) were individually incorporated.

Each mix and control were then pasteurized at 85°C /30min and cooled to approximately 40°C.Then, each portion was divided into three equal portions. Starters were added as follows:

1-Treatment *S.thermophilus*, *L. bulgaricus* and *L.johnsonii* B-2178 ($TI_1$&$TII_1$ $C_1$).
2-Treatment ($TI_2$ &$TII_2$ &$C_2$) *S.thermophilus*, *L.bulgaricus* and *L. gasseri* B-14168.
3-Treatment ($TI_3$&$TII_3$&$C_3$) *S.thermophilus*, *L. bulgaricus* and *L.salivarius* B-1950.

All starters were added at level of 3%. Inoculated yoghurt bases were then poured into 70ml sterile plastic cups and incubated at 42°C until pH reached 4.5, followed by cooling to 4°C and storing for two weeks.

Analytical procedures:

Yoghurt was sampled when fresh and after 15days of storage.

Microbiological analysis:

Viability of lactobacillus strains was monitored after production (zero time) and 15days of storage. To

this end, 10g portions of duplicate yoghurt samples were blended with 90ml of simulated gastric juice prepared as described above and enumerated after incubation at 37°C for 2h, reflecting the time spent by food in the stomach. Then samples were submitted to serial dilutions of peptone water.Viability of *L. johnsonii*, *L. gasseri* and *L. salivarius* were determined on MRS-raffinose agar (Abd El-Khalek *et al*,2004), MRS agar (Salem *et al*, 2006) and MRS mannitol agar (Salem *et al*, 2007) respectively. The plate's incubation was done at 37°for 72h, in an anaerobic environment (BBL Gas Pak Becton Dickinson,Cockeysville MA, USA) for all lactobacilli.

Sensory evaluation of yoghurts:

Sensory evaluation of yoghurts was carried out when fresh and after 15ays of storage at 4°C. A panel consisting of 20 members evaluated the yoghurt samples presented in cooled cups in individual booths at room temperature. Samples were evaluated for flavour (50 points), body and texture (40 points) and appearance (10 points) according to (Abd El-Khalek et al, 2004).

**3. Results**

Comparative survival of probiotic lactobacilli in simulated gastric juice containing protective nutrients:

The effects of simulated gastric juice containing protective materials in vitro on viability of tested probiotic lactobacilli strains are presented in Figs (1, 2, 3, &4).

In general, each strain showed lower viability in simulated gastric juice either in control or containing protective materials at pH 2.0 than in simulated gastric Juice with pH 3.0 or pH 4.0.

When the simulated gastric juice was at pH 2.0, all the strains showed progressive reduction in viability during 180 min of simulated gastric transit, especially *L. bulgaricus*, which lost total viability after 180 min of simulated gastric transit in control (Fig.1.a.).

When the simulated gastric juice was at pH 3.0, *L. salivarius* had the highest survival rate over the 180 min of exposure to simulated gastric juice for control treatments.While the poorest survivor was *L.bulgaricus*, whose concentration declined to undetectable levels after 180 min of exposure (Fig 1.b).

When the simulated gastric juice was at pH 4.0, all of the tested four strains retained the same level of viability during 180 min of simulated gastric juice transit in the absence of a protective matrix, such as sodium caseinate, whey protein or starch (Fig.1c.).

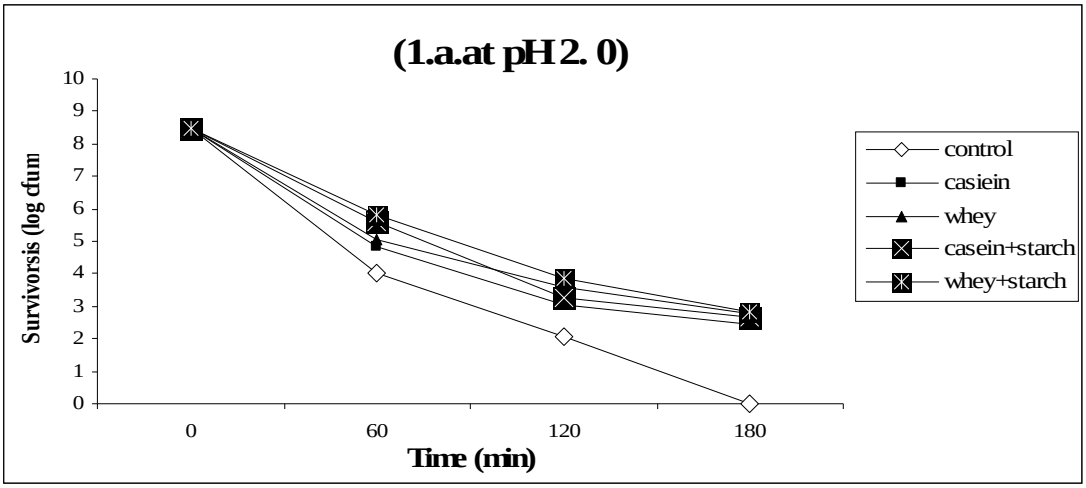
The effect of sodium caseinate and whey protein addition, singly and in combination with starch on viability during simulated gastric transit with pH 2.0, 3.0 and 4.0 simulated gastric juice is presented in Figs(1,2,3 and 4). In general, sodium caseinate and whey protein addition improved simulated gastric transit tolerance. In this regard, all tested strains exhibited complete tolerance to simulated gastric transit in the presence of sodium caseinate or whey protein singly and in combination with starch. The results showed that the greatest survival effect attributable to sodium caseinate plus starch occurred in *L. salivarius* followed by *L. johnsonii* at pH 4.0.

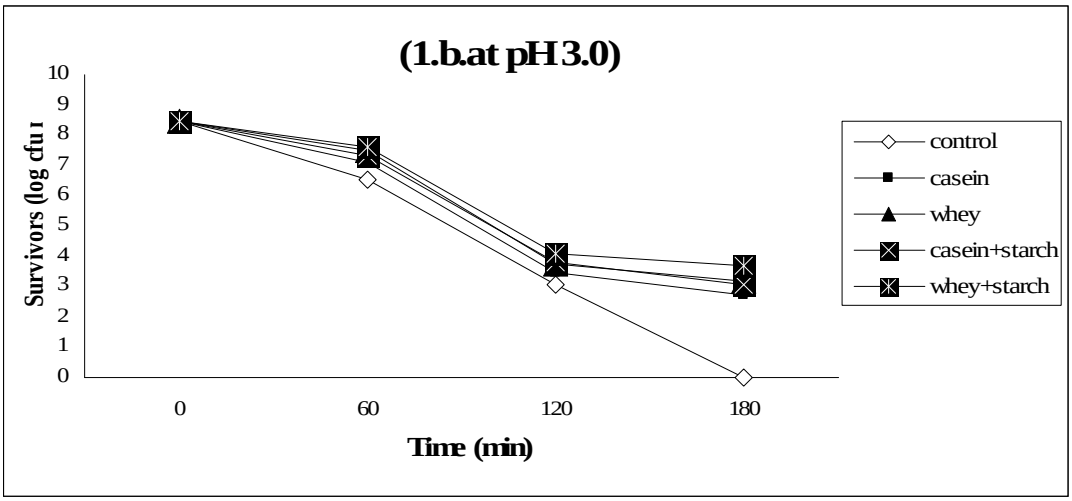
The strain of L. *bulgaricus* had a poor survival during 180 min at pH 2.0 and 3.0.

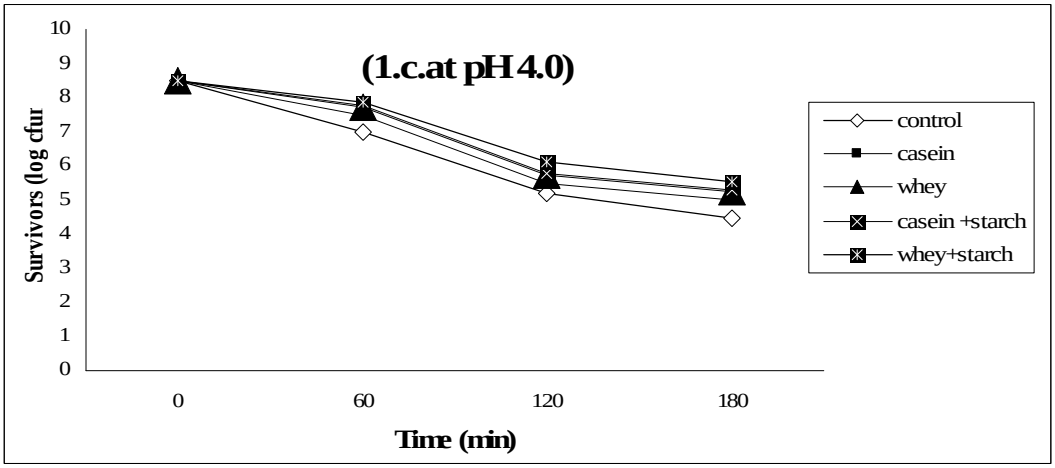
The intrinsic resistance to acid of *L. bulgaricus* is poor (Conway *et al*, 1987 and Charteris, *et al*, 1998). In this study, intrinsic resistance to gastric transit tolerance was observed to be rare probiotic property among the strains examined and to be influenced by the presence of milk proteins (sodium caseinate and whey protein, singly and in combination with starch).

Although pH could be used as, a suitable direct measure for selection of probiotic strains, most probiotics are consumed in food products.The presence of food and food ingredients has been reported to improve viability of microorganisms during gastric transit (Huang and Adams, 2004). The suggested mechanism for the beneficial effect of food ingredients is the pH increase of the gastric contents resulting from the addition of the food (Zarate *et al*, 2000). In the current study, the presence of milk proteins, singly and in combination with starch at pH 2.0 and pH 3.0, exerted a major effect on the gastric tolerance of some strains but not others. In this regard, *L. johnsonii*, *L. gasseri* and *L. salivarius* were capable of undiminished survival during simulated gastric transit in the presence of sodium caseinate, whey protein and their combination with starch. These data indicate that some strains of lactobacillus species may survive passage through the human stomach, particularly when ingested with milk products or milk protein- based foodstuffs.

Survival of lactic acid bacteria in human gastric juice adjusted to low pH has been previously shown to be enhanced by the addition sodium caseinate, whey protein and skim milk (Conway *et al*, 1987 and Charteris *et al*, 1998).

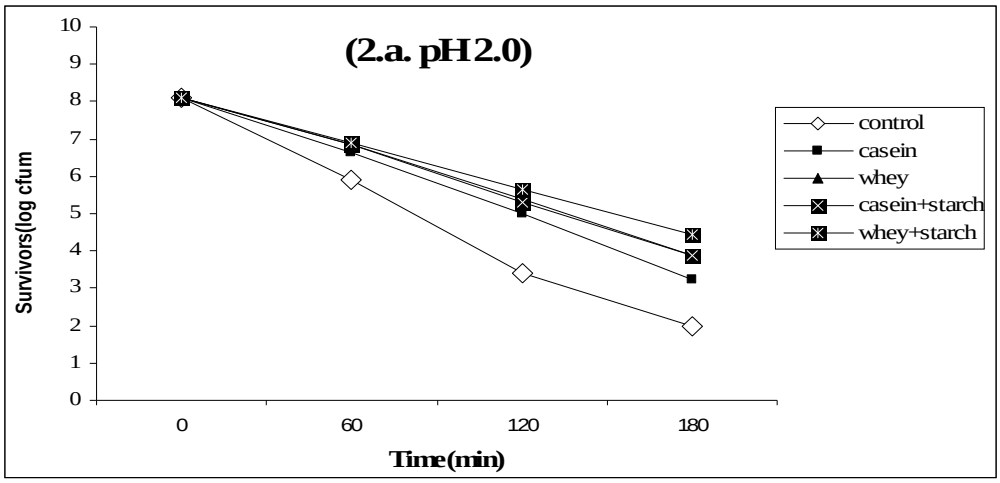


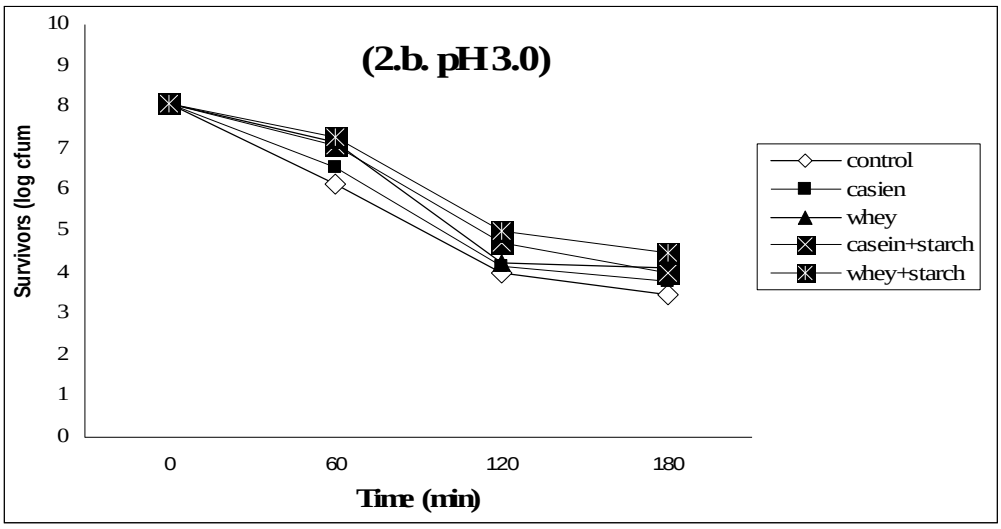



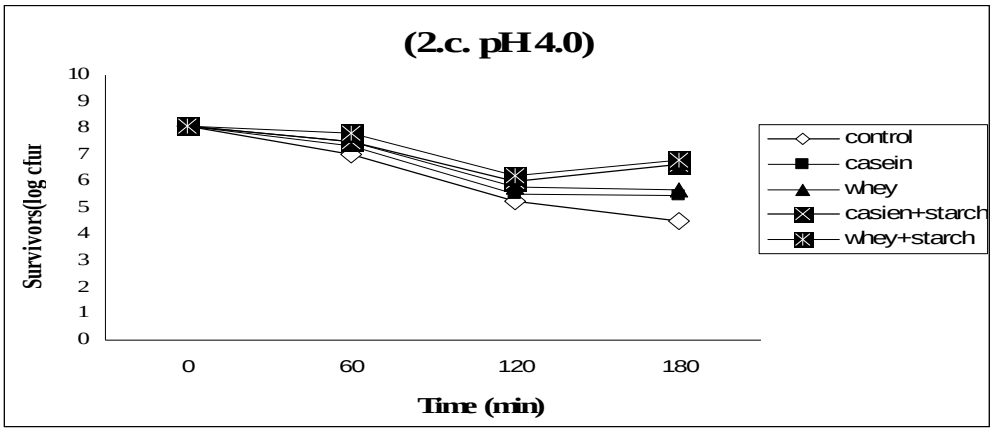




The data are the means of triplicate experiment

**Fig(1)**: Survival of *L.bulgaricus* in simulated gastric juice containing sodium caseinate, whey Protein, (sodium caseinate and starch) and (whey protein and starch) at pH 2.0, pH 3.0 and pH 4.0.

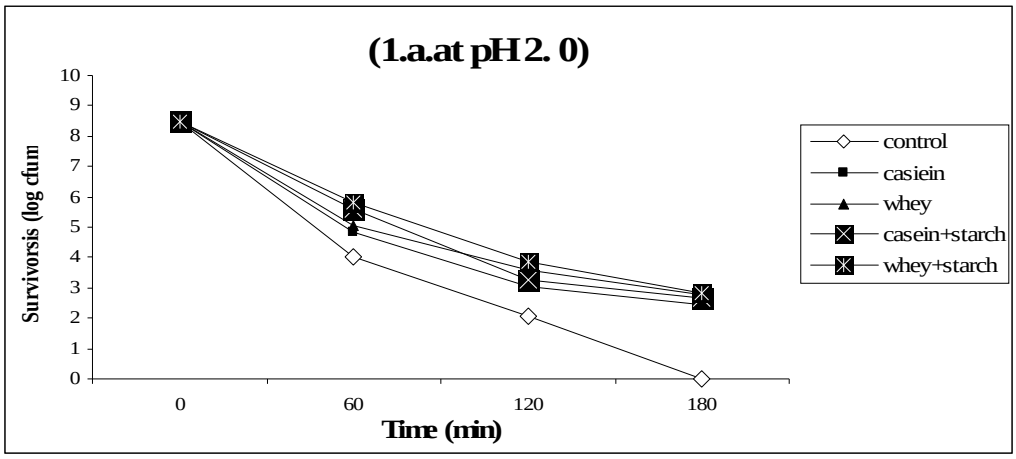


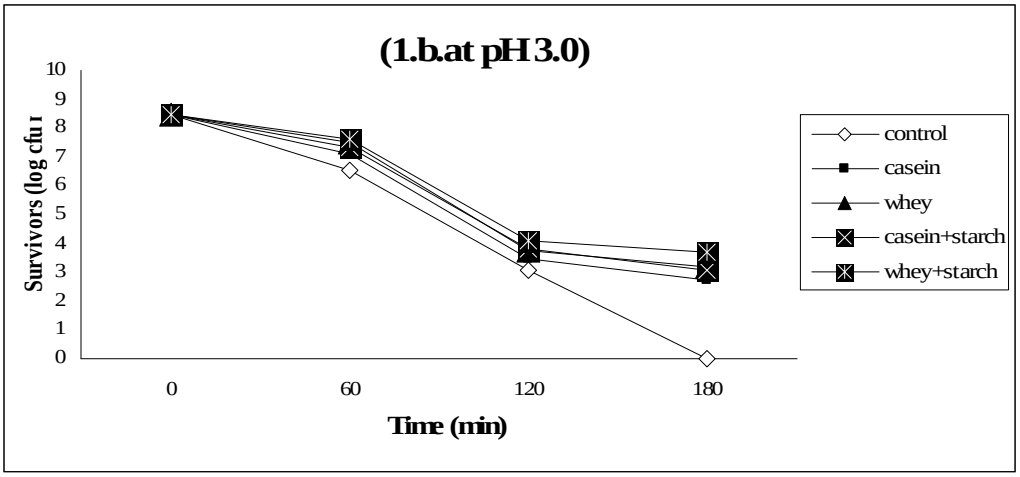



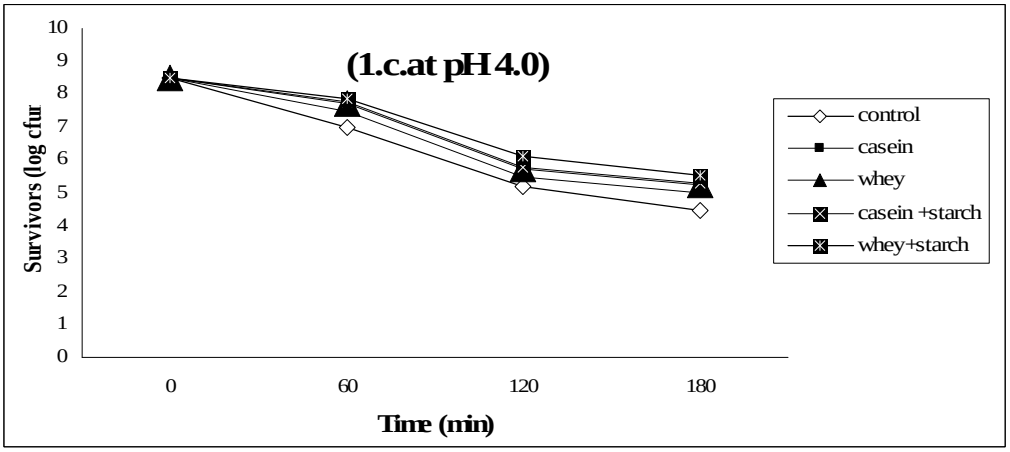




The data are the means of triplicate experiment

**Fig(2)**: Survival of *L.johnsonii* in simulated gastric juice containing sodium caseinate, whey Protein, (sodium caseinate and starch) and (whey protein and starch) at pH 2.0,pH 3.0 and pH 4.0.

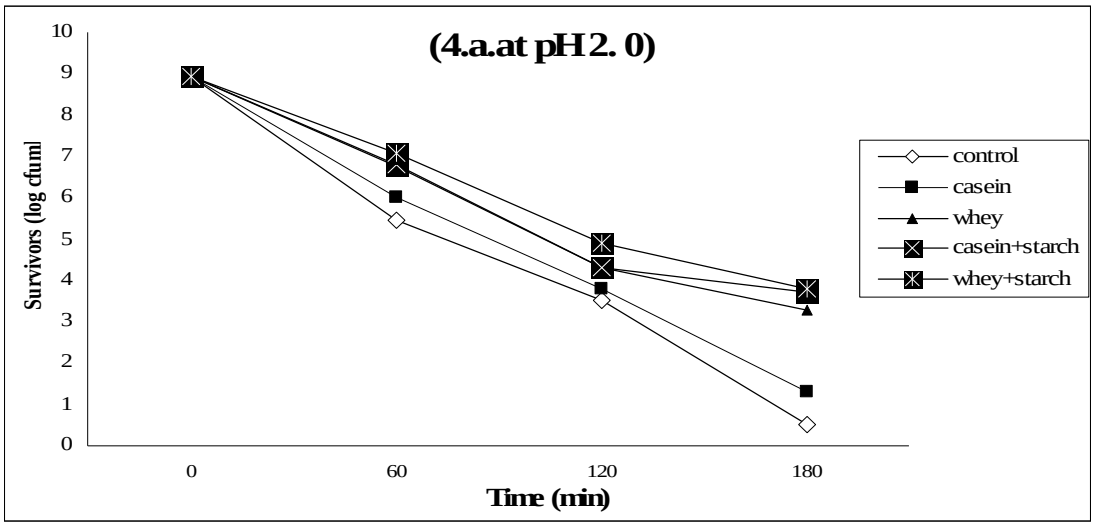


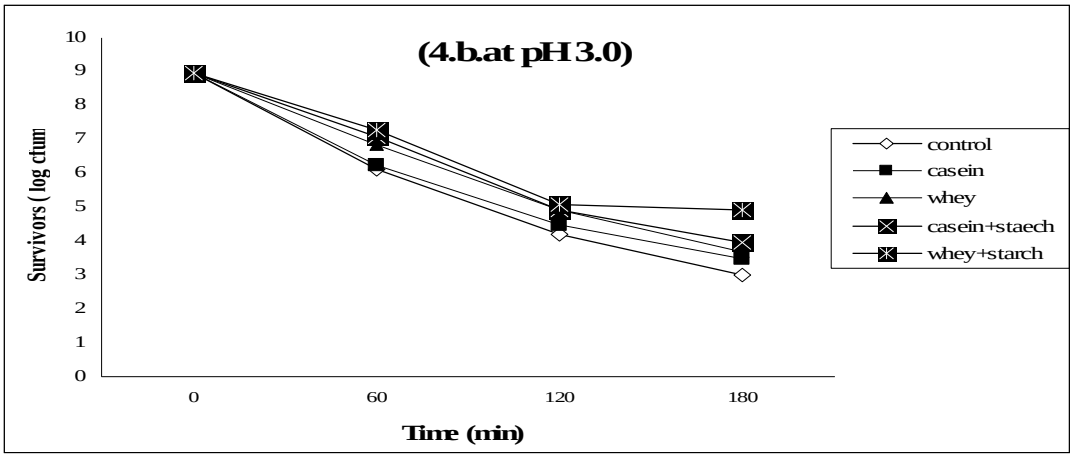



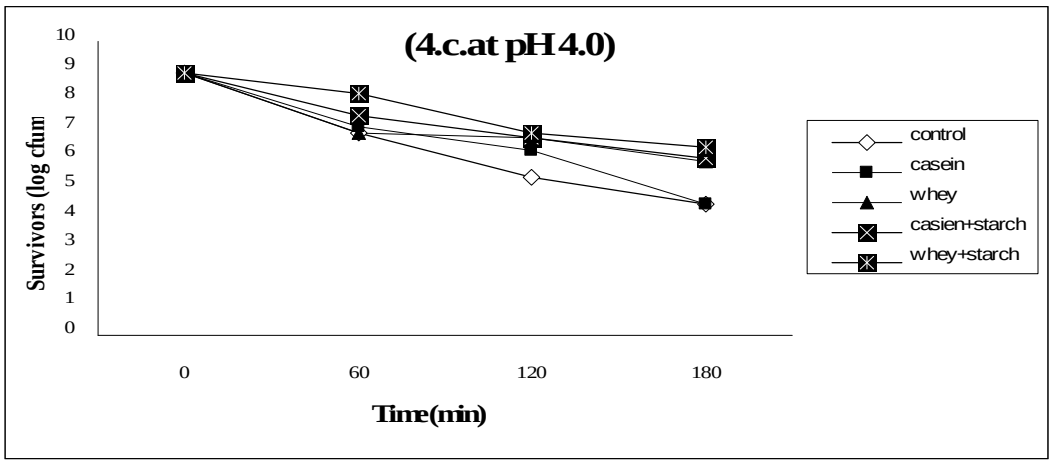




The data are the means of triplicate experiment

**Fig(3)**: Survival of *L.gasseri* in simulated gastric juice containing sodium caseinate and whey (sodium caseinate and starch) and (whey protein and starch) at pH 2.0,pH 3.0 and pH 4.0.




The data are the means of triplicate experiment

**Fig(4):** Survival of *L. salivarius* in simulated gastric juice containing sodium caseinate, whey Protein, (sodium caseinate and starch) and (whey protein and starch) at pH 2.0,pH 3.0 and pH 4.0.

Viability of probiotic lactobacillus strains in yoghurt supplemented with prebiotics :

The viability of the three strains *L. johnsonii*, *L. gasseri* and *L. salivarius* in yoghurt containing milk proteins (whey protein or sodium caseinate) in combination with starch stored at a refrigerator (~4°C) for two week is shown in Table (1). In general, the viability of all three strains decreased during storage. However, the viability was in many cases higher than that of the control, without protective materials. On an average, best viability was observed with whey protein plus starch.

The highest viability of 84.04% was recorded for *L. johnsonii* with whey protein plus starch. Overall, sodium caseinate plus starch was the least effective in maintaining viability, with average viabilities of 60.6%, 52.27 and 59.37%. The lowest viability was recorded by *L. gasseri* with an average viability of 52.27 %.

The control samples containing no protective materials had average survival rate of 34.32%, 22.69 % and 40% for *L. johnsonii*, *L. gasseri* and *L. salivarius*, respectively. Sodium caseinate and whey protein plus Hi-maize starch were only helpful in improving viability of probiotic organisms in yoghurt during storage (Table 1).The improved viability is possibly due to prebiotics providing extra solids, which tend to protect cells from injury (Capela *et al*, 2006).

Viability of lactobacilli is affected because of several factors including acid produced during fermentation, oxygen content in the product and oxygen permeation through the packaging material (Desai *et al*, 2004).

**Table (1):** Viability of Lactobacillus strains in yoghurt supplemented with milk proteins plus starch during storageat 4°C for days.

| Lactobacillus strains | Reading interval | Control | Whey + starch | Casein +starch |
|---|---|---|---|---|
| | | Count cfu/m | | |
| *L. johnsonii* | Zero time | $6.7 \times 10^8$ | $9.4 \times 10^8$ | $8.6 \times 10^8$ |
| | 15day | $23 \times 10^6$ | $7.9 \times 10^8$ | $5.2 \times 10^8$ |
| | % viability | 34.32 | 84.04 | 60.6 |
| *L. gasseri* | Zero time | $2.6 \times 10^8$ | $5.7 \times 10^8$ | $4.4 \times 10^8$ $2.3 \times 10^8$ |
| | 15 days | $5.9 \times 10^7$ | $3.3 \times 10^8$ | |
| | % viability | 22.7 | 57.9 | 52.27 |
| *L. salivarius*, | Zero time | $4.5 \times 10^8$ | $6.3 \times 10^8$ | $6.4 \times 10^8$ |
| | 15 days | $1.8 \times 10^7$ | $4.2 \times 10^8$ | $3.8 \times 10^8$ |
| | % viability | 40.0 | 66.66 | 59.37 |

**%** of viability = (count after 15 days cfu/g / zero time count cfu/g ×100).

**Table (2):** Organoleptic properties of probiotic yoghurt containing starch in combination with whey or casein throughout storage course on refrigerator.

| Properties | Storage period (days) | Yoghurt treatments | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | $C_1$ | $TI_1$ | $TII_1$ | $C_2$ | $TI_2$ | $TII_2$ | $C_3$ | $TI_3$ | $TII_3$ |
| Flavor(50) | 0 | 49 | 49 | 49 | 49 | 49 | 49 | 48 | 48 | 48 |
| | 15 | 48 | 48 | 48 | 48 | 48 | 48 | 46 | 46 | 46 |
| Body&texture (40) | 0 | 36 | 37 | 37 | 38 | 38 | 37 | 35 | 36 | 36 |
| | 15 | 37 | 38 | 38 | 38 | 38 | 38 | 30 | 30 | 30 |
| Appearance (10) | 0 | 9 | 9 | 9 | 9 | 9 | 9 | 7 | 7 | 7 |
| | 15 | 8 | 8 | 8 | 8 | 8 | 8 | 6 | 6 | 6 |
| Total (100) | 0 | 94 | 95 | 95 | 96 | 96 | 95 | 90 | 91 | 91 |
| | 15 | 93 | 94 | 94 | 94 | 94 | 94 | 82 | 82 | 82 |

$C_1$: Contol with *S. thermophilus , L.bulgaricus* and *L.* johnsonii.

$TI_1$*: S. thermophilus , L.bulgaricus* and *L. johnsoni* containing starch and whey protein (each, 0.5% w/v).

$TII_1$: *S. thermophilus , L.bulgaricus* and *L. johnsonii* containing starch and sodium caseinate (each 0.5% w/v).

$C_2$: Control with *S. thermophilus , L.bulgaricus* and *L. gasseri* B-14168.

$TI_2$: *S. thermophilus , L.bulgaricus* and *L. gasseri* B-14168 containing starch& whey protein (each 0.5% w/v).

$TII_2$: *S. thermophilus , L.bulgaricus* and *L. gasseri* B-14168 containing starch & sodium caseinate (each 0.5% w/v).

$C_3$: Control with *S. thermophilus , L.bulgaricus* and.*L.salivarius* B-1950.

$TI_3$: *S. thermophilus , L.bulgaricus* and.*L. salivarius* B-1950 containing starch& whey protein (each 0.5% w/v).

$TII_3$: *S. thermophilus , L.bulgaricus* and.*L. salivarius* B-1950 containing starch & sodium caseinate (each 0.5% w/v).

Sensory evaluation

In recent years, per capita consumption of yoghurt has increased drastically because many consumers associate yoghurt with good health (Hekmat and Reid 2006). Yoghurt is characterized as a fermented milk product with a refreshing flavor, a smooth viscous gel and a slight sour taste (Bodyfelt *et al*, 1988). These sensory properties offer quality control criteria, and therefore, yoghurt should be evaluated for appearance, flavor, texture and overall quality.

Data presented in Table (2) show that the addition of starch in combination with whey or casein did not alter the mean appearance score of all samples of probiotic yoghurt either when fresh or after 15 days of storage. There were no flavor differences among $TI_1$,$TII_1$,$TI_2$,$TII_2$ and $TI_3$,$TII_3$ Table (2).These results indicate that the addition of *L. johnsonii*, *L. gasseri* and *L. salivarius* did not affect the flavor of the yoghurt. Yoghurt flavor is influenced by the presence of lactic acid and other flavoring compounds produced by culture bacteria during fermentation process.

The three probiotic strains did not inhibit the standard yoghurt cultures or overtly contribute to acid production from conversion of lactose to lactic acid.

The addition of starch in combination with casein did not alter texture of T4 and T5 in comparison to other treatments and control samples. The texture of yoghurt is affected by the rate of acid production during the fermentation process. Also, the heating processes of the mix at 85°C for 30 minutes affect the texture of the yoghurt. Heating the mix denatures whey protein, increases the water holding capacity of milk protein and reduces syneresis in yoghurt. Panelists rated the texture of $T_4$ and $T_5$ samples higher than other treatments.

This current study has demonstrated that although the viability of lactobacilli is affected by pH 2.0, most of the tested strains survived well at pH 3.0 and pH 4.0.

Furthermore, survival of lactobacilli in simulated gastric juice at pH 2.0 is enhanced by the addition of milk proteins singly or in combination with starch. Moreover, this study indicates that there are potential benefits of adding starch (0.5% w/v) plus sodium caseinate (0.5%w/v) to milk-based media aimed to preparing probiotics

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