Collagen binding by vaginal aggregative lactobacilli

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ABSTRACT: Ten autoaggregating vaginal *Lactobacillus* strains (five of these strains were selected among isolates from sows' vaginal swabs and the other five among isolates from cows' vaginal swabs) were investigated for their ability to bind type I collagen (Cn-I). All 10 autoaggregating strains in the range of A_{570nm} readings 0.118–1.806 bound to immobilised Cn-I (at concentration of 100 µg/ml) in wells of microtitre plates, however, *Lactobacillus acidophilus* SV31 was much more adherent than the rest of the tested strains. The influence of culture medium on Cn-I binding was confirmed only in 50% of the tested strains when agar-grown cells bound significantly more Cn-I than broth-grown cells. The specificity of the binding was confirmed since the Cn-I binding by lactobacilli was abolished after their preincubation with this protein. The effect of heparan sulphate and hyaluronic acid was tested on 5 vaginal strains displaying the best Cn-I binding in microtitre plates after their cultivation on MRS agar plates. Both selected inhibitors significantly ($P < 0.001$ or $P < 0.01$) reduced Cn-I binding by the majority of strains. The presence of the gene coding APF (aggregation-promoting factor) was detected in seven strains (all five sows' and two cows' *Lactobacillus* strains) by PCR.

Keywords: vaginal *Lactobacillus*; collagen; aggregation; extracellular matrix; probiotic use

INTRODUCTION

The presence of lactobacilli as a constituent of normal microflora of the reproductive tract is generally considered to be beneficial to their host. It is known that autoaggregation is one of the important lactobacillar activities in the human vagina (Kmeť and Lucchini, 1997). It can also be connected with the ability of lactobacilli to exclude intestinal or urogenital pathogens, e.g. *E. coli* (Puzová *et al*., 1994) by the mechanism of coaggregation (Kmeť *et al*., 1995; Kmeť and Lucchini, 1999). That is why *Lactobacillus* strains with autoaggregation ability were selected for Cn-I binding experiments. One of the factors mediating autoaggregation is APF (aggregation-promoting factor) described earlier by Reniero *et al*. (1992) which was also screened in our selected vaginal *Lactobacillus* strains.

It is known that ECM (extracellular matrix) may serve as a substrate not only for the adhesion of the cells of the host organism but also for the attachment of colonizing microorganisms. Many microorganisms express cell surface adhesins that mediate microbial adhesion to the ECM of host tissues (Ljungh and Wadström, 1995). Bacterial binding to ECM proteins is also considered as a virulence factor in pathogens, however, only a few reports offer an evidence of this fact. For example, the binding of *S. aureus* to collagen was described as the virulence factor in experimental endocarditis (Hienz *et al*., 1996).

One third of the animal protein is collagen, but different tissues contain different types of collagen. For instance, type II collagen is located primarily in the cartilage while type IV collagen occurs exclusively in basement membranes (Patti *et al*., 1994). Type I collagen (Cn-I) is the most abundant collagen molecule in soft body tissues (Aleljung, 1994). Lactobacilli were shown to interact only with three types of collagens (type I, II and IV), however, the binding of collagen types II and IV was generally lower than the binding to Cn-I, when 80% of 110 strains bound only Cn-I (Aleljung *et al*., 1991; Aleljung, 1994).

Collagen binding was described in intestinal (Aleljung *et al*., 1991; Štyriak *et al*., 1999b) and oral lactobacilli (McGrady *et al*., 1995), however, such information should be useful also for lactobacilli considered for probiotic use in the vaginal ecosystem. From our point of view, specific strains may be selected that confer health-promoting effects with the capability to colonise the site threatened by infection. That is why we decided to investigate aggregative vaginal isolates for binding to Cn-I which is often bound by several pathogens.

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MATERIAL AND METHODS

Sources and cultivation of strains

A number of *Lactobacillus* strains were initially isolated from sows' and cows' vaginal swabs using Rogosa agar (Oxoid, Basingstoke, England) plates.

The strains of two phenotypes with different autoaggregation abilities were isolated from these vaginal swabs – autoaggregating ones, forming a precipitate resulting in a clear solution, and non-autoaggregating ones, producing constant turbidity in a tube.

Five autoaggregating *Lactobacillus* strains (*L. acidophilus* SV22, *L. acidophilus* SV31, *L. gasseri* SV42, *L. reuteri* SV81 and *L. reuteri* SV82) were selected among isolates from sows' vaginal swabs and the other five autoaggregating strains among isolates from cows' vaginal swabs (signed CV1 – CV5) for our experiments. The species identification of the strains was carried out by phenotypic methods (growth at defined temperatures, carbohydrate fermentation patterns, etc.) using Anaero-23 test kits (Lachema, Brno, Czech Republic). The human intestinal strain of *Lactobacillus gasseri* 4B2 with a strong autoaggregative phenotype mediated by APF (aggregation-promoting factor; Lucchini *et al*., 1998) was obtained from the Instituto di Microbiologia stock collection as a control strain for PCR. Two growth media were examined for their influence on the expression of the surface receptors of all tested strains. Lactobacilli were grown overnight in Man-Rogosa-Sharpe (MRS) broth (Difco Laboratories, Detroit, MI, USA) and on MRS agar plates (Difco) at 37°C in 5% CO_2 atmosphere.

Chemicals

Bovine serum albumin (BSA), heparan sulphate and fucoidan were purchased from Sigma Chemicals Co., St Louis (Missouri, USA). Collagen (Cn-I) was purchased from Serva (Heidelberg, Germany), crystal violet from Loba (Austria) and Nunc-Immuno microtitre plates with Maxi Sorp surface (96 wells) from Nunc International (Roskilde, Denmark). All buffers and chemicals were of analytical grade.

Microtitre plate binding assays

Nunc-Immuno microtitre 96-well plates were coated with type I collagen solution (100 µl) at a concentration of 100 µg per ml and subsequently incubated overnight at 4°C. Protein solutions were removed and plates were washed three times with PBS. Then PBS with BSA (200 µl of a 2% solution in PBS) was added to each well to prevent non-specific bacterial binding. After 2 hours incubation at 25°C, BSA was removed and wells were washed twice with PBS. Finally, bacterial suspensions (100 µl; 10^9 cfu per ml) of individual strains were added and the plates were incubated on an orbital platform shaker for 2 hours at 37°C. All unbound bacteria were removed by washing the wells three times with PBS. Bacteria in the wells were then fixed at 60°C for 20 minutes and stained with crystal violet (95 µl per well) for 45 minutes. Wells were subsequently washed six times with PBS to remove excessive stain. After adding 100 µl of citrate buffer (pH 4.3) to each well and 45 min incubation at room temperature to release the stain bound to bacteria, the absorbance values (A_{570nm}) were determined in a Multiscan enzyme-linked immuno-sorbent assay reader and the averages of ten absorbance values were calculated. Each batch of assays also included one or more control strains with known Cn-I binding level as well as blank wells. Two *Staphylococcus aureus* strains (ISP 546 and Cowan 1) and *Staphylococcus haemolyticus* SM 131 (Paulsson and Wadström, 1990) were used as positive controls. Lactobacilli were classified as strongly adherent ($A_{570nm} > 0.3$), weakly adherent ($0.1 < A_{570nm} > 0.3$), or nonadherent ($A_{570nm} < 0.1$).

The specificity of binding was tested by 1-hour preincubation of bacteria with an equal volume of type I collagen solution at concentration of 100 µg per ml and subsequent washing followed by examination of bacterial binding to the same protein.

Inhibition of bacterial binding in microtitre plates

Bacterial suspensions (100 µl; 10^9 cfu per ml) of the selected *Lactobacillus* strains with the highest percentage of collagen binding (SV22, SV31, SV42, CV4 and CV5) were preincubated with an equal volume of heparan sulphate or hyaluronic acid (both inhibitors at a concentration of 1 mg/ml) for 1 hour at room temperature and incubated in microtitre plates for 2 hours at 37°C parallelly with non-treated bacteria of these strains. Binding in microtitre plates was assayed as above and the averages of ten absorbance values were compared with those of the same non-treated strains.

PCR amplification

The specific PCR amplification protocol was used for chromosomal DNA extracted from overnight broth-cultures of all 10 investigated autoaggregative *Lactobacillus* strains according to the method described by Kmeť and Lucchini (1999). Specific primers APF3 and APF4 were used as described previously by Lucchini *et al*. (1998) for the demonstration of *apf* gene. PCR reaction mixture (25 µl) contained *Lactobacillus* DNA (10 ng), Taq buffer 1×, Taq polymerase 1.0 U (Promega), $MgCl_2$

1.5 mM, dNTPs 200 µM each, primers 0.5 µM each. The human intestinal strain of *Lactobacillus gasseri* 4B2 (Lucchini *et al*., 1998) was used as positive control strain for PCR. PCR reaction mixture without template DNA was used as negative control.

PCR amplifications were carried out in a Progene (Techne, Cambridge) thermocycler. The tubes were subjected to the following thermal cycling conditions: 5 min at 94°C for one cycle, then 60 s at 94°C, 60 s at 55°C and 60 s at 72°C for 35 cycles. After cycling 10 µl of each reaction was analysed by electrophoresis on 1.5% agarose gel at 7 V/cm.

Table 1. Collagen (Cn-I) binding by 10 *Lactobacillus* (isolates from sows signed as SV and cows' isolates as CV) strains cultivated on MRS agar and in MRS broth

MRS agar-grown lactobacilli (except the SV 81 strain) bound significantly more to Cn-I than MRS broth-grown cells. Statistical evaluation of the results was done by one-way analysis of variance (ANOVA). Significance of differences between means was calculated by Tukey's test.
All values are expressed as the mean ± SD (n = 10). The significance level between MRS agar- and MRS broth-grown cells of the same strain is indicated by stars ($^{***}P < 0.001$; $^{**}P < 0.01$; $^{*}P < 0.05$)

Strains	MRS agar	MRS broth	*apf* gene
SV 22	0.313 ± 0.046	0.234 ± 0.035	+
SV 31***	1.806 ± 0.223	0.669 ± 0.072	+
SV 42	0.263 ± 0.035	0.233 ± 0.046	+
SV 81	0.066 ± 0.026	0.137 ± 0.039	+
SV 82	0.122 ± 0.033	0.052 ± 0.033	+
CV 1*	0.128 ± 0.015	0.078 ± 0.035	–
CV 2**	0.118 ± 0.023	0.055 ± 0.030	+
CV 3***	0.195 ± 0.050	0.092 ± 0.032	+
CV 4	0.165 ± 0.043	0.140 ± 0.033	–
CV 5***	0.213 ± 0.030	0.124 ± 0.036	–

Statistical analysis

Statistical evaluation of the results was done by one-way analysis of variance (ANOVA). Significance of differences between means was calculated by Tukey's test.

RESULTS

Ten autoaggregating strains bound to immobilised Cn-I (at a concentration of 100 µg/ml) in the range of A_{570nm} readings 0.118–1.806 (Table 1). The majority of strains displayed A_{570nm} readings maximally up to the value 0.313. However, *Lactobacillus acidophilus* SV31 was much more adherent after its cultivation on MRS agar (A_{570nm} = 1.806) as well as in MRS broth (A_{570nm} = 0.669) in comparison with the rest of the tested strains.

The influence of culture medium on Cn-I binding was confirmed only in 50% of the tested strains (Table 1) when agar-grown cells bound significantly more Cn-I than broth-grown cells. Significantly higher binding of Cn-I was observed in 5 vaginal strains (SV31, CV1, CV2, CV3 and CV5) grown on MRS agar than in MRS broth-grown cells. The other 5 vaginal strains showed no significant influence of culture medium on their Cn-I binding.

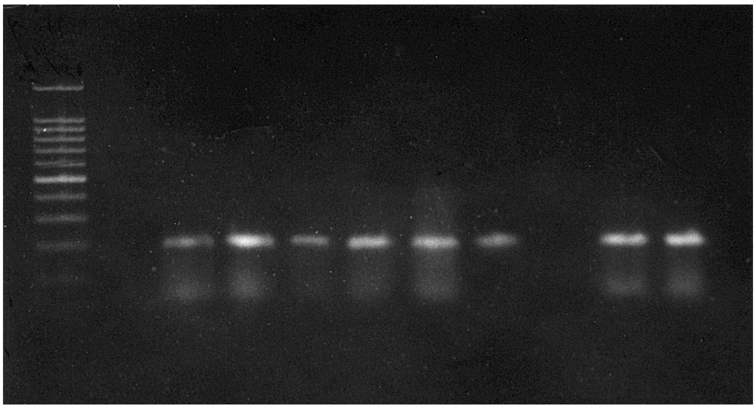
The specificity of lactobacilli binding to the Cn-I was confirmed by their preincubation with this protein. Such pretreatment abolished the adhesion (Table 2).

The effect of heparan sulphate and hyaluronic acid on Cn-I binding in microtitre plates was tested on 5 selected vaginal strains with the highest percentage of collagen binding after their cultivation on MRS agar plates. Both selected inhibitors significantly ($P < 0.001$ or $P < 0.01$) reduced Cn-I binding by these strains with two exceptions (strain CV5 after heparan sulphate pretreatment and SV42 strain after its preincubation with hyaluronic acid) as shown in Table 2.

Table 2. Inhibition effect of heparan sulphate and hyaluronic acid on Cn-I binding by 5 *Lactobacillus* strains in microtitre plates

Statistical evaluation of the results was done by one-way analysis of variance (ANOVA). Significance of differences between means was calculated by Tukey's test. The values are expressed as mean ± SD (n = 10). All inhibition values were compared to control (untreated bacteria). The significance level is indicated by stars ($^{*}P < 0.001$; $^{**}P < 0.01$; $^{*}P < 0.05$; ns – no significance)

Strains	Control	Collagen	Heparan sulphate	Hyaluronic acid
SV 22	0.313 ± 0.046	0.124 ± 0.034***	0.115 ± 0.033***	0.149 ± 0.036***
SV 31	1.806 ± 0.223	0.131 ± 0.041***	0.731 ± 0.210***	0.122 ± 0.029***
SV 42	0.263 ± 0.035	0.070 ± 0.033***	0.071 ± 0.028***	0.211 ± 0.037ns
CV 4	0.165 ± 0.043	0.012 ± 0.007**	0.026 ± 0.013**	0.024 ± 0.008**
CV 5	0.213 ± 0.030	0.010 ± 0.005***	0.104 ± 0.024ns	0.029 ± 0.012***



Lane 1 – 100 bp DNA ladder (Promega, USA)
Lane 2 – negative control (only PCR buffer without template DNA)
Lane 3 – *Lactobacillus acidophilus* SV22
Lane 4 – *Lactobacillus acidophilus*
Lane 5 – *Lactobacillus gasseri* SV42
Lane 6 – *Lactobacillus reuteri* SV81
Lane 7 – *Lactobacillus reuteri* SV82 SV31
Lane 8 – *Lactobacillus gasseri* 4B2 (positive control)
Lane 9 – free lane
Lane 10 – *Lactobacillus* sp. CV2
Lane 11 – *Lactobacillus* sp. CV3

Figure 1. Detection of an apf gene by PCR analysis of DNAs of 7 positive vaginal strains isolated from sows and cows. A single product of the expected 183 bp size was obtained from the amplification of all sows' and two cows' *Lactobacillus strains*. The strains CV1, CV4 and CV5 are not shown in the figure because no product was amplified from their DNA

Using primers APF3 and APF4, a single product of the expected 183 bp size was obtained from the amplification of all five sows' and two cows' *Lactobacillus* strains (Figure 1).

DISCUSSION

Adherence of pathogens to the extracellular matrix of various host tissues has often been investigated, demonstrating the important role of these interactions in the establishment of many infections (Westerlund and Korhonen, 1993; Ljungh and Wadström, 1995). However, little is known about members of the indigenous microflora, including lactobacilli, and their ability to colonize epithelium lesions and to bind to ECM proteins (Aleljung *et al*., 1991).

The major constituents of the ECM are collagenous proteins and that is why they may represent a major target site for many microorganisms (Patti *et al*., 1994). The idea that also non-pathogenic bacteria might utilize an analogous strategy for colonization is especially attractive in the context of probiotic bacteria, where competitive exclusion of pathogens through occupation of common binding sites on mucosal surfaces was discussed (Reid *et al*., 1990). In fact, a number of interactions exists between non-pathogenic bacteria and host components. The Cn-I binding ability is also one feature of autoaggregating lactobacilli which could be considered in the selection of strains for the preparation of therapeutic products able to antagonize the colonization of Cn-I binding sites by vaginal pathogens.

Bacterial binding to collagen is affected by several factors such as culture medium, presence of different compounds, etc. The influence of culture medium on Cn-I binding was confirmed in 50% of our tested strains when agar-grown cells bound significantly more Cn-I than broth-grown cells. These results are similar to the observations of Aleljung *et al.* (1991) that agar-grown cells usually bind around 15–25% more Cn-I than broth-grown cells. Some earlier studies also showed that the cell envelope is dependent on the composition and form (solid or liquid) of the culture medium (Al-Hiti and Gilbert, 1983). Moreover, the expression of high molecular-weight proteins by *Staphylococcus aureus* was shown to be enhanced when the bacterium was grown on a solid medium compared with growth on the same medium in the liquid form (Cheung and Fischetti, 1988).

It was earlier confirmed that heparin and other sulphated glycosaminoglycans, for example dextran sulphate and fucoidan, strongly inhibit ECM proteins to bind cells of some pathogenic strains like staphylococci (Štyriak *et al*., 1999a; Pascu *et al*., 1996), however, non-sulphated compounds do not have such effects (Štyriak *et al*., 1999a; Pascu *et al*., 1996). It indicates that the oligosaccharide structures are not as important as the density of sulphate groups on these polymers. However, hyaluronic acid, a nonsulphated compound, also significantly inhibited the binding of Cn-I to gut lactobacilli (Štyriak *et al*., 1999b) as well as to vaginal *Lactobacillus* strains presented here. It suggests that the sulphation of polymers is much less important for an inhibition effect on lactobacilli in comparison with pathogens. This fact should also be a promising advantage for possible use of sulphated inhibitors for a reduction of ECM binding by pathogens, however, their inhibition effects on ECM binding by beneficial microorganisms complicate these considerations. On the other hand, it should be possible to find by screening of *Lactobacillus* strains a suitable strain with high Cn-I binding ability also in the presence of inhibitors. Our hypothesis is based on the fact that large differences between individual lactobacilli were observed in the present study as far as the influence of culture medium and the effect of tested inhibitors are concerned. It suggests the occurrence of different molecules on individual strain surfaces. Some of such structures, for example a collagen-binding S-layer protein in *Lactobacillus crispatus* (Toba *et al*., 1995) or a 29 kDa collagen-binding protein from *Lactobacillus reuteri* (Roos *et al*., 1996) as well as other glycoprotein molecules were described previously. APF coded by *apf* gene is also one of such surface molecules. This gene was detected in 7 of the 10 vaginal *Lactobacillus* strains in our study, however, its role in Cn-I binding was not ascertained. The presence of an APF was detected in 5 vaginal *Lactobacillus* strains from sows and two from cows (Figure 1). However, there were also reported other aggregation-promoting factors such as a 2 kDa hydrophilic peptide produced by a *Lactobacillus gasseri* isolate (Boris *et al*., 1997) or a putative DEAD-box helicase (a 56 kDa protein) produced by a *Lactobacillus reuteri* strain 1063 (Roos *et al*., 1999). It means that various factors are responsible for aggregation of lactobacilli which should be important in the vaginal ecosystem. It is probable that the presence and expression of aggregation-promoting factor have no coherence with Cn-I binding by *Lactobacillus* strains, however, from our point of view both properties are useful for probiotical strains.

We would like to note in conclusion that the *L. acidophilus* SV31 with the highest percentage of collagen binding was chosen for a field experiment and it is nowadays applied to sows' vaginae with the aim to decrease the number of pathogens in their reproductive tracts. This experiment should confirm the ability of the *L. acidophilus* SV31 to antagonize colonization of Cn-I binding sites by vaginal pathogens also in vivo and to support our contention about the convenience of Cn-I binding lactobacilli in probiotical preparations.

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