Lectin-Like Binding of Four Animal Lactobacilli Considered for their Use in Probiotical Preparations

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Abstract: Four gut lactobacilli (*Lactobacillus plantarum* L5, *Lactobacillus paracasei* L81, *Lactobacillus fermentum* L 670 and *Lactobacillus casei* subsp. *pseudoplantarum* L.c.) were examined by particle agglutination assay (PAA) for their lectin-like binding activity after their cultivation on Rogosa agar and in MRS broth. Seven ECM (extracellular matrix) molecules (bovine mucin, porcine mucin, bovine fibronectin, porcine fibronectin, fetuin, bovine lactoferrin and heparin) were selected for this assay. Moreover, haemagglutination tests with pig, cattle, sheep, and hen erythrocytes were performed. However, none of the four *Lactobacillus* strains examined did react with any of the erythrocytes tested. The differences between individual strains were observed in their binding to immobilised ECM molecules. The best adherent were the *Lactobacillus plantarum* L5 and *Lactobacillus paracasei* L81, however, the other two strains showed also good ECM binding of some ECM proteins. With regard to an influence of cultivation medium on lectin-like binding activity, binding of all ECM molecules was expressed in *Lactobacillus paracasei* L81 to significantly higher degree after cultivation on Rogosa agar than in MRS broth. Similarly, strains *Lactobacillus fermentum* L670 and *Lactobacillus casei* subsp. *pseudoplantarum* L.c. displayed significantly higher binding of fibronectin and mucin after growth on Rogosa agar in comparison with MRS broth cultivation. The influence of cultivation medium on fetuin binding by *Lactobacillus fermentum* L670 was also not significant while *Lactobacillus casei* subsp. *pseudoplantarum* L.c. bound fetuin significantly better after growth on Rogosa agar.

Heparin pretreatment increased the binding of the ECM molecules by the *Lactobacillus fermentum* L 670 strain significantly with the exception of porcine fibronectin when the strain was cultivated in MRS broth. Similar positive effect of heparin was observed also in the other three lactobacilli. This result is important especially in the connection with the observations that heparin decreased ECM binding of enteropathogens as staphylococci or clinical enterococcal isolates. Following up on some earlier strain characteristics, these results confirm that the selected lactobacilli are suitable for probiotic purposes.

Keywords: *Lactobacillus*, ECM proteins, extracellular matrix, collagen, fibronectin, albumin, vitronectin, lactoferrin, fetuin, mucin, probiotic use.

INTRODUCTION

Both indigenous and pathogenic bacteria are able to attach to host cells, and attachment is assumed to be a critical parameter for colonization of mucosal surfaces by microorganisms [1, 2].

Lactobacilli are members of the normal mucosal microflora of most animals. Hundreds of papers report the use of various *Lactobacillus* strains as probiotic agents for human and animals. Moreover, several requirements have been identified as important properties for lactobacilli to be effective probiotic organisms [3, 4]. Their effects are considered to include the prevention of gastrointestinal infections [5, 6], enhance immune response [7, 8], and antimutagenic as well as anticarcinogenic activity [9, 10].

When selecting strains for probiotics, it is necessary to respect the origin of the strain used, its ability to survive and grow in the respective ecological unit [11]. Recently, the idea has emerged to select among lactic acid bacterial strains those which are able to be incorporated into the resident flora and to demonstrate beneficial potentialities, then to investigate their biological effects both *in vitro* and *in vivo*, and finally use them in probiotical products offering health benefits [12, 13].

The extracellular matrix (ECM) is a mixture of secreted proteins composed primarily of collagens, fibronectin, laminin, and proteoglycans located on epithelial and endothelial cell surfaces [14]. Adherence of pathogens to ECM of various host tissues has been often investigated, demonstrating the important role of these interactions in the establishment of many infections [2, 15]. However, very little is known about members of the indigenous microflora, including lactobacilli, and their ability to bind ECM proteins despite some studies demonstrating collagen [16-20] and fibronectin binding [14, 20].

Lactobacillus populations are very interesting within the gastrointestinal tract of piglets, due to their purported benefits for gut function and health [21]. While, these *Lactobacillus* populations establish early

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in the piglet, succession occurs throughout the pig's lifetime, with lactobacilli remaining a predominant portion of the population [22]. The strains for our study selected show a series of features which make them particularly promising for the preparation of probiotic products. The characteristic features of the strains used are strong adherence to epithelial gut cells as well as inhibitory activity against enteropathogenic *E.coli* under *in vitro* conditions [5]. These lactobacilli were also confirmed as producers of organic acids that generated an inhibitory barrier against digestive tract pathogens on the mucosa of the small intestine [23]. Hydrogen peroxide, an inhibitory substance to pathogens may be one of the responsible factors in the dominance of lactobacilli [24] except organic acids and bacteriocins. *Lactobacillus plantarum* L 5 and *Lactobacillus casei* subsp. *casei* L 81 produce hydrogen peroxide in detectable amounts [25]. The adherence of lactobacilli to gut cells *in vitro* correlated with their ability to adhere to the mucosa of both jejunum and ileum in gnotobiotic piglets [5]. Gnotobiotic piglets were used in some previous experiments because they provide epithelial surfaces in a defined, living system in which a single attribute of an organism (i.e. the ability to colonize an epithelium) can be tested. However, this model is not suitable for experiments directed to the investigation of lectin-like binding ability of bacteria.

The study of haemagglutination properties has been considered as a convenient and effective way to investigate the presence of adhesins on bacteria [26, 27]. Moreover, Adlerberth *et al*. (1996) [28] demonstrated that the carbohydrate-binding ability leading to haemagglutination of *Lactobacillus plantarum* is closely related to the ability to adhere to intestinal epithelial cells. That is why we decided to examine a haemagglutination activity of four lactobacilli which were investigated also for their ECM-binding properties and are considered for their use in probiotical preparations.

MATERIALS AND METHODS

Sources and Cultivation of Strains

Three *Lactobacillus* spp strains (*Lactobacillus plantarum* L 5, *Lactobacillus paracasei* L 81 and *Lactobacillus fermentum* L 670) originally isolated from the jejunum and ileum of piglets as well as *Lactobacillus casei* L.c. from the intestinum of a calf were used in this study. They were obtained from Dr. Radomíra Nemcová from Veterinary University in Košice (Slovakia). Two growth media were examined for their influence on the expression of the surface receptors of all strains tested. Lactobacilli were grown overnight in Man-Rogosa-Sharpe (MRS) broth (Difco Laboratories, Detroit, MI, USA) and on Rogosa agar plates (Difco) at 37°C in 5% CO_2 atmosphere. *Staphylococcus haemolyticus* SM 131 as well as *E. coli* strains On-6, AX 139, AX 35 and AX 16 from the Collection of Microorganisms of the Medical Faculty of the UPJŠ (Pavol Jozef Šafárik University) Košice (Slovakia) were used as positive controls in haemagglutination tests.

Chemicals

ECM molecules (bovine mucin (BM), porcine mucin (PM), bovine fibronectin (BFIB), fetuin (FET), bovine lactoferrin (BLACT) and heparin (HEP), porcine plasma fibronectin (PFIB) from BioInvent International AB (Lund, Sweden). All buffers and chemicals were of analytical grade.

Adsorption of ECM Proteins to Latex Beads

Proteins were adsorbed to the Difco latex beads by electrostatic and hydrophobic interactions as described previously [19, 29].

Particle Agglutination Assay (PAA)

The protein-coated latex beads (15µl) were mixed on a glass slide with an equal volume of a bacterial cell suspension of 10^{10} cfu ml^{-1}. These two drops were gently mixed and the agglutination reaction was scored after 2 min as a PAA value from strongly positive (3) to weakly positive (1) or negative (0) as previously described by Štyriak *et al*. (1999b) [30].

Erythrocytes and Haemagglutination Tests

After being collected, pig, cattle, sheep, and hen erythrocytes were washed three times with phosphate-buffered saline (PBS), pH 7.0. Bacterial cells grown on Rogosa agar plates (Difco) at 37°C in 5% CO_2 atmosphere were harvested and washed once with PBS and resuspended in the same buffer to a final concentration of 10^9 cfu per ml. Moreover, bacteria grown in MRS broth were centrifuged and washed once with PBS and resuspended in the same buffer to a final concentration of 10^9 cfu per ml. Haemagglutination (HA) tests were carried out as described previously by Mukai *et al*. (1998) [27] at either 4°C or 37°C in 96-well U-bottom microtitre plates. *Staphylococcus haemolyticus* SM 131 as well as *E. coli* strains On-6, AX 139, AX 35 and AX 16 were used as positive controls in haemagglutination tests.

Table 1: Binding (PAA Score) of Four Animal Lactobacilli to ECM as Tested by Particle Agglutination Assay

Strain	BM	PM	BFIB	PFIB	FET	BLACT	HEP
Lactobacillus plantarum L 5	0	1	3	0	1	0	3
Lactobacillus paracasei L 81	0	1	3	0	1	0	3
Lactobacillus fermentum L 670	0	1	2	0	0	0	2
Lactobacillus casei L.c.	0	0	1	0	1	0	2

RESULTS

The PAA score (Table **1**) displays some differences among lectin-like binding ability of 4 selected strains tested by PAA.

With regard to an influence of cultivation medium on ECM binding, Rogosa agar permits in most cases a better expression of bacterial surface structures than MRS broth because a higher bacterial binding was observed after cultivation of the strains on Rogosa agar plates than in MRS broth. However, in some cases no significant or opposite effect of medium was observed.

Concerning the study of haemagglutination properties, none of the four *Lactobacillus* strains examined did react with any of the erythrocytes tested.

DISCUSSION

It is more and more recognized that the resident microflora of the gastrointestinal tract plays an important role in inhibiting gut colonization by incoming pathogens [31, 32]. Probiotic agents, live microorganisms with beneficial effects for the host, may offer an alternative to conventional antimicrobials in the treatment and prevention of enteric infections. This alternative is very actual especially nowadays when increasing antibiotic resistance may soon render conventional therapy inadequate for many infections. While the expression of the factors listed in some review articles (e.g. Reid, 1999) [4] is likely important for probiotical activity, it is not easy to grade the extent to which any given property is essential or of greatest importance *in vivo*. However, it seems that adherence and expression of some antagonistic activity against pathogens, especially against their adhesion, belongs to the most critical factors, but that fact does not exclude other properties [4].

Neeser *et al*. (2000) [32] corroborated by their findings the hypothesis that selected probiotic bacterial strains could be able to compete with enteropathogens for the same carbohydrate receptors in the gut. Mack *et al*. (1999) [33] proposed on the basis of their *in vitro* studies the hypothesis that the ability of probiotic agents to inhibit adherence of attaching and effacing organisms to intestinal epithelial cells is mediated through their ability to increase expression of MUC2 and MUC3 intestinal mucins. The capacity to adhere to components of the mucosa and thus avoid rapid exclusion from a beneficial environment must be a high-priority task for an intestinal organism [34]. These findings put a new light on the way by which probiotic bacteria can provide protection to the gut against microbial pathogens.

Four gut lactobacilli, investigated in this study by PAA for lectin-like binding activity, were previously examined for their collagen binding [19] and now we extended our study by screening of these strains for lectin-like binding abilities. As shown in Table **1**, many significant differences between individual strains were observed in the binding of these immobilised proteins. The ECM structures may be expressed on the surfaces of eucaryotic cells such as epithelial, endothelial, fibroblasts, erythrocytes, etc.

Mucin from porcine stomach was used as a model to confirm a mucin-binding activity of lactobacilli because it is known that mucin molecules serve as initial binding sites for most of enteric and other bacteria [35]. Since cell surface lectins could be involved in recognition events associated with many bacterial diseases, fetuin called also “lectin screening protein” was also used in our study.

Several glycosaminoglycans form part of the extracellular matrix and that is why heparin, a representative of this group, was chosen as a model to study glycosaminoglycan-binding ability. Heparin is distributed widely in the human body [1], stored intracellularly in mast cell granules and released on mast cell degranulation in an allergic response [36].

In order to compare ECM binding of the strains after their cultivation on solid and in liquid medium, the strains were grown on Rogosa agar plates and in MRS broth. Growth on solid medium as compared to liquid

medium induces changes in the expression of cell surface proteins in *Staphylococcus aureus* [37], some lactobacilli [16, 38] and Streptococcus bovis strains [30]. That is why the similar result with three of four *Lactobacillus* strains tested is not surprising.

In our previous study [19], three selected inhibitors significantly reduced Cn-I binding by *Lactobacillus plantarum* L 5 strain. However, a possible positive effect of a substance on bacterial binding was not tested previously. The PAA results presented previously on the International symposium on anaerobic microbiology in Prague in November 2000 [39] show that heparin was bound by all our lactobacilli tested.

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