Characterization of adhesion properties to the cellular substratum of some *Enterococcus* strains selected for potential use in probiotic products or food products

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Abstract

Due to bile resistance Enterococcus faecium strains are used as probiotics in the management of diarrheal illnesses (Kimoto et al., 1999). However, enthusiasm for enterococci as probiotics has waned since the emergence of vancomycin resistant enterococci.

The purpose of this work was to investigate the capacity of adherence to the cellular substratum of some Enterococcus faecium strains isolated from Robinia pseudacacia and Chelidonium majus flowers and grass and to investigate their antibioresistance pattern, in order to select some strains with potential benefit for use in probiotic products and inoculant for silage.

Our results showed that all tested Enterococcus strains exhibited a strong ability to attach to HeLa cells, showing an aggregative adherence pattern (Fig. 1). This could be a strong argument for the potential use of these strains as probiotics, having in view that one of the most important mechanisms of action cited for probiotics is the competition with the pathogenic microorganisms for the adhesion sites at the intestinal level.

The intensity of the adherence rate was higher when bacterial cultures were used, comparatively with bacterial washed sediments, meaning that the intensity of adherence to the cellular substratum is depending on the extracellular compounds acting as adhesins, partially removed by washing step, but may be also on the gradient of some bacterial compounds secreted and accumulated in culture medium.

The tested strains proved to be fully sensitive to glycopeptidic antimicrobial agents (vancomycin and teicoplanin), thus, the use of these strains as food products or probiotics do not exihibit the risk of dissemination of glycopeptides resistance mechanisms in comensal, clinical or environmental strains.

Keywords**:** *Enterococcus, adherence to HeLa cells, probiotics, inoculant for sialge, vancomycin resistance*

Introduction

Probiotics are defined as live microorganisms, that may beneficially affect the host upon ingestion by improving the balance of the intestinal microbiota (Arunachalam, 1999). The dietary use of live microorganisms has a long history (Short, 1999). Mention of cultured dairy products is found in the Bible and the sacred books of Hinduism. Soured milks and cultured dairy products, such as kefir, koumiss, leben and dahi, were often used therapeutically before the existence of microorganisms was recognized. The use of microorganisms in food fermentation is one of the oldest methods for producing and preserving food. Much of the world depends upon various fermented foods that are staples in the diet (Saxelin et al., 1999).

The bacteria which colonise the gut must overcome the intestinal barriers and have the ability to proliferate at a rate that resists washout. Adhesion capacity to the entorocytes may be a major factor, being regarded a prerequisite for colonization and is one of the main selection criteria for new probiotic strains (Havenaar et al., 1999).

Enterococci are Gram-positive, facultative anaerobic cocci of the *Streptococcaceae* family. They are spherical to ovoid and occur in pairs or short chains. Enterococci are catalase-negative, non-spore forming and usually nonmotile. Enterococci are part of the intestinal microbiota of humans and animals. Due to the bile resistance *Enterococcus faecium* strains are used as probiotics in the management of diarrheal illnesses (Kimoto et al., 1999). However, enthusiasm for enterococci as probiotics has waned since the emergence of vancomycin resistant enterococci.

The purpose of this work was to investigate the capacity of adherence to the cellular substratum of some *Enterococcus faecium* strains isolated from *Robinia pseudacacia* and *Chelidonium majus* flowers and grass and to investigate their antibioresistance pattern, in order to select some strains with potential benefit for use in probiotic products and inoculant for silage.

Material and methods

Bacteria and culture conditions *Enterococcus faecium* strains no. VL 43, VL 47 and GM-8 were used. These bacteria were originally isolated from from *Robinia pseudacacia* and *Chelidonium majus* flowers and grass and grown in MRS medium (Merck, 2000). All strains were stored in the laboratory collection at -70°C in appropriate medium represented by MRS supplemented with 20% glycerol Primary cultures were obtained and after cultivated in MRS liquid medium in order to obtain mid-logarithmic phase cultures that were further used in our experiments.

Antibiotic susceptibility testings were perfomed using the standardized disc difussion method (following NCCLS recommendations). The following Oxoid discs were used: oxacillin (OXA) (30μg), cephuroxime (CXM) (30μg), cephtriaxone (CRO) (30μg), imipenem (IMP) (30μg), norfloxacin (NOR) (30μg), streptomycin (STR) (30μg), tobramycin (TOB) (30μg), vancomycin (VA) (30μg), teicoplanin (TEC) (30μg), erythromycin (ERY) (30μg).

The adherence capacity to the cellular substratum represented by HeLa cells was tested by Cravioto's adapted method. In this purpose, bacterial mid-logarithmic phase cultures of *Enterococcus faecium* tested strains were centrifuged at 4000 rmp/min for 10 minutes, and the pellet was washed three times in phosphate buffered saline (PBS) and resuspended in Eagle's minimum essential medium (MEM). Bacterial suspension density was adjusted at 10^7

CFU/ml. HeLa cells were routinely grown in MEM enriched with 10% heat-inactivated (30 min at 56°C) fetal bovine serum (Gibco BRL), 0.1 mM nonessential amino acids (Gibco BRL), and supplemented 0.5 ml of gentamicin (50 µg/ml) (Gibco BRL) and incubated at 37°C for 24 hrs. HeLa cells monolayers grown in 6 multi-well plastic plates were used at 80-100% confluency. For the adherence assay, the HeLa cell monolayers were washed 3 times with PBS; 1 ml of fresh Eagle MEM without antibiotics was added to each well and 1 ml of bacterial suspension was used for the inoculation of each well. The inoculated plates were incubated for 2 hrs at 37ºC. After incubation, the monolayers were washed 3 times with PBS, briefly fixed in cold ethanol (3 min), stained with Giemsa stain solution (1:20) (Merck, Darmstadt, Germany) and left to incubate for 30 min. The plates were washed, dried at room temperature overnight, and examined microscopically (magnification, ×2500) with I.O. and photographed with a Contax camera adapted for Zeiss microscope.

Results and discussion

The ability of probiotic bacteria to colonise the intestinal tract has been heavily debated in relation to effectiveness and physiological effect of these microorganisms. There has been some confusion as to the meaning of the word "colonisation". In the very strict meaning of the word, colonisation would mean a permanent implantation of the organism after a single ingestion. Hardly any of the probiotic bacteria process the ability to colonise in this permanent way. Although some recent research results have shown that some probiotic bacteria - especially Bifidobacteria - are able to persist in the intestine for more that 100 days after ceasing ingestion, the general perception is that probiotic bacteria have to be supplied on a continuous basis to secure a population of the gastro-intestinal system.

It is probably more correct to speak about adherence of LAB bacteria to the gut epithelial cells than it is to speak about colonisation. Whether speaking of adhesion or colonisation, the following factors have been identified as being of importance for the ability of probiotic bacteria to enter, persist and exert their physiological effect in the gastrointestinal system: acid resistance, bile resistance, adhesion ability, oxygen sensitivity, indigestible but fermentable carbohydrates in diet, species relatedness (human, animal or vegetable origin) and other environmental factors (diet and lifestyle of the host individual).

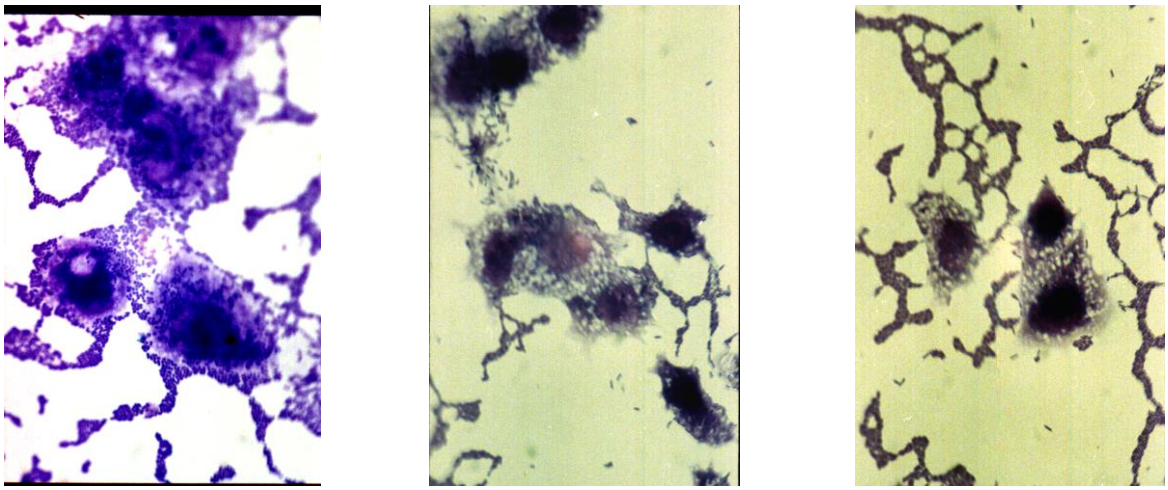
Having entered the intestinal tract it is important that the bacteria process the ability to adhere to the epithelial cells to avoid being washed out by the passing foods, mucus flow and peristaltic movements. Our purpose in the present work was to investigate the adherence properties to the cellular substratum of some *Enterococcus sp.* strains, and also to analyze the natural and acquired antibiotic resistance patterns in these strains.

Survival in and temporary colonization of the human gastro-intestinal tract have been demonstrated for some probiotics. However, *in vivo* testing is expensive and time consuming and requires approval by ethical committees (Blum et al., 1999). Therefore, reliable *in vitro* methods for selection of promising strains are required. Eukariotic cell lines (CaCo-2, HeLa, HEp-2) have been successfully used for *in vitro* studies on the mechanism of cellular adhesion of nonpathogenic lactobacilli and bifidobacteria (Jacobsen et al., 1999).

In this study, three strains of *E. faecium* isolated from from *Robinia pseudacacia* and *Chelidonium majus* flowers and grass, were tested for their ability to adhere to the HeLa cells monolayers. Probably due to the acid pH resulted from the metabolic activity of these bacteria, a strong monolayer detaching effect was noticed. Thus, eventhough a 50-80% confluency of cell monolayer is indicated for *in vitro* adherence studies, we used 100% confluent monolayers in order to be able to appreciate the adherence pattern and intensity.

Our results showed that all tested *Enterococcus* strains exhibited a strong ability to attach to HeLa cells, showing an aggregative adherence pattern (Fig. 1). This could be a strong argument for the potential use of these strains as probiotics, taking into account the fact that one of the most important mechanisms of action cited for probiotics is the competition with the pathogenic microorganisms for the adhesion sites at the intestinal level.

Fig. 1. Aggregative adherence of of VL-43, 47 and GM-8 strains (from left to the right) to HeLa cells (Giemsa staining, x2500).



The adherence rate was tested comparatively for bacterial mid-logarithmic phase cultures as well as for washed bacterial cells resuspended in Eagle MEM. Paradoxically, the intensity of the adherence rate was higher when live bacterial cultures were used, comparatively with bacterial washed sediments, meaning that the intensity of adherence to the cellular substrate is depending on the gradient of some bacterial compounds secreted and accumulated in culture medium directly or indirectly implicated in the cell to cell signalling.

Despite the actual tendency of expanding the range of bacterial cultures being used in food products and increased interest in probiotics and health benefits, a major issue of concern is the safety of cultures that can be consumed live and in large quantities (O'Brien et al., 1999). Enterococci are the most controversial probiotics and probably represent the largest risk to human health of any species currently used in this way (Franz et al., 1999). The differences between an enterococcal pathogen and an apparently safe food use strain is unclear, and the potential for the latter to acquire virulence factors by gene transfer has not been investigated (Huycke et al., 1992; Heaton et al., 1996). It is already established that the molecular taxonomy of enterococci does not lead to a distinction between these two types of strains (Chow et al., 1999). Our knowledge of virulence in enterococci is incomplete, in part due to the fact that they are normal human commensals and, as such, have subtle virulence traits that are not easily identified. Virulence traits include adherence to host tissue, invasion and abscess formation, modulation of host inflammatory responses, and secretion of toxic products, but also antimicrobial resistance (Eaton & Gasson, 2001).

Enterococci are noted for their capacity to exchange genetic information by conjugation and these processes are known to take place in the gastrointestinal tract (Clewell, 1990).

Having in view the emergence of vancomycin resistance among *Enteroocccus* strains and the danger of horizontal transmission of this complex resistance mechanism to other Gram-positive cocci, especially clinical *Enterococcus* strains and methicilin resistant *S. aureus* (Noble et al., 1992) we also investigated in this study the antibioresistance patterns in these strains.

Our results showed that the tested strains exhibited, as expected, natural low resistance levels to aminolgycosides and probably aquired resistance to beta-lactams (including carbapenems and 3rd generation cephalosporins) (table 1).

Table 1. Antibiotic susceptibility testing results (by Kirby Bauer method)

Antibiotic	**Beta-lactams**				**Quinolones**	**Macolides**	**Aminoglycosies**		**Glycopeptides**	
Bacterial strain	**OXA**	**CRO**	**CXM**	**IMP**	**NOR**	**ERY**	**TOB**	**STR**	**VA**	**TEC**
GM-47	**R**	**S**	**R**	**S**	**S**	**S**	**R**	**R**	**S**	**S**
GM43	**R**	**R**	**R**	**S**	**S**	**S**	**R**	**R**	**S**	**S**
GM8	**R**	**S**	**R**	**R**	**S**	**S**	**R**	**R**	**S**	**S**

Further studies will be needed in order to establish the genetic support of this resistance, the biochemical mechanisms and the transferability to other Gram-positive or even Gram-negative genera.

Fortunately, the tested strains proved to be fully sensitive to glycopeptidic antimicrobial agents (vancomycin and teicoplanin), thus, the use of these strains as food products or probiotics do not exihibit the risk of dissemination of glycopeptides resistance mechanisms in environmental, comensal or clinical strains.

In conclusion, the tested enterococci strains exhibited high adherence rate to the cellular substratum represented by HeLa cells and did not carry vancomycin resisatnce genes, aspects that are pleading for their use as probiotics as well as food products.

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