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In vitro Evaluation of Probiotic Activities of Lactic Acid Bacteria Strains Isolated From Novel Probiotic Dairy Products

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Abstract: Due to their increasing popularity among consumers the number of probiotic dairy products on the local market has increased tremendously during the last few years. Probiotic bacteria used in commercial products today are mainly members of the genera *Lactobacillus* and *Bifidobacterium.* Twenty lactic acid bacterial strains were isolated and identified by phenotypic method from samples of probiotic dairy products as fermented milk drinks, yoghurts and infant milk powder. These strains were grouped at species level as *L. casi, L. acidophilus, L. plantarum and Bifidobacteria species*. These strains were further tested for the presence of functional traits useful for probiotic applications, such as resistance to acidic condition at pH 2, bile salt hydrolytic activity and ability for adhesion to Caco-2 cells as well as ability to inhibit the adhesion of *E. coli, Salmonella typhimurium and Shigella flexneri* to caco-2 cells. Our obtained results showed that most of tested strains exhibited characteristics suggesting that they would survive in the gastrointestinal tract and also had the capability for adhesion to *caco-2 cells.* Greater variability was observed for the other traits analyzed. These data suggest that these probiotic strains had characteristic and differential functions traits. Therefore, results from our present study are expected to encourage people to consume more probiotic dairy products, as it was revealed that these products contain some probiotic *lactic acid bacteria* which play a major role for the beneficial health effects of consumers.

Key words: Dairy products · Lactic acid bacteria and probiotic activities

INTRODUCTION

Lactic acid bacteria (LAB) have a long history of safe use, especially in the dairy industry and play a major role in the production of fermented milk products. Over the past few decades, an increased drive has existed for the isolation of novel Lactobacillus strains that exert a beneficial health effect when ingested by humans. Such strains are termed probiotic. According to Guarner and Shaafsma [1] probiotics are ‘‘living micro-organisms, which upon ingestion in certain numbers, exert health benefits beyond inherent basic nutrition’’.

Beneficial effects conferred by lactobacilli include inhibition of pathogenic organisms, such as *Salmonella, Shigella* and *Helicobacter* [2-5]. Furthermore, lactobacilli have been associated with numerous other health benefits, such as reduction of lactose intolerance [6] and increased immune response [7]. A beneficial role for *lactobacilli* has also been implied in cancer [8, 9] and especially in the case of colon cancer [10, 11].

Although the concept of using bacteria as bio therapeutic agents is not new, probiotic *lactic acid bacteria (LAB)* have attracted enormous attention only in recent years. The renewal of interest in the health-promoting properties of certain LAB inhabiting the human gastrointestinal tract has stimulated the innovative development by the food industry of functional food products containing probiotic strains. As a consequence, the manufacture and marketing of probiotic yoghurts and other fermented milk products have increased dramatically worldwide in recent years.

With regard to the health-promoting effects of these products, claims such as ‘‘helps maintain a healthy balance of beneficial bacteria’’ or ‘‘stabilizes the intestinal microflora and modulates its function’’ are made on the labels of the products and in advertising brochures of the dairy industry. However, the information on the bacterial strains used in these products and on their particular properties, is scarce.

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Recently, a number of novel fermented dairy products and infant milk powder have been developed and are being marketed under the name of probiotic products. Due to their increasing popularity among consumers the number of probiotic dairy products on the local market has increased tremendously during the last few years. The application of *LAB* in the manufacture of fermented milk products is not a new concept. In the last few years, strains of *L. acidophilus*, *L. casei* complex and *Bifidobacterium lactis* predominate in commercial probiotic products [12-14].

In order for a probiotic strain to exert its beneficial effect on the host, it has to be able to survive passage through the host's digestive tract. So far, in order to survive in and colonize the gastrointestinal tract, probiotic bacteria should express high tolerance to acid and bile and have the ability to adhere to intestinal surfaces [15, 16]. Survival ability and temporary colonization of the human gastrointestinal tract have been demonstrated for some *lactic acid bacteria* [17, 18]. However, *in vivo* testing is expensive, time consuming and requires approval by ethical committees Therefore, reliable *in vitro* methods for selection of promising strains are required. Enterocyte-like *Caco-2* cells [19] have been successfully used for *in vitro* studies on the mechanism of cellular adhesion of nonpathogenic *lactobacilli* [20, 21]. Also, *Caco-2* cells have been used to examine the antimicrobial activity of *lactobacilli* [3] against pathogenic bacteria.

Therefore, in our study, *LAB* strains isolated from novel probiotic dairy products were examined for tolerance to acidity and bile salt hydrolase (BSH) activity, as well as for their ability to adhere to *caco-2 cell* and *in vitro* inhibition of some of enteric pathogens.

MATERIALS AND METHODS

Samples Collection: Thirty samples of different probiotic dairy products, fermented milk drinks, probiotic yoghurt and infant milk powder (10 of each) were separately collected from local markets in Riyadh city, Saudi Arabia and sent to the laboratory. The samples were kept for 2-4 h in the refrigerator until analysis was conducted.

Isolation and Phenotypic Characterization of LAB Strains: It was adopted according to Therzaghi and Sandine [22], De Man *et al*. [23], Florez *et al*. [24] and Roissart and Luquet [25].

Bacterial Strains and Growth Conditions: Identified strains of LAB isolated from examined samples were kept and stored at-80 C in MRS broth, supplemented with 20% glycerol for further testing. Enteric pathogens as *E. coli* (*ATCC* 8739), *Salmonella typhimurium* (*ATCC* 49416) and *Shigella flexneri* (*ATCC* 12022) were obtained from central lab for drug and food analysis, MOH, KSA and used as test strains. These pathogens were enumerated after 24 h incubation on MacConkey agar (Oxoid) and SS agar (Oxoid) at 37°C.

Evalution of the Probiotic Activities of Isolated Strains
Acid Tolerance: The resistance of isolated strains in a low pH environment was tested as previously described by Conway *et al*. [26].

Screening for Bile-Salt Hydrolytic (BSH) Activity: It was adopted according to Dashkevicz and Feighner [27].

Adhesion to *Caco*-2 Cells: The method was carried out according to Jacobsen *et al.* [28].

Inhibition of Pathogen Adhesion to Caco-2 Cells: The cell infection assay was conducted as previously reported by Coconier *et al.* [29].

RESULTS AND DISCUSSION

Probiotics are defined as live microorganisms which when administered in adequate amounts confer a health benefit in the host [30]. However, the minimum amount of probiotics needed to obtain a clinical effect has not been established. As more information on probiotics is available, it seems likely that numbers will vary as a function of the strain and the health effect desired [31].

Twenty probiotic *lactobacillus* and *Bifidobacteria* strains were isolated and have been categorized to four groups, depending on the phenotyping or biochemical identification and as stated in the label of manufacturers as *L. casi*, *L. acidophilus*, *L. plantarum* and *Bifidobacterium* spp. (*B. lactis*). Similar findings were reported by Schillingerand [32] and Tabasco *et al.* [33]. The presence of multiple and closely related species in these products made the differential enumeration of probiotic and yoghurt starter bacteria difficult due to similarity in growth requirements and overlapping biochemical profiles of the species. For instance on the labels of some yoghurts the name *Lactobacillus bifidus* still is used for *Bifidobacterium bifidum* which is not a *Lactobacillus* species. Moreover, strains used in the manufacture of yoghurt are not always indicated on the label. For these reasons, it seemed to us that the identity and taxonomy of *LAB* used in the manufacture of novel-type probiotic yoghurts need clarification.

Table 1: Mean count, phenotyping characterization and BSH activity of LAB strains revealed from examined samples

Type of samples (n= sample number)	LAB Mean count Log cfu/ ml± SD))	Groups of revealed probiotic LAB isolates	BSH
Probiotic fermented milk drink (n=10)	7.5± 0.4	*L. casi*	-
		L. acidophilus	+
		L. plantarum	+
		B. lactis	+
Probiotic yoghurt (n=10)	6.2±0.7	*L. casi*	-
		L. acidophilus	+
		B. lactis	+
Infant milk powder (n=10)	7.9±0.7	*L. casi*	-
		B. lactis	+

Viable numbers of the *LAB* present in the examined samples of commercial novel-type probiotic dairy products were determined and are given in Table 1. Mean count (log cfu/ml) of total *LAB* in examined samples were 7.5±0.4, 6.2±0.7 and 7.9±0.7 in fermented milk drinks, probiotic yoghurt and infant milk powder, respectively.

It seems reasonable to assume that adequate numbers of the probiotic bacteria are need to be consumed to exert a health-promoting effect for the consumer and it has been suggested that to have any therapeutic effect, the minimal number of probiotic bacteria in a product should be above 10^5 or 10^6 per gram [34, 35]. Moreover, it is important that the *lactobacilli* remain viable during refrigerated storage of the product for a certain period as yoghurts may be consumed after storage in the refrigerator for several weeks.

Regarding the bile salt hydrolase activity (BSH), all tested strains of *L. acidophilus*, *L. plantarum* and *Bifidobacterium* spp. exhibited positive bile salt hydrolase activity, recorded as precipitation zones in the BSH plate assay whereas the strains of *L. casi* group were BSH negative (Table 1).

These results are in agreement with those obtained by Moser and Savage [36] and Maragkoudakisa *et al.* [37]. Resistance to bile salts is generally considered as an essential property for probiotic strains to survive the conditions in the small intestine. Bile salt hydrolytic (BSH) activity may contribute to resistance of *LAB* to the toxicity of conjugated bile salts in the duodenum and therefore is an important colonization factor [38].

The *in vitro* criteria used in our study for the evaluation of candidate probiotic have been described in previous studies and are referred to as selection guidelines by the FAO/WHO committee [39]. The *in vitro* screening of the survival of *lactobacilli* in simulated GI tract conditions may only have value in predicting the actual *in vivo* survival of a strain when consumed in a non-protected way.

The tested *LAB* strains isolated from examined samples of probiotic dairy products differed considerably in their resistance to acid. After 3h of exposure to pH2, the best survival was observed with strains of *L. acidophilus and B. lactis* (>2.0 log cycles reduction). While other strains (*L. casi and L. plantarum*) displayed loss of viability of more than <2 log cycles (Figure 1).

Our findings on the viability of *LAB* strains at pH 2 are in agreement with results stated by Pennacchia *et al.* and Zoumpopoulou *et al.* [40, 41]. It should be mentioned, however, that probiotic *LAB* are mostly consumed in fermented dairy products and milk proteins may provide a protective matrix enhancing and supporting survival of bacteria in the gastric juice of the stomach [42, 43].

All tested probiotic strains revealed from examined samples were able to adhere to *Caco-2* cells (Figure 2). *L. plantarum* showed the strongest adhesion ability (237.6±2.5 adhesive bacteria), while the least adhesive strains was *L. acidophilus* (138.7± 2.1 adhesive bacteria). The mean numbers of adhesive bacteria of *L. casi* and *B. lactis* were 166.8±3.1 and 199.5±3.9 respectively.

Our results are similar with the data recorded by Maragkoudakisa *et al.* and Duary *et al.* [37, 44]. While different findings were reported by Pan *et al.* [45] who found that *L. acidophilus* showed the strongest adhesion ability among other tested probiotic strains. Adhesion of *LAB* has been claimed to be essential for the exertion of a beneficial (probiotic) effect in the large intestine.

Three *lactobacilli and one B. lactis* strains were used in the adhesion inhibition assay, *LAB* strains were chosen on the basis of their adhesion ability to *caco-2cell.* The adhesion of *E. coli*, *S. typhimurium* and *Shigella flexneri* to caco-2cells was found to be 63%, 67% and 57 % respectively (Data not shown). The adherence ability of all the tested pathogens were obviously reduced by co-cultured with *LAB* tested strains.

The adhesion of *E. coli* to *Caco-2cells* was reduced by percentage of 30, 22, 42 and 38 % when *E. coli* was treated with strains of *L. casi, L. acidophilus,*

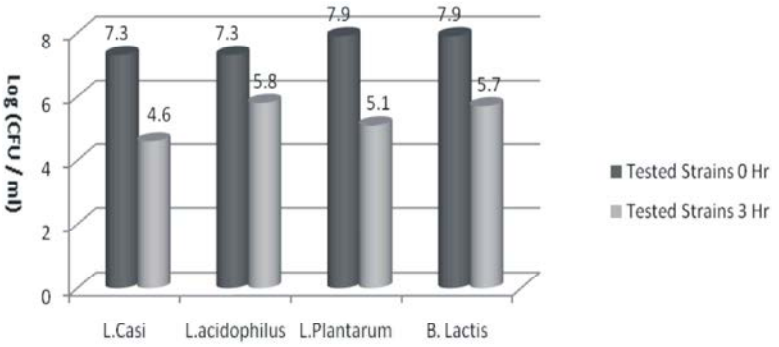

Fig. 1: Acid tolerance activity of isolated LAB strains (Mean ± SD) (Survival of LAB strains was compared by plate counting after exposure to pH2 in PBS for 0 and 3 h)

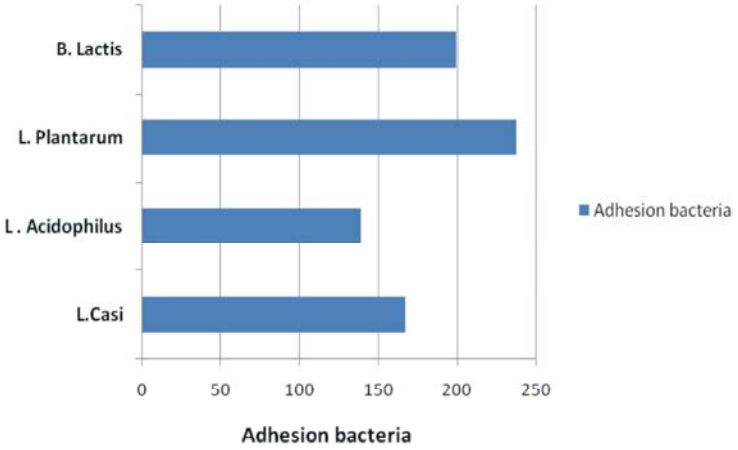

Fig. 2: Adhesion ability of revealed LAB strains to Caco-2 cell (Results were shown as number of adhering LAB in 20 microscopic fields ±SD)

Table 2: Percentage of inhibition (decrease) of pathogen adhesion to caco-2 cells co-cultured with different *LAB* strains revealed from examined samples

	% of pathogens inhibition		
Tested Strains	*E. coli*	*S. typhimurium*	*Shigella flexneri*
L. casi	30.8±0.7	37.5±1.2	15.6±0.9
L. Acidophilus	22.1±1.8	41.3±1.2	32.9±2.6
L. plantarum	42.4±1.2	12.9±2.0	31.3±1.2
B. lactis	38.7±1.2	37.1±2.5	22.9±o.7

L. plantarum and *Bifidobacterium* spp. (*B. lactis*) respectively (Table 2). As far as, adhesion of *S. typhimurium and Shigella flexneri* was reduced with percentages of (37, 41, 12 and 37) and (15, 32, 31, 22) with strains of *L. casi*, *L. acidophilus*, *L. plantarum* and *Bifidobacterium* spp. (B. lactis), respectively.

Generally, our results presented in (Table 2) revealed that adhesion of tested enteric pathogens (*E. coli*, *S. typhimurium* and *Shigella flexneri*) to caco-2cells was reduced even up to 50% with tested probiotic strains. Inhibition of Gram-negative pathogens to eukaryotic cell lines, has already been reported for strains such as *L. acidophilus* and *Bifidobacterium* spp. [46, 47] as well as for strains as *L. casi* and *L. plantarum* [37].

The antimicrobial activity of *lactobacilli* may be due to a number of factors. The adhesion of *lactobacilli* to host intestinal epithelium might result in the competitive or exclusion of adhesion of pathogenic bacteria [48]. The mechanism of inhibition on the pathogen invasion might also be due to steric hindrance of human enterocytic pathogen receptors by whole cell *lactobacilli* rather than

to a specific blockade of receptors [49]. On the other hand several other mechanisms for *lactobacilli* to inhibit *E. coli* and *S. typhimurium* infection have also been suggested. Among these were: contribution to mucosal barrier function, modulation of the immune response, competition for substrates, co aggregation with pathogens, decreasing of the luminal pH via the production of lactic acid and secretion of specific compounds such as bacteriocins [50].

In conclusion, *LAB* strains isolated from examined samples in this study presented interesting probiotic characteristics, especially greater resistance to acid and bile conditions, as well as good adhesion capacity to Caco-2 cells. These strains also showed greater enteropathogens growth inhibiting activity and interference with pathogens adhesion to *Caco-2* cells. These characteristics may enable them to establish themselves in the intestinal tract and to compete with other bacterial groups. Therefore, results from our present study are expected to encourage the people to consume more probiotic dairy products, as it was revealed that these products contain some probiotic lactic acid bacteria which play a major role for the beneficial health effects of consumers.

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