

Short Communication

Inhibition of *Clostridium difficile* strains by intestinal *Lactobacillus* species

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Indigenous intestinal microflora (including lactobacilli) has an important role in protection against *Clostridium difficile* infection. To assess *in vitro* interaction between lactobacilli and *C. difficile*, antagonistic activity of 50 intestinal *Lactobacillus* spp. strains against 23 pathogenic *C. difficile* strains was determined. Phenotypic properties of *C. difficile* strains [production of short-chain fatty acids (SCFAs) and toxin A, and antimicrobial susceptibility] and lactobacilli (production of SCFAs and H₂O₂) were investigated. Five lactobacilli (*Lactobacillus paracasei* and *Lactobacillus plantarum* species) were antagonistic to all *C. difficile* strains, 18 were antagonistic to some *C. difficile* strains and 27 showed no antagonistic activity. This antagonistic activity was strain-specific and seemed to correlate with H₂O₂ and lactic acid production. *C. difficile* strains that were more sensitive to lactobacilli ($n = 9$) usually produced higher toxin levels and more SCFAs, and were more resistant to antibiotics, than strains that were resistant to lactobacilli ($n = 14$). Compatibility of *C. difficile* strain properties (resistance to lactobacilli or antibiotics) with intestinal microecological conditions (e.g. presence of antagonistic lactobacilli, concentration of antibiotics) may determine expression of disease.

Introduction

Clostridium difficile infection is a frequent complication of antibiotic therapy in hospitalized patients. Impairment of the composition of intestinal microflora is an important prerequisite for colonization by *C. difficile* and development of infection. It is known that up to 50 % of hospitalized patients become asymptomatic carriers of toxigenic strains and, in symptomatic patients, clinical manifestations vary greatly (Bartlett, 1992; Johnson & Gerding, 1998). The reasons for this may lie in variations in virulence factors of *C. difficile* strains on one hand and different degrees of disruption of indigenous intestinal microflora and colonization resistance on the other.

Our previous findings indicate the importance of lactobacilli in maintenance of colonization resistance against *C. difficile* (Naaber, 1997; Naaber *et al.*, 1997). For prevention and therapy of antibiotic-associated diarrhoea and *C. difficile* infection, several strains of lactobacilli have been used, but have had varied efficacies (Kotz *et al.*, 1992; Biller *et al.*, 1995; Roffe, 1996; McFarland, 1998; Ouwehand, 1998; Vandeplass, 1999; Pochapin, 2000; D'Souza *et al.*, 2002). Inhibitory potency of different *Lactobacillus* spp. against *C. difficile* and mechanisms involved in these interactions have not yet been elucidated.

The aim of our study was to evaluate the inhibitory activity of various intestinal lactobacilli against different pathogenic *C. difficile* strains *in vitro*.

Methods

Twenty-three *C. difficile* strains that belonged to different genotypes and were isolated from faeces of 21 hospitalized patients with sporadic cases of antibiotic-associated diarrhoea were included. Strains were typed with arbitrarily primed PCR as described by Tang *et al.* (1995). From two patients, two different strains were isolated simultaneously. Strains of intestinal lactobacilli ($n = 50$) were isolated from faeces of young, healthy children during our previous studies (Mikelsaar *et al.*, 2002).

In vitro toxin A production by *C. difficile* strains was detected with the Oxoid toxin A test, according to the manufacturer's instructions. Strains were considered to be low toxin producers if the test was positive with undiluted or 1 : 10 diluted sample and high producers if the test was positive in a 1 : 100 or higher dilution.

MICs of chloramphenicol, clindamycin, rifampicin, tetracycline, ampicillin, erythromycin, metronidazole and vancomycin were detected with E tests, according to the manufacturer's instructions (AB Biodisk), and interpreted according to the current break-points of the National Committee for Clinical Laboratory Standards.

For determination of production of short-chain fatty acids (SCFAs), *C. difficile* strains were cultivated in peptone/yeast/glucose broth and lactobacilli in MRS broth for 48 h. *In vitro* production of SCFAs was measured by GC as a concentration of these compounds, as described by Holdeman & Moore (1972). Production of H₂O₂ by lactobacilli was detected on tetramethyl benzidine medium, as described by Eschenbach *et al.* (1989).

Abbreviation: SCFA, short-chain fatty acid.

Antagonistic activity of lactobacilli was detected on agar plates as inhibition of *C. difficile* growth. Wilkins–Chalgren blood agar was inoculated with a 10 µl aliquot of a 48 h culture of lactobacilli in MRS broth by streaking the plates. After 48 h incubation in a 10% CO₂ environment, 1 µl *C. difficile* saline suspension (0.5 on the McFarland turbidity scale) was streaked perpendicularly with a lactobacillus growth streak. Inhibition assays with each *Lactobacillus* sp. and *C. difficile* combination were repeated at least four times on two separate plates. Plates were incubated for 24 h in an anaerobic environment and inhibition zones (mm) of *C. difficile* growth from the *Lactobacillus* sp. streak were measured.

Data were analysed with Jandel SigmaStat 2.0 software by using Fisher, Spearman correlation and Mann–Whitney tests.

Results and Discussion

Based on antagonistic activity against *C. difficile* strains, we divided the lactobacilli into three groups: (i) those able to inhibit growth of all *C. difficile* strains investigated (LB⁺); (ii) those able to inhibit growth of some (nine of 23) *C. difficile* strains (LB^{+/-}); and (iii) those without any antagonistic activity (LB⁻). Species composition of these groups is shown in Table 1.

Five strains of lactobacilli were able to inhibit growth of all *C. difficile* strains tested. These belonged to *Lactobacillus paracasei* and *Lactobacillus plantarum* [facultatively heterofermentative (FHEL) group]. At the same time, all obligately heterofermentative (OHFL) group species (except one) did not show antagonistic activity against any *C. difficile* strain. However, the antagonistic activity of lactobacilli was not strictly correlated with a particular species of lactobacilli.

Based on the interaction with LB^{+/-} group lactobacilli, *C. difficile* strains could be divided into two groups: nine strains were sensitive to all these lactobacilli and 14 *C. difficile* strains were resistant. We found that 11 *C. difficile* strains were high toxin producers, 11 were low toxin producers and one strain did not produce toxin A *in vitro*. High toxin producers were usually sensitive to LB^{+/-} group lactobacilli, in contrast to low toxin producers ($P < 0.001$). All *C. difficile* strains tested were highly sensitive to ampicillin, vancomycin

and metronidazole. With other antibiotics, MIC values varied greatly. Strains with higher MICs to chloramphenicol, rifampicin, tetracycline and erythromycin were usually sensitive to the antagonistic activity of LB^{+/-} lactobacilli and were also highly toxigenic ($P < 0.001$). Thus, based on these phenotypic properties, *C. difficile* strains could be divided into two typical groups: phenotype A (antibiotic-resistant, more susceptible to antagonistic activity of lactobacilli and highly toxigenic) and phenotype B (sensitive to antibiotics, more resistant to inhibition by lactobacilli and low toxin A producers).

By comparing quantitative production of SCFAs, we found that *C. difficile* strains of phenotype A produced significantly higher amounts of isobutyric (mean, 1.84 versus 1.16 mg l⁻¹; $P < 0.001$), butyric (mean, 6.56 versus 5.15 mg l⁻¹; $P < 0.05$), isovaleric (mean, 2.14 versus 1.68 mg l⁻¹; $P < 0.001$) and valeric (mean, 1.85 versus 1.31 mg l⁻¹; $P < 0.01$) acids, compared to phenotype B strains. There was no significant difference in production of acetic or isocaproic acids. All lactobacilli tested produced acetic and lactic acids. We found that LB⁺ group strains usually produced more lactic acid (median, 20.9 mg l⁻¹) than LB^{+/-} (median, 18.5 mg l⁻¹; $P < 0.005$) and LB⁻ (median, 10 mg l⁻¹; $P < 0.005$) strains. No differences were found in production of acetic acid.

H₂O₂ was produced by 42% of lactobacilli *in vitro*. We found that LB⁺ and LB^{+/-} strains were more commonly H₂O₂ producers (14 of 23) than LB⁻ strains (seven of 27; $P = 0.02$).

In our study, we found that both *in vitro* antagonistic activity of lactobacilli against *C. difficile* and susceptibility of *C. difficile* strains to this antagonism was strain-dependent. Previous studies that used only a single indicator *C. difficile* strain to screen several lactobacilli or a single *Lactobacillus* sp. strain against different *C. difficile* strains did not describe this important phenomenon (Bogovič-Matijašić *et al.*, 1998; Forestier *et al.*, 2001; Strus *et al.*, 2001).

The important role of *in vitro* antagonistic activity of lactobacilli against potential pathogens and also the ability

Table 1. Antagonistic activity of different species of lactobacilli against *C. difficile*

Lactobacillus group*	Species	No. strains		
		LB ⁺ (n = 5)	LB ^{+/-} (n = 18)	LB ⁻ (n = 27)
OHOL	<i>Lactobacillus acidophilus</i>		4	1
	<i>Lactobacillus delbrueckii</i> subsp. <i>delbrueckii</i>		1	1
OHFL	<i>Lactobacillus buchneri</i>		1	6
	<i>Lactobacillus brevis</i>			5
	<i>Lactobacillus fermentum</i>			5
	<i>Lactobacillus coprophilus</i>			1
FHEL	<i>Lactobacillus paracasei</i> subsp. <i>paracasei</i>	2	7	7
	<i>Lactobacillus plantarum</i>	3	5	1

*FHEL, Facultatively heterofermentative lactobacilli; OHFL, obligately heterofermentative lactobacilli; OHOL, obligately homofermentative lactobacilli.

to sustain colonization resistance in microecosystems *in vivo* has been attributed by several investigators to the production of H₂O₂ and SCFAs (Eschenbach *et al.*, 1989; Brashears *et al.*, 1998; Ouwehand, 1998; Brashears & Durre, 1999; Malakar *et al.*, 1999). Others have found that, in some cases, where lactobacilli show high antagonistic activity against pathogens, these mechanisms are not involved or have only a supportive effect (Su *et al.*, 1987; Coconnier *et al.*, 1997). Bogovič-Matijašić *et al.* (1998) suggested the importance of bacteriocins of lactobacilli in inhibition of *C. difficile*. Our finding that lactobacilli that were more antagonistic to *C. difficile* produced H₂O₂ and high levels of lactic acid more frequently indicates the probable role of these compounds in lactobacilli–*C. difficile* interactions. However, the role of other mechanisms of antagonism, such as production of bacteriocins, should be investigated and the relevance of *in vitro* findings should be evaluated by *in vivo* studies.

In *C. difficile* strains, we found an inverse relationship between toxicity, resistance to antibiotics and susceptibility to antimicrobial agents that were produced by lactobacilli. Correlation (positive or negative) between virulence and antibiotic resistance has been described in several other species. On one hand, these properties could be selected by the same environmental factors or mediated by related mechanisms; on the other hand, expression of resistance may require extra energy and thus reduce virulence and metabolic activity (Martínez & Baquero, 2002). In the case of antibiotic-associated diarrhoea, concentration of antibiotics and the antagonistic effect of indigenous microflora are the main selective factors for *C. difficile* strains in the intestine. Based on our findings, we suggest that if antibiotic concentration is high and the indigenous microflora is deeply suppressed, antibiotic-resistant (but lactobacilli-sensitive) and more toxigenic strains could have an advantage. In cases of milder alteration of indigenous microflora and lower concentrations of antibiotics, strains of *C. difficile* that are lactobacilli-resistant and more antibiotic-sensitive, but less toxic, have the advantage to survive and cause infections. If this is true, administration of some antibiotics (e.g. clindamycin) induces more serious *C. difficile* infection, not only due to deeper alteration of indigenous microflora, but also by selection of more toxigenic *C. difficile* strains.

We have found a substantial difference in antagonistic activity of intestinal lactobacilli against *C. difficile* strains with different virulence. Evaluation of the *in vivo* effect of highly antagonistic strains of lactobacilli and description of the mechanisms involved enable a more complete understanding of the pathogenesis of *C. difficile* diarrhoea and may introduce new possibilities in an ecological approach to its prophylaxis and therapy.

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