Anaerobe xxx (2014) 1–6

Contents lists available at ScienceDirect

Anaerobe

journal homepage: www.elsevier.com/locate/anaerobe

Pathogenesis and toxins

Effects of single- and multi-strain probiotics on biofilm formation and in vitro adhesion to bladder cells by urinary tract pathogens

C.M.C. Chapman*, G.R. Gibson, I. Rowland

Department of Food and Nutritional Sciences, University of Reading, P.O. Box 226, Whiteknights, Reading RG6 6AP, United Kingdom

ARTICLE INFO

Article history:
Received 14 November 2013
Received in revised form
29 January 2014
Accepted 17 February 2014
Available online xxx

Keywords:
Probiotic
Multi-species
Urinary
Crystal Violet
Cell culture

ABSTRACT

Purpose: There is increasing evidence that probiotic bacteria can inhibit and/or prevent urinary tract infections. Possible mechanisms include prevention of adhesion of pathogens to the bladder epithelium and inhibition of biofilm formation. Currently there is interest in the comparative efficacy of single probiotics vs. strain mixtures. We have therefore tested the inhibitory activity of four single probiotics and four probiotic mixtures towards the urinary tract pathogens *Escherichia coli NCTC 9001 and Enterococcus faecalis NCTC 00775*.
Methods: Inhibition of biofilm formation by cell-free supernatants was tested using the Crystal Violet assay, while prevention of pathogen adhesion to host cells was tested by using bladder cancer cells as a model for the human urinary tract.
Results: Under pH-controlled conditions, there was no significant inhibition of biofilm formation by any treatment. Without pH control, 5/8 treatments significantly inhibited biofilm production by *E. coli*, while 5/8 treatments inhibited production by *E. faecalis*. Using data from all Crystal Violet assays, there was no significant difference in the ability of single- and multi-strain probiotics to inhibit biofilm formation. In the cell culture assays, all treatments were able to significantly reduce numbers of pathogenic cells adhering to host cells by 2.5–3.5 logs. No significant difference was observed between the displacement caused by single strains and mixtures for either pathogen.
Conclusions: Inhibition of biofilm seems to be a major mechanism of urinary tract pathogen exclusion, related to, and possibly dependent upon, the probiotic ability to reduce environmental pH. Exclusion via competition of binding sites is a possible *in vivo* mechanism for these probiotics. If an additive or synergistic effect exists between strains within a mixture, it does not manifest itself in a greater effect through these two inhibitory mechanisms.

1. Introduction

Urinary tract infections (UTIs) are considered the most common bacterial infections with an estimated annual worldwide incidence of 250 million cases, with great impact on patients and healthcare systems [1]. Indigenous vaginal microbiota have an important role to play in the maintenance of a healthy urogenital tract, with some 50 species inhabiting the area [2]. High levels of lactobacilli have been identified within the vaginal microbiota of healthy women [3,4], with *Lactobacillus acidophilus, Lactobacillus fermentum, Lactobacillus plantarum,* and *Lactobacillus rhamnosus* being among the most common species observed [5]. Conversely, women suffering from recurring UTIs have been observed to have lower levels of such species in the vagina and urethra [6].

There is great interest in the use of probiotics as treatment for UTI for several reasons. Uropathogenic species are showing greater resistance to antibiotics [7], suggesting that an alternative to pharmaceutical therapies may be required. Given that some lactic acid bacteria, such as lactobacilli [8] are resistant to common antibiotics such as vancomycin and tetracycline [9], it is suggested that probiotics could be used as an adjunct to antibiotic therapy, and may also restore gut microbiota disrupted by antibiotic therapies. The lack of side-effects of probiotic treatment [10] together with the frequency of UTI recurrence [11] indicate a treatment which can be used frequently, and with greater safety. Favourable results have been seen using probiotics as a treatment alongside antibiotics to treat UTI and restore healthy vaginal microbiota [12,13], although the use of probiotics alone as a treatment has the potential benefit of reducing the disruption to commensal flora.

In our previous studies, we have shown that despite the potential for mutual antagonism between constituent probiotic

* Corresponding author. Tel.: +44 (0) 118 378 8700; fax: +44 (0) 118 931 0080.
E-mail address: chapmancmc@hotmail.com (C.M.C. Chapman).

http://dx.doi.org/10.1016/j.anaerobe.2014.02.001
1075-9964/

strains within a mixture [14], multi-strain probiotics are able to inhibit growth of UTI-causing species *Escherichia coli* NCTC 9001 and *Enterococcus faecalis* NCTC 00775 when the probiotics are present as both viable cells and cell-free supernatants [15]. This suggests that competition for nutrients and production of antagonistic compounds are potential mechanisms for the inhibition of UTI by probiotics, and that multi-strain treatments may provide extra benefits due to their species-specific effects on different pathogenic strains.

Adherence to host cells is thought to be a major contributor to both *E. faecalis* [16] and *E. coli* pathogenesis [17,18], particularly with regard to UTI-causing strains [19]. Formation of biofilms is a major mechanism of attachment, in which a group of bacterial cells adhere to the host epithelium, covered with an extracellular polymer which protects the colony from environmental stresses such as changes to pH and temperature [20] and antibiotics [21]. The glycocalyx is an extracellular matrix composed of proteins, exopolysaccharides (EPS), and sometimes nucleic acids [22], and is a common feature to bacterial biofilms. Biofilm development occurs in response to extracellular signals, both environmental and self-produced [23]. An association has been observed between the ability of uropathogenic *E. coli* to produce haemolysin and its formation of biofilms, believed to be connected to the ability of the pathogen to persist in the urinary tract [24]. Biofilm formation by *E. faecalis* is believed to be a mechanism by which this organism resists the effects of antibiotics. Probiotic inhibition of biofilms has been previously observed, and is believed to be dependent upon reduction of environmental pH [25].

The Crystal Violet assay [26] is a surrogate model of biofilm formation by which the biofilm is stained, the intensity of the staining corresponding to degree of biofilm formation. It is widely used as a screening mechanism for biofilm formation by such pathogens. It is therefore possible to test the inhibition of biofilm formation by probiotics, by observing a reduction in model biofilm formation by pathogens when incubated with probiotic supernatants.

The complex process of bacterial adhesion involves the bacterial cell membrane coming into contact with host cell surfaces. Cell surface hydrophobicity is evidently an important factor in the strength of bacterial adhesion, as is the degree of charge on cell surfaces. It is believed that two different mechanisms come into play during bacterial cell adhesion, those of specific and non-specific binding. Non-specific binding involves interactions of a lower affinity than in specific binding [27]. Duary et al. have observed the correlation between increased hydrophobicity and increased adherence to human epithelial cells [28].

The ability of probiotics to adhere to host cell binding sites is one of the main benefits of probiotics. This adhesion to epithelial cells provides competition with pathogens and can therefore inhibit pathogenic adhesion [29], reducing the risk of pathogen-derived illnesses.

Cell adhesion studies have been carried out to determine the ability of many single-species probiotic treatments to adhere to both human mucus and epithelial cells, and to inhibit adhesion of pathogens to such cells [30]. Atassi et al. [31] observed a strain-specific pattern of inhibition of various uropathogens including *E. coli* IH11128, using HeLa cells as a model. Similar strain-specificity was observed by Mastromarino et al. [32]. Chen et al. [33] observed a 35% reduction of adhesion of pathogens to HeLa cells when co-incubated with *Lactobacillus crispatus.* For studying potential effects of urinary tracts in the bladder a more appropriate cell line would be one derived from bladder epithelium such as T24 cells used by Karlsson et al. [34]. It appears that few studies has been done to determine what effect multi-species probiotic preparations might have in these areas, or to compare the effect of single- and multi-strain treatments on such species.

Our previous study focussed on the ability of single and multi-strain probiotic treatments to inhibit growth of urinary tract pathogens [15]. This study continues that research by using the same single probiotic strains and mixtures. In this study the focus shifts from the inhibition of growth overall, to concentrate on two major mechanisms of probiotic interference with pathogenic ingress, namely the inhibition of protective biofilm formation, and competition for binding sites on bladder epithelium. The first aim was to assess the degree to which these eight probiotic treatments were able to affect these two mechanisms. The second main aim of this study was to compare the efficacy of single and multi-strain probiotic treatments in the inhibition of urinary tract pathogens via the mechanisms mentioned above.

2. Materials and methods

2.1. Organisms used

Probiotics used were *L. acidophilus* NCIMB 30184 (PXN 35), *L. fermentum* NCIMB 30226 (PXN 44), *L. plantarum* NCIMB 30187 (PXN 47), and *L. rhamnosus* NCIMB 30188 (PXN 54). The following mixtures were tested: 2-lactobacilli mixture (*L. acidophilus* and *L. plantarum*), 3-lactobacilli mixture (*L. acidophilus, L. fermentum,* and *L. rhamnosus*), 4-lactobacilli mixture (*L. acidophilus, L. fermentum, L. rhamnosus* and *L. plantarum)*, and the commercially-available Bio-Kult® mixture. Its constituent strains are – at varying % of total volume – *L. acidophilus, L. delbrueckii subsp. Bulgaricus, Lactobacillus casei, L. plantarum, L. rhamnosus, Lactobacillus salivarius ssp. Salivarius, Lactobacillus helveticus, Bifidobacterium bifidum, Bifidobacterium breve, Bifidobacterium infantis, Bifidobacterium longum, S. thermophilus, Lac. Lactis, Bacillus subtilis*.

Pathogens tested were *E. faecalis* NCTC 00775 and *E. coli* NCTC 9001.

2.2. Preparation of probiotic supernatants

Overnight probiotic cultures in de Man Rogosa Sharpe (MRS) broth, incubated at 37 °C in anaerobic conditions, were centrifuged at 2050 ×*g* for 10 min. The resulting cell-free supernatant was removed and the pellet discarded. Half of each supernatant was neutralised to pH7 using 1 M Sodium Hydroxide. Neutralised and non-neutralised supernatants were frozen in aliquots and defrosted overnight at room temperature before use.

2.3. Crystal Violet assay

The method of Wakimoto et al. [35] was adapted. Pathogen culture medium (100 μl of Nutrient broth for *E. coli, or* Tryptone Soy broth for *E. faecalis*) was added to each well of a 96-well plate (Greiner Bio-one, Stonehouse, Great Britain). Exponential growth phase pathogen culture (5 μl) was inoculated into each well, along with 100 μl of probiotic supernatant. Plates were then incubated at 37 °C for 18 h. After washing with dH_20, 150 μl of 0.5% Crystal Violet was added to each well, incubated for 5 min, and then discarded. Biofilm was quantified by enzyme-linked immunosorbent assay reader (Tecan, Männendorf, Switzerland) at 595 nm. As a control, the pathogen was incubated without probiotic supernatant. Higher absorbance values were taken to represent greater quantity of biofilm, while values below that of the biofilm for the pathogen-only control were taken to represent inhibition of biofilm formation.

2.4. Cell adhesion assays

2.4.1. Cell culture conditions

Human urinary bladder epithelium carcinoma cells were obtained from LGC Standards (Teddington, Middlesex) and cultured in

Please cite this article in press as: Chapman CMC, et al., Effects of single- and multi-strain probiotics on biofilm formation and in vitro adhesion to bladder cells by urinary tract pathogens, Anaerobe (2014), http://dx.doi.org/10.1016/j.anaerobe.2014.02.001