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EPIDEMIOLOGY AND BACTERIAL TYPING

Characterisation and differentiation of lactobacilli by lectin typing

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***Lactobacillus* isolates from healthy Estonian and Swedish children were characterised by a lectin typing technique; 56 isolates from six species (*L. acidophilus*, *L. paracasei*, *L. plantarum*, *L. fermentum*, *L. brevis* and *L. buchneri*) were tested. The typing system was based on an agglutination assay with a panel of six commercially available lectins, which were chosen on the basis of their carbohydrate specificities. The isolates were also subjected to proteolytic degradation before lectin typing to decrease auto-agglutination of whole cells in the assay. The 56 isolates were divided into 15 different lectin types by their lectin agglutination patterns. Proteolytic treatment reduced auto-agglutination for the majority of species, apart from *L. acidophilus*, which remained predominantly auto-agglutinating (eight of nine strains). The system produced stable and reproducible results under standardised culture conditions. Lactobacilli are important bacteria for use as probiotics and this system may supplement current molecular typing techniques and may help in identification of strains that could be useful in this role.**

Introduction

Lactobacilli are widespread components of the normal healthy human intestinal micro-flora [1]. Their therapeutic value has been highlighted by their effectiveness against both viral and bacterial gastrointestinal disorders through normalisation of the microbiota [2, 3]. Some lactobacilli have also been associated with pathology in immunocompromised patients [4]. Therefore, it is important to characterise groups of strains that are beneficial for probiotic use.

The genus *Lactobacillus* is a very diverse genus consisting of >50 recognised species [5]. Traditionally, based on metabolic pathways, the genus has been divided into three groups [6], which do not correlate with 16S rRNA phylogeny. Previous investigations have used many genotype-based typing systems to characterise and to group lactobacilli [7, 8]. However, classical phenotypic characterisation remains important for discerning groups of strains based on their common properties.

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The analysis of cell-surface components of bacteria by lectin agglutination is a useful method for differentiating groups of strains based on their carbohydrate expression [9]. Lectins are oligomeric and multimeric plant or animal proteins or glycoproteins of non-immune origin with binding specificities toward a particular carbohydrate structure. This multimeric structure gives lectins their ability to agglutinate cells or form precipitates with glycoconjugates in a similar manner to antigen–antibody interactions [10]. The use of lectin-reactive sites as a marker(s) for bacterial strain differentiation has the advantage of being a simple and economical method for grouping strains or characterising their cell surface, or both [11–15].

Lactobacilli have a typical gram-positive cell envelope. Carbohydrates are present in the cell envelope as teichoic acids, lipoteichoic acids and polysaccharides. Monosaccharides also decorate the glycocalyx. The monosaccharide composition of cell hydrolysates has been used as a criterion for the chemical differentiation of gram-positive bacteria [16]. Previous studies involving carbohydrate analysis of lactobacilli have shown that monosaccharides such as *N*-acetylglucosamine and *N*-acetylgalactosamine are present in the glyocalyx in significant amounts [17]. Moreover, the presence of these and other monosaccharides in glycopolymers on

the glycocalyx of lactobacilli has been linked to the adhesive properties of the strains [17]. Thus far, the use of lectins to study the surface of lactobacilli has been limited. Gorskaia *et al.* [18] showed that a high proportion of lactobacilli bound to the mannose-specific lectin Concanavalin A (Con A) and that this was useful as an additional marker for strain characterisation.

The aim of the present study was to assess the use of lectin–carbohydrate interactions for strain characterisation and differentiation of lactobacilli. Different pretreatment strategies were evaluated to eliminate interference from non-specific auto-agglutination of cells.

Materials and methods

Bacterial isolates and culture conditions

A total of 52 intestinal isolates of *Lactobacillus* spp. from faecal samples of 26 healthy children aged 1–2 years old from Estonia and Sweden was used in this study: *L. acidophilus* (8 isolates), *L. paracasei* (17), *L. plantarum* (8), *L. fermentum* (5), *L. brevis* (7) and *L. buchneri* (7); 28 isolates were from 15 Estonian children and 24 isolates were from 11 Swedish children. The isolates were identified as lactobacilli according to morphological and cultural properties, catalase test (negative) and the API 50 CHL System (bioMérieux, Marcy l'Etoile, France) as reported by other investigators [4]. Four culture collection strains (*L. paracasei* DSM 5622, *L. paracasei* DSM 20020, *L. buchneri* ATCC 4005 and *L. acidophilus* ATCC 4356) were also used in the study. All lactobacilli were cultured routinely in Man-Rogosa-Sharpe (MRS) broth for 24 h, in CO_2 10% in air at 37°C. For preparation of suspensions, the isolates were grown on MRS agar for 24 h, in air with CO_2 10% at 37°C.

Preparation of whole cells

Lactobacillus isolates were harvested from one agar plate in 0.01 M sodium phosphate buffer, pH 7.6, containing 0.15 M NaCl (PBS). After centrifugation, first at 3000 rpm for 10 min and then at 4500 rpm for 15 min, the bacterial cells were resuspended in PBS to an OD_{550} of 0.9 before exposure to lectins [14].

Proteolytic treatment of bacteria

Before proteolytic treatment, cells were washed once with PBS and centrifuged at 3000 rpm for 10 min. The pellet was resuspended in 0.2 M glycine-HCl buffer (pH 2.2) and then heated at 100°C for 15 min to remove cell-surface proteins [19]. After washing with PBS at 3000 rpm for 10 min, the pellet was resuspended in PBS containing proteinase K (Sigma) 0.1 mg/ml. The suspension was incubated for 1 h at 60°C and then for 5 min at 100°C to denature the proteinase K. Each suspension was then centrifuged at 4500 rpm for 15 min. The pellet of cell debris was suspended in PBS to an OD_{550} of 0.9 before reaction with lectins.

Lectins

Six commercially available lectins (Sigma) (Table 1) were selected on the basis of their carbohydrate specificities as given by the manufacturer. Before use in an agglutination assay, the lectins were suspended at 0.5 mg/ml in PBS supplemented with 0.1 mM $CaCl_2$ and 1 mM $MgCl_2$ [14]. Working samples of lectin solutions were stored at −20°C until required.

Lectin agglutination assay

Bacterial suspensions (40 μl) were mixed with 10 μl of lectin solution (0.5 mg/ml) in U-shaped microtitration wells for 5 s or with 10 μl of PBS (negative control) and allowed to settle, undisturbed overnight at 20°C [14]. Results were read by visual inspection. A positive result was indicated by a carpet of aggregated cellular material on the bottom of the wells, whereas a negative result was indicated by a dot of cellular material in the centre of the well. Negative results were confirmed by tilting wells at an angle of >45° and observing movement of cellular material. Auto-agglutination was deemed to have occurred when a positive result was observed in the negative control well. As positive controls, lectins were shown to agglutinate a 0.75% v/v solution of human type O erythrocytes after incubation at 20°C for 2 h. Lectin reaction patterns were observed and arbitrarily assigned lectin types (Table 2).

Table 1. Lectin panel used in the present study and associated specificities

Lectin		
Source (Taxonomic name)	Abbreviation	Specificity*
Canavalia ensiformis	Con A	D-Manα1-linked, D-Glcα1-linked
Bandeiraea simplicifolia I	BS-I	D-Gal, D-GalNAc
Bandeiraea simplicifolia II	BS-II	D-GlcNAc
Arachis hypogaea	PNA	D-Galβ1-3D-GalNAc
Vicia sativa	VSA	D-Glc, D-Man
Triticum vulgaris	WGA	$(D\text{-GlcNAc})_2$, NeuNAc

*According to manufacturer.

Table 2. Lectin reaction patterns (arbitrary lectin types)

Lectin type	Reaction with lectin						
	Con A	BS-I	BS-II	PNA	VSA	WGA	PBS*
1	−	−	−	−	−	−	−
2	+	−	−	−	−	−	−
3	−	+	−	−	−	−	−
4	−	−	−	−	−	+	−
5	+	−	−	−	+	−	−
6	+	+	−	−	−	−	−
7	+	−	+	−	−	−	−
8	−	+	−	+	−	−	−
9	+	−	−	−	−	+	−
10	+	+	−	−	−	+	−
11	+	+	−	+	−	−	−
12	+	−	−	−	+	+	−
13	+	−	+	−	−	+	−
14	+	+	−	−	+	+	−
15	+	+	+	−	+	+	−
AA†	+	+	+	+	+	+	+

See Table 1 for explanation of abbreviations.
*PBS (pH 7.2) was used as a negative control.
†Auto-agglutination occurred in the negative control well.

Results

Lectin reaction patterns of intestinal Lactobacillus isolates

All intestinal isolates were tested at least in triplicate with fresh cultures; lectin patterns remained both stable and reproducible under standard culture conditions. However, lectin type may vary when isolates are cultured in liquid media (Mikelsaar and Annuk, unpublished data). Lectin patterns were not species-specific for the lactobacilli tested (Table 3), although some of the species examined did have distinct characteristics in their lectin patterns.

Unlike other lactobacilli tested, for which auto-agglutination in the control wells was either eliminated or reduced after proteolytic treatment, *L. fermentum* showed an increase in auto-agglutination. Furthermore, all eight intestinal *L. acidophilus* isolates tested showed auto-agglutination with whole-cell preparations and after proteolytic treatment; seven of the eight isolates remained untypable because of auto-agglutination (Table 3). *L. paracasei* and *L. plantarum* were the only species with strains belonging to lectin type 1, i.e., no reaction with any of the lectins in the panel (Table 2). When whole-cell samples of *L. paracasei* and *L. plantarum* were used, 48% of isolates were lectin type 1, whereas after proteolytic treatment only 8% of strains remained as lectin type 1 (Table 3). An increased number of binding sites to Con A was revealed (58% to 85%) after proteolytic pre-treatment of *L. brevis*, *L. buchneri*, *L. plantarum*, *L. fermentum* and *L. paracasei* isolates.

Effects of proteolytic treatment on bacteria–lectin interactions

The main effect of the proteolytic pre-treatment of lactobacilli before reaction with lectins was to uncover additional lectin-reactive sites. More lectin types were noted for intestinal isolates after proteolytic treatment than when whole cells were used (12 *versus* 8), indicating that lectin-reactive sites were exposed by the treatment (Fig. 1). Furthermore, the number of isolates that did not react with any of the lectins in the panel was reduced from 12, when whole cells were used, to two isolates when samples were used after proteolytic treatment (Fig. 1). Furthermore, when whole cells were tested, a large number of intestinal isolates (20 of 52) were untypable because of non-specific auto-agglutination in the negative control well (Fig. 1). However, the number of untypable strains due to auto-agglutination was decreased to 9 of 52 when isolates were treated with proteinase K before reaction with lectins (Fig. 1).

Lectin types of culture collection strains

Representative culture collection strains of *L. acidophilus*, *L. paracasei* and *L. buchneri* were assessed for lectin types with whole cells. One culture collection strain of *L. paracasei* was assessed as lectin type 1, which corresponded with most dominant lectin type observed with the other *L. paracasei* isolates tested, whereas the other culture collection *L. paracasei* strain auto-agglutinated (Table 3). Similarly, the culture collection strains of *L. acidophilus* and *L. buchneri* auto-agglutinated, in common with all other *L. acidophilus* isolates and the majority of *L. buchneri* isolates tested. After proteolytic treatment the culture collection strains of *L. paracasei* and *L. buchneri* had the same lectin types as the most dominant type for their species (types 10 and 2 respectively). In common with most other *L. acidophilus* isolates tested, the culture collection strain of this species auto-agglutinated after proteolytic treatment. These results indicate the suitability of culture collection strains of lactobacilli as phenotypic representatives of their species.

Table 3. Lectin reaction patterns observed for individual lactobacilli, culture collection strains are indicated in parentheses

	Number of isolates (culture collection strains) of											
	L. acidophilus: 8 (1)		*L. paracasei*: 17 (2)		*L. plantarum*: 8		*L. fermentum*: 5		*L. brevis*: 7		*L. buchneri*: 7 (1)	
Lectin type	Whole cells	Pk*	Whole cells	Pk	Whole cells	Pk	Whole cells	Pk	Whole cells	Pk	Whole cells	Pk
1†	–	–	8 (1)	–	4	2	–	–	–	–	–	–
2	–	–	1	2	1	3	–	–	3	4	–	5 (1)
3	–	–	–	–	–	–	5	–	–	–	2	–
4	–	–	–	–	–	1	–	–	–	–	–	–
5	–	–	–	–	–	–	–	–	–	3	–	–
6	–	–	–	–	–	–	–	2	–	–	–	–
7	–	–	1	–	1	–	–	–	–	–	–	–
8	–	–	3	3	–	–	–	–	–	–	–	2
9	–	–	–	2	1	–	–	–	–	–	–	–
10	–	–	1	4 (2)	–	–	–	1	–	–	–	–
11	–	–	1	–	–	–	–	–	–	–	–	–
12	–	–	–	1	–	2	–	–	–	–	–	–
13	–	–	–	1	–	–	–	–	–	–	–	–
14	–	–	–	4	–	–	–	–	–	–	–	–
15	–	1	–	–	–	–	–	–	–	–	–	–
AA‡	8 (1)	7 (1)	2 (1)	–	1	–	–	2	4	–	5 (1)	–

*Proteolytically treated bacterial samples used to test lectin interactions.
†No reactions with any of the lectins in the panel.
‡Auto-agglutination occurred in the negative control well.

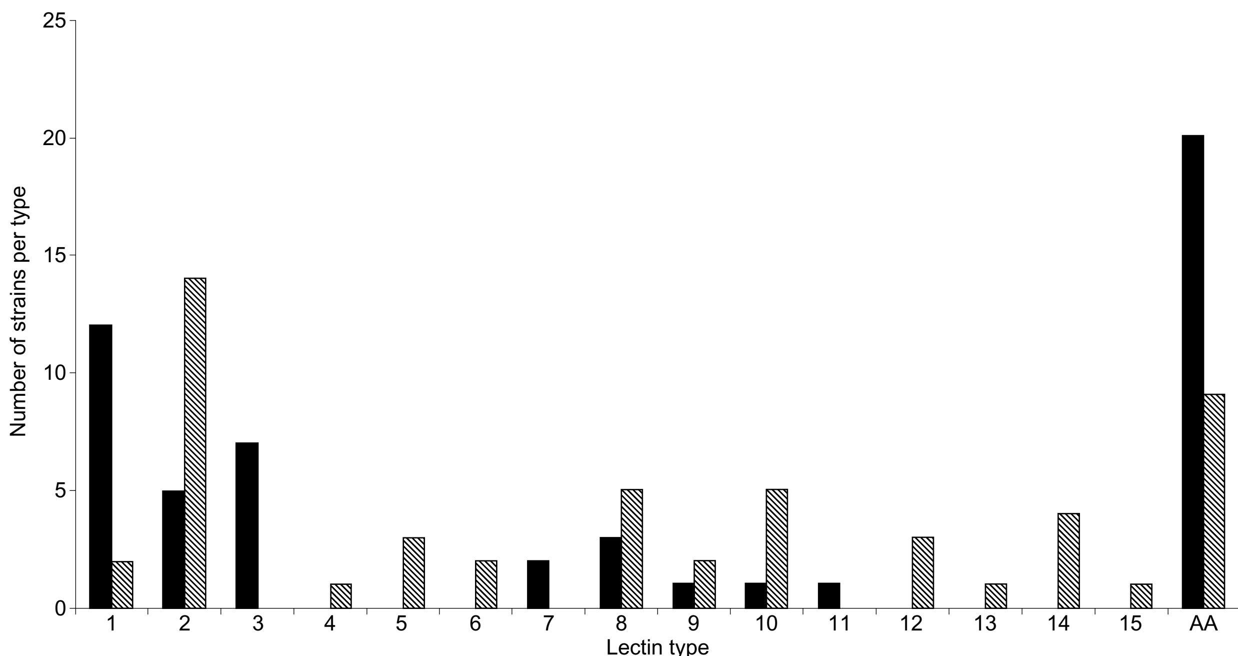

Fig. 1. Differences in lectin pattern frequencies of intestinal *Lactobacillus* strains when whole cells (■) and proteolytically treated cells (▧) were allowed to interact with lectins.

Lectin types of Lactobacillus isolates from the same host

Multiple isolates of lactobacilli were available from 12 subjects. When whole cells were tested, the majority of isolates from the same host had identical lectin types (9 of 12). However, some subjects had multiple isolates with different lectin types, indicating the presence of multiple phenotypes amongst the lactobacilli colonising these subjects. A similar result was seen with 7 of the 12 subjects colonised by a single phenotype when the isolates were treated with proteinase K before lectin typing. However, the latter method detected a greater number of subjects colonised by multiple phenotypes of lactobacilli than when whole cells were used, five *versus* three, respectively.

Discussion

This study developed a simple microtitration assay for differentiating *Lactobacillus* isolates based on their carbohydrate composition demonstrated with lectins. The use of microtitration wells to assess bacteria–lectin interactions allowed a high rate of processing for strains and, in agreement with other studies, no major problems were encountered with the microtitration wells [14].

It has been established that strains of *L. reuteri* auto-agglutinate because of the expression of a helicase on the cell surface [20]. Therefore, in addition to the use of whole cells, lactobacilli were exposed to proteolytic digestion before examination by lectin typing and a decrease in auto-agglutination followed the proteolytic treatment. However, the majority of *L. acidophilus* strains retained their auto-agglutinating properties after proteolytic treatment. These strains may be resistant to proteolytic treatment because of the presence of a tightly bound S-layer [21]. *L. acidophilus* is commonly used as a probiotic because of its colonising ability. Therefore, its adhesive qualities and auto-agglutination may be due to its S-layer, as reported for *L. crispatus* [22]. Some strains that were successfully lectin typed as whole cells auto-agglutinated after proteolytic treatment. This may be a result of cell lysis and the interactions of nucleic acids with lectins, as proposed previously [23]. This result suggests that both strategies of lectin typing could be utilised in combination.

In common with *Campylobacter* spp. [24], there was no indication that lectin patterns were species-specific, as some lectin types were common to more than one species. However, some group-specific characteristics were noted. Both *L. paracasei* and *L. plantarum*, which belong to the group of facultatively heterofermentative lactobacilli [25], were the only species where lectin type 1 was observed. This indicates that the presence of carbohydrates in their glycocalyx, undetectable by the current panel of lectins, is a common feature of this group. The presence of multiple phenotypes in the same host subject for most of the species tested is an important consideration in choosing successful colonising strains. These results may also be important in assessing the ecology of intestinal microflora as an addition to current molecular biology techniques.

The typing system developed in the present study used lectins with specificities for carbohydrates previously detected in the glycocalyx and cell envelope of lactobacilli [17]. In agreement with the previous study,

the lectin typing assay used in the present study demonstrated the presence of mannose, glucose and *N*-acetyl-D-glucosamine in the glycocalyx of *L. plantarum*. Furthermore, these sugars along with galactose and *N*-acetylgalactosamine were commonly encountered in the glycocalyx of the other lactobacilli tested. The sugars detected in the current study have been encountered previously on surface-exposed macromolecules of lactobacilli, which may act as binding sites for the bacterial S-layer [26, 27]. However, previous reports of the presence of sialic acids on the surface of *L. plantarum* [28] could not be confirmed by this system, even with a further test with the *Maakia amurensis* lectin, which has a specificity for terminal α-2,3-linked sialic acid (data not shown). Nevertheless, expansion of the lectin panel in future studies may allow a more detailed examination of carbohydrate expression by lactobacilli.

In agreement with studies involving pathogenic bacteria [11–15], this study found lectin typing to be a simple and reproducible method for typing lactobacilli under standard conditions available to any laboratory. The *Lactobacillus* genus is large and diverse [5] and the use of both phenotypic and genotypic typing systems has already been established in the area of food technology to identify useful lactobacilli [29]. Therefore, the grouping of strains by a phenotypic typing system may complement current genotyping techniques in identifying species or phenotypes that could be useful as probiotics. Such strains would be beneficial in counteracting various pathogenic bacteria in the gastrointestinal tract.

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