

Effect of Prebiotics on *Lactobacillus acidophilus* Growth and Resulting pH Changes in Skim Milk and a Model Peptone System

DW Olson¹ and KJ Aryana^{1,2*}

¹School of Animal Sciences, Louisiana State University Agricultural Center, Baton Rouge, LA 70803, USA

²Department of Food Science, Louisiana State University Agricultural Center, Baton Rouge, LA 70803, USA

Abstract

The effect of Raftiline® GR, Raftiline® HP-Gel, and Raftilose® P95 prebiotics on growth curves of the probiotic *Lactobacillus acidophilus* LA-K and resulting pH changes in skim milk and a model system consisting of 0.1% peptone was investigated. Skim milk (100 mL) and 0.1% peptone (100 mL) each containing either 1% or 3% Raftiline® GR, Raftiline® HP-Gel, or Raftilose® P95 prebiotics were autoclaved, cooled to 37°C, and inoculated with 0.132 mL of *L. acidophilus* LA-K. Both the growth of *L. acidophilus* and the changes in pH were followed for 16 h at 37°C. The growth was determined by measuring the optical density (turbidity) at 600 nm. Prebiotics had a greater influence on the growth of *L. acidophilus* and a greater pH lowering effect in a peptone model system than in skim milk. This observation seems logical since more nutrients are available in skim milk compared to peptone. The system, skim milk or peptone, in which prebiotics are used, does influence the overall desirable effect of the prebiotic.

Keywords: Prebiotic; Probiotic; *Lactobacillus acidophilus*; Growth curve

Introduction

Probiotics have been defined as “live microorganisms which when administered in adequate amounts confer a health benefit on the host” [1]. Probiotics include many different species, especially species of the genera *Lactobacillus* and *Bifidobacterium* [2]. A commonly used probiotic bacterium is *L. acidophilus*.

Health benefits provided by probiotics include improving gastrointestinal health [3], modulating immune function [4], possibly helping to prevent colon cancer [5], possibly mediating an anti-hypertensive effect [6], reducing urinary tract infections in women [7], improving lactose digestion [8], reducing serum cholesterol levels [9], and possibly preventing many other health problems. The probiotic properties of survival in an *in vitro* gastro-intestinal tract model system and adhesion to Caco-2 cells have been shown for *L. acidophilus* LA5 [10].

Gibson et al. [11] defined prebiotics as “a selectively fermented ingredient that allows specific changes, both in the composition and/or activity in the gastrointestinal microflora that confers benefits upon host well-being and health”. Prebiotic intake has been reported to be effective against bowel cancer, inflammatory bowel disease, pathogenic agents, coronary heart disease, necrotizing enterocolitis, and obesity and can improve mineral availability. Insulin and oligofructose, transgalacto-oligosaccharides, and lactulose can be classified as prebiotics [11]. Inulin and oligofructose are found in many types of plants as plant storage carbohydrates [12]. Inulin has a linear chain consisting of either α -D-glucopyranosyl- $[\beta$ -D-fructofuranosyl]_{n-1}- β -D-fructofuranoside or a β -D-fructopyranosyl- $[\beta$ -D-fructofuranosyl]_{n-1}- β -D-fructofuranoside [11]. The fructosyl-glucose linkage is β (2 \leftrightarrow 1) and the fructosyl-fructose linkages are β (1 \leftrightarrow 2). Chicory inulin has a degree of polymerization ranging from 2 to approximately 60. Oligofructose (fructo-oligosaccharides) is obtained by partial enzymatic hydrolysis of inulin with endo-inulinase (EC 3.2.1.7) and has a degree of polymerization ranging from 2 to 7 with an average of 4 [11]. Oligofructose and inulin that are commercially available have variable chain lengths. The prebiotics used in the present study have a degree of polymerization of 4 (P95) (a short chain oligofructose) [13], an average degree of polymerization of at least 10 (GR) (a medium

chain inulin) and a degree of polymerization greater than 23 (HP-Gel) (a long chain inulin) [14]. Prebiotic chain lengths influence various characteristics. Rats had greater bioavailability of calcium upon consumption of long chain fructan (inulin) compared to short chain oligofructose [15]. In contrast with medium and long chain fructo-oligosaccharides, the short chain fructo-oligosaccharides are the first to be consumed by bifidobacteria resulting in greater lactate and acetate production [16].

Products that contain both probiotic cells and prebiotic substances are called synbiotic products. Inulin and oligofructose can be used as an ingredient in various products including probiotic yogurt [17] and probiotic ice cream [18]. Araujo et al. [19] developed a cottage-like synbiotic cheese that contained *Lactobacillus delbrueckii* UFVH2b20 and inulin and this product maintained a microorganism count high enough allowing it to be considered a probiotic throughout its shelf life. Consuming synbiotic yogurts containing *Bifidobacterium animalis* subsp. *lactis* and inulin can increase bifidobacterial numbers and decrease clostridial numbers in some people [20].

Milk is an excellent medium for microbial growth and hence it is heavily regulated and has legal limits for bacteria [21]. Peptone (0.1%) is often used to make dilution blanks to enumerate microorganisms [22,23]. It is not known if prebiotics would have a greater beneficial effect on growth of *L. acidophilus* in a system devoid of milk such as in a 0.1% peptone model system than in a skim milk system.

Various procedures exist to count bacterial cells. Plate counting is

***Corresponding author:** Kayanush Aryana, Department of Food Science, Louisiana State University Agricultural Center, Baton Rouge, LA 70803 USA, E-mail: KAryana@agcenter.lsu.edu

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common, but it can be labor intensive. Turbidity is a rapid procedure, but it does not distinguish between viable and dead cells. Fluorescent dye staining has been successfully used [24] to measure bacterial viability.

The objective of the present study was to compare the 16 hour growth curves of *L. acidophilus* and resulting pH changes in skim milk and in a model system consisting of 0.1% peptone each containing either 1% or 3% oligofructose or inulin with various chain lengths.

Materials and Methods

Materials

Bacto™ peptone (Becton, Dickinson and Company, Sparks, MD, USA) was obtained from University Stores at Louisiana State University. Raftiline® GR, Raftiline® HP-Gel, and Raftilose® P95 prebiotics were obtained from BENEIO-Orafti (Oreye, Belgium). *L. acidophilus* LA-K was obtained from Chr. Hansen (Milwaukee, WI, USA) and had a total cell count of greater than 9×10^9 cfu/g in the pure culture (communication with the manufacturer).

Sample preparation

For each replicate, skim milk and 0.1% peptone were each poured into five milk dilution bottles to a volume of 100 mL. Three types of prebiotics (Raftiline® GR, Raftiline® HP-Gel, and Raftilose® P95) at either 1% or 3% (w/v) were individually added to skim milk and 0.1% peptone in milk dilution bottles. One set of controls for both skim milk and 0.1% peptone contained *L. acidophilus* but no prebiotics while the other set of controls contained neither prebiotics nor *L. acidophilus*. The treatments and controls for the skim milk and 0.1% peptone were autoclaved at 121°C for 15 minutes and cooled to 37°C in an incubator. An aliquot of 0.132 mL of *L. acidophilus* LA-K was added to each treatment and one controls each for the skim milk and 0.1% peptone. Three replicates for both the 1 and 3% prebiotic levels were performed.

Growth curves

Immediately after addition of *L. acidophilus* LA-K to each sample, a 3 mL aliquot was aseptically taken from the milk dilution bottle and placed into a disposable cuvette for preparation of the growth curve. Cuvettes were kept within the 37°C incubator for 16 hours except for analysis once every 40 minutes. Growth curves were prepared by measuring the optical density (turbidity) at 600 nm in a disposable cuvette with a Nicolet Evolution 100 spectrophotometer (Thermo Electron Corporation, Madison, WI, USA).

pH measurements

Milk dilution bottles containing the samples and the control used for determining the pH were kept within the 37°C incubator for 16 hours except for analysis once every 40 minutes. The pH was measured with an Ultra Basic pH/mV meter (Denver Instrument Company, Arvada, CO, USA). The pH meter was standardized with pH 4 and 7 buffers before use.

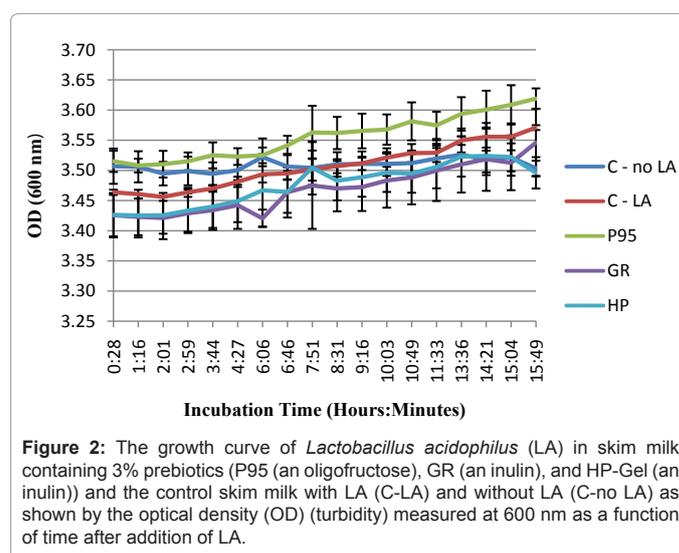
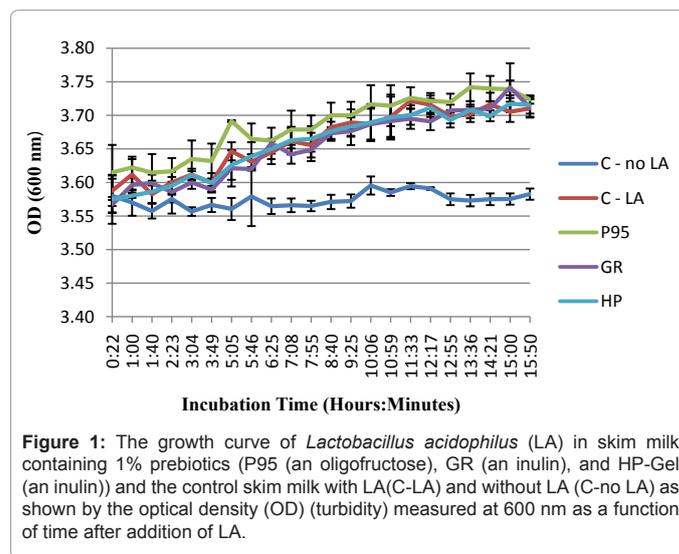
Results and Discussion

Optical density of skim milk containing prebiotics

The optical densities of skim milk containing 1% prebiotics and their controls are presented in figure 1. For samples containing 1% prebiotics, there was typically less than 0.1 difference in optical densities of samples containing *L. acidophilus*. The optical densities of the P95 containing samples tended to be slightly higher than the optical

densities of the remaining samples and the control containing *L. acidophilus*. The optical densities of skim milk containing 3% prebiotics and their controls are shown in figure 2. It appeared that *L. acidophilus* LA-K entered the stationary phase after about 12 hours of incubation for samples containing either 1 or 3% prebiotics. Although there were larger differences in optical densities among samples containing 3% prebiotics compared to samples containing 1% prebiotics, slopes of the optical densities versus incubation time curves for all of the samples and the control containing *L. acidophilus* still appeared to be approximately similar. The rate of growth of *L. acidophilus* in all of the *L. acidophilus* containing samples and the control containing *L. acidophilus* appeared to be approximately similar making it difficult to show the stimulating effect of these prebiotics within 16 hours of measurement. However, it is possible that there could have been a growth stimulating effect by the prebiotics later in the stationary phase or in the death phase that occurred beyond the approximate 16 hours of measurement.

Growth curves of *L. acidophilus* have been reported in the literature. In a recent study regarding *L. acidophilus* LA-K homogenized at various pressures, it was found that this culture stayed in the logarithmic phase of growth for at least 10 hours of incubation [25]. Liang and Shah [26]



reported that various strains of *L. acidophilus* and *Lactobacillus casei* had rapid growth for the first 9 to 15 hours of incubation and then slower growth afterwards to 24 hours when monitored by measuring absorbance at 620 nm and grown in a medium without cholesterol.

L. acidophilus can utilize various oligosaccharides and polysaccharides. All of the tested strains of *L. acidophilus* (33200, 837, DDS-1, and NCFM) were able to grow on an MRS-fructooligosaccharide agar [27]. *L. acidophilus* originating from swine feces degraded (arabino) galactooligosaccharides and xylooligosaccharides, partially degraded arabinogalactan enriched polysaccharide fraction and fructooligosaccharides, but did not degrade arabinan enriched polysaccharide fraction, arabinooligosaccharides, rhamnogalacturonan enriched polysaccharide fraction, rhamnogalacturonooligosaccharides, galacturonooligosaccharides, arabinoxylan polysaccharide from wheat flour, and arabinoxylooligosaccharides [28]. *L. acidophilus* NCFM can utilize isomaltooligosaccharides [29]. *L. acidophilus* DSM 20079 was able to grow on MRS media containing pectin or inulin since these counts of *L. acidophilus* were nearly as high as counts when grown on MRS media containing glucose after 24 hours [30].

Variable results have been reported as to the effect of prebiotics on the growth of various strains of *L. acidophilus* and *L. acidophilus* 4461 did not have higher counts in reconstituted skim milk containing 1, 2, or 3% Raftiline HP compared to reconstituted skim milk without Raftiline HP after 6 hours of incubation at 37°C [31]. When fermenting reconstituted skim milk with *L. acidophilus* LAC4 and *S. thermophilus* TA040, increased *L. acidophilus* counts were obtained when reconstituted skim milk was supplemented with 4% (w/w) lactulose compared to reconstituted skim milk without lactulose [32] and with maltodextrin, polydextrose, and oligofructose added individually compared to reconstituted skim milk without these prebiotics. Russo et al. [33] showed that the simultaneous availability of D-glucose and exopolysaccharides stimulated the growth of *L. acidophilus* NCFM by extending the logarithmic phase and delaying its entrance into the stationary phase. They also found that *L. acidophilus* reached a maximum cell concentration after 24 hours and a death phase after 30 hours when grown in a chemically defined medium supplemented with D-glucose. Nazzaro et al. [34] found that inulin did not affect the growth of *L. acidophilus* DSM 20079 compared to its growth in glucose. However, Saminathan et al. [35] found large differences in specific growth rates in representative *Lactobacillus* species in media supplemented with different oligosaccharides. Variations in specific growth rates with different strains of *L. acidophilus* show the importance of using the proper type of prebiotics while preparing synbiotics.

Optical density of 0.1% peptone containing prebiotics

The *L. acidophilus* growth curves are presented in figure 3 when measured in 0.1% peptone containing 1% prebiotics and their controls and in figure 4 when measured in 0.1% peptone containing 3% prebiotics and their controls. Compared to the curves in Figure 1 and 2, the curves in Figure 3 and 4 had lower starting and ending OD values. This was caused by the peptone model system being devoid of casein micelles that are naturally present in skim milk which would increase OD values. The optical densities usually decreased with incubation time, and this may have been due to the growth curve being in the death phase. The decrease was slower for the peptones that contained prebiotics compared to the control. This observation was probably due to slower death of *L. acidophilus* in the peptones containing prebiotics compared to the control. The greater effectiveness of prebiotics in stimulating *L. acidophilus* growth in 0.1% peptone compared to

skim milk may be explained by more nutrients being available for *L. acidophilus* in the skim milk compared to peptone, thereby reducing the need for additional nutrients provided by the prebiotics.

pH of skim milk containing prebiotics

The pH values of skim milk containing 1% prebiotics and their controls are presented in Figure 5. Although the pH values of the P95-containing samples tended to be slightly lower than the pH values of the remaining samples, the pH values of all of the samples containing prebiotics and the control containing *L. acidophilus* were very similar to each other. This similarity indicated that the prebiotics did not appear to have a major effect on rate of decrease of pH by *L. acidophilus* in skim milk. The pH values of skim milk containing 3% prebiotics and their controls are shown in figure 6. Likewise, there was not much difference in rate of decrease of pH values in the skim milks that contained different types of prebiotics. The decrease in pH over time results from the breakdown of lactose to form lactic acid [36]. The lack of differences in pH of the treatments compared to the control may be caused by the buffering capacity of skim milk arising from the caseins, whey proteins, phosphate and citrate.

In addition to growth, variable results have also been reported as

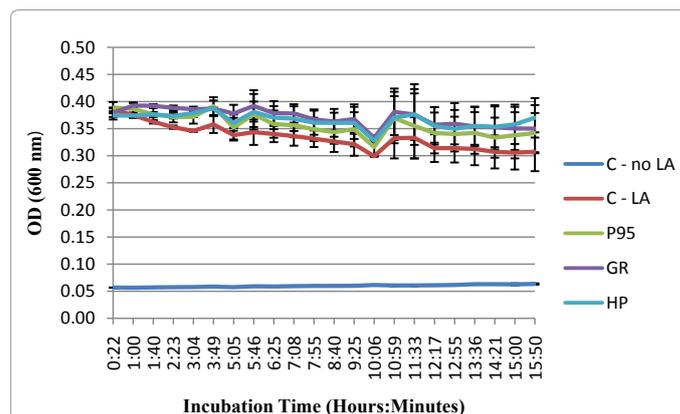


Figure 3: The growth curve of *Lactobacillus acidophilus* (LA) in 0.1% peptone containing 1% prebiotics (P95 (an oligofructose), GR (an inulin), and HP-Gel (an inulin)) and the control skim milk with LA (C-LA) as shown by the optical density (OD) (turbidity) measured at 600 nm as a function of time after addition of LA.

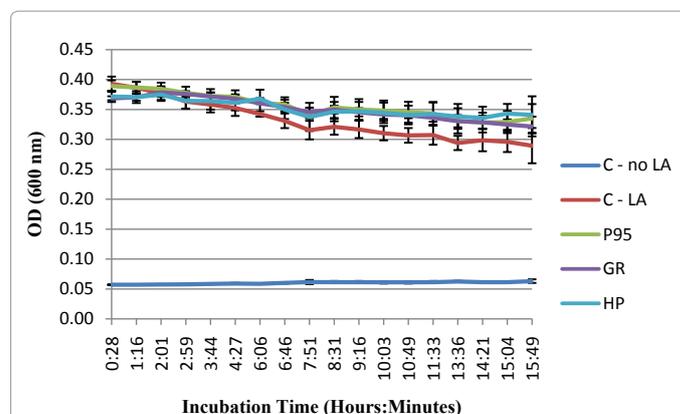


Figure 4: The growth curve of *Lactobacillus acidophilus* (LA) in 0.1% peptone containing 3% prebiotics (P95 (an oligofructose), GR (an inulin), and HP-Gel (an inulin)) and the control skim milk with LA (C-LA) as shown by the optical density (OD) (turbidity) measured at 600 nm as a function of time after addition of LA.

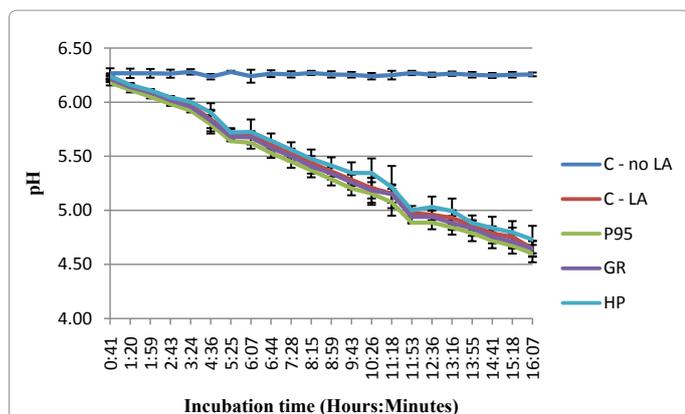


Figure 5: The pH of skim milk containing 1% prebiotics (P95 (an oligofructose), GR (an inulin), and HP-Gel (an inulin)) and the control skim milk with *L. acidophilus* (LA) (C-LA) and without LA (C-no LA) as a function of time after addition of LA.

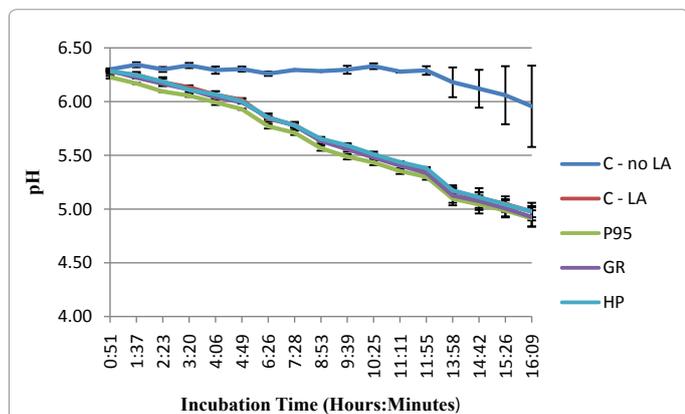


Figure 6: The pH of skim milk containing 3% prebiotics (P95 (an oligofructose), GR (an inulin), and HP-Gel (an inulin)) and the control skim milk with *L. acidophilus* (LA) (C-LA) and without LA (C-no LA) as a function of time after addition of LA.

to the effect of prebiotics on the decrease in pH of various strains of *L. acidophilus*. The pH did not drop faster in reconstituted skim milk containing 1, 2, or 3% Raftiline HP than in reconstituted skim milk without Raftiline HP after 6 hours of incubation with *L. acidophilus* 4461 at 37°C [31]. Supplementing reconstituted skim milk that was fermented with *L. acidophilus* LAC4 and *S. thermophilus* TA040 with 4% (w/w) lactulose increased the lactic acid acidity after day 1, day 7, and day 35, increased the maximum acidification rate, decreased the time to reach the maximum acidification rate and the time to reach pH 4.5, and decreased the pH after 7 and 35 days [32]. When skim milk was supplemented with maltodextrin, oligofructose, and polydextrose individually, similar effects as with lactulose supplementation for lactic acid concentration, maximum acidification rate, and time to reach pH 4.5 were also found [37].

pH of 0.1% peptone containing prebiotics

The pH values of 0.1% peptone containing 1% prebiotics and their controls are presented in Figure 7. The pH values of the samples containing prebiotics were lower than the pH value of the control sample containing *L. acidophilus*. Both the GR- and HP Gel-containing samples had early decreases in pH and lower pH values than the P95-containing samples. It did appear that prebiotics stimulated the rate

of decrease of pH in 0.1% peptone. The pH values of 0.1% peptone containing 3% prebiotics and their controls are shown in Figure 8. Although there was an initial decrease in pH for the control that did not contain the *L. acidophilus*, this decrease in pH was smaller than the decrease in pH for the GR-containing and HP-Gel-containing samples. The P95-containing samples generally had less variation than both the control containing *L. acidophilus* and the GR-containing and HP-Gel-containing samples. Therefore, the GR-containing and HP-Gel-containing samples, but not P95-containing samples, were effective in stimulating the growth of *L. acidophilus*. There was a distinct pH-lowering effect in peptone compared to skim milk because peptone is a simple system and not as nutritionally dense as skim milk. Kunová et al. [38] showed that inulin was usually the most effective for stimulating the growth of various lactobacilli measured after 24 hours of incubation.

Conclusion

Prebiotics had a greater influence on the growth of *L. acidophilus* and a greater pH lowering effect in a peptone model system than in skim milk. The system, skim milk or peptone, in which prebiotics are used, does influence the overall desirable effect of the prebiotic.

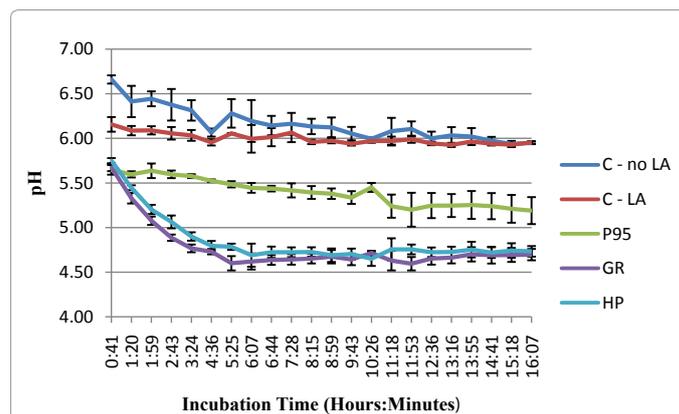


Figure 7: The pH of 0.1% peptone containing 1% prebiotics (P95 (an oligofructose), GR (an inulin), and HP-Gel (an inulin)) and the control skim milk with *L. acidophilus* (LA) (C-LA) and without LA (C-no LA) as a function of time after addition of LA.

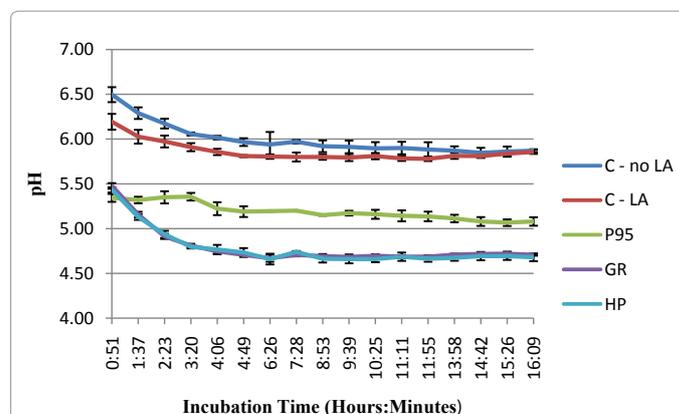


Figure 8: The pH of 0.1% peptone containing 3% prebiotics (P95 (an oligofructose), GR (an inulin), and HP-Gel (an inulin)) and the control skim milk with *L. acidophilus* (LA) (C-LA) and without LA (C-no LA) as a function of time after addition of LA.

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