EFFECT OF PROBIOTICS FEED AS ADDITIVES ON THE ACTIVITY OF ISOLATED AND CHARACTERIZED LACTIC ACID INTESTINAL BACTERIA TO INHIBIT *Escherichia coli*-10 IN SHEEP

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SUMMARY

Lactobacillus plantarum-2LMB and *Enterococcus faecium*-1LMB as lactic acid intestinal bacteria (LAIB) were selected on the basis of characteristics indicating that they would be good candidates for a competitive exclusion product that would inhibit *Escherichia coli*–10 in the intestinal tract of live sheep. Fecal samples from healthy Barki sheep that were culture negative for *E. coli* were collected. LAIB were isolated from sheep feces by repeated plating on MRS and M17 agar medium to select the pure colonies of *Lactobacillus sp.* and *Enteroccocus sp.* respectively. SDS-PAGE Gel Electrophoresis and API 50 (Analytical Profile Index) were used to identify the strains. *Bacillus subtilis and Saccharomyces cerevisiae* were used as a source of probiotics. They were added to broth media of *L. plantarum* and *E. faecium* by 1:1 or 2:1 to predict their positive effect on LAIB. Inhibitory effect of the two intestinal bacteria strain on *E. coli* growth, LAIB growth and their lactic acid production were determined during regular intervals. Cell dry weight was also determined at the end of incubation time. Duplicate of the probiotic (especially *B. subtilis*) dose increased the inhibitory effect of *L. plantarum* more than *E. faecium* to *E. coli* growth. Adding the probiotics singly or in double dose to growth medium of LAIB in absence of *E. coli* showed a significant increase ($P<0.001$) in their growth activity. In presence of *E. coli*, supplementation of probiotics displayed the same behaviour. Higher values of net effectiveness of probiotics was observed on LAIB growth activity by adding *B. subtilis* (2:1 in absence of *E. coli*) and *S. cerevisiae* (1:1 in presence of *E. coli*) to the media. Probiotics had more net effect on *L. plantarum* growth than *E. faecium* in presence or absence of *E. coli*. Net effectiveness of probiotics had a clear positive effect on lactic acid production produced by LAIB in presence or absence of *E. coli*. Cell dry weight was significantly increased ($P<0.001$) by adding *S. cerevisiae* to growth medium of *L. plantarum* by single dose than other treatments in absence of *E. coli*. The higher ($P<0.001$) values observed, in the case of *E. faecium*, with adding the *B. subtilis* by single dose. Duplicating the dose of probiotics in presence of *E. coli* had increased the cell dry weight of LAIB. Our results suggested that probiotics (bacterial or yeast) had a positive effect on lactic acid intestinal bacteria activities and it could improve their

inhibition to unfriendly bacteria such as *E. coli*–10 in the intestine of sheep.

Keywords: *L. plantarum, E. faecium, B. subtilis, S. cerevisiae, E. coli, lactic acid, sheep.*

INTRODUCTION

The use of probiotics as farm animals feed supplements dates back to the 1970,s. the word probiotic is derived from the Greek meaning "for life". Probiotics are mono- or mixed culture of live microorganisms which, when applied to animals or human,they display a beneficial affect on the host by improving the properties of indigenous microflora. The common terms for probiotics are "friendly", "beneficial" or healthy" microorganisms. Many strains of bacteria including *Bacillus subtilis* and yeast's such as *Saccharomyces cerevisiae* had the Generally Recognised As Safe (GRAS) status from the US Food and Drug Administration.

Probiotcs have many beneficial effects at the level of animal host and at the level of intestinal micro-flora. On the animal host, it can maintain and regenerate the state of residence (Smirnov *et al.*, 2002) and improve their performance (Dawson, 1995). The nutritional effect of probiotics is characterized by an improvement of the utilization of nutrients by animal host. This effect can be monitored by digestibility measurements (Roberton and Chevalier, 1997). Administration of bacteria as a probiotic would be of great usefulness, probably because its antigenic stimulation would favour the maturation of secretory immune system thus preventing infection (Perdigon and Alvarez, 1992). Pollman *et al.*, (1980) observed that oral inoculation of animals with *Lactobacilli* led to elevated levels of total serum proteins, globulin and increased white blood cell count. Probiotic bacteria species such as *B. subtilis* have been shown to produce digestive enzymes, amylase, protease and lipase, which may enrich the concentration of intestinal digestive enzymes (Moon and Kim, 1989; Lee and Lee, 1990).

At the level of intestinal microfolra, many of the beneficial productive responses associated with the use of propiotic supplements can be directly related to their effects on the microbial population in the digestive tract (Mc Cormick, 1984; Nahashon *et al.*, 1992 and Dawson, 1995). Probiotics regulate and improve the balance of the microbial environment of the intestine, decrease digestive disturbances, inhibit pathogenic intestinal microorganisms (Brashears *et al.*, 2003) and improve feed conversion rate and health performance (Windschitl, 1992).

The role of gut flora is multifarious. Fermentation of feed compounds, induction of anatomical and physiological changes in the intestinal cell wall structure, increase animal resistance against entero-pathogenic bacteria (Vanbelle *et al.*, 1999). It has long been known that LAIB inhibit pathogenic bacteria especially *Escherichia coli* in laboratory media. *Escherichia coli* is an important pathogen, causing bloody diarrhea (hemorrhagic colitis) and renal failure (hemolytic uremic syndrome) (Salmon *et al.*, 1989). Several LAIB, most commonly strains from genera *Lactobacillus plantarum* and *Enterococcus faecium* have been tested as a competitive exclusion product (Brashears *et al.*, 2003).

The overall objective of this study was to develop competitive exclusion product produced by lactic acid intestinal bacteria (LAIB) (*L. plantarum* and *E.*

faecium) isolated from sheep in presence of two doses of each one of two different organisms of probiotic; bacteria (*B. subtilis*) or baker's yeast (*S. cerevisiae*) that would potentially reduce the intestinal colonization, growth and fecal shedding of *E. coli*–10 as pathogen intestinal bacteria in sheep.

MATERIALS AND METHODS

Isolation and culture medium of Lactic Acid Intestinal Bacteria (LAIB) from healthy Barki sheep

Three healthy, adults and females Barki sheep housed in the farm of the Department of Animal Production, Faculty of Agriculture, Alexandria University showed that fecal culture negative for *E. coli*–10 was selected to supply fecal samples for the isolation of LAIB that were to be screened for their inhibitory activity against *Escherichia coli* -10.

One gram of each fecal sample was added to 10 ml of sterile De Man Rogosa Sharpe, (1960) (MRS) broth media (composition in g/liter: 10.0 universal peptone; 5.0 yeast extract; 20.0 D(+)glucose; 2.0 di-potassium hydrogen phosphate; 1.0 polyoxyethylene sorbitan monooleate; 2.0 di-ammonium hydrogen citrate; 5.0 sodium acetate; 0.1 magnesium sulfate; 12.0 agar-agar "not present in MRS broth") for cultivation and enumeration of *Lactobacillus sp.* Another one gram of fecal sample was added to 10 ml of M17 broth medium (composition in g/liter: 5.0 peptone from soymeal; 2.5 peptone from meat; 2.5 peptone from casein; 2.5 yeast extract; 5.0 meat extract; 5.0 D(+)lactose; 5.0 ascorbic acid; 19.0 sodium β-glycerophosphate; 0.25 magnesium sulfate; 12.75 agar-agar "not present in M17 broth") for cultivation and enumeration of *Enteroccocus sp.* this media was proposed by Terzaghi and Sandine (1975). All samples were then mixed thoroughly. The fecal material was then streaked onto MRS and M17 agar plates for *Lactobacillus sp.* and *Enteroccocus sp.* selection, respectively. The plates were incubated at 37 °C for 48 h in plastic bags flushed with CO_2 for 30 s. Approximately 10 to 15 well-isolated colonies were picked up from each plate and transferred to individual tubes containing 10 ml of MRS or M17 broth medium, which were further incubated at 37 °C for 18 to 72 h to obtain maximum growth of the cultures. The isolated cultures were re-streaked onto MRS or M17 agar plates for *Lactobacillus sp.* and *Enteroccocus sp.* selection and incubated at 37°C for 48 h until isolated colonies of one form were obtained. Pure colonies were Gram stained for preliminary identification. The isolated cultures were maintained at frozen (-70°C) stocks in MRS broth supplemented with 10% (vol/vol) sterile glycerol. Isolates were sub-cultured in MRS or M17 broth medium at 37°C for 24 to 48 h (culture activation) before they were used for further studies.

E. coli-10 was already isolated from sheep intestine and characterized and identified morphologically and genetically at the Department of Microbiology, Faculty of Science, Alexandria University.

Identification of LAIB isolates

Lactobacillus plantarum-2LMB and *Enterococcus faecium*-1LMB were identified by morphologically and physiological testes (Sharpe, 1979; Garvie, 1986; Hardie, 1968: Kandler and Weiss, 1986). Gram positive cocci (*Enterococcus faecium*-1LMB) were tested for growth in M17 broth medium. Gram positive rod shaped isolate (*Lactobacillus plantarum*-2LMB) was tested for growth in MRS broth and also

No	Treatment
1	0.5 ml *Lactobacillus plantarum*, alone (Control)
2	0.5 ml *Bacillus subtilis* + 0.5 ml *Lactobacillus plantarum* (1:1)
3	1.0 ml *Bacillus subtilis* + 0.5 ml *Lactobacillus plantarum* (2:1)
4	0.5 ml *Saccharomyces cerevisiae* + 0.5 ml *Lactobacillus plantarum* (1:1)
5	1.0 ml *Saccharomyces cerevisiae* + 0.5 ml *Lactobacillus plantarum* (2:1).
6	0.5 ml *Enterococcus faecium*, alone (Control).
7	0.5 ml *Bacillus subtilis* + 0.5 ml *Enterococcus faecium* (1:1).
8	1.0 ml *Bacillus subtilis* + 0.5 ml *Enterococcus faecium* (2:1).
9	0.5 ml *Saccharomyces cerevisiae* + 0.5 ml *Enterococcus faecium* (1:1)
10	1.0 ml *Saccharomyces cerevisiae* + 0.5 ml *Enterococcus faecium* (2:1).
11	0.5 ml *Bacillus subtilis*, alone (Control).
12	0.5 ml *Saccharomyces cerevisiae*, alone (Control).
13	0.5 ml *Escherichia coli*-10, alone (Control).

CO_2 production. The identification was completed by sugar fermentation patterns obtained with the *API 50* galleries (bioMerieux, Vercieu, France); results were analyzed by computerized database software provided by the manufacturer. The identity of LAIB isolates was confirmed by the SDS-PAGE (sodium dodecyle sulfate) technique of whole-cell proteins as described by Pot *et al.*, (1994) and Ausubel *et al.*, (1995).

The purity of the isolated bacteria was evaluated by Tricine-sodium dodecyle sulfate (SDS)-polyacrylamide gel electrophoresis. Gels (10% acrylamide were silver stained for visualization of peptides. Bands patterns of isolates were scanned. Normalized and compared to database of normalized protein fingerprints of LAIB reference strains by use of Gel compare 4.0 software (Applied Maths, Kortrijk, Belgium) which was also used for generation of cluster analysis.

Mixing of probiotics with LAIB

To predict the effect of probiotic on the activity of lactic acid intestinal bacteria (*Lactobacillus plantarum*-2LMB and *Enterococcus faecium*-1LMB) isolated and characterized by the previous methods to inhibit *Escherichia coli*-10 as a pathogen. They were mixed with the two types of probiotics (*Bacillus subtilis* and *Saccharomyces cerevisiae* in different ratios to 9.0 ml of individual broth medium as indicated above..

The all treatment was also tested in presence of 0.5 ml *E. coli*. *B. subtilis* strain was obtained in powdered form from a German company (Versuchsprapart: BS03; 2 $X10^9$). All bacterial strains (*L. plantarum*, *E. faecium*, *B. subtilis* and *E. coli*) and yeast (*S. cerevisiae*) were activated by their incubation at 37°C for 24 and their turbidity was adjusted to OD_{600} = 1 with sterilized broth medium before used in the inoculation experiments.

Determination of the inhibitory effect of LAIB on growth of Escherichia coli–10

Toxicity effect of the two identified strains (*L. plantarum* and *E. faecium*) and their mixtures with the different types of probiotics was determined by disc diffusion assay (Thornberry, 1950). Filter paper discs (Watman No. 1, 3-mm diameter) were impregnated with 10µl of each one of the LAIB and its mixture with probiotics. Discs were applied to the surface of agar plates that were previously inculated with activated culture of *Escherichia coli*–10. The plates were flushed with CO_2 for 30 sec. and incubated at 37°C.The diameter of

inhibition zone (*mm*) formulated around each disc was recorded after 72h. Control disks were impregneted with 10 µl of MRS or M17 broth medium. Determination of inhibition zone of each one of the previous treatments on *Escherichia coli*–10 growth was replicated three times.

Determination of LAIB growth

Growth of LAIB in absence or presence of *Escherichia coli*–10 was measured turbidimeterically as change in optical density (*OD*) at *600* nm (Spectronic spectrophotometer 20D$^+$, Laboratory of Animal Nutrition, Faculty of Agriculture, Alexandria University). Aliquots of culture fluid were taken at regular intervals during growth (after 3, 6, 24, 48 and 72 h of incubation) for measurements of OD_{600} and lactic acid concentration. All measurements were carried out under sterilized and anaerobic conditions. Determinations were replicated three times for each sample.

Lactic acid assay

A simple colorimetric assay was used to determine lactic acid concentration proposed by Taylor (1996) in intestinal bacteria medium of lower gut in ruminant's animals. For a standard curve, it was added 0-30 µg lactic acid to 16 X 150-mm borosilicate tubes. The curve should be in 5-µg increments or less. This volume was transfered in tubes up to 0.5 ml with double distilld water. Three millitre of concentrated H_2SO_4 were added and mixed on a vortex mixer. The quantity of acid was difined here as 82% acid. Samples were incubated at 95-100°C for 10 min (in a steam water bath). Extraneous water was kept out of the tube and then they were cooled under room tempreture. $CuSO_4$ reagent was added and then 100 µL of *p*-phenylphenol reagent. The mixture was mixed well in a vortex mixer and tubes were left at room temprature for at least 30 min and then the absorbance was read at 570 nm (Spectronic spectrophotometer 20D$^+$). Blanks were showed values of 0.2-0.5 compared with water. One sample of medium growth during the time of incubation (after 3, 6, 24, 48 and 72 h of incubation) was taken to determine lactic acid production.

Biomass assay

After 72 h of incubation, the cell dry weight (g/L) or biomass production of each one of LAIB samples and their previous mixtures with probiotics used was determined. The rest volume of growth medium after the 72 h of incubation was harvested and centrifuged (Heraeus Christ GmbH, Model Osrterode/Harz-Cryofuge 20-3) at 4000 X g for 10 min at 4°C.The obtaind pellet was washed twice with quarter strength of Ringers solution (compositon in g/L: 2.25 sodium chlorid; 0.105 potassium chlorid; 0.12 calcium chlorid; 0.05 sodium bicarbonate). The washed pellet in the centrifuge cups was then dried at 105°C for 4 h to a constant weight. Acording to the yield of biomass, the cultures were divided into three groups; good: >1.3 g/L, fair: 1-0.6 g/L and poor <0.6 g/L.

Efficiency of the association between LAIB and probiotics

The absorbance at 650 nm of the supernatants that resulted after the centrifugation of the growth media was used to express the association efficiency between LAIB and probiotics in absence or presence of *Escherichia coli*–10. Absorbance (OD_{650}) with a range of 0-0.1 unit indicated a good association between LAIB and probiotics while absorbance of 0.2 to 0.3 and more than 0.3 unit indicated a fair and poor association, respectively.

Statistical analysis

Data of bacterial growth and lactic

acid production during different times of incubations and biomass production and efficiency of biomas separation in response to occurrence of probiotics types in absence or presence of the pathogen (*E. coli*-10), were statistically analyzed by ANOVA (Steel and Torrie, 1980) using GLM procedure (SAS, 1993).

RESULTS AND DISCUSSION

Mean values of inhibition zone formulated around discs impregnated with each one of LAIB and their mixture with *B. subtilis* or *S. cerevisiae* by 1:1 or 2:1 are presented in Table 1 and illustrated in Figure 1. Higher inhibition zone was observed by adding *B. subtilis* and *S. cerevisiae* than control (*L. plantarum* or *E. faecium* alone). Duplicate doses of probiotic to LAIB have increased extend of inhibition zone in the pathogen (*E. coli*). However, adding the probiotics to the media improved the effectiveness of LAIB in their inhibition of *E. coli* growth by 120,140,60 and 80% in *L. plantarum* by adding *B. subtilis* 1:1 and 2:1, and *S. cerevisiae* 1:1 and 2:1, respectively. In the case of *E. faecium*, probiotics had improved the inhibition by 40,60,60 and 60% by adding *B. subtilis* 1:1 and 2:1, and *S. cerevisiae* 1:1 and 2:1, respectively. *B. subtilis* as a bacterial probiotic had a good positive effect than *S. cerevisiae* on the activity of the lactic acid intestinal bacteria to inhibit *E. coli* growth (Figure 2). Probiotics showed more positive effect on *L. plantarum* than *E. faecium* activity in their inhibition of the pathogen.

The inhibition observed was probably due to competitive exclusion products. Because, it is likely that the LAIB that inhibited *E. coli* were producing substances that were inhibitory to the pathogen (Brashears *et al.*, 2003). Generally, the mode of action may be due to producing antibiotic substance and inhibiting harmful bacteria metabolism and decrease intestinal pH (De-Blas *et al.*, 1991 and Brashears *et al.*, 2003). Benmark, (1998) reported that *L. plantarum* has the ability to reduce and eliminate potentially the lower intestine pathogenic microorganisms, these results confirmed the present results.

Mixing LAIB with probiotics was more effective in their inhibition of *E. coli* than the strain tested singly. However, increasing the ability of LAIB to inhibit the *E. coli* by adding single or double dose of *B. subtilis* or *S. cerevisiae* may be due to their positive effects in improving lactic acid production in intestinal media. Probiotics have a beneficial affect on LAIB in lower gut. Because they had the ability to increase their activity to produce lactic acid which may lower the pH in small intestine to the levels that inhibit the growth of pathogenic microbes (De-Blas *et al.*, 1991; Brashears *et al.*, 2003 and Perdigon and Alvarez, (1992). It was assumed that the effect of probiotics was linked to the gastrointestinal tract and causes the incidence of diarrhea and other gut infections. *L. casei* and *L. acidophilus* were tested singly and together by Perdigon and Alvarez, (1992) for stimulation of phagocytic activity. It was found that the mixture was more effective than the strains given singly. This suggest that a mixture of bacteria was more effective and the individual effects of the component strains may be additive. Brashears *et al.* (2003) reported that LAIB isolates from cattle were effective in decreasing *E. coli* in laboratory media, ruminal fluid, and manure, indicating that they might be effective in decreasing the pathogen load in the live animal and, ultimately, in the feedlot environment.

In Table (2), adding the probiotics (*B.*

Table (1) : Effect of probiotics supplementation (1:1 or 2:1 of *B. subtilis* or *S. cerevisiae*) with the two strains of intestinal bacteria isolated from sheep on inhibition[a] of *E. coli*-10 growth as a pathogenic bacterium.

Identified Strains (*API*† profile)	Control	*Bacillus subtilis*		*Saccharomyces cerevisiae*	
		1:1	2:1	1:1	2:1
Lactobacillus plantarum -2LMB	5	11	12	8	9
Enterococcus faecium -1LMB	5	7	8	8	8

[a]Mean for three separate trials. Inhibition was measured as the size (*mm*) of the clear zone around the discs impregnated with each one of LAIB on agar surface (see *Figure 1*).

† *Analytical Profile Index* (API) *profile.*

Table (2) : Effect of probiotics supplementation‡ on the growth activity[a] (OD_{600}) of the two identified strains of lactic acid intestinal bacteria isolated from sheep in presence or absence of *E. coli* -10 as a pathogenic bacterium.

Identified Strains (*API*† profile)	Control	Control + *E. coli*	*Bacillus subtillis*				*Saccharomyces cerevisiae*			
			1 : 1		2 : 1		1 : 1		2 : 1	
			- *E coli*	+ *E coli*	- *E coli*	+*E coli*	- *E coli*	+*E. coli*	- *E. coli*	+*E.coli*
L. planterum - 2LMB	0.58^{f} ±0.000	0.59^{f} ±0.001	0.70^{d} ±0.001	0.79^{c} ±0.038	0.83^{b} ±0.001	0.76^{d} ±0.001	0.79^{c} ±0.001	0.90^{a} ±0.001	0.80^{b} ±0.001	0.58^{f} ±0.000
E. faecium -1LMB	0.59^{f} ±0.001	0.67^{f} ±0.000	0.72^{g} ±0.000	0.79^{d} ±0.001	0.81^{c} ±0.001	0.76^{l} ±0.009	0.70^{h} ±0.001	0.90^{a} ±0.001	0.74^{e} ±0.001	0.59^{J} ±0.001

Means within each row having different letters were significantly different at (P<0.001).

†: Analytical Profile Index (*API*) profile.

‡: Probiotics (*B. subtillis* or *S. cerevisiae*) were added to the medium that was inoculated with the intestinal bacteria by the portion 1:1 or 2:1of probiotic to intestinal bacteria.

a: The mean values of all times of incubation 0,3,6,24,48 and 72h. Mean ± stander division.

Each of the data points is an average of three measurements (n=3).

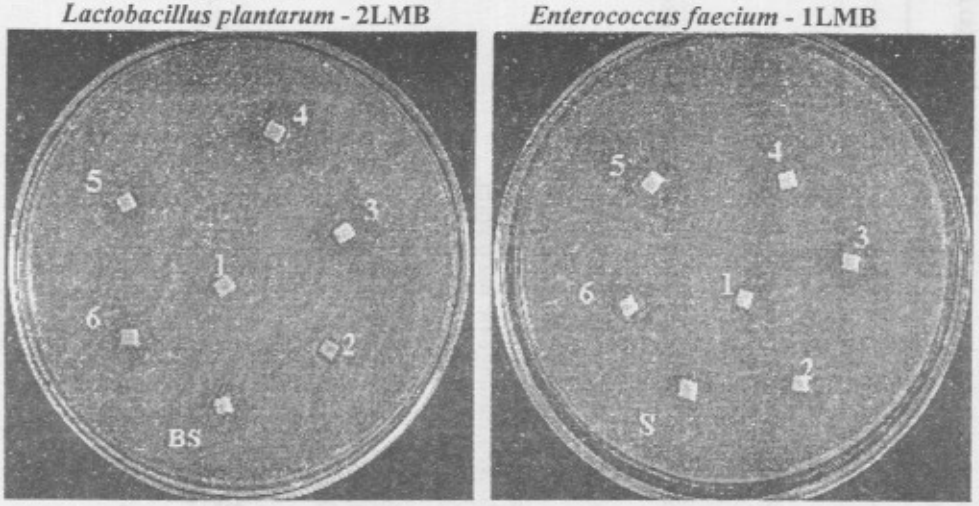

Figure 1. Forming the clear inhibition zone around the discs impregnated with 10 µl of activated culture of each one of LAIB isolated from sheep and their mixtures with each one of probiotics in petri dishes inoculated with *E. coli* - 10 after 72h of incubation at 37 °C. BS (*B. subtillis*), SC (*S. cerevisiae*)

1. Control (disc impregnated with 10 µl of sterilized medium)
2. *L. plantarum*, alone or *E. faecium*, alone (control)
3. *B. subtilis* + *L. plantarum* (1:1) or *B. subtilis* + *E. faecium* (1:1)
4. *B. subtilis* + *L. plantarum* (2:1) or *B. subtilis* + *E. faecium* (2:1)
5. *S. cerevisiae* + *L. plantarum* (1:1) or *S. cerevisiae* + *E. faecium* (1:1)
6. *S. cerevisiae* + *L. plantarum* (2:1) or *S. cerevisiae* + *E. faecium* (2:1).

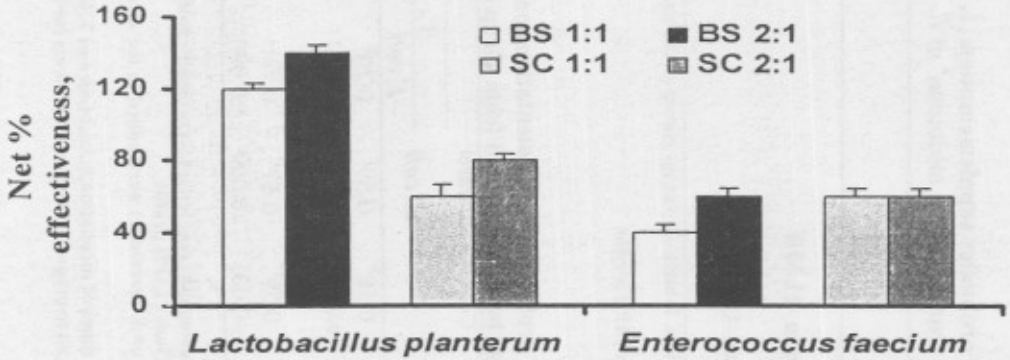

Figure 2. Net effectiveness[1] of the two different types of probiotics used (*B. subtilis* (BS) and *S. cerevisiae* (SC)) on the activity of LAIB identified from sheep in their inhibition of *E. coli*-10 growth.

[1]Net effectiveness was calculated as a percentage by removal of the inhibition zone value of the control discs (*L. plantarum* or *E. faecium*, alone) from their values when it was mixed with *B. subtilis* or *S. cerevisiae*.

subtilis or *S. cerevisiae*) singly or in twice dose to growth medium of LAIB in absence of *E. coli* showed an significantly increase (P<0.001) in their growth activity. In presence of *E. coli*, supplementation of probiotics had the same behaviour. Higher values of net effectiveness (Table 4) of probiotics was observed on LAIB growth activity by adding *B. subtilis* (2:1 in absence of *E. coli*) and *S. cerevisiae* (1:1 in presence of *E. coli*) to the medium. Probiotics had more net effectiveness on *L. plantarum* growth than *E. faecium* in presence or absence of *E. coli*.

Changes in growth activity of the two identified bacterial strains (*L. plantarum* and *E. faecium*) with *B. subtilis* or *S. cerevisiae* in presence or absences of *E. coli* during different time of incubations are shown in *Figures 3 and 4*. Adding probiotics to the growth media increased dramatically *L. plantarum* and *E. faecium* growth especially during stationary phase (first 24h). Duplicating the dose of *B. subtilis* to *L. plantarum* and to *E. faecium* was more effective than a single dose on intestinal bacterial growth in absence and presence of *E. coli*. At presence of *E. coli*, a single dose of *B. subtilis* was more effective for *E. faecium* than the other dose. According to the supplementation of *S. cerevisiae* to the growth medium, beaker's yeast alone showed higher growth rate in comparison with the other treatment. Anyway, duplicating the dose of *S. cerevisiae* in absence of *E. coli* was more effective on growth rate of *L. plantarum* and *E. faecium* during the time of incubations. In presence of *E. coli*, no significant difference was observed (P>0.001) between single and twice doses of *S. cerevisiae* to *L. plantarum* in growth media. In *E. faecium* growth media, it was observed that, single dose of *S. cerevisiae* increased (P<0.001) intestinal bacterial growth.

The results of lactic acid production by the tested strains were in the same trend of bacterial growth data (Tables 3 and 5; Figures 5 and 6). It is clear that net effective of probiotics had a clear positive effect on lactic acid produced by LAIB in presence or absence of E. coli, (Table 5).

Lactic acid intestinal bacteria in ruminant animals are able to "balance" unfriendly bacteria by producing lactic acid. For example, *L. plantarum* has been reduced clinically for its effect on irritable bowel syndrome and pen (Griffin *et al.*, 1988). *L. plantarum* has generally showed higher values of lactic acid production than *E. faecium*. This finding may be due to the different bacterial characterization among the two bacterial strains. Dako *et al.*, (1995) reported that peptidase activities of *Lactobacilli* were generally highest when compared to *Enterococci* strain. Brashears *et al.*, (2003) found that *Lactobacilli* strain has a higher toxicity effect on *E. coli* growth than *Enterococci* strain. These results may be due to a higher lactic acid produced by *Lactobacilli* than *Enterococci*.

According to the mean values of biomass production (Table 6), all strains and their mixtures with probiotics had a good (>1.3g cell dry weight/L) biomass production after 72h of incubation. The biomass production was significantly increased (P<0.001) by adding *S. cerevisiae* to growth medium of *L. plantarum* by a single dose than other treatments in absence of *E. coli*. The higher (P<0.001) values was observed, in the case of *E. faecium*, with adding the *B. subtilis* by single dose. Duplicating the dose of probiotics in presence of *E. coli* had increased the biomass production of LAIB. Good association between LAIB and probiotics used after 72h of incubation were observed in Table 7. These results may indicate the beneficial

Table (3) : Effect of probiotics supplementation‡ on total acidity produced on lactic acid production[a] basis (g/L) of the two identified strains of lactic acid intestinal bacteria isolated from sheep in presence or absence of *E. coli* -10 as a pathogenic bacterium.

Identified Strains (*API*† profile)	Control	Control + *E. coli*	*Bacillus subtillis* 1 : 1		*Bacillus subtillis* 2 : 1		*Saccharomyces cerevisiae* 1 : 1		*Saccharomyces cerevisiae* 2 : 1	
			- E coli	*+ E coli*	*- E coli*	*+ E coli*	*- E coli*	*+E. coli*	*- E. coli*	*+E. coli*
L. planterum -2LMB	1.13^{e} ±0.134	0.93^{f} ±0.155	1.50^{d} ±0.064	1.51^{bcd} ±0.975	1.45^{d} ±0.962	1.44^{d} ±0.009	1.42^{de} ±0.059	1.58^{b} ±0.081	1.56^{bc} ±0.966	1.73^{a} ±0.173
E. faecium -1LMB	1.50^{c} ±0.030	1.40^{d} ±0.050	1.78^{b} ±0.067	1.75^{b} ±0.061	1.87^{a} ±0.024	1.54^{c} ±0.869	1.61^{c} ±0.150	1.43^{d} ±0.039	1.70^{b} ±0.030	1.53^{c} ±0.042

Means within each row having different letters were significantly different at (P<0.001).

†: Analytical Profile Index (*API*) profile.

‡: Probiotics (*B. subtillis* or *S. cerevisiae*) were added to the medium that was inoculated with the intestinal bacteria by the portion 1:1 or 2:1of probiotic to intestinal bacteria.

a: The mean values of all times of incubation 0,3,6,24,48 and 72h. Mean ± stander division.

Each of the data points is an average of three measurements (n=3).

Table (4) : Net effectiveness[a] (calculated) of probiotic supplementation on growth of the two identified strains of lactic acid intestinal bacteria isolated from sheep in presence or absence of *E. coli* -10 as a pathogenic bacterium.

Identified Strains (*API*[†] profile)	*Bacillus subtillis*				*Saccharomyces cerevisiae*			
	1 : 1		2 : 1		1 : 1		2 : 1	
	- E coli	*+ E coli*	*- E coli*	*+ E coli*	*- E coli*	*+ E coli*	*- E coli*	*+ E coli*
L. planterum -2LMB	20.48	33.73	43.37	27.99	35.63	51.94	38.21	53.63
E. faecium -1LMB	21.08	17.06	36.42	12.61	18.21	33.53	24.28	24.48

a: was calculated (as a percentage) by remove the effect of *L. planterum* or *E. faecium* supplemented with or without 0.5ml of activated culture of *E coli.*

Table (5) : Net effectiveness[a] (calculated) of probiotic supplementation on lactic acid production by the two identified strains of lactic acid intestinal bacteria isolated from sheep in presence or absence of *E. coli* -10 as a pathogenic bacterium.

Identified Strains (*API*[†] profile)	*Bacillus subtillis*				*Saccharomyces cerevisiae*			
	1 : 1		2 : 1		1 : 1		2 : 1	
	- E coli	*+ E coli*	*- E coli*	*+ E coli*	*- E coli*	*+ E coli*	*- E coli*	*+ E coli*
L. planterum -2LMB	30.09	62.37	28.32	54.84	25.66	69.89	38.05	86.02
E. faecium -1LMB	18.57	25.00	24.67	10.00	7.33	2.14	13.33	9.29

a: was calculated (as a percentage) by remove the effect of *L. planterum* or *E. faecium* supplemented with or without 0.5ml of activated culture of *E coli.*

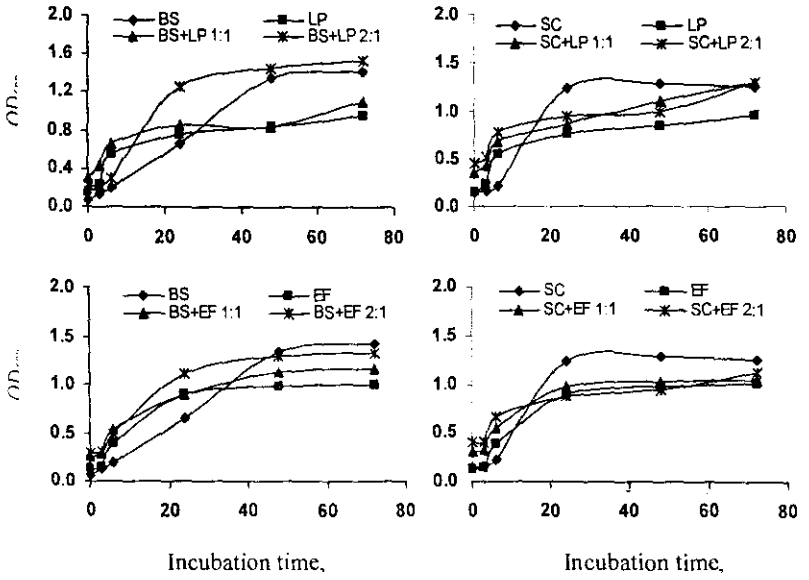

Figure 3. Effect of different type of probiotics (*B. subtilis* (BS) and *S. cerevisiae* (SC)) on the growth (OD_{600}) of the identified LAIB in sheep (*L. plantarum* (LP) and *E. faecium* (EF)) during different times of incubations in absence of *E. coli* - 10.

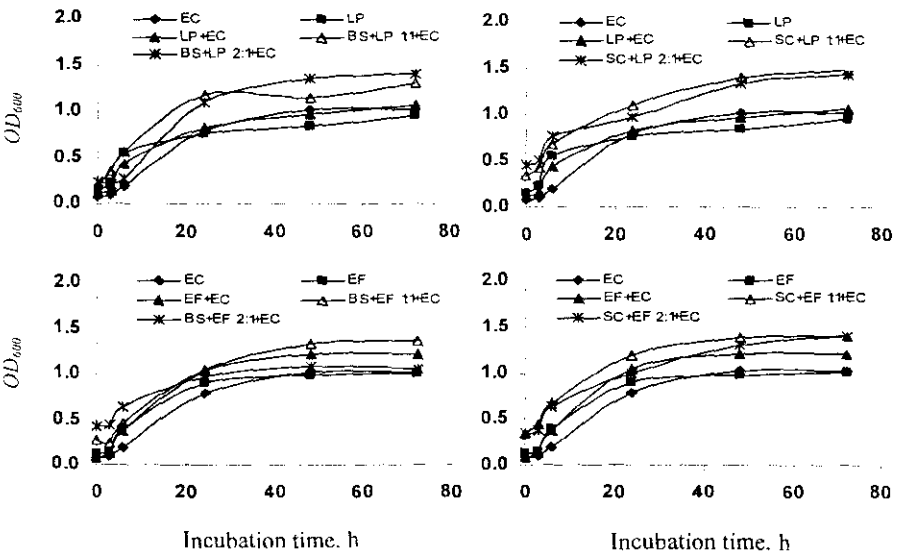

Figure 4. Effect of different type of probiotics (*B. subtilis* (BS) and *S. cerevisiae* (SC)) on the growth (OD_{600}) of the identified LAIB in sheep (*L. plantarum* (LP) and *E. faecium* (EF)) during different times of incubations in presence of *E. coli* – 10 (EC).

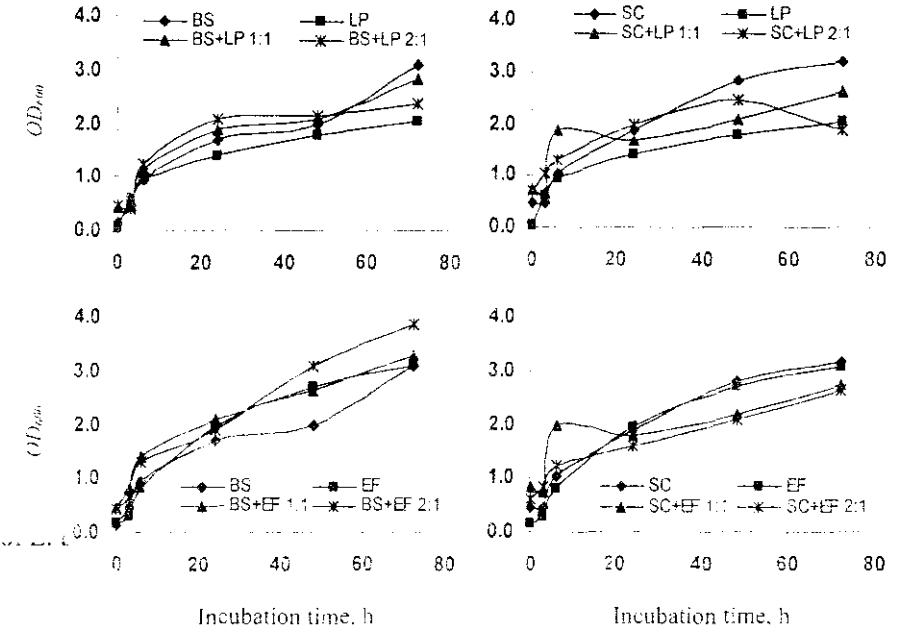

Figure 5. Effect of different type of probiotics (*B. subtilis* (BS) and *S. cerevisiae* (SC)) on lactic acid production (g/L) of the identified LAIB in sheep (*L. plantarum* (LP) and *E. faecium* (EF)) during different times of incubations in absence

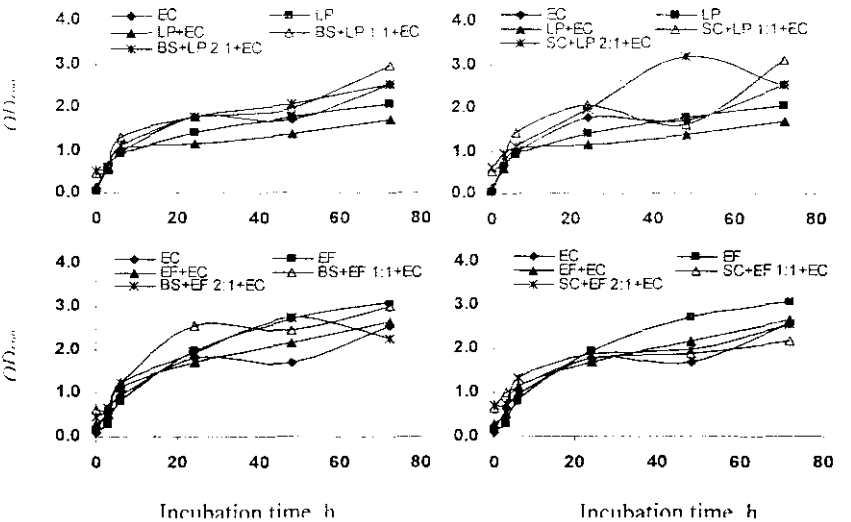

Figure 6. Effect of different type of probiotics (*B. subtilis* (BS) and *S. cerevisiae* (SC)) on lactic acid production (g/L) of the identified LAIB in sheep (*L. plantarum* (LP) and *E. faecium* (EF)) during different times of incubations in presence of *E. coli* – 10 (EC).

Table (6) : Effect of probiotics supplementation‡ on bacterial biomas production[a] (g/L) of the two identified strains of lactic acid intestinal bacteria isolated from sheep in presence or absence of Escherichia coli -10 as a pathogenic bacterium after 72hr of incubation.

Identified Strains (*API*† profile)	Control	Control + *E. coli*	*Bacillus subtillis*				*Saccharomyces cerevisiae*			
			1 : 1		2 : 1		1 : 1		2 : 1	
			- E coli	*+ E coli*	*- E coli*	*+ E coli*	*- E coli*	*+E. coli*	*- E. coli*	*+E. coli*
L. planterum -2LMB	1.12^{h} ±0.284	1.06^{f} ±0.023	1.89^{f} ±0.010	1.17^{h} ±0.020	2.20^{e} ±0.100	1.52^{g} ±0.236	4.82^{a} ±0.007	1.80^{f} ±0.004	2.82^{d} ±0.002	1.12^{h} ±0.284
E. faecium -1LMB	1.31^{h} ±0.201	1.278^{h} ±0.000	4.61^{a} ±0.010	1.37^{h} ±0.010	2.17^{f} ±0.010	1.43^{h} ±0.004	4.30^{b} ±0.004	2.95^{e} ±0.005	3.55^{c} ±0.008	1.31^{h} ±0.201

Means within each row having different letters were significantly different at (P<0.001). Mean ± stander division, *†: Analytical Profile Index* (API) *profile*, ‡: Probiotics (*Bacillus subtillis* or *Saccharomyces cerevisiae*) were added to the medium that was inoculated with the intestinal bacteria by the portion 1:1 or 2:1of probiotic to intestinal bacteria, a : Bacteria biomas production was determined quantitatively after the centrifugation of growth medium after 72h of incubation. The culture were divided into three groups; good >1.3g/L, fair 1-0.6 g/L and poor < 0.6 g/l, Each of the data points is an average of three measurements (n=3).

Table (7) : Effect of probiotics supplementation‡ on the efficiency of biomas separation[a] (OD_{650}) of the two identified strains of lactic acid intestinal bacteria isolated from sheep in presence or absence of *Escherichia coli* -10 as a pathogenic bacterium after 72hr of incubation.

Identified Strains (*API*† profile)	Control	Control + *E. coli*	*Bacillus subtillis* 1 : 1		*Bacillus subtillis* 2 : 1		*Saccharomyces cerevisiae* 1 : 1		*Saccharomyces cerevisiae* 2 : 1	
			- *E coli*	+ *E coli*	- *E coli*	+ *E coli*	- *E coli*	+*E. coli*	- *E. coli*	+*E. coli*
L. planterum -2LMB	0.007^{c} ±0.000	0.002^{c} ±0.000	0.039^{c} ±0.001	0.050^{c} ±0.001	0.151^{b} ±0.000	0.229^{a} ±0.120	0.055^{c} ±0.000	0.055^{c} ±0.001	0.051^{c} ±0.001	0.007^{c} ±0.000
E. faecium -1LMB	0.112^{a} ±0.000	0.039^{d} ±0.001	0.036^{d} ±0.000	0.033^{d} ±0.001	0.051^{c} ±0.001	0.033^{d} ±0.001	0.013^{e} ±0.015	0.033^{d} ±0.000	0.032^{d} ±0.005	0.112^{a} ±0.000

Means within each row having different letters were significantly different at ($P<0.001$). Mean ± stander division.

†: Analytical Profile Index (*API*) profile.

‡: Probiotics (*Bacillus subtillis* or *Saccharomyces cerevisiae*) were added to the medium that was inoculated with the intestinal bacteria by the portion 1:1 or 2:1of probiotic to intestinal bacteria.

a : The absorbance OD_{650} of the growth media after the centrifugation was used to express the efficiency of the association between lactic acid bacteria and each one of the probiotic used in this study in the growth medium. The absorbance of supernatants after centrifugation of culture were divided into three groups; good association of 0-0.1; fair 0.2 and poor > 0.3.

Each of the data points is an average of three measurements (n=3).

effect of probiotics on LAIB activities in sheep.

CONCLUSION

The present findings indicate that probiotics (bacterial or yeast) had a positive effect on lactic acid intestinal bacteria activities and it could improve their inhibition to unfriendly bacteria such as *Escherichia coli*–10 in the intestine of sheep.

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تأثير ال Probiotics كاضافات غذائية على نشاط البكتيريا المعزولة و المعرفة من الأمعاء و المنتجة لحمض الاكتيك في تثبيط نشاط بكتيريا ال *Escherichia coli*-10 في الأغنام

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أجريت هذه الدراسة لمعرفة تأثير إضافة ال Probiotics على نشاط بكتيريا الأمعاء (ال *Lactobacillus plantarum* و ال *Enterococcus faecium*) في تثبيط نشاط بكتيريا ال *Escherichia coli*-10 كبكتيريا ممرضة في أمعاء الأغنام. تم عزل و تعريف سلالتين من بكتيريا الأمعاء المنتجة لحمض اللاكتيك من أمعاء الأغنام البرقي من مزرعة الكلية و هي ال *L. plantarum* و ال *E. faecium*. في هذه الدراسة تم استخدام بكتيريا ال *Bacillus subtilis* و خميرة الخباز (*Saccharomyces cerevisiae*) كأمثلة لل Probiotics و تم وإضافتهم بنسبة ١:١ أو ١:٢ إلى بكتيريا الأمعاء المعزولة. تم تقدير معدل النمو و كمية حمض اللاكتيك المنتجة بعد تحضين كل سلالة بكتيريا معزولة مع كل نوع من أنواع ال Probiotics بنسبة المختلفة (١:١ و ١:٢) حتى ٧٢ ساعة من التحضين في حالة وجود أو عدم وجود البكتيريا الممرضة (ال *E. coli*-10) . كذلك تم تقدير الكتلة الحيوية لبكتيريا الأمعاء تحت الظروف المختلفة من التحضين فى نهاية فترة التحضين بعد ٧٢ ساعة. تم أيضا تقدير كفاءة ارتباط هذه البكتيريا المعزولة مع ال *B. subtilis* أو *S. cerevisiae* في حالة وجود أو عدم وجود البكتيريا الممرضة.

أظهرت النتائج أن إضافة ال Probiotics في بيئة البكتيريا المعزولة أدت إلى زيادة نموها في حالة وجود أو عدم وجود بكتيريا ال *E. coli*. و لقد لوحظ أن أعلى قيمة معنوية للتأثير الفعال الصافي لل Probiotics على نمو بكتيريا الأمعاء في حالة إضافة ال *B. subtilis* كان بنسبة ١:٢ في غياب ال *E. coli* و كذلك ال *S. cerevisiae* بنسبة ١:١ في حالة وجود ال *E. coli* في البيئة. لوحظ أيضا أن ال Probiotics كان لها تأثير إيجابي و فعال في زيادة إنتاج حمض اللاكتيك من بكتيريا الأمعاء المعزولة و لوحظ ارتفاع معنوي في الكتلة الحيوية للبكتيريا باضافة ال *S. cerevisiae* لبيئة بكتيريا ال *L. plantarum* بنسبة ١:١ بالمقارنة ببافي المعاملات في حالة عدم وجود ال *E. coli*. فى حالة بكتيريا ال *E. faecium* لوحظ أن أعلى قيمة للكتلة الحيوية كانت بإضافة ال *B. subtilis* بنسبة ١:١ في البيئة و أيضا في حالة عدم وجود *E. coli*. أما في حالة و جود ال *E. coli* لوحظ أن إضافة ال Probiotics بنسبة ١:٢ كان لها تأثير ايجابي على الكتلة الحيوية للسلالتين المعزولتين من أمعاء الأغنام.

من هذه الدراسة يمكن استنتاج أن ال Probiotics سواء كانت بكتيريا أو خمائر يمكن أن يكون لها تأثير إيجابي على نشاط بكتيريا الأمعاء المنتجة لحمض اللاكتيك في الأمعاء. كذلك يمكنها أن تحسن من تأثير بكتيريا الأمعاء على تثبيط البكتيريا الممرضة الموجودة في أمعاء الأغنام.