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GLYCOCONJUGATE-RECOGNIZING SYSTEMS IN CULTURE FLUIDS OF HUMAN PROBIOTIC STRAINS OF BIFIDOBACTERIA AND LACTOBACILLI

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ABSTRACT

Chemiluminescent and fluorescence analyses were used to obtain and identify visual patterns of protein glycoconjugate-binding/sensitive/recognizing ordered sets or systems and subsystems (including lectin and glycol-oxidase ones) in culture fluids of probiotic strains of bifidobacteria and lactobacilli isolated from healthy human donors. Glycoconjugate-binding systems recognized glycoconjugates in a mosaic unique way in accordance with the principles: a) "one lectin"-"a limited set of glycoconjugates ranked/ordered by affinity"; b) "one glycoconjugate"-"a limited set of lectins ranked/ordered by affinity"; c) "variable (sub)system of lectins"—"a variable (sub)system of glycoconjugates corresponding to the variation of lectins". Glycoconjugate-binding subsystems of lectins and enzymes of carbohydrate metabolism of probiotic bacteria were characterized by similarities and differences in recognition (the presence of similar and unique components in subsystems), binding (including relative severity, in comparison with other components of the strain subsystem and subsystems of other strains) and distinguished synthetic polymer universal antigens-similar structures of the broad medical importance and applications.

KEYWORDS: bifidobacteria, lactobacilli, typing, cultural fluid, proteins, lectins, enzymes, glycoconjugates, antigens, probiotics, human.

ABBREVIATIONS

CF culture fluid(s)
CY-5s casein-yeast medium with five salts
GC glycoconjugates
HBM hydrolyzed bovine milk
LL lectins of lactobacilli
LB lectins of bifidobacteria
LPB lectins of probiotic bacteria
LS lectin system(s)
pI isoelectric points
PSB phosphate-salt buffer pH 7.4

1. INTRODUCTION

Lectins belong to glycoconjugates (GC)-binding proteins of a non-immunoglobulin nature. They are important regulators of metabolism.[1] Human lectins of bifidobacteria and lactobacilli (LB and LL) mimic the action of probiotics, exhibit synergistic antimicrobial effects (including together with antibiotics), and co-function with the human body's defense systems.[2-5] In this regard, it is still relevant to search for sources of new systemic lectins of probiotic bacteria (LPB).

The aim is to continue the search and research of sources of promising LPB from probiotic bifidobacteria and lactobacilli using a wide range of synthetic standard polymer GC (www.lectinity.com).

2. MATERIALS AND METHODS

B. bifidum 791; *B. longum* B379M, X; *B. pseudocatenulatum* OV-2, *B. angulatum* OV-15; *Lactobacillus helveticus* NK_1 and *L. amylovorus* BT-24/88 were studied. The strains are from the Collection of microorganisms at the *G.N. Gabrichevsky* Reseach Institute for Epidemiology & Microbiology. Bacteria were grown 18-24 h at 37°C in casein-yeast medium with salts (CY-5s) or in low-fat hydrolyzed bovine milk (HBM) with additives. The liquid phase of the microbial cultural fluid was demulsified by boiling for 30 minutes, frozen, the top layer of cream was completely removed, and thawed. The filler liquid (supernatant) was sterilized by vacuum membrane microfiltration in a *Steriflip* cartridge (Millipore) and the microfiltrate components (> 27 kD) were concentrated by membrane ultrafiltration in a *Centricon Plus-20* centrifuge cartridge (Millipore). The proteins in the samples were

controlled by the *Waddel* spectrophotometric method in our modification [6]. Protein concentrates with lectins were separated using the *Lasne* method [7] by high–voltage isoelectrofocusing in a polyacrylamide gel plate in the region of a linear pH gradient of 2-6 in the presence of 7-8 M urea and 5% (w/w) sucrose at 10° C. The components were electroblotted onto a membrane sandwich comprising a hydrophilic membrane - *Durapore* (Millipore) and a hydrophobic membrane *Immobillon P* (Millipore).

0.005%-Tween in a pH 7.4 phosphate-salt buffer (PSB) was used to block and wash the blots.

After treatment of the blots with acetic acid aqueous methanol solution, the proteins were treated with a fluorescent dye based on ruthenium (Ru^{+}) (**method-1**) - SYPRO Ruby Blot Protein Stain (www.probes.com/syprodyes). The fluorescence of the dye was excited at 254 nm (to obtain a set of bands with increased fluorescence intensity) or at 365 nm (to type strains).

Among the identified proteins by method-1, GC-binding proteins by **method-2** were further identified. A set of biotinylated GC based on a linear polyacrylamide chain with multiple side branches of short chains with mono- or disaccharide residues was used (www.lectinity.com). The blots (before or after treatment of with an acidic methanol solution [the latter option is the most effective]) were treated with GC-biotin (1 mcg/ml in PSB, incubation overnight at 4°C with stirring). Then streptavidin peroxidase in optimal concentration was added to the washed blot. Then a chemiluminescent peroxidase substrate with increased sensitivity, *BioWest* (Pierce, USA), was added to the blot surface.

The final fluorescence patterns (method-1) or chemiluminescence patterns (method-2) were recorded in live image mode in the *EPI Chemi Dark Room II* camera of the *BioChemi System* (UVP, USA) in the form of a series of stepwise kinetic patterns using an *Ethydium Bromide* light filter. The paintings were analyzed using *Labworks4* software.

The following synthetic GC were used (www.lectinity.com) - imitators of polysaccharides, antigens, proteins, and peptidoglycans (synonyms in parentheses):

*Fuc-α-1- [pseudo-(α-L-fucan)],

*Gal-β-1- [pseudo-(β-D-galactan)],

*GaNAc-α-1- [poly(T_n-antigen)-containing polymer],

*GalNAc-α-1,3-Gal-β-1- [A_{di}; poly(AII-blood group-antigen)-containing polymer],

*GalNAc-α-1,3GalNAc-β-1- [Fs; poly(Forsman antigen)-containing polymer],
*GalNAc-α-1,3GalNAc-α-1- [mucin-similar polymer]
*Gal-α-1,3GalNAc-α-1- [poly($T_{\alpha\alpha}$-antigen)-containing polymer],
*GalNAc-β-1- [desialysed pseudo-mucin],
*Gal-β-1,4GlcNAc-β-1- [poly(LacNAc)-containing pseudo-mucin],
*Man-α-1- [pseudo-(α-D-mannan)],
*(MurNAc-L-Ala-D-isoGln)-β-1- [MDP-; poly(muramyl dipeptide)-containing a polymer; pseudo-peptidoglycan],
*Rha-α-1- [pseudo-(α-L-ramnan)].

3. RESULTS AND DISCUSSION

Strain-dependent protein sets identified by the method-1 have been identified (figure 1-A). When blocked proteins were revealed by the GC set, the differences between strains, species, and genera of *Gram* positive bacteria increased (figure 1-B). Using the example of lactobacilli (strain NK_1), it can be seen that growth in CY-5s leads to the formation of more pronounced sets of proteins and lectins compared with growth in HBM (figure 1).

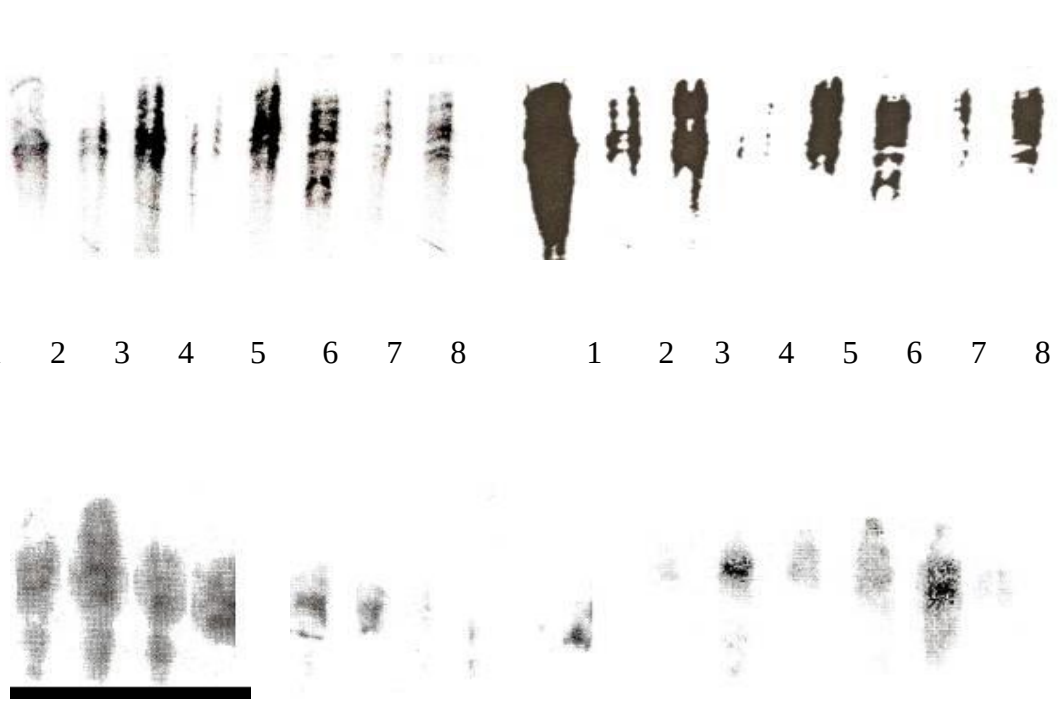

Figure 1: Proteins of probiotic bacterial CF, separated in the pH range 4-5.

A. Two patterns (left and right) of proteins identified by method-1 (on the right are the contours of the entire protein array with maximum contrast).
B. GC-binding proteins identified by method-2 using the following type of GC (from left to right): A_{di} (2-5), GalNAc (1-6), F_s (1-7). Strains: 1- *L. helveticus* NK_1 (growth in the medium CY-5s), 2- *L. helveticus* NK1 (рост в среде HBM), 3- *L. amylovorus* BT 24/88 (growth in the medium HBM), 4- *B. longum* X (growth in the medium HBM), 5- *B. longum* B379M (growth in the medium HBM), 6- *B. bifidum* 791 (growth in the medium HBM), 7- *B. pseudocatenulatum* OV-2 (growth in the medium HBM), 8- *B. angulatum* OV-15 (growth in the medium HBM).

Major LL forms of the NK1 strain with specificity to pseudo-mannan (with exposed clusters of Man-α-residues) in the region of isoelectric points (pI) 4.6-4.8, pseudo-mucin (with exposed clusters of GalNAc-β-residues) with a pI of about 4.2 in the region of 4.0-4.4 (weaker than in bifidobacteria), pseudo-fucan (with exposed clusters of L-Fuc-α-residues) with pI in the range 4.0-4.4, pseudo-peptidoglycan (with exposed clusters of muramyl-dipeptide(MDP)-residues), polymer A-disaccharide (Adi) (multiply mimic of the surface antigen of erythrocytes of human blood group A(II)).

There was practically no binding of LL and LB with GC to disaccharide residues Gal-α-1,3-GalNAc-α-, as well as to pseudo-rhamnan (with exposed clusters of L-Rha-α-residues).

Major forms of LB from *B. longum* have been identified with specificity to pseudo-mannan (with available Man-α-residues) with pI in the range 4.5-4.6, pseudo-mucin (GalNAc-α-) with pI in the range 4.0-4.2, pseudo-fucan (L-Fuc-α-) with pI in the range 4.0-4.4. The LB binding differs from that of the LL strain of NK_1 by the position of the pI bands and the distribution of the dominant binding intensity.

The binding of LB to GC reflected the genus characteristics of the tested species and strains of bifidobacteria, which differed from the LL characteristics of the tested species and strains of lactobacilli.

The binding patterns of pseudo-polysaccharides, blood group A(II) antigens and Forsman (both found on erythrocytes), pseudo-peptidoglycans (mimics of bacterial peptidoglycans) depended on the belonging of bacteria to the genus, species and strain.

The similarity of GC-specific lectin systems (LS) with exposed N-acetyl-D-galactosamine residues (D-GalNAc-β-; A-disaccharide; D-GalNAc-α-D-GalNAc-β-) was found.

Similarity in the protein array region with pI 4.5-5.0 was found in LS specific to pseudo-mannan or pseudo-peptidoglycan. α-L-fucan-binding LPB (pI 3.7-4.3; block 2-3 strips closer to 3.8-3.9) in lactobacilli were more pronounced and more extended than that in bifidobacteria.

The absence of this type of lectins was observed in the OV-15 strain. Prior to treatment of the blot with an acidic methanol solution, the pI 4.0-4.4 region in the case of bifidobacteria was masked (one band at pI 3.9 in case of *B. bifidum* 791). At the same time, two additional less pronounced regions were identified: pI 3.6-3.7 (in the NK_1 strain, not in the BT-24/88 strain) and 4.8-4.9 (stronger in lactobacilli compared with bifidobacteria).

There was no binding of poly(Gal-α-1,3-GalNAc-α-) pseudo-polysaccharide to the proteins of *L. helveticus, L. amylovorus* and *B. longum*. There was also no binding of poly(α-L-Rha-) pseudo-polysaccharide to the acid-methanol-treated blot.

Other individual qualitative and quantitative features of LS of the studied strains of lactobacilli and bifidobacteria were also identified (figure 1).

In some cases, depending on the selected type of GC, fading or a decrease in the intensity of certain protein GC-treated bands on the blots could be observed, which depended on the presence of a carbohydrate metabolism enzyme (oxidoreductase [EC 1.11.1.7-like] or glycosidase [EC 3.2.1. : β-D-galactosidase or α-L-rhamnosidase]), modifying the bound GC as a substrate.[8]

The results indicate a layered (gradient, vector) arrangement of lectins, the participation of LPB in directed supra-molecular assemblies that promote the co-functioning of LPB with other proteins, including in variants of LPB-organized delivery of effectors from solution to the solid phase.

Separated LS within the protein array of the same strain were arranged in a mosaic manner (major and minor LPB forms were observed), and the nature of the mosaic itself depended on the type of GC taken for the manifestation of LS (figure 1-B).

The presence of several selected probiotic strains will make it possible to expand the range of LPB oriented to a selected/specified target or selected targets (GC polymer, antigen polymer). This may be one of the principles of designing a probiotic consortium of probiotic strains.[9, 10]

Figure 1-B also shows that the same lectin (or a group of closely localized, cohesive lectin forms) of probiotic bacteria can recognize to a greater or lesser extent (recognize in a ranked, ordered manner) a set or system of GC targets of different types.

Thus, the approach and methodology proposed and used in this work and the results obtained point to the prospects for LPB systemic research[11-14], when one lectin (or one GC) is opposed by a ranked/ordered affinity GC (sub)system (or ranked/ordered by affinity LPB (sub)system). At the same time, the unique LPB mosaic is contrasted with the (and vice versa), which is the case in the microbiocenoses of human biotopes.

The key examples are the results presented in Fig. 1-B of LPB systems distinguishing synthetic mucin-type clustered polyvalent polymer with exposed carbohydrate residues of D-GalNAc- in antigens of wide medical significance.

4. CONCLUSION

The results indicate the prospects of using GC (or LPB) systems for.

*identification of new LPB (or new GC) systems with potential new biological activities;

*prediction and use of adhesive, antigen-recognizing, antitumor and other biological properties of strains and their metabolites;

*functional typing of *Gram* positive bacteria;

*selection of strains and their combinations in the design of multi-strain synbiotics and probiotic consortia.

The proposed electrophoresis-blotting models of interactions between protein(s) and glycoconjugate(s) systems are simple, adequate, and reliable alternatives to using cellular model systems for similar purposes.

The identified major forms of LPB, selectively binding specific types of GC, have prospects for preparative preparation and use.

Disclosure of conflict of interest

The authors declare no conflict of interest.

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