

## Screening of Antagonistic Activity of Probiotic Bacteria Against Some Food-Borne Pathogens

M. El-Kholy<sup>a</sup>, S.H. El-Shinawy<sup>a</sup>, A. M. S. Meshref<sup>a\*</sup>, A. M. Korny<sup>a</sup>

<sup>a</sup> Food Hygiene Department, Faculty of Veterinary Medicine, Beni-Suef University, Egypt.

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**ABSTRACT:** The objectives of this study were to assess the microbiological quality of Egyptian yoghurt and to investigate the antagonistic activity of some probiotic bacteria against some food borne pathogens *in vitro* and in yoghurt. The results indicated a poor microbiological quality of yoghurt, since the microbiological parameters recorded higher values than the admissible levels. Results from the agar spot test and well diffusion assay showed the capability of probiotic bacteria to inhibit the growth of *S. aureus*, *E. coli O157:H7* and *L. monocytogenes in vitro*. Yoghurt produced from milk artificially contaminated with *S. aureus* (8.48 log cfu mL<sup>-1</sup>) and *E. coli O157:H7* (8.3 log cfu mL<sup>-1</sup>) was used for studying the ability of *L. acidophilus La-5* and *B. longum ATCC15707* to inhibit the growth of such pathogens. During the refrigerated storage the counts of both pathogens decreased significantly in the probiotic yoghurt than in control one. Survival of both *L. acidophilus La-5* and *B. longum ATCC15707* in yoghurt was satisfactory as both bacteria remained viable at levels > 10<sup>7</sup> cfu g<sup>-1</sup> after 15 d of storage at 4 °C, which indicate that the yoghurt would be a suitable vehicle for probiotics.

**Keywords:** *E. coli O157:H7*, Probiotics, Quality, *S. aureus*, Yoghurt.

### Introduction

Yoghurt is the most popular fermented dairy product consumed in Egypt. It is produced by fermentation of heat treated milk. Fermentation processes promote the development of essential and safe microflora, which play a vital role in preventing the outgrowth of pathogenic and spoilage microorganisms (Tsfaye *et al.*, 2011).

"Probiotics" are defined as "live microorganisms which when administered in adequate amounts confer a health benefit on the host" (FAO/WHO, 2001). *Lactobacillus* and *Bifidobacterium* species are the most commonly used as probiotics. Dairy products have been used as carrier foods for probiotic bacteria. Among dairy products, fermented milks were the most used to deliver the probiotic bacteria (Bergamini *et al.*, 2005). The consumption of such

products has health promoting effects, including immunomodulation, alleviation of lactose intolerance, anti-tumor properties, reduction in cholesterol level and anti-infection properties (Kasimoglu and Akgun, 2004).

In order to produce health benefits, a sufficient number of viable microorganisms must be present throughout the entire shelf life of the product. In this regard, minimum levels for probiotic bacteria in yoghurt ranging from 10<sup>5</sup>-10<sup>6</sup> cfu mL<sup>-1</sup> (Dave and Shah, 1997; Gueimonde *et al.*, 2004). Therefore viability and activity of the probiotic bacteria in yoghurt are important considerations. Several studies reported varying viability of various probiotic bacteria in yoghurt (Klaver *et al.*, 1993; Modler and Villa-Garcia, 1993; Medina and Jordano, 1994; Samona and Robinson, 1994; Nighswonger *et al.*, 1996; Shah *et al.*, 1995; Dave and Shah, 1997; Shah and

\*Corresponding Author: [foodhyg@yahoo.com](mailto:foodhyg@yahoo.com)

Lankaputhra, 1997; Canganella *et al.*, 2000; Donkor *et al.*, 2006).

Probiotic bacteria have been shown to possess antagonistic activity against food-borne disease agents such as *S. aureus*, *salmonella spp.*, *E. coli*, *L. monocytogenes* and *Cl. Perfringens* (Kasimoglu and Akgun, 2004; Karagozlu *et al.*, 2007; Millette *et al.*, 2007). This inhibition may primarily be due to the production of inhibitory compounds such as bacteriocins, hydrogen peroxide or organic acids as well as competitive adhesion to epithelium (Karagozlu *et al.*, 2007).

Although fermented milks are generally considered to be safe, owing to its acidic nature, process failure and contaminated raw materials have resulted in their being involved in food-borne illness. During past decades, microorganisms such as *S. aureus*, *E. coli O157:H7*, *Salmonella spp.*, and *L. monocytogenes* were reported as the most common food-borne pathogens that are present in many foods and able to survive in fermented milk products (Pazakova *et al.*, 1997; Dineen *et al.*, 1998; Gulmez and Guven, 2003; Tsegaye and Ashenafi, 2005; Karagozlu *et al.*, 2007). Several studies were conducted to determine the growth and survival of *S. aureus* and *E. coli O157: H7* in yoghurt (Canganella *et al.*, 1998; Dineen *et al.*, 1998; Zuniga-Estrada *et al.*, 1999; Halawa and Abouzeid, 2000; Bachrouri *et al.*, 2002; Ogwaro *et al.*, 2002). However, there is little information on the effects of probiotic bacteria on survival and activity of *S. aureus* and *E. coli O157:H7* in yoghurt (Massa *et al.*, 1997; Kasimoglu and Akgun, 2004).

The main objectives of this study were to assess the microbiological quality and safety of locally produced plain yoghurt in Beni-Suef governorate, Egypt, to investigate the possible antagonistic activity of selected probiotic bacteria against *S. aureus*, *E. coli O157:H7* and *L. monocytogenes in vitro* and

to investigate the effect of *L. acidophilus La-5* and *B. longum ATCC15707* on survival of *S. aureus* and *E. coli O157:H7* in yoghurt during the fermentation and refrigerated storage. Moreover, the viability of *L. acidophilus La-5* and *B. longum ATCC15707* over the duration of storage was investigated to assess the possible use of yoghurt as a carrier for probiotic bacteria.

## Materials and Methods

### - Microbiological and chemical analysis of yoghurt

Fifty plain yoghurt samples were randomly obtained from local markets in Beni-Suef city, Egypt. All samples were transported to the laboratory under refrigerated conditions ( $4 \pm 2$  °C) in retail packages within 1-2 h of collection and analyzed immediately upon arrival.

For microbial counts, 10 gm of yoghurt samples were homogenized with 90 mL of a 0.1 % sterile peptone water (Oxoid, UK) for initial dilution. Decimal dilutions were prepared with the same diluent (9 mL) and appropriate dilutions were used to enumerate coliform, faecal coliform, *E. coli*, *S. aureus* and yeast and mold according to Roberts and Greenwood (2003).

Regarding the chemical analysis of yoghurt, titratable acidity (TA; as lactic acid %) of yoghurt samples was determined according to the method of Bradley *et al.*, (1992).

- *In vitro* antimicrobial activity assay:
- Bacterial strains

*Lactobacillus acidophilus* La-5 and *Bifidobacterium longum* ATCC15707 were kindly provided by CRIFS (Canada). Strains of *Lactobacillus plantarum* (IFPL33, IFPL81, IFPL106, IFPL119, IFPL124, IFPL150, IFPL156, IFPL162, IFPL189, IFPL207, IFPL250, IFPL252, IFPL379 and IFPL935), *S. aureus*, *L. monocytogenes* ATCC5672 and *E. coli O157:H7* were

provided by CSIC (Madrid, Spain). Probiotic strains were propagated in de Man Rogosa and Sharpe (MRS) broth supplemented with 0.05 % L-cysteine hydrochloride (Sigma) at 37 °C for 24 h under an atmosphere of 5 % CO<sub>2</sub> for *L. acidophilus* La-5, anaerobically for *B. longum* ATCC15707 and aerobically for the others. All pathogenic strains were propagated in 10 ml of Brain Heart infusion (BHI) broth (Oxoid) at 37 °C for 24 h. Growth of bacterial cultures was routinely monitored by optical density determinations at 600 nm (OD<sub>600</sub>).

- *Agar spot test*

Antibacterial activity was investigated by an agar spot test, using a colony overlay assay according to Tejero-Sarinena *et al.* (2012). Overnight cultures ( $10^7$ - $10^9$  cfu mL<sup>-1</sup>) of probiotic strains were spotted (5 ul) on the surface of MRS agar and incubated at 37 °C for 24 h under appropriate conditions, to allow colonies to develop. Subsequently, the plates were overlaid with 10 mL of 0.7 % (w/v) BHI agar at 45 °C, previously inoculated with 100 ul ( $10^7$ - $10^9$  cfu mL<sup>-1</sup>) of an overnight culture of the indicator pathogen strain. MRS agar plates without any probiotic spot were also poured with BHI agar containing indicator pathogenic strain as a control. All the plates were incubated aerobically at 37 °C for 24 h. Zones of inhibition around the spots were examined and scored.

- *Well diffusion assay*

Cell free culture supernatants (CFCS) were obtained by centrifugation of overnight culture ( $10^9$  cfu mL<sup>-1</sup>) of probiotic strains at 12000 xg for 10 min at 4 °C. The pellet was discarded and the CFCS was neutralized to pH 6.2 with 1M NaOH and filter sterilized through 0.45 um pore size filter. Aliquots (30 uL) of the CFCS were placed in wells (7 mm diameter), cut in cooled MRS agar plates. The supernatant was allowed to dry

for 1 h inside the wells at room temperature. The plates were overlaid with 10 mL of 0.7 % BHI agar at 45 °C, previously inoculated with 100 ul of an overnight culture of the selected pathogen strain ( $\sim 10^7$  cfu mL<sup>-1</sup>). The plates were left to solidify and then incubated at 37 °C for 24h. Inhibition zones around the wells were measured in mm from the edge of the wells (Ayeni *et al.*, 2009).

- *Impact of probiotic bacteria on growth and survival of S. aureus and E. coli O157:H7 in yoghurt*

Cow milk was heated in a water bath at 90 °C with agitation for 30 min then cooled to 45 °C and inoculated with 2 % (v/v) yoghurt starter cultures (Yoflex<sup>®</sup>, which contained *S. thermophilus* and *L. delbrueckii* ssp. *bulgaricus*, Chr. Hansen). Immediately following the addition of starter, the inoculated milk was divided into 5 portions. The first and second portions of milk were separately inoculated with *L. acidophilus* La-5 and *B. longum* ATCC15707 to give an initial inoculum of  $4 \times 10^8$  and  $4 \times 10^8$  cfu mL<sup>-1</sup>, respectively. Each portion was subdivided into two batches, one inoculated with *S. aureus* and the other inoculated with *E. coli* O157:H7 at rate of  $3 \times 10^8$  and  $2 \times 10^8$  cfu mL<sup>-1</sup> respectively. Another two portions of milk were separately inoculated with  $3 \times 10^8$  and  $2 \times 10^8$  cfu mL<sup>-1</sup> of *S. aureus* and *E. coli* O157:H7 respectively (control). The last portion was maintained without any further inoculation. All portions were incubated at 43 °C until firm coagulum was formed (4-5 h). After fermentation infected and control yoghurts were stored at 4 °C for 15 days. The samples were taken from milk after inoculation (0 h), at termination of fermentation (4 h) and daily during the storage period for chemical and microbiological evaluation.

The number of colony forming units (cfu/g) was determined by surface spread technique onto Sorbitol Mac Conkey agar and Eosin Methylene Blue agar for

enumeration of *E. coli* O157:H7 and onto Baird Parker agar for enumeration of *S. aureus*. Plates were incubated at 37 °C for 24 h and then counted for viable organisms (Roberts and Greenwood, 2003).

To enumerate *L. acidophilus* La-5 and *B. longum* ATCC15707, 0.1 mL of the serially diluted samples were pour-plated on MRS agar supplemented with 0.05 % L-cysteine hydrochloride. Colonies on the plates were counted after 48 h of incubation at 37 °C under appropriated conditions.

- *Statistical analysis*

SPSS pocket program for windows (version 16, 2007) was used for the statistical analysis. Values of different parameters were expressed as the mean ± standard error (SE). One-way analysis of variance (ANOVA) and Duncan's multiple range tests were used to determine significant differences in the measured attributes at  $P < 0.05$ .

## Results and Discussion

The microbiological quality of yoghurt is influenced by the initial flora of raw milk, the processing conditions and post-processing contamination. The presence of coliform bacteria in milk products generally provides an index of the hygienic standards of the product and its keeping quality. Results given in table 1 show that the total coliforms and faecal coliforms could be detected in 74 and 48 % of the examined yoghurt samples with a mean value of  $2.47 \times 10^2 \pm 5.69 \times 10$  and  $1.91 \times 10^2 \pm 5.15 \times 10$  MPN  $g^{-1}$ , respectively. Many reports dealing

with the occurrence of coliforms in yoghurt have been accumulated. In those studies, various rates of coliforms were reported as 83.33, 82, 10, 46.7, 20 and 76 % of examined yoghurt samples by Hassan (2003), El-Kasas (2004), El-Diasty and El-Kaseh (2009), El-Prince *et al.* (2010), Shafa (2012) and El-Malt *et al.* (2013) respectively.

According to the Egyptian Standards (2005), coliforms count of yoghurt should not exceed 10 cfu  $g^{-1}$ , 30 (60 %) samples were found to be highly contaminated with coliforms over this limit. The existence of coliforms in yoghurt may not necessarily indicate a direct faecal contamination, but is more likely as an indicator of insufficient heat treatment of milk, use of unclean equipment, poor personnel hygiene, and water being contaminated or good manufacturing practice being not followed.

*E. coli* is an indicator of faecal contamination and the possibility of the presence of enteric pathogens. *E. coli* was isolated from 16 (32 %) yoghurt samples with a mean count of  $9.29 \times 10 \pm 3.75 \times 10$  MPN  $g^{-1}$  (Table 1). The contamination rate in yoghurt samples was generally higher than those reported by Hassan (2003), Abd Elaal (2008), Mezyed *et al.* (2008), El-prince *et al.* (2010) and Shafa (2012) as they found 26.67, 20, 20, 20 and 0 % of their samples were contaminated by *E. coli* respectively. On the contrary levels of contamination as high as 47.6, 40 and 60 % were reported by Aboul-Khier *et al.* (1985), El-Kasas (2004) and El-Malt *et al.* (2013), respectively.

Table 1. Microbial load of yoghurt samples (/g)

	No. of positive samples		Min	Max	Mean ± SE
	No	%			
Total coliform	37	74	3	$1.1 \times 10^3$	$2.47 \times 10^2 \pm 5.69 \times 10$
Faecal coliform	24	48	3	$1.1 \times 10^3$	$1.91 \times 10^2 \pm 5.15 \times 10$
<i>E. coli</i>	16	32	3	$1.1 \times 10^3$	$9.29 \times 10 \pm 3.75 \times 10$
<i>S. aureus</i>	19	38	$1 \times 10^2$	$1.5 \times 10^5$	$1.73 \times 10^4 \pm 5.02 \times 10^3$
Yeast and Mold	42	84	$4 \times 10^2$	$3 \times 10^5$	$1.55 \times 10^4 \pm 8.68 \times 10^3$

All positive samples failed to comply with the Egyptian standards (2005), of freedom of 1 g of yoghurt from *E. coli*. Yoghurt can be easily contaminated by infected food handlers who practice poor personal hygiene or by water containing human discharges.

In the present study, 19 (38 %) yoghurt samples were contaminated with *S. aureus* ranging from  $10^2$  to  $1.5 \times 10^5$  cfu g<sup>-1</sup>. All positive samples do not comply with the Egyptian standards (2005), of freedom of 1 g of yoghurt from *S. aureus* (Table 1). There are a number of studies concerning the incidence of *S. aureus* on yoghurt. Hassan (2003), Abd Elaal (2008), El-Shinawy *et al.*, (2011) and El-Malt *et al.*, (2013) reported 40, 28, 5 and 72% of the yoghurt samples examined in Egypt were contaminated with *S. aureus*. It obvious from the previous and the present data that yoghurt samples are frequently subjected to *S. aureus* contamination, which may indicate unhygienic handling and inadequate personal hygiene.

*S. aureus* is one of the most common causes of food poisoning in humans worldwide. Although all samples have lower counts of *S. aureus* than  $10^6$  -  $10^8$  cfu mL<sup>-1</sup> levels that are regarded as significant for human food poisoning to occur (Kerouanton *et al.*, 2007 ; Quinn *et al.*, 2002), they still present a public health hazard. Therefore, the general hygienic practices aimed at minimizing bacterial contamination of yoghurt should be emphasized, as well as the growth of *S. aureus* must be prevented to avoid potential risk.

Neither the absence of *S. aureus* nor the presence of small numbers of organism can provide complete assurance that the yoghurt is safe, since conditions inimical to the survival of *S. aureus* may result in a diminished population or death of viable microbial cells, while sufficient toxins remains to elicit symptoms of staphylococcal food poisoning (Bennett and

Monday, 2003).

The result for molds and yeasts in yoghurt are shown in table 1. Counts for molds and yeasts ranged between  $4 \times 10^2$  to  $3 \times 10^5$  with an average count of  $1.55 \times 10^4 \pm 8.68 \times 10^3$  cfu g<sup>-1</sup>. According to Egyptian standards (2005), 42 (84 %) out of 50 samples tested were considered having unacceptable hygienic quality, since they were above the limit of 10 cfu g<sup>-1</sup>. Nearly similar result was reported by Hassan (2003) who found 83.33 % of the yoghurt samples tested was contaminated by molds and yeasts. In recent studies El-Diasty and El-Kaseh (2009), El-Shinawy *et al.*, (2011) and El-Malt *et al.*, (2013) reported that 50, 70 and 98 % of the yoghurt samples tested were contaminated by molds and yeasts, respectively.

Contamination by molds and yeasts is one of the main limiting factors for the stability and the commercial value of yoghurt (Canganella *et al.*, 1998). The presence of molds and yeasts in yoghurt may be attributed to contamination through air, contaminated packaging material (dust) or poor hygiene on the processing area. The presence of molds and yeasts in yoghurt are objectionable, as they grow at a wide range of temperature and pH values resulting surface discoloration, poor appearance, blowing, off-flavours and off-odours (ICMSF 2005). However, of even more serious concern is that molds are capable of producing toxic metabolites known as mycotoxins. Some of these toxins, such as aflatoxins, are known carcinogens (Bullerman, 1981).

Titrateable acidity (TA) is normally used to estimate the freshness of milk and to monitor the production of lactic acid during fermentation. Results shown in table 2 revealed that the TA was ranged between 0.55 and 1.2 % with a mean value of  $0.76 \pm 0.018$  %. Nearly similar TA was reported in previous study on small scale yoghurt by El-Shinawy *et al.*, (2011) stating that the mean

value of TA 0.75%. On the other hand, higher values of TA (1, 0.92 and 1.23 %) were reported in earlier studies by Musaiger *et al.*, (1998), Hassan (2003) and El-Kasas (2004) respectively. All samples surveyed had TA of the recommended  $\leq 1.5$  % by Egyptian standards (2005). The development of acidity during fermentation is necessary for a well balanced aroma, texture and flavour of yoghurt as well as control the growth of harmful bacteria and some pathogens (Ozer and Kirmaci, 2010).

Generally, Fermented milks, like the fresh milk from which they are produced, are liable to contamination. The microbial contamination of yoghurt at levels above the safety levels may be due to a certain extent to the use of traditional culture (starter) and manufacturing process carried out under very poor hygienic conditions.

- *Antimicrobial activity of probiotic strains*

All of the lactobacilli and *B. longum* ATCC 15707 tested showed a zone of inhibition against all pathogens. *L.*

*acidophilus* La-5 showed the largest inhibition zone ( $> 17$  mm) against all pathogens. *B. longum* ATCC 15707 and *L. plantarum* IFPL 150 showed strong inhibition activity (a clear zone of inhibition between 11 and 17 mm) against all pathogens. The remaining strains of *L. plantarum* tested showed the same degree of inhibition (+; zone of inhibition between 5 and 10 mm) against all pathogens (Table 3).

The same probiotic strains were also tested by a well diffusion agar test, to study their ability to produce antimicrobial substances. We observed that the inhibitory effect against the pathogenic strains by a well diffusion assay were in accordance with the agar spot test (Table 4). The controls showed no inhibitory activity.

*In vitro* tests has proven useful for selecting isolates that have the ability to inhibit or compete with harmful bacteria and can provide an insight into possible mechanisms by which probiotics exert their antagonistic effects towards pathogens. The antagonistic activities demonstrated by

Table 2. Results of titratable acidity of examined yoghurt (%)

Number of samples	Min	Max	Mean $\pm$ SE
50	0.55	1.2	0.76 $\pm$ 0.018

Table 3. *In vitro* antimicrobial activity of probiotic strains (agar spot test)

Strain	<i>S. aureus</i>	<i>L. monocytogenes</i> ATCC 5672	<i>E. coli</i> O157:H7
<i>L. acidophilus</i> La-5	+++	+++	+++
<i>B. longum</i> ATCC15707	++	++	++
<i>L. plantarum</i> IFPL150	++	++	++
<i>L. plantarum</i> IFPL (33, 81, 106, 119, 124, 156, 162, 189, 207, 250, 252, 379, 935)	+	+	+

Table 4. *In vitro* antimicrobial activity of probiotic strains (well diffusion assay)

Strain	<i>S. aureus</i>	<i>L. monocytogenes</i> ATCC 5672	<i>E. coli</i> O157:H7
<i>L. acidophilus</i> La-5	+++	+++	+++
<i>B. longum</i> ATCC15707	++	++	++
<i>L. plantarum</i> IFPL150	++	++	++
<i>L. plantarum</i> IFPL (33, 81, 106, 119, 124, 156, 162, 189, 207, 250, 252, 379, 935)	+	+	+

probiotics may be due to the production of substances with antibacterial properties in particular: organic acids (e.g. lactic acid and acetic acid), hydrogen peroxide and bacteriocins (Tejero-Sariñena *et al.*, 2012). They can also produce biosurfactants and other adhesion inhibitors and stimulate the immune response (Ayeni *et al.*, 2009).

The potential of some *Lactobacillus* and *Bifidobacterium* spp. and their by products to control pathogens and spoilage microorganisms *in vitro* and in food systems has been evaluated by several researchers (Goktepe, 2006; Millette *et al.*, 2007). The results found in those studies and present work demonstrate the capability of the selected probiotics to inhibit the growth of several pathogens such as *S. aureus*, *E. coli*

*O157:H7* and *L. monocytogenes in vitro* and the potential mechanism of action are specific to a particular strain (Tejero-Sariñena *et al.*, 2012).

- *Impact of probiotic bacteria on growth and survival of S. aureus and E. coli O157:H7 during fermentation and storage of yoghurt*

The selection of *L. acidophilus* La-5 and *B. longum* ATCC15707 for incorporation in yoghurt production was based on the results of a series of *in vitro* trials regarding their antimicrobial activities.

The counts of *S. aureus* and *E. coli O157:H7* in yoghurts made with probiotic and without probiotic strains culture, during manufacture and storage at 4 °C are given in Fig. 1 & 2.

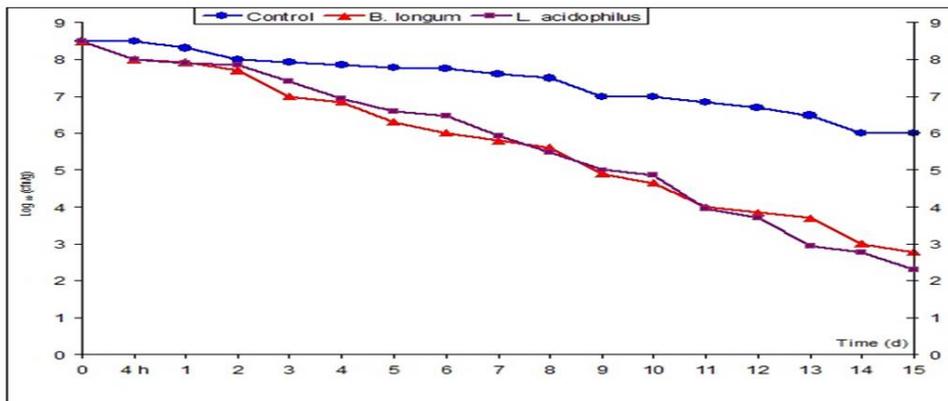


Fig. 1. Behaviour of *S. aureus* during the fermentation and storage of probiotic yoghurt (*L. acidophilus* La 5 *B. longum* ATCC15707)

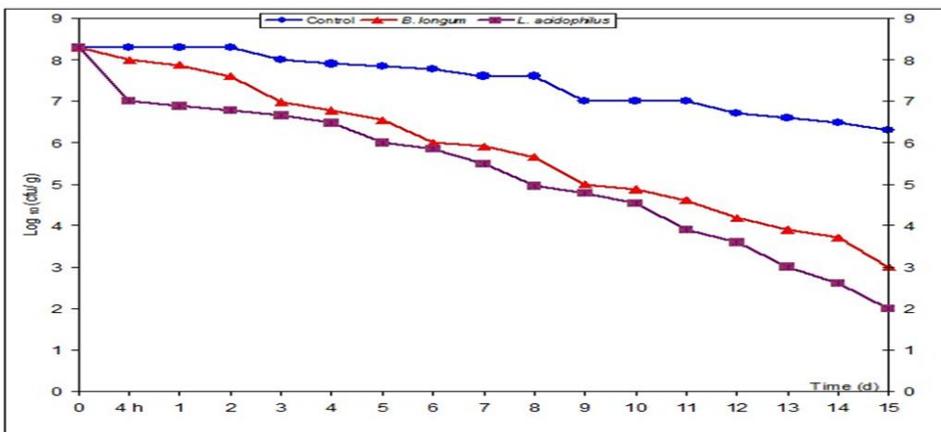


Fig. 2. Behavior of *E. coli* 0157 during the Fermentation and storage of Probiotic yoghurt (*L. acidophilus* La 5 and *B. longum* ATCC15707)

The behaviour of *S. aureus* and *E. coli* O157:H7 during the fermentation and storage of yoghurt was similar. The viable population of the *S. aureus* and *E. coli* O157:H7 in yoghurt declined as the storage period extended. The counts of *S. aureus* decreased significantly ( $P < 0.05$ ) from 8.48 log cfu mL<sup>-1</sup> to 2.3 and 2.78 log cfu g<sup>-1</sup> for the yoghurt co-inoculated with *L. acidophilus* La-5 and *B. longum* ATCC15707 respectively, whereas they decreased to 6 log cfu/g in yoghurt made without probiotic strains. The counts of *E. coli* O157:H7 decreased significantly ( $P < 0.05$ ) from 8.3 log cfu mL<sup>-1</sup> to 2 and 3 log cfu g<sup>-1</sup> for the yoghurt co-inoculated with *L. acidophilus* La-5 and *B. longum* ATCC15707 respectively, while they decreased to 6.3 log cfu g<sup>-1</sup> in yoghurt made without probiotic strains.

During the storage period, survival of *S. aureus* and *E. coli* O157:H7 were significantly lower ( $P < 0.05$ ) in yoghurt with probiotic compared to that without probiotic strains that indicates the antagonistic activity against both pathogens.

The behaviour of many pathogens of concern to food safety has been extensively investigated in traditional yoghurt. In previous studies, the cells of *E. coli* O157:H7 and *S. aureus* have been shown to survive in yoghurt stored at 4 °C from few days to several weeks (Canganella *et al.*, 1998; Zuniga-Estrada *et al.*, 1999; Halawa and Abouzeid, 2000; Bachrouri *et al.*, 2002; Ogwaro *et al.*, 2002). On the other hand, Dineen *et al.* (1998) showed that *E. coli* O157:H7 was not recovered after the curd formation step in yoghurt manufactured with milk inoculated with 10<sup>5</sup> cfu mL<sup>-1</sup>. However, there are a limited number of studies about the effect of probiotic bacteria on the behaviour of *S. aureus* and *E. coli* O157:H7 in yoghurt. Massa *et al.* (1997) reported a slight decrease in number of *E. coli* O157:H7 after 7 d of storage at 4 °C. The

counts decreased from 7.08 to 5.32 log cfu mL<sup>-1</sup> in traditional yoghurt, and from 7.38 to 5.41 log cfu mL<sup>-1</sup> in "bifido" yoghurt. Kasimoglu and Akgun (2004) reported that 10<sup>6</sup> cfu g<sup>-1</sup> of *E. coli* O157:H7 were eliminated after 48 and 72 h in acidophilus and traditional yoghurt respectively. Al-Delaimy and Hamamdeh (2013) showed that *S. aureus* was inactivated after 12 and 9 d of storage at 4 °C in traditional and probiotic yoghurt respectively. The different obtained by various workers might be due to differences in strain sensitivity to pH and temperature, type of starter cultures used, inoculums' level, acid adaptation conditions and properties of food system tested (Tsegaye and Ashenafi, 2005; Hsin-Yi and Chou, 2001).

The viability of probiotic bacteria in yoghurt must be kept sufficiently high to ensure that consumers receive health benefits. These benefits include the prevention of diarrhea, balancing of intestinal microflora, stimulation of the immune system, antitumor properties and alleviation of lactose intolerance (Goktepe, 2006). Of particular importance, is the capacity of probiotics to antagonize pathogens (Tejero-Sariñena *et al.*, 2012).

In order to produce these benefits, the minimum level of probiotic bacteria in yoghurt has been suggested to be 10<sup>5</sup>-10<sup>6</sup> viable cells per mL or g of product (Dave and Shah 1997; Gueimonde *et al.*, 2004).

The changes in the viable counts of *L. acidophilus* La-5 and *B. longum* ATCC15707 in yoghurt during manufacture and storage are given in Fig. 3. Survival of both probiotics in yoghurt was satisfactory and the microbial counts remained stable with values around 8 log cfu g<sup>-1</sup> throughout the storage period. Generally speaking, over 15 days storage at 4 °C there was some decline in the numbers of *L. acidophilus* La-5 and *B. longum* ATCC15707. Similar observations were reported by Shah *et al.*,

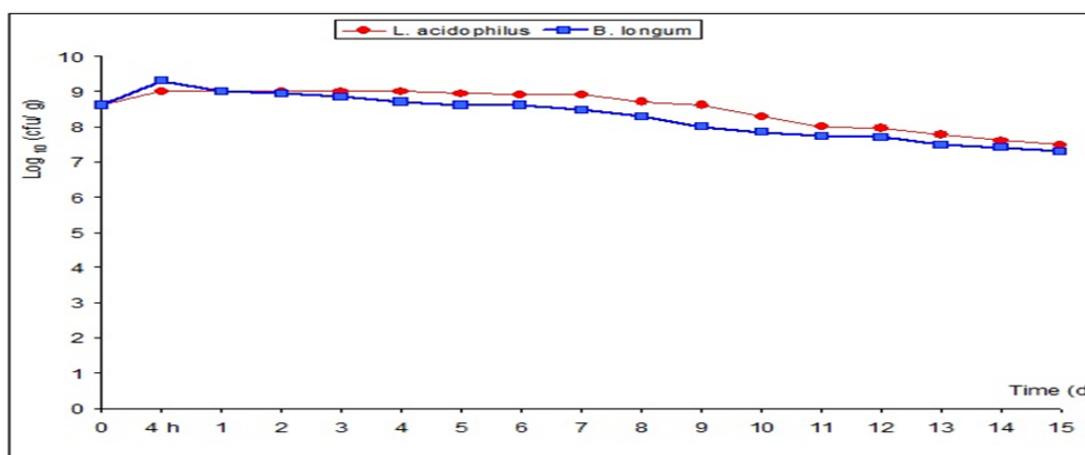


Fig. 3. Survival of *L. acidophilus* La 5 and *B. longum* ATCC15707 during the fermentation and Storage of yoghurt

(1995), Dave and Shah (1997), Shah and Lankaputhra (1997), Canganella *et al.*, (2000) and Gilliland *et al.*, (2002). After 15 days of storage at 4 ° C, the yoghurt still contained ca. 7.48 and 7.3 log cfu g<sup>-1</sup> of *L. acidophilus* La-5 and *B. longum* ATCC15707 respectively, thus satisfying the criteria for probiotic bacteria.

The viability of lactobacilli and bifidobacteria in fermented milks in the literature is variable. A poor survival for bifidobacteria in yoghurts was reported by Klaver *et al.*, (1993) and Modler and Villa-Garcia (1993). In contrast, a satisfactory viability was reported by Medina and Jordano (1994) and Samona and Robinson (1994). Nighswonger *et al.*, (1996) found that, although the viable counts of some of the *L. acidophilus* and *L. casei* used as adjuncts in yoghurt production declined significantly after storage, most probiotic populations were between 5 and 7 log cfu g<sup>-1</sup> at the end of the products shelf life. Donkor *et al.*, (2006) concluded that the ability of probiotic to survive in yoghurt was strain dependent. In addition *L. acidophilus* could survive in yoghurt at sufficient levels (> 10<sup>6</sup> cfu g<sup>-1</sup>) for up to 28 days. Variation in the probiotic viability data among different authors may probably be attributed to strain

variation, acid accumulation, interaction with starter cultures and storage condition.

The data suggests that yoghurt can be a suitable carrier food to supply consumers with lactobacilli and bifidobacterium having potential health and nutritional benefits. A similar observation was previously reported for *L. acidophilus* and *B. bifidum* by Kailasapathy and Rybka (1997), on the contrary Gilliland and Speck (1977) and Modler and Villa-Garcia (1993) considered yoghurt as unsuitable vehicle for *L. acidophilus* and bifidobacteria respectively. However, care should be used in strain selection to provide maximal survival during refrigerated storage of the yoghurt.

The acid production in yoghurt depends on the growing of microorganisms and their ability for fermentation of the lactose in milk. Yoghurt is manufactured by the addition of a culture containing *S. thermophilus* and *L. delbrueckii ssp. Bulgaricus*. These organisms are active even at refrigerated temperatures and still can produce small amounts of lactic acid by fermentation of lactose resulting in noticeable TA increase (Shah *et al.*, 1995). The TA increased over time from an initial 0.70 % at termination of fermentation (4 h) to 1.2, 1.4 and 1.5 % by 15 d of storage at 4 °

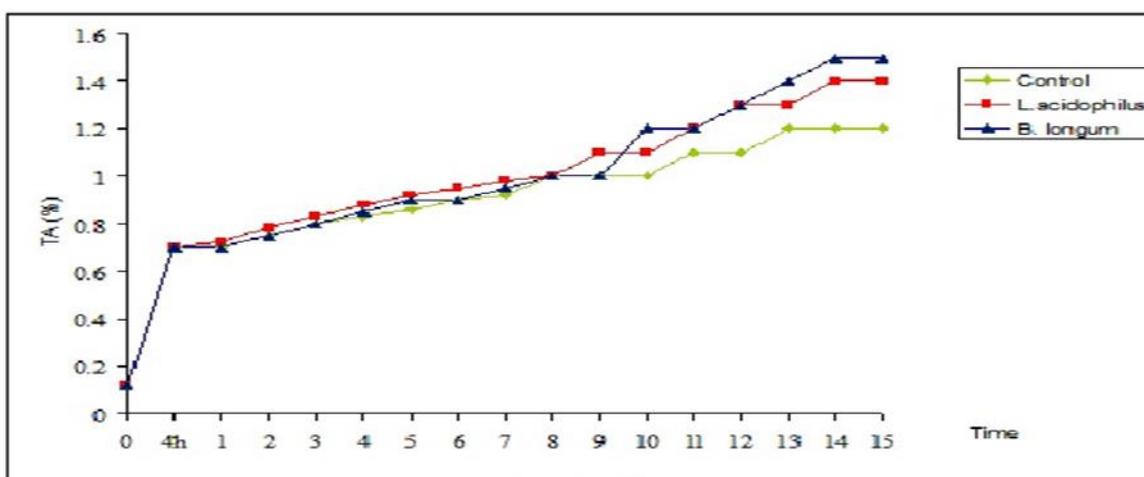


Fig. 4. Changes in Titratable acidity during the fermentation and storage of control and probiotic yoghurt

C, for control, *L. acidophilus* La-5 and *B. longum* ATCC15707 yoghurts respectively (Fig. 4).

The concentration of lactic acid produced in control yoghurt was not significantly ( $P > 0.05$ ) different than that in the probiotic yoghurts from day 1 until the end of storage period. A similar observation was previously reported by Donkor *et al.* (2006). This might imply that the inactivation of *S. aureus* and *E. coli* O157:H7 in yoghurt during cold storage is not only governed by the acidity of the yoghurt but also by the presence of other inhibitory substances produced by the probiotic strains (Kasimoglu and Akgun, 2004). Moreover, the lactic acid content appeared to have no effect on the viability of probiotic organisms (Akalın *et al.*, 2004; Donkor *et al.*, 2006).

### Conclusion

Results of the study clearly indicate that the microbiological quality of locally produced plain yoghurt was inferior. The microbial contamination of yoghurt represents not only spoilage of the product but also indicate the presence of potential human pathogens. The presence of pathogenic bacteria as *E. coli* and *S. aureus* pose a risk for public health. Therefore, the hygienic standard needs to be strengthened during manufacture and storage to ensure the

production of safe, high quality yoghurt. The results obtained in this study also demonstrated the capability of the selected probiotic bacteria to inhibit the growth of *E. coli*, *S. aureus* and *L. monocytogenes* *in vitro*. Survival of both *L. acidophilus* La-5 and *B. longum* ATCC15707 in yoghurt was satisfactory as both bacteria remained viable at levels  $> 10^7$  cfu g<sup>-1</sup> after 15 d of storage at 4 °C, which indicate that the yoghurt would be a suitable vehicle for probiotic bacteria. Both *L. acidophilus* La-5 and *B. longum* ATCC15707 have been shown to possess inhibitory activity toward the growth of *E. coli* O157:H7 and *S. aureus* during the fermentation and storage of yoghurt.

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