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Reuteri yoghurt against Diarrheagenic *Escherichia coli* and shiga toxin production

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ABSTRACT

Yoghurt containing *L. reuteri*, as a probiotic milk product was prepared and questioned to study for the growth effect of *Lactobacillus reuteri* on the growth and inhibition ratio of Enteroaggregative *E.coli* (EAEC O111:H2) and its shiga toxin (diarrhea cases) in milk, yoghurt and in Reuteri-yoghurt during 14 days of cold storage. Results of growth curves showed very little effect of *Lactobacillus reuteri* on EAEC in the first 7days, while more inhibition effect was shown during the rest 7 days. Furthermore, Inhibition ratio of EAEC O111:H2 growth due to the effect of *L. reuteri* (R) and yoghurt starter culture (Y) in Di-cultures (R&Y) and in Tri-cultures in Reuteri yoghurt, was estimated and calculated. Results reveal similar and weak inhibition % of EAEC due to *L. reuteri* (Di-culture, DCR) and yoghurt culture (DCY). While in Tri-cultures (TriC) of yoghurt and reuteri cultures, together, Inhibition ratio growth of EAEC reveal very little effect in the first 7 days of storage, while it increased remarkably after 14 days of storage. At the same time, results for shiga-toxin (Stx1) production by EAEC O111:H2 in pasteurized milk (trail 1), reuteri- milk (trail 2), yoghurt (trail 3) and reuteri- yoghurt cultures (trail 4) revealed that shiga-toxin (Stx1) were produced by the 5 strains (100%) of EAEC O111:H2 during the 14 days of storage in milk (trail 1). The highest effect of *L. reuteri* on EAEC Stx production was detected in Reuteri-yoghurt (trail 4), as all of the strains (100%) lost their toxicity after 7 days of storage. The lower effect was noted in reuteri culture (trail 2), as 1 strain (20%) was positive for Stx production at the end of storage, While, the lowest effect was recorded for yogurt cultures on Stx production, as 3 strains (60%) were positive at the end of storage period. Hence, it is obvious the anti-toxic activity of *Lactobacillus reuteri* on shiga-toxin (Stx1) production of EAEC particularly in the presence of yoghurt starter cultures. Reuteri yoghurt sensory evaluation was quite acceptable by panelist people. Hence, Reuteri-yoghurt could be considered good fermented milk product, due to anti-shiga infant diarrhea recommended drink, with good sensory properties and shelf life and may be attractive for entering the Egyptian growing market of probiotics, as a competitor new fermented milk product.

Keywords: Yoghurt, *Lactobacillus reuteri, growth, inhibition, E.coli*

Introduction

At the turn of the 20th century, *L. reuteri* was recorded in scientific classifications of lactic acid bacteria, though at this time it was mistakenly grouped as a member of *Lactobacillus fermentum*. Reuter reclassified the species as "*Lactobacillus fermentum* biotype II" and it has been isolated from many foods, especially meat, milk and natural environments (Reuter, 1965). Moreover, *Lactobacillus reuteri* have been considered important bacteria for human health; the main probiotic effects attributed to these bacteria include: improvement in lactose utilization, prevention of diarrhea, colon cancer, hypercholesterolemia, improvement of vitamin synthesis and calcium absorption (Taranto *et al.,* 2000, and Vinderola, et al., 2000), and production of substances of low molecular mass with antimicrobial agent termed reuterin (Axelsson *et al.,* 1989 and Casas *et al.,* 1998). Also, one of the well-documented effects of *L. reuteri* is in the treatment of diarrheal diseases in children, where it significantly decreases symptom duration (Urbanska *et al.,* 2016). It is therefore understandable that lately there has been an increasing interest in the incorporation of these species into fermented milk products (Claude *et al.,* 2016).

Yoghurt is one of the most common dairy products consumed around the world. Traditional yoghurt is defined as "Coagulated milk product obtained by lactic acid fermentation through the

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action of *Lactobacillus delbreukii spp bulgaricus and Streptococcus thermophilus*". Yoghurt should contain sufficient number (10 ^{7} cfu/g$^{)}$ of the used starter culture alive until it reaches the consumer gut (Tamime and Death, 1980). Therefore, the survival of yoghurt microorganisms during storage is important criterion for the quality and health characteristics of the product (Granatto *et al.,* 2010).

On the other hand, *Escherichia coli* were classified into six pathotypes, Enteropathogenic *E.coli* (EPEC), Enterotoxigenic *E.coli* (ETEC), Enteroinvassive *E.coli* (EIEC), Enteroaggregative *E.coli* (EAEC), diffusely adherent E.coli (DAEC) and Enterohemorraghic *E.coli* (EHEC) (Torres *et al.,* 2005). *Escherichia coli* that produces one or more types of cytotoxins known as Shiga toxin (Stx) or Verocytotoxin (VT) is referred to as Shiga toxin-producing *E. coli* (STEC) or Verocytoxion producing *E. coli* (VTEC) (Nataro and Kaper 1998). In Egypt, El Gamal *et al.,* (2015a and b) and Abdelhamid *et al.* (2017) studied Diarrheagenic and shiga Toxin-Producing *Escherichia coli* in retailed raw meat, hospitalized diarrheal children and in retailed milk and soft cheese.

In respect of using probiotics in milk products to combat pathogenic bacteria, Amal *et al.,* (2016) and Azizkhani and Tooryan, (2016); Alistair *et al.,* (2016) and Oluyege, Adekemi *et al.,* (2018) studied the antimicrobial impact of some probiotic bacteria, *Lactobacilli* strains, and yoghurt starter culture against selected pathogenic bacteria including *E.coli*.

Currently and more recently, probiotics is being increasingly studied and have come to attention as a potentially beneficial as an alternative therapy for diarrheal disease. The main probiotic bacteria used for clinical purposes include bacteria of the species *Lactobacillus, Bifidobacterium, and Enterococcus* (De Vrese and Marteau, 2007 and Ishibashi and Yamazaki , 2001).

Therefore this work amid to prepare Reuteri yoghurt that containing *L. reuteri*, as a probiotic milk product. Reuteri yoghurt, was questioned for studying the growth effect and inhibition ratio of *Lactobacillus reuteri* as probiotic bacteria on Enteroaggregative *Escherichia coli* (EAEC) in milk and Reuteri yoghurt with yoghurt starter culture (*Lactobacillus delbreukii spp bulgaricus and Streptococcus thermophilus*), during 14 days of cold storage. At the same time, the effect of *Lactobacillus reuteri*, Streptococcus *thermophilus and Lactobacillus bulgaricus* on the production of shiga toxin produced by EAEC strains during 14 days of cold storage was studied.

Materials and Methods

Materials:

Milk for Reuteri yoghurt:

Fresh cow's and buffalos milk (mixed 1:1, fat adjusted to ~4%) were obtained from a private farm, Giza province.

Bacterial cultures:

Streptococcus Salivarius subsp. thermophilus and Lactobacillus delbrueckii subsp. bulgaricus as yoghurt starter culture were obtained from Chr. Hansen laboratories, Copenhagen, Denmark. While, *Lactobacillus reuteri* culture was obtained from Dairy microbiology Laboratory at the National Research Center (NRC, Dokki, Egypt). Starter cultures were maintained and sub-cultured in sterile reconstituted skim milk which incubated at 42C for ~4hs for gelation and cold stored until use (Wael *et al.,* 2017).

Strains of Enteroaggregative *E. coli*, EAEC O111:H2, (producing Stx 1 toxin) were isolated in previous study from infant diarrheal cases, identified, maintained and sub-cultured onto MacConkey broth and agar at 37C for 24h (El Gamal *et al.,* 2016 a and b).

Lactobacillus delbrueckii subsp. bulgaricus strain was sub-cultured on MRS broth & agar (De Man, Rogosa and Sharp obtained from Lab M) and incubated anaerobically at 37C until use. While Streptococcus *thermophilus* was maintained on MRS broth and sub-cultured on M17 agar (Oxoid) aerobically at 37C until use (Claude *et al.,* 2016 and ISO/IDH2003).

Enteroaggregative *E. coli* strains (in mono -culture) and with *Lactobacillus reuteri* (in Di - culture) and with yoghurt starter culture (in Tri -culture):

Enteroaggregative *E. coli* (EAEC O111:H2) strains, (in mono-culture):

Pasteurized milk in test tubes (10ml) were inoculated with Enteroaggregative *E.coli* (EAEC O111:H2 strains, separately) and incubated 37^0C for 24hs. *E. coli* were enumerated and counted onto violet red bile agar (VRBA) and Levine eosin methylene blue (EMB) agar (Becton Dickinson, Sparks, MD, USA) (FDA, 2002 and Abdelhamid *et al.,* 2017) at intervals of zero time (after 4 h incubation, simulates yoghurt end of fermentation ~4 h) and at intervals of 3,7,10 and 14 days in milk under cold storage (5-7 ^{0}C).

Lactobacillus reuteri affected Enteroaggregative *E.coli* (EAEC O111:H2 strains), (in Di-CR-mixed cultures):

Pasteurized whole milk (4% fat) in 250ml flasks were inoculated with the Enteroaggregative *E.coli* (EAEC O111:H2 strains, separately, and incubated 37C for 4hs. EAEC cultures were counted on MacConkey (Becton Dickinson) ager plates (FDA, 2002) at intervals of 0, 3,7,10 and 14 days of the di-cultures in milk under cold storage. *L. reuteri* in mixed cultures were also counted onto MRS agar (Difco), (Claude *et al.,* 2016), simultaneously at the same intervals.

Yoghurt cultures affected EAEC strains (in Di-CY-mixed cultures):

Pasteurized whole milk (4% fat) in 250ml flasks were inoculated with Enteroaggregative *E.coli* (EAEC O111:H2 strains, separately, and incubated 42C for 4hs. EAEC cultures were counted on MacConkey (Becton Dickinson) ager plates (FDA) at intervals of 0, 3,7,10 and 14 days of the Di-cultures in yoghurt under cold storage. *Lactobacillus delbrueckii subsp. bulgaricus* and Streptococcus *thermophilus* in mixed cultures were also counted onto MRS agar (Oxoid), and M17 (Oxoid) agar, respectively (Claude *et al.,* 2016 and ISO/IDH2003), simultaneously at the same intervals.

Yoghurt culture and *Lactobacillus reuteri* (Reuteri yoghurt) affected EAEC strains (in Tri-C-mixed cultures):

Pasteurized whole milk (4% fat) in 250ml flasks were inoculated with Enteroaggregative *E. coli* (EAEC O111:H2 strains, separately, with yoghurt liquid starter cultures, as recommended by Claude *et al.* (2016). The inoculated mixtures were aliquot into cups and incubated at 42 °C for fermentation and the incubation was terminated when pH reached 4.5 (approximately 6 h), then yogurt cups were cooled rapidly in a refrigerator at 4±1 °C for storage in order to obtain a product with a good texture and culture cells in a homogenous physiological state.

Enumeration of *L. reuteri,* yoghurt starter culture and *E. coli*:

Samples of Yogurt and Reuteri-yoghurt, 10 g each was blended with 90 mL of a dissolution medium (1 $g.L^{-1}$ tryptone, 8.5 $g.L^{-1}$ sodium chloride, and 10 $g.L^{-1}$ sodium citrate dehydrate) in 25ml flasks and blended well. After a 10-min incubation period at room temperature, 1 mL of the homogenate was transferred to 9 mL of 1 g.L−1 peptone water (Oxoid, London, UK) and homogenized 30 s. Serial dilutions in 1 g.L−1 peptone water were then pour plated in MRS-T agar (tetracycline 0.9 mg.mL−1, Sigma, St. Louis, MO, USA) for enumeration of *L. reuteri* (Hekmat *et al.,* 2009) and in M17 agar for *Str. thermophilus* counts. The MRS-T plates were then incubated in anaerobic conditions (*L. reuteri)* (5% CO_2, 10% H_2, and 85% N_2) at 37 °C for 48 h, while M17 plates were incubated aerobically (*Str. thermophilus*) (ISO/IDH2003). Plating for counting *Lb. bulgaricus* was carried out onto MRS (Oxoid, London, UK) and incubated as previously described for *L. reuteri.* Enteroaggregative *E.coli* (EAEC O111:H2 strains were counted onto MacConkey (Becton Dickinson) ager, which were incubated at 37C for 24h (FDA, 2002).

Kinetics of inhibition ratio (InhR, %) of EAEC *E.coli* due to *Lactobacillus reuteri:*

The inhibition ratio (%) of pathogenic strains (PS) of EAEC *E.coli* (Enteroaggregative *E. coli* O111:H2) due to *Lactobacillus reuteri* (in DiC-culture-DCR) and yoghurt cultures (*Streptococcus thermophilus and Lactobacillus. bulgaricus*), (in DiC-culture-DCY) and mixtures of *L. reuteri* and yoghurt cultures (in Tri-culture-Tri C) was carried out according to following equation as reported by Cheikhyoussef *et al.* (2008) and Amal *et al.,* 2016):

PS (pathogenic strain) inhibition (%) for Di- cultures (Di-C) =
= (log cfu g-1 PS MC- log cfu g-1 PS DC X 100) / log cfu g-1 PS MC
PS inhibition (%) for Tri- cultures (Tri-C) =
= (log cfu g^{-1} PS MC- log cfu g^{-1} PS Tri X 100) / log cfu g^{-1} PS MC
*PS MC monoculture of EAEC strains.
*PS DCR: Di- cultures of pathogenic EAEC strains with *L. reuteri*.
*PS DCY: Di-cultures of pathogenic EAEC strains with yoghurt cultures.
*PS Tri-C Tri-cultures of pathogenic strains EAEC strains with *L. reuteri* and yoghurt cultures.

Shiga toxin of EAEC detection test:

Five *E. coli* (EAEC) colonies were picked up from one plate, from each of mono, Di and Tri-cultures plates and were tested for shiga toxin production using the Immune-Card STAT EHEC kit (Meridian Bioscience, Inc., USA). This is a rapid test for detection of shiga toxin production by *E. coli* and for discrimination of the two types of shiga toxin (ST1 and ST2) in broth methods (Abdelhamid *et al.,* 2017).

Determination of pH:

The pH values of the samples were determined during fermentation and storage at 4 ˚C. Each yogurt sample (1 g) was mixed with distilled water (1:1), and pH was measured using a digital pH meter, calibrated routinely with fresh pH 4.0 and 7.0 standard buffers (Azizkhani and Tooryan, 2016).

Sensory evaluation:

The organoleptic properties including flavor 40 points; body and texture 30 points and appearance 30 points was carried out for the Reuteri yoghurt free pathogen strains according to Mehanna *et al.* (2000). The organoleptic evaluation was done by 10 of the dairy department staff member.

Statistical analysis:

Statistical analyses were performed using the GLM procedure with SAS (2004) software. Duncan's multiple comparison procedure was used to compare the means. A probability to P≤0.5 was used to establish the statistical significance.

Results and Discussion

Effect of *Lactobacillus reuteri* on growth of Enteroaggregative *Escherichia coli*, EAEC (in Di-culture R in pasteurized milk):

Results as shown in Figure (1) reveal that EAEC in single culture increased 1.1 log cfu/g^{-1} in pasteurized milk, while EAEC in Di-culture (mixed with *L. reuteri*) decreased only 0.3 log cfu/g^{-1}, while PH decreased from 6.5 to 4.5. Similar behavior was noticed for the growth of *L. reuteri* during cold storage as it decreased only 1 log cfu/g^{-1}, this might be considered as a good response from side of probiotic characters of the strain.

The strongest inhibition effect was observed against *E. coli* by *Lactobacillus acidophilus DSM 20079* by SAS (2004) which exhibited antibacterial activity of some strains of probiotic bacteria (including some Lactobacillus spp.). Moreover, they reported that some Lactobacillus spp as probiotic bacteria had reduced the population of the pathogenic bacteria by 4.7 log CFU /g, higher than was obtained by *L. reuteri* in our study. Also, Parthiban *et al.* (2003 found high activity of *L. reuteri* at 6 log CFU/g as it could reduced *E. coli* O157:H7 to undetectable levels by 10 days and 20 when the initial *E. coli O157:H7* levels were 3 and 6 log CFU/g, respectively. On the other hand, counts of *L. reuteri* in the present study was compatible with a guide of the International Dairy Federation (IDF) in order to exhibit positive health effect of probiotics as it has a to deliver in certain numbers minimum of 10^7 cfu of probiotic / g product (Ouwehand and Salminen, 1998). Furthermore, results for the growth of *L. reuteri* during storage were in agreement with Hekmat *et al.* (2009); Liu and Tsao (2009) they reported data show limited growth *of L. reuteri* in milk and low stability during storage. Also, Granatto *et al.* (2010) reported that the number of probiotic survived microorganisms decreased with extended storage, but several factors determine the rate of their decrease.

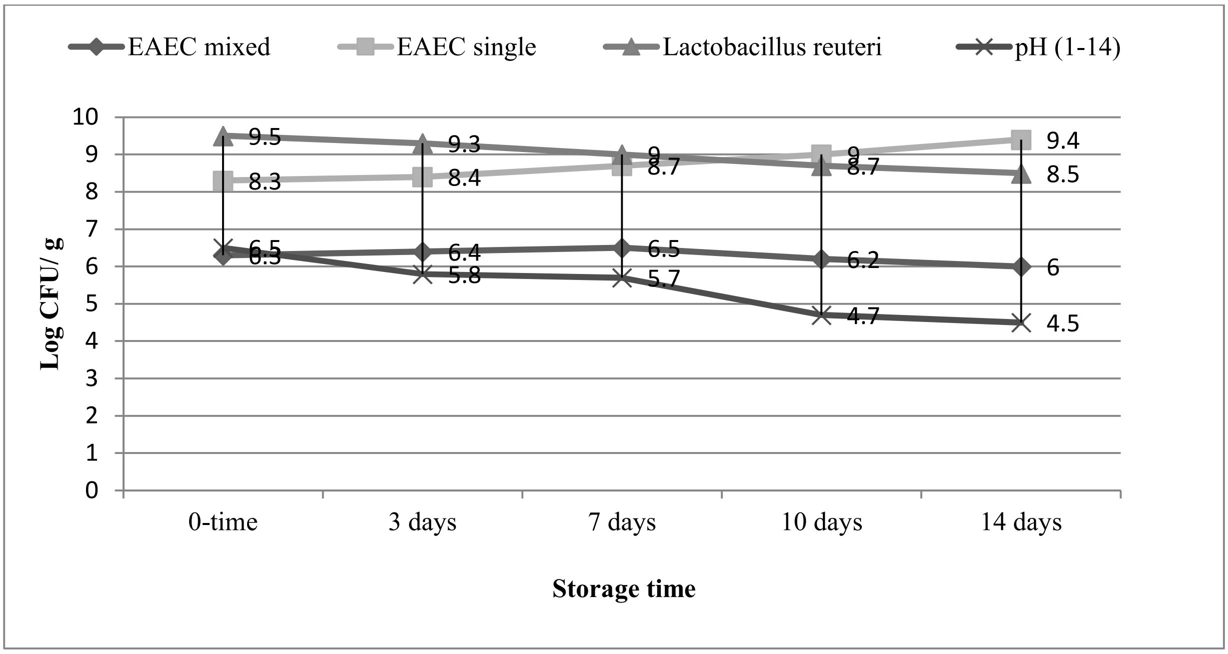

Fig. 1: Effect of *Lactobacillus reuteri* on growth of EAEC O111:H2 in pasteurized milk

Effect of yoghurt starter culture (*Streptococcus thermophilus and Lactobacillus bulgaricus*) on the growth of EAEC in yoghurt (in Di-culture Y):

Results in Figure (2) show the effect of yoghurt stature culture (S*treptococcus thermophilus and Lactobacillus bulgaricus*) on the growth of EAEC O111:H2 (Di-culture, yoghurt culture with DEC, DiC-Y). Results reveal very little effect of yoghurt starter culture on EAEC in mixed culture (DiC-Y) in yoghurt during 14 days of cold storage, as it decreased only 0.5 log cfu/g^{-1}, while PH decreased from 6.4 at the start point to 3.5 at the end of cold storage. On the other hand EAEC in single culture increased as previously described 1.1 log cfu/g^{-1} in pasteurized milk during the 14 days of cold storage. Yoghurt stature culture, *Streptococcus thermophilus and Lactobacillus bulgaricus*, grew well in ratio about 1: 1 with counts fluctuated between 7.3 to 9 log cfu/$g^{-1,}$ during cold storage.

Hence, the obtained results agree with Granatto *et al.* (2010) who reported that survival of yoghurt microorganisms during storage is important criterion for the quality and health characteristics of the product. Also, they reported that the number of survived microorganisms decreased with extended storage as appeared in the current results, but several factors determine the rate of their decrease. On the other hand, counts of EAEC in yoghurt in the current study were much lower than was found by Abd Elaal (2008), and El-prince *et al.* (2010) as their samples were contaminated by *E. coli*, respectively. Contrarily, levels of contamination as high as 60% were reported by El-Malt *et al.* (2013). All positive samples failed to comply with the Egyptian standards (2005) for the presence of *E. coli*.

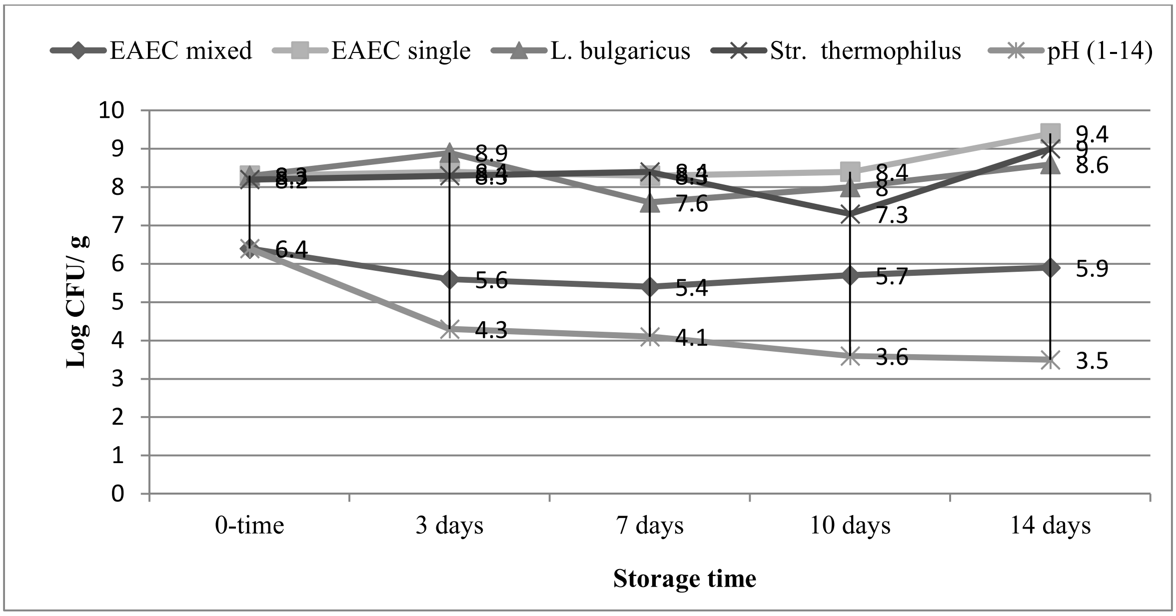

Fig. 2: Effect of stature culture (*Streptococcus thermophilus and Lactobacillus bulgaricus*) on growth of EAEC O111:H2 stx1 in yoghurt

Effect of *Lactobacillus reuteri* on growth of Enteroaggregative *E.coli* (EAEC O111:H2 strains, in Reuteri yoghurt (in Tri-culture) :

Figure (3) show the effect of *Lactobacillus reuteri* on the growth of EAEC O111:H2 in Reuteri yoghurt. Results reveal that no effect up to 7days cold storage on EAEC, while a little inhibitor effect was shown during the 7 days rest of storage, as it decrease 1.3 log cfu/g^{-1}, with decrease of the PH values from 6.5 to 4.3 at the end of storage .As previously mentioned EAEC in single culture increased as previously described 1.1 log cfu/g^{-1} in pasteurized milk during the 14 days of cold storage. The R (L. reuteri) and Y (*Streptococcus thermophilus and Lactobacillus bulgaricus*) cultures grew well in a relatively equal ratio 1:1:1 with counts in a range of 8.2 to 9.3 log cfu/g^{-1}, indicating healthy and positive relation, equilibrium, between the cultures.

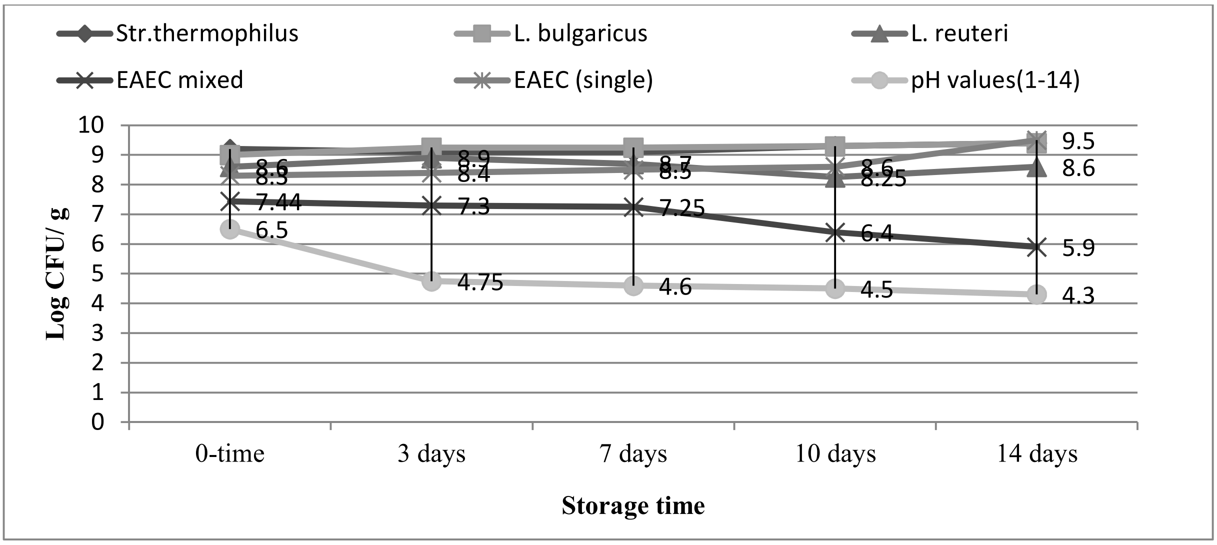

Fig. 3: Effect of *Lactobacillus reuteri* on growth of EAEC O111:H2 in Reuteri yoghurt

The obtained results could be considered far from that found by Amal et al., (2016) concerning inhibition effect of some *Lactobacillus spp.* against pathogenic strains of *E. coli*, as the probiotic bacteria had reduced the population of the pathogenic bacteria by 4.7 log units Also, Pieter *et al.,*

(2016) and Sherman *et al.* (2005) reported that *L. reuteri* limiting occurrence of E.coli (EIEC) and reduce counts of EHEC and EPEC.

In the triple cultures with R (*L. reuteri*) and Y (*Streptococcus thermophilus and Lactobacillus bulgaricus*) starter cultures grew well in a relatively equal ratio 1:1:1 with counts close to the findings by Champagne *et al.* (2016), Claude *et al.* (2016) and Parthiban *et al.* (2003) for the growth of *L. reuteri* with yoghurt cultures. Also, the obtained results showed no significant effect of *L. reuteri* on milk acidification, even when combined with yogurt starter culture that agrees with Hekmat *et al.* (2009). This could be due to the low proteolytic capacity of the probiotic bacteria or to the relatively low concentration of nutrients available for the growth of these microorganisms (Hidalgo-Morales *et al.,* 2005).

However, the current results were in concomitant with Tharmaraj and Shah (2009) who stated that co-culturing of target organisms with probiotic bacteria in reconstituted skim milk showed more inhibitory effect against the target *E. coli*. Furthermore, Pieter *et al.* (2016) reported that the strong antimicrobial effect of *L. reuteri 1063* was plausibly due to the combined action of undissociated L-lactic acid and reuterin. Also, Yoshibumi *et al.* (2010), Alistair *et al.* 2016) and Philip *et al.* (2005), reported the antimicrobial effect of *L. reuteri and Lactobacillus spp*. against, EAEC, EPEC and EHEC *E. coli.*

Effect of Lactobacillus reuteri on shiga-toxin (Stx1) produced by EAEC O111:H2:

Results in table (1) show up the effect of *Lactobacillus reuteri* on shiga-toxin (Stx1) production by EAEC O111:H2 in pasteurized milk (trail 1), reuteri- milk (trail 2), yoghurt (trail 3) and reuteri-yoghurt cultures (trail 4). Results reveal that shiga-toxin (Stx1) was produced by the 5 strains (100%) of EAEC O111:H2 in the beginning of the experiment for the 4 trails and (100%) of the 5 strains in trail 1, up to the 14 days of storage in milk. The highest effect of *L. reuteri* on EAEC Stx production was detected in Reuteri-yoghurt (trail 4), as the 5 strains lost their toxic activity after 10 days , and decreasingly during the 3 and 7 days of storage period as only 40 and 20% were positive for Stx production, respectively. The lower effect was noticed in reuteri culture (trail 2), as only 1 strain (20%) was positive for Stx production at the end of storage. While, the lowest effect was recorded for yogurt cultures on Stx production, as 3 strains (60%) were positive. Hence, it is obvious the anti-toxic activity of *Lactobacillus reuteri* on shiga-toxin (Stx1) production of EAEC particularly in the presence of yoghurt starter cultures.

Table 1: Effect of *Lactobacillus reuteri* on shiga-toxin (Stx1) produced by EAEC O111:H2 in milk, yoghurt, reuteri-milk and Reuteri yoghurt.

Storage time (days)	Stx by EAEC in milk (trail 1)		Stx by EAEC in yoghurt (trail 2)		Stx by EAEC with *Lactobacillus reuteri* (trail 3)		Stx by EAEC in Reuteri yoghurt (trail 4)	
	No	% +	No	% +	No	% +	No	% +
0	5	100	5	100	5	100	5	100
3	5	100	4	80	3	60	2	40
7	5	100	4	80	2	40	1	20
10	5	100	3	60	1	20	0	0
14	5	100	3	60	1	20	0	0

Mohamed Zeinhom *et al.* (2012), reported an active fraction extracted from *Lactobacillus acidophilus* cell-free spent medium (LAla-5AF) showed antivirulent effect against enterohemorrhagic *Escherichia coli* (EHEC) whic down-regulate several virulence genes including stxB2. Moreover, El Gamal *et al.* (2015a) reported that 12% stool samples of diarrheal children cases, were identified as Enteroaggregative Escherichia coli (EAEC) and 7 of them were Shiga toxins producing strains. Furthermore, Enteroaggregative *E. coli* was found in most human strains in Enterotoxigenic strains (O6:H16), Enteroaggregative strains (O126:H27) and shiga toxins Enterohemorraghic strains (O157:H7) and (O26:H11), (Shabana *et al.* (2014) and O111:H2 (Tim Dallman *et al.,* 2012).

Also, El Gamal *et al.* (2015b) isolated Enteroaggregative *Escherichia coli* from Cairo and Giza retailed meat and meat product samples, but any of the isolated strains were found to produce shiga toxins. Meanwhile, Mojtaba *et al.* (2014) found Enteroaggregative *Escherichia coli* strains and in raw milk (48%) and unpasteurized cheese (48%). Furthermore, Abdelhamid *et al.* (2017) isolated EAEC from raw milk and some soft cheese samples. They reported that Shiga toxin stx1, shiga toxin stx2, were detected in 66.6% of EAEC isolated strains.

Inhibition ratio (%) of EAEC O111:H2 due to the effect of *L. reuteri*:

Inhibition ratio (%) of EAEC O111:H2 due to the effect of *L. reuteri* (R) and yoghurt starter culture (Y) in Di-cultures (R&Y) and in Tri-cultures in Reuteri yoghurt, during 14 days of cold storage, was shown in figure (4). Data show that inhibition % of *L. reuteri* on EAEC (Di-culture, DCR) increased from 24 to 36.2% similar to the effect of yoghurt culture (DCY), as it fluctuated between 22.9 to 34.9% to end of storage. Moreover, results of Inhibition (%) in Tri-cultures (TriC) of yoghurt and reuteri cultures, together, on EAEC in Reuteri yoghurt show very little effect as inhibition % fluctuated between 10.8 and 13 %, in the first 7 days of storage, while its Inhibition % increased more than 24 % during storage the period to reach 37.2 % after 14 days.

Amal *et al.* (2016) observed stronger inhibition effect (%) of *Lactobacillus acidophilus DSM 20079* against *E. coli* than that was found for *L. reuteri* in the current study. Since they reported 93.32% and 84.63% degree of inhibition in triple culture (yoghurt +probiotic + pathogenic bacteria) and Di-culture (probiotic + pathogenic bacteria), respectively. Moreover, they add that probiotic bacteria had reduced the population of the pathogenic bacteria by 4.7 log units. On the other hand, the current results were in agreement with Cheikh youssef *et al.* (2008) who found that the tested probiotic bacteria (*L. reuteri*) and yoghurt cultures (*S. thermophilus and L. bulgaricus*) possess varying degrees of inhibition against pathogenic strains of *E. coli.*

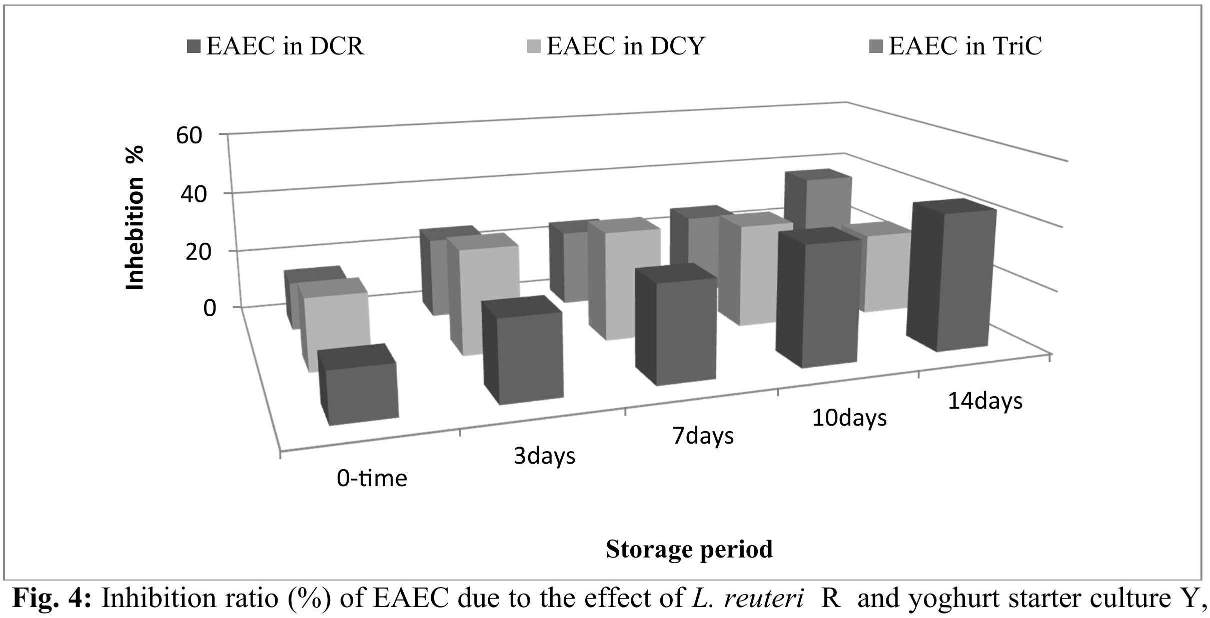

Fig. 4: Inhibition ratio (%) of EAEC due to the effect of *L. reuteri* R and yoghurt starter culture Y, in Di-culture (R&Y) and Tri-culture during storage.

Sensory evaluation:

Results of Reuteri yoghurt sensory evaluation as shown in Table (2) reveal that there were very small significant differences among the panelists for the properties of fresh reuteri yoghurt and during the 14 days of cold storage. Furthermore, results for total sensory evaluation as shown in table (3) indicate that fresh reuteri yoghurt show acceptable quality and gained high score (average 96.4%), while total sensory evaluation score decreased slowly to reach 89.5% at the end of 14 days storage.
Reuteri yoghurt product showed higher quality than was recorded by Wael *et al.* (2017), for fresh yoghurt control, 93% and 94.5% after 10 days. The decrease of total scores in the current study my due to the development of acidity and or to the microbial metabolites produced by *L. reuteri* affecting the sensory properties.

Table 2: Sensory properties of yoghurt containing *Lactobacillus reuteri* strain during storage period at 5 ±2 C^{o} for 15 days

No. of Person	Appearance 30%				Body & texture 30%				Flavor 40%			
	1 day	5 day	10 day	15 day	1 day	5 day	10 day	15 day	1 day	5 day	10 day	15 day
1	29.40^{A} ±0.18	28.51^{B} ±0.09	27.21^{A} ±0.9	27.25^{A} ±0.12	30.00^{A} ±0.21	29.73^{A} ±.021	28.23^{B} ±0.21	26.71^{B} ±0.20	39.45^{A} ±0.22	38.63^{B} ±0.15	38.92^{B} ±0.19	37.03^{B} ±0.19
2	28.10^{B} ±0.23	28.47^{B} ±0.12	27.22^{A} ±0.7	27.26^{A} ±0.19	29.09^{B} ±0.03	28.40^{B} ±0.25	27.26^{C} ±0.14	26.91^{B}± 0.27	39.92^{A} ±0.25	39.01 ^{A} ±0.17	37.53^{C} ±0.36	37.77^{B} ±0.24
3	29.00^{A} ±0.22	29.59^{A} ±0.31	26.20^{A} ±0.40	26.19^{B} ±0.41	29.25^{B} ±0.18	27.74^{C} ±0.01	27.30^{C} ±.03	26.87^{C} ±0.21	39.39^{A} ±0.41	38.22^{B} ±0.40	37.80^{C} ±0.19	37.86^{B} ±0.19
4	28.41^{B} ±0.10	27.55^{C} ±0.12	26.35^{B} ±0.33	26.20^{B} ±0.08	28.09^{C} ±0.16	26.36^{D} ±0.06	26.41^{D} ±0.12	26.84^{C} ±0.19	39.50±^{A} 0.33	37.25^{C} ±0.48	37.42^{C} ±0.32	36.74^{C} ±0.47
5	30.00^{A} ±0.19	28.49^{B} ±0.14	26.32^{B} ±0.41	26.29^{B} ±0.31	30.08^{A} ±0.13	26.38^{D} ±0.21	27.26^{C} ±0.10	27.71^{B} ±0.23	37.80^{C} ±0.25	37.00^{c} ±0.45	37.50^{C} ±0.28	37.77^{B} ±0.22
6	29.40^{A} ±09±	28.50^{B} ±0.15	27.26^{A} ±0.99	27.17^{A} ±0.09	30.21^{A} ±0.29	29.74^{A} ±0.13	29.00^{A} ±0.14	29.96^{A} ±0.23	38.50^{B} ±0.37	38.60^{B} ±0.58	37.70^{C} ±0.22	37.69^{B} ±0.28
7	28.39^{B} ±0.02	27.39^{C} ±0.08	26.24^{A} ±0.87	25.19^{C} ±0.26	29.02^{B} ±0.03	28.69^{B} ±0.13	28.01^{B} ±0.13	27.79^{B} ±0.25	37.55^{C} ±0.18	37.60^{C} ±0.58	38.30^{B} ±0.22	37.90^{B}± 0.18
8	29.39^{A} ±0.08	27.35^{C} ±0.16	26.32^{B} ±0.54	25.18^{C} ±0.19	29.23^{B} ±.021	28.40^{B} ±0.16	27.13^{C} ±0.20	27.80^{B} ±0.32	38.79^{B} ±0.27	37.65^{C} ±0.46	37.74^{C} ±0.16	37.70^{B} ±0.21
9	29.44^{A} ±0.02	28.31^{B} ±0.12	27.29^{A} ±0.24	26.26^{B} ±0.29	30.01^{A} ±.023	29.41^{A} ±0.14	290.30 ^{A} ±0.01	29.75^{A} ±0.22	39.54^{A} ±0.31	39.71^{A} ±0.38	39.74^{A} ±0.17	39.92^{A} ±0.39
10	29.40^{B} ±0.21	27.39^{C} ±0.12	27.24^{A} ±0.15	26.23^{B} ±0.09	29.21^{B} ±0.11	28.71^{B} ±0.15	27.09^{C} ±0.21	27.92^{B} ±0.25	40.71^{A} ±0.25	39.25^{A} ±0.1	38.74^{B} ±0.15	37.05^{B} ±0.22

Means (±SE, n=3) with the same capital letters in the same column are not significantly different at $P\leq0.05$

Table 3: Total Sensory scoring of yoghurt containing *Lactobacillus reuteri* strain during storage period 14 days at 5 ±2 C$^{o.}$ ±2.

Storage time	Panelists /points %										
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**Means**
Fresh	98	96	97	95	97	96	94	96	98	97	96.4
3 days	96	95	94	90	91	95	92	92	96	94	93.5
10 days	93	91	90	89	90	93	92	90	94	92	91.4
14 days	90	90	88	88	90	92	89	89	90	89	89.5

Total sensory scoring of the 10 panelists (regardless the decimal fractions but not for means)

The obtained results indicate the enhancement addition of *L. reuteri* to the flavor, body & texture and appearance of yoghurt even for 14 days under cold storage. This could be in close to the findings of Rodas *et al.* (2002) who reported that the sensory evaluation of buttermilk containing *L. reuteri* indicated that the judges were able to perceive differences after 12 days of storage buttermilk. Also, Adrian *et al.* (2007) found that acidity and pH values did not change appreciably and no sensory changes were found during the first 14 days of storage and the final product preserved an acceptable flavor. Parthiban *et al.* (2003) reported that *L. reuteri* did not produce any odor or color change produced in ground beef during 25 days storage at 4°C.

Contrarily, the obtained results contradict Claude *et al.* (2016), and van Niel *et al.* (2012) who stated that when a population above 8.17 Log CFU.g^{-1} of *L. reuteri,* that has a heterofermentative metabolism, was attained, sensory problems were noted , as generated texture defects, and this would be difficult to manage in a set-style yogurt production. Moreover, Davidson *et al.* (2000) reported that in a fermented frozen yogurt, the balance of flavoring systems may be significantly affected by varying levels of organic compounds. Also, several researches reported that when a population above 8.17 Log CFU.g^{-1} of *L. reuteri* was attained, sensory problems were noted (van Niel *et al.* 2012).
In conclusion, out of risk of antibiotic Susceptibility and Plasmid Profile of *Lactobacillus* Species Isolated from Yoghurt (Oluyege *et al.,* 2018), Reuteri-yoghurt could be addressed as a competitor new milk product with, safe (anti-shiga *E. coli* diarrhea), good shelf life and acceptable sensory properties my allow it for entering the growing Egyptian market of probiotics.

Conclusion

Reuteri-yoghurt could be considered good fermented milk product, due to anti-shiga infant diarrhea recommended drink, with good sensory properties and shelf life and may be attractive for entering the Egyptian growing market of probiotics, as a competitor new fermented milk product. So we recommended that Reuteri-yoghurt must be main meal food for children, old age and pregnant which surfing from immune deficiency.

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