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Effect of *Lactobacillus gasseri* and *Lactobacillus plantarum* on *Escherichia coli* and *Bacillus cereus* in yoghurt model

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Abstract
Background: *Probiotics are living microorganisms that confer health benefits, especially when used as food preservative due to its known antimicrobial effect against many of the recorded foodborne pathogens. Dairy products have been recorded that may harbor many food poisoning bacteria such as E. coli and B. cereus.*
Material and methods: *Investigate the antimicrobial effect of two lactobacilli strains represented by L. plantarum (1%) and L. gasseri (1%) lonely and/or combined with their extracted bacteriocins (300ppm) on experimentally inoculated pathogenic E. coli and B. cereus in yoghurt model along seven days in chilling conditions by traditional bacteriological evaluation and statistical analysis of variance.*
Results: *Significant reduction of the tested organisms in yoghurt samples was observed when treated with the used lactobacilli strains and their bacteriocins; L. plantarum (1%) showed higher antimicrobial effect than L. gasseri, furtherly, combined lactobacillus strain with its bacteriocin showed higher inhibitory effect. In addition, E. coli showed more susceptibility to the used L. plantarum, L. gasseri and their bacteriocins in comparison with the susceptibility of B. cereus. Maximum reduction (%) of the tested E. coliwas observed after the 7th day of the incubation to be 94.26 and 93.52%, while in B. cereus were 94.35 and 92.47% of the treated groups of combined L. plantarum with its bacteriocin, and combined L. gasseri with its bacteriocin, respectively.*
Conclusion: *Application of L. plantarum, L gasseri and their bacteriocins possessed a potential significant antimicrobial effect against the tested E. coli and B. cereus in yoghurt model in refrigerating conditions; so, its not only recommended to be used in yoghurt production for safe product manufacturing, but also for their beneficial health effects to the consumers.*

Keywords: *Probiotics, Bacteriocins, Foodborne pathogens, Dairy products.*

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I. Introduction

Milk and processed milk products have been considered as one of the most preferable and nutritious foods since long time ago.Not only for their high protein, minerals, and energy content that acts as a good promotive of human health, immunity and wholesome, but also for their flexible processing varieties that suits all consumer's preferences[1].

On the other hand,they are an ideal enrichment media for the multiplication of many pathogenic food poisoning bacteriasuch as *Escherichia coli, Staphylococcus aureus,* and *Bacillus cereus* that may reach milk and milk products through some intrinsic and extrinsic factors mainly after using of mastiticraw milk in dairy production, improper sanitation of production lines, contaminated equipment, production room air flow, and inadequate workers personal hygiene[2, 3].

Further dairy production of fermented products such as cheese and yoghurt must contain fermentation processes that promote the development of essential acidity for curd production. Fermentation process is mainly conducted through addition of some beneficial lactic acid bacteria (LAB) encoded as starter culture for their functional and bio-preservation role of fermented dairy products processing [4].

One of the most promising areas of development in the human nutritional field over the last decades has been the use of probiotics and recognition of their role in human health and disease. Lactic acid bacteria are the most commonly used probiotics in foods. It is well known that probiotics have a number of beneficial health effects in humans and animals. They play an important role in the protection of the host against harmful microorganisms and also strengthen the immune system. Some probiotics have also been found to improve feed digestibility and reduce metabolic disorders[5].

Probiotics are defined by **FAO/WHO**[6] as live microorganisms which when administered in adequate amounts confer a health benefit on the host**.** Since the therapeutic role of probiotics depends on the count of viable cells of minimum threshold of viable probiotic bacteria between 10^6-10^7CFU/g at the time of consumption should be achieved in order to provide their benefits and comply with standards of legislation.

Due to its promising antagonistic activity against some foodborne pathogens such as *S. aureus* and *E. coli*, it was recommended to use LAB as food additive due to their fermentative ability, health and nutritional benefits, and its production of inhibitory compounds such as bacteriocins, hydrogen peroxide or organic acids as well as competitive adhesion to the intestinal epithelium *in vivo*[7, 8].

For that, global interests of using probiotics and bactriocinsas food additive for their productivity and health concern are increasing. Therefore, the present study was planned to determine the antimicrobial activities of some probiotics (L. gasseri *andL. plantarum*) and their bacteriocins on the viability of some isolated pathogenic strains (*Escherichia coli*and*Bacillus cereus*) in sterilized yoghurt medium.

II. Material and Methods

2.1. Lactobacillus used strains:

Lactobacillus gasserianditsbacteriocin, *Lactobacillus planterum*and its bacteriocin were prepared and obtained ready-to-use from MIRCEN (Microbiological Resource Center), Faculty of Agriculture, Ain-Shams University, Cairo, Egypt.

2.1.1. Preparation of probiotic bacteria:

One ml of eachL. gasseri and *L. plantarum* was cultured overnight in 20 ml MRS brothat 37°C under anaerobic condition.

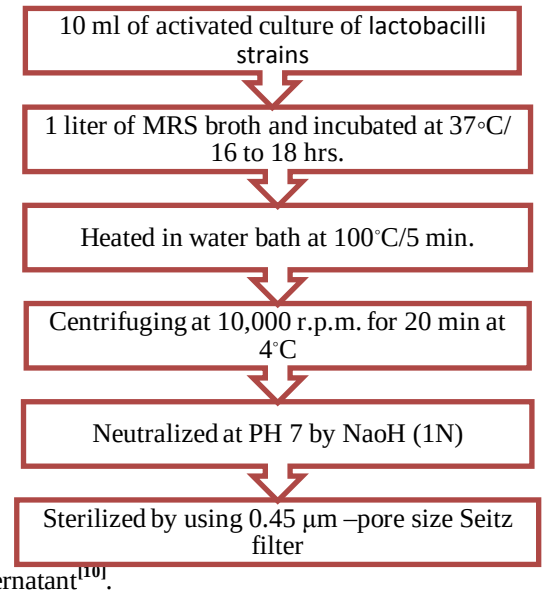
Anotherone ml of the previously prepared culture was sub-cultured again overnight in 20 ml MRS broth till obtain concentration of 10^{10} CFU/ml.

2.1.2. Extraction of crude bacteriocn oflactobacilli (Fig.1):

Preparation of cell free culture supernatant (CFS) (bacteriocin extraction) was done by taking one ml of lactobacilli strains and cultured overnight in 20 ml MRS brothat 37°C under anaerobic condition.Another one 1ml of the previously prepared culture was sub-cultured again overnight in 20 ml MRS broth.

Ten ml of activated culture of lactobacilliwith a concentration of 10^{10} CFU/mlwas inoculated into 1 liter of MRS broth and incubated at 37°C for 16 to 18 hrs under anaerobic condition[9].

Cells were removed by heating cultured broth in water bath at 100°C for 5 min to get rid of H_2O_2 then centrifuging at 10,000 r.p.m. for 20 min at 4°C. The supernatant was collected and neutralized at PH 7 by NaoH (1N) sterilized by using 0.45 µm –pore size Seitz filter with single sheet to eliminate the possible presence of viable bacterial cells and obtain cell free supernatant[10].



Figure, 1:Preparation of cell free culture supernatant (CFS)

2.2. Inoculum preparation:

The pathogenic strains (*E. coli* and *B. cereus*) were activated on TSB (tryptic soya broth) at 37°C/24 hrs. The organisms were activated for 3 successive sub-cultures till obtaining the concentration of 10^6 CFU/ml[11].

2.3. Preparation of group's samples: Yoghurt preparation according to**Corrieu and Be´al**[12]

The skim milk was heated to 85°C for 30 min and immediately cooled to 45°C, and was inoculated by the activated starter cultures obtained from MIRCEN (Microbiological Resource Center), Faculty of Agriculture, Ain-Shams University, Cairo, Egypt (*Lactobacillus bulgaricus* and *Streptococcus thermophilus*).
Samples were grouped as follows:

- ***E.coli* groups**:

G1: Yoghurt made with 2%yoghurt starter cultures + 1% *E.coli*+ 1% *L. planterum*.
G2: Yoghurt made with 2% yoghurt starter cultures + 1% *E.coli*+ 300ppm*L. planterum*bacteriocin.
G3: Yoghurt made with 2% yoghurt starter cultures + 1% *E.coli*+ 1% *L. planterum* + 300ppm *L. planterum* bacteriocin.
G4: Yoghurt made with 2% yoghurt starter cultures + 1% *E.coli*+ 1% L. gasseri.
G5: Yoghurt made with 2% yoghurt starter cultures + 1% *E.coli*+300pp L. gasseribacteriocin.
G6: Yoghurt made with 2% yoghurt starter + 1% *E.coli*+ 1% L. gasseri+ 300ppmL. gasseribacteriocin.
G7: Yoghurt made with 2%yoghurt starter cultures + 1% *E.coli*(Control positive).
G8: Yoghurt made with 2%yoghurt starter cultures (Control negative).

- ***B.cereus* groups**:

G1: Yoghurt made with 2%yoghurt starter cultures + 1% *B.cereus*+ 1%*L. planterum.*
G2: Yoghurt made with 2% yoghurt starter cultures + 1% *B.cereus* + 300 ppm *L. planterum*bacteriocin.
G3: Yoghurt made with 2% yoghurt starter cultures + 1% *B.cereus* + 1% *L. planterum* + 300 ppm *L. planterum* bacteriocin.
G4: Yoghurt made with 2% yoghurt starter cultures + 1% *B.cereus* + 1% L. gasseri.
G5: Yoghurt made with 2% yoghurt starter cultures + 1% *B.cereus* +300pp L. gasseribacteriocin.
G6: Yoghurt made with 2% yoghurt starter + 1% *B.cereus* + 1% L. gasseri+ 300 ppm L. gasseribacteriocin.
G7: Yoghurt made with 2%yoghurt starter cultures + 1% *B.cereus* (Control positive).
G8: Yoghurt made with 2%yoghurt starter cultures (Control negative).

Samples from each group were then mixed, put into sterile cups (100 ml) and incubated at 42°C until curd formation, and were then kept in a refrigerator at 4°C.

2.4. Bacteriological examination of the tested bacteria

2.4.1. Preparation of samples was performed according to ISO 6887-1[13]:Twenty-five grams of each sample was taken under aseptic condition to sterile stomacher bag, and then 225 ml sterile peptone water 0.1% was added.The contents were homogenized; 1ml was transferred into separate tubes containing 9ml sterile peptone water 0.1%, from which ten-fold serial dilutions were prepared. The prepared samples were subjected to the following bacteriological examination:

2.4.2. Enumeration of *E. coli* viable count according to ISO 16649-2[14]

One ml of previously prepared serial dilutions of each sample was poured into each Petri dish with approximately 15 ml of the tryptone-bile-glucuronic medium (TBX) medium, mixing the inoculum with the medium and allow the mixture to solidify, incubation at 44 °C for 18 hrs. to 24 hrs.
The average numbers of morphologically typical colonies (greenish-blue colony) were multiplied by dilution factor to get the count of *E. coli*CFU/g of the yoghurt sample.

2.4.3. Enumeration of *B. cereus*was performed according to FDA[15]

0.1 ml of previously prepared serial dilutions of each sample was spread onto *Bacillus cereus* agar plates, and incubated at 37°Cfor 24-48 hrs.
The average numbers of morphologically typical colonies were multiplied by dilution factor to get the count of *B. cereus*CFU/g of the yoghurt sample.
The experiment was repeated 3 times.

2.4.4. Statistical analysis: application of Analysis of Variance (ANOVA) test of the obtained data was performed according to **Feldman *et al.*[16]**.The result was the mean of triplicates ± standard error.

III. Results

Referring to the obtained results of the inhibitory effect of *L. planterum* and *L. gasseri*and/or their bacteriocins on experimentally inoculated pathogenic *E. coli* in yoghurt samples were recorded in **Table (1)**; overall, treated groups showed lower*E. coli* counts than control group. *Lactobacillus plantarum* and its bacteriocin had more bactericidal activity on the tested *E. coli*with higher reduction percent than *L. gasseri*. In addition, the treated groups with lactobacillus strain plus its bacteriocin (G3 and G6) showed higher antibacterial effect with higher reduction percent than other treated groups, where *E. coli* counts were reduced from 6.8 $\log_{10}$CFU/g in zero day to 0.39 and 0.44 $\log_{10}$CFU/g in the 7^{th} day of cold incubation for *L. planterum* combined with its bacteriocin (G3) and *L. gasseri* combined with its bacteriocin (G6) with reduction percent of 94.26 and 93.52%, respectively.

Table no. 1: Mean ± S.E of *E. coli* counts (log_{10}CFU/g) in the examined yoghurt groups during their refrigeration storage (4℃).

Storage (day)	G1	R %	G2	R %	G3	R %	G4	R %	G5	R %	G6	R %	G7	R %	G 8
0	6.80± 0.36^{ab}	-	6.80± 0.36^{ab}	-	6.80± 0.36^{ab}	-	6.80± 0.36^{ab}	-	6.80± 0.36^{ab}	-	6.80± 0.36^{ab}	-	6.80± 0.36^{ab}	-	0. 0
1	1.01±0.0 6^{cd}	85.14	1.09±0.0 3^{c}	83.97	1.14±0.0 5^{c}	83.23	1.64±0.0 3^{b}	75.88	1.11±0.0 5^{c}	83.67	1.11±0.0 6^{c}	83.67	6.48±0. 00^{a}	4.70	
3	0.80±0.0 1^{cd}	88.23	0.76±0.0 8^{cd}	88.82	0.71±0.0 13de	89.55	1.05±0.0 1^{cd}	84.55	0.93±0.0 4^{cd}	86.32	0.71±0.0 1de	89.69	6.48±0. 00^{a}	4.70	
7	0.59±0.0 6^{ef}	91.32	0.46±0.0 8^{fg}	93.23	0.39±0.0 2^{g}	94.26	0.77±0.0 15^{cd}	88.67	0.59±0.0 1^{ef}	91.32	0.44±0.0 2^{fg}	93.52	6.48±0. 00^{a}	4.70	

G1:Yoghurt made with 2%yoghurt starter cultures + 1% *E.coli*strain + 1% *l.b planterum*strain.
G2: Yoghurt made with 2% yoghurt starter cultures + 1% *E.coli*strain + 300pp *l.b planterum*bacteriocin.
G3: Yoghurt made with 2% yoghurt starter cultures + 1% *E.coli*strain + 1% *l.b planterum strain* + 300pp*l.b planterum* bacteriocin.
G4: Yoghurt made with 2% yoghurt starter cultures + 1% *E.coli*strain + 1% *l.b gasseri*strain
G5: Yoghurt made with 2% yoghurt starter cultures + 1% *E.coli*strain +300pp *l.b gasseri*bacteriocin.
G6: Yoghurt made with 2% yoghurt starter 1% *E.coli*strain + 1% *l.b gasseri*strain + 300pp *l.b gasseri*bacteriocin.
G7: Yoghurt made with 2%yoghurt starter cultures + 1% *E.coli*strain
G8: Yoghurt made with 2%yoghurt starter cultures
*S. E.= Standard Error.
^{ab} values within a column with different superscript letters were significantly different at ($P \leq 0.05$).
R%: Reduction %

On the other hand, anti-*B. cereus* effect of tested probiotics and/or their bacteriocins as mentioned in **Table (2)** revealed that the treated groups showed lower *B. cereus* counts than control group. The treated groups with *L. planterum* and *L. gasseri* combined with their bacteriocin (G3 and G6) showed higher antibacterial effect with higher reduction percent than other treated groups, but it is worthily noted that the lactobacillus strains alone had a greater inhibitory effect than their bacteriocins alone where the reduction percent of *B. cereus* counts were 94.21 and 88.85% for *L. planterum* and *L. gasseri* when added alone (G1 and G4), while were 85.96 and 87.26% after their bacteriocins inoculation alone, respectively.

Moreover, it is worth noting recorded significant differences ($P \leq 0.05$) between the obtained results between groups and within same tested group.

Table no. 2: Mean ± S.E. of *B.cereus* counts (log_{10}CFU/g) in the examined yoghurt groups during their refrigeration storage (4℃).

Storage (days)	G1	R %	G2	R %	G3	R %	G4	R %	G5	R %	G6	R %	G7	R %	G 8
0	6.91±0.0 0^{a}	-	6.91±0.0 0^{a}	-	6.91±0.0 0^{a}	-	6.91±0. 00^{a}	-	6.91±0.0 0^{a}	-	6.91±0.0 0^{a}	-	6.91±0. 00^{a}	-	0. 0
1	1.15±0.0 5^{c}	83.35	1.49±0.0 3^{b}	78.43	1.04±0.0 2^{cd}	84.94	1.56±0. 06^{b}	77.42	1.59±0.0 6^{b}	76.98	1.15±0.0 2^{c}	83.35	6.9±0.0 0^{a}	-	
3	0.85±0.0 7de	87.69	1.14±0.0 3^{c}	83.50	0.79±0.0 4de	88.56	1.13±0. 07^{c}	79.30	1.00±0.0 7^{cde}	85.52	0.85±0.0 5de	87.69	6.9±0.0 0^{a}	-	

7	0.40±0.01^{f}	94.21	0.97±0.09^{cd}	85.96	0.39±0.02^{f}	94.35	0.77±0.04^{e}	88.85	0.88±0.09^{f}	87.26	0.52±0.04^{f}	92.47	6.9±0.00^{a}	'	

G1:Yoghurt made with 2%yoghurt starter cultures + 1% *B.cereus*strain + 1% *l.b planterum*strain.
G2: Yoghurt made with 2% yoghurt starter cultures + 1% *B.cereus*strain + 300pp *l.b planterum*bacteriocin.
G3: Yoghurt made with 2% yoghurt starter cultures + 1% *B. cereus*strain + 1% *l.b planterum strain* + 300pp*l.b planterum* bacteriocin.
G4: Yoghurt made with 2% yoghurt starter cultures + 1% *B.cereus*strain + 1% *l.b gasseri*strain
G5: Yoghurt made with 2% yoghurt starter cultures + 1% *B.cereus*strain +300pp *l.b gasseri*bacteriocin.
G6: Yoghurt made with 2% yoghurt starter 1% *B.cereus*strain + 1% *l.b gasseri*strain + 300pp *l.b gasseri*bacteriocin.
G7: Yoghurt made with 2%yoghurt starter cultures + 1% *B.-cereus*strain
G8: Yoghurt made with 2%yoghurt starter cultures
*S. E.= Standard Error.
R%: Reduction %

IV. Discussion

Lactobacilli are important organisms recognized for their fermentative ability as well as their health and nutritional benefits. These bacteria are the major component of the starters used in fermentation, especially for dairy products. They produce various compounds such as organic acids, diacetyl, hydrogen peroxide and bacteriocin or bacterial proteins during lactic acid fermentations which can antagonize the growth of some pathogenic bacteria in foods. The use of lactic acid bacteria and their metabolites to improve microbiological safety and extend the shelf life of foods is defined as bio-preservative. Bacteriocins are extra-cellularly released peptides or protein molecules, with a bactericidal or bacteriostatic mode of action against closely related species.

Referring to the current observations, *L. planterum* and *L. gasseri* and their bacteriocins showed a promising significant inhibitory effect on the examined *E. coli* and *B. cereus* strains that is in line with the previously recorded findings of **Mami et al.[17],Tharmaraj and Shah[18], Fahad and Ahmed[19] andMarie et al.[20]**who attributed the antimicrobial effect of lactobacillus species strains to that probiotic bacteria produce lactic and acetic acids as a metabolic byproducts which play a complementary role in inhibiting pathogenic and spoilage bacteria, as they were reported to have inhibitory activity against common human pathogens. They are able to produce antimicrobialsubstances such as bacteriocins which have great potential to be used in therapeutics and as food bio-preservatives.

Moreover, in groups 3 and 6, where lactobacillus strain was combined with its extracted bacteriocin, showed more reduction rates in comparison with the used strains and/or its bacteriocins alone which came in agreement with the recorded observations by **Perezet al.[21]**who recorded that combination of bacteriocins with other food additives including LAB starter cultures revealed higher antimicrobial effect in comparison with its application alone because of synergizing its action as bactericidal additive, with or without cell lysis, or bacteriostatic, inhibiting cell growth**[22]**. Most of the bacteriocins produced from LAB, in particular those inhibiting Gram-positive bacteria, exert their antibacterial effect by targeting the cell envelope-associated mechanisms**[23].** Several antibiotics and some class II bacteriocins target Lipid II, an intermediate in the peptidoglycan biosynthesis machinery within the bacterial cell envelope and, by this way they inhibit peptidoglycan synthesis**[24]**.

Bacteriocins are generally defined as peptides or proteins ribosomal synthesized by bacteria that inhibit or kill other related or unrelated microorganisms. Bacteriocins have attracted considerable interest for their use as safe food preservatives, as they are easily digested by the human gastrointestinal tract. The use of bacteriocins as natural food preservatives fulfills consumer demands for high quality and safe foods without the use of chemical preservatives**[25].**

Therefore, application of lactobacilli and/or their bacteriocins have been considered as safe food preservatives especially in dairy products.

V. Conclusion

Regarding to the obtained results, it could be concluded that the used lactobacillus strains revealed promising significant inhibitory effect on the tested strains, where *L. planterum* and/or its bacteriocin showed higher potent efficacy against*E. coli* and *B. cereus* reduction than L. gasseri; in addition, combination of lactobacillus strains with its bacteriocin showed higher inhibitory effect on the tested organisms. Application of *L. planterum* in combined with its bacteriocin is strongly recommended as bio-preservatives in yoghurt industry for more safe and beneficial production.

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