

## Clinical Microbiology

## Intestinal lactoflora in Estonian and Norwegian patients with antibiotic associated diarrhea

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## ABSTRACT

The disruption of intestinal microbiota is an important risk factor for the development of *Clostridium difficile* caused antibiotic associated diarrhea (AAD). The role of intestinal lactoflora in protection against *C. difficile* is unclear. Fecal samples ( $n = 74$ ) from AAD patients were investigated for *C. difficile* and lactobacilli by culture and real-time PCR. Lactobacilli were identified by enterobacterial repetitive intergenic consensus PCR (ERIC-PCR) and sequencing of 16S rRNA. In *C. difficile* negative cases we found somewhat higher counts of intestinal *Lactobacilli* (5.02 vs. 2.15 CFU log<sub>10</sub>/g;  $p = 0.053$ ) by culture and more frequently *Lactobacillus plantarum* (33.3% vs. 9.4%;  $p = 0.03$ ) as compared with positive ones. Results of total counts of lactobacilli comparing Estonian and Norwegian samples were conflicting by culture and PCR. We found higher colonization of Norwegian AAD patients with *L. plantarum* (21% vs. 5%,  $p = 0.053$ ) and Estonians with *Lactobacillus gasseri* (19% vs. 2%,  $p = 0.023$ ). Particular lactobacilli (e.g. *L. plantarum*) may have a role in protection against *C. difficile*, whereas the meaning of total counts of lactobacilli remains questionable. In different persons and nations, different lactobacilli species may have a protective role against *C. difficile*.

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## 1. Introduction

Antibiotic associated diarrhea can develop due to infection caused by a specific pathogen (in the majority of cases by *Clostridium difficile*) or due to dysbiosis of intestinal microbiota. *C. difficile* associated AAD is clinically more severe and could be associated with high mortality. AAD due to dysbiosis without a specific pathogen is usually mild and self limited [1].

It is a general agreement that the most important risk factor for establishment of *C. difficile* in the intestinal tract is the alteration of indigenous intestinal microbiota. However, it is not known which microbial groups are mainly responsible for maintenance of colonization resistance against *C. difficile*. The role of intestinal lactobacilli in colonization resistance is controversial [2]. Also, the usage of probiotic lactobacilli for prophylaxis or treatment of *C. difficile* infection has been only partially successful [3,4]. Reasons for these

controversial results could be due to the individuality of intestinal lactoflora and the differences in disturbance of the intestinal microbial ecosystem after antibiotic treatment.

The aim of our study was to compare counts and species composition of intestinal lactoflora in (1) *C. difficile* positive and negative AAD cases and (2) Estonian and Norwegian AAD patients using culture based and molecular methods.

## 2. Material and methods

Consecutive fecal samples routinely sent for *C. difficile* diagnostics in Estonia (4 hospitals) and in Norway (Stavanger University Hospital) were collected during 2008. After initial screening for *C. difficile* in local labs, consecutive 31 *C. difficile* culture positive and 43 culture negative cases with documented diarrhea and antibiotic usage (AAD cases) were included, and fecal samples were stored at  $-80\text{ }^{\circ}\text{C}$ . Of these 74 AAD patients (44 female and 30 male; age 3–89; median 72 years), 41 were from Norway and 33 from Estonia (age medians 76 and 61 years;  $p = 0.005$ ).

*C. difficile* was detected by (1) cultivation of samples on Brazier's CCEY Agar with cefoxitin and cycloserine supplement (LabM, UK) in

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anaerobic environment (Concept, UK; with gas mixture 5 CO<sub>2</sub>, 5%, H<sub>2</sub>, 90% N<sub>2</sub>) and (2) real-time PCR as described by Rinttilä et al. [5]. Culture and/or PCR positive samples considered as *C. difficile* positive cases.

For quantification of lactobacilli by culture, weighed fecal samples were homogenized in prerduced buffer, and serial dilutions were seeded on Man–Rogosa–Sharpe agar (MRS; Oxoid, UK) and Rogosa (Oxoid, UK) agar. Rogosa agar was incubated in anaerobic cabinet (Concept, UK; with gas mixture 5 CO<sub>2</sub>, 5%, H<sub>2</sub>, 90% N<sub>2</sub>) and MRS agar in microaerophilic environment (Joan, France; with gas mixture 10% CO<sub>2</sub>). Colony forming units per gram (CFU log<sub>10</sub>/g) were calculated. The detection limit of quantitative culture was 2 CFU log<sub>10</sub>/g.

For quantification of lactobacilli by real-time PCR, bacterial DNA from fecal samples was extracted using a QIAamp DNA stool mini kit (QIAGEN, Hilden, Germany). Real-time PCR was performed with the ABI PRISM 7500 HT (Applied Biosystems) as described previously [5]. Data analysis was conducted with Sequence Detection Software version 1.6.3, supplied by Applied Biosystem.

From each cultivated sample, up to 5 morphologically different dominating strains of lactobacilli were isolated, typed by enterobacterial repetitive intergenic consensus PCR (ERIC-PCR) and identified by 16S rRNA sequencing as described previously [6,7]. Comparison of the 16S rRNA sequences obtained by using the GeneBank database BLAST program (<http://www.ncbi.nlm.nih.gov/BLAST/>) allowed the assignment of a strain to a particular species. In general, when 16S rRNA similarity values exceed 97%, the strains are considered to belong to the same species.

Data were analyzed with SigmaStat (Jandel Scientific, USA) using the Spearman Rank correlation test, Wilcoxon Signed Rank test, Mann–Whitney Rank Sum Test, and Chi square test.

### 3. Results

We detected in total 62 *C. difficile* positive cases by PCR and/or culture. Lactobacilli were found in 44 cases by culture and 68 cases by PCR (Table 1). Comparing counts in PCR and/or culture positive cases ( $n = 74$ ), PCR detected significantly higher counts of lactobacilli (medians 8.2 vs. 2.95 CFU log<sub>10</sub>/g,  $p < 0.001$ ), but no correlation in counts between these methods was found ( $r = 0.09$ ;  $p = 0.4$ ). In total, 91 strains of lactobacilli were isolated and identified: *Lactobacillus rhamnosus* ( $n = 34$ ), *Lactobacillus gasseri* ( $n = 16$ ), *Lactobacillus plantarum* ( $n = 15$ ), *Lactobacillus casei* ( $n = 11$ ), *Lactobacillus acidophilus* ( $n = 8$ ), *Lactobacillus paracasei* ( $n = 2$ ), *Lactobacillus zeae* ( $n = 2$ ), *Lactobacillus amylovorus* ( $n = 1$ ), *Lactobacillus fermentum* ( $n = 1$ ) and *Lactobacillus brevis* ( $n = 1$ ).

Comparing total counts of lactobacilli by culture in *C. difficile* positive and negative cases, we found somewhat lower counts in *C. difficile* positive samples than in negative ones (medians 2.15 vs. 5.02 CFU log<sub>10</sub>/g). However this difference was not statistically significant ( $p = 0.053$ ). No differences were found comparing these counts detected by PCR (medians 8.25 vs. 7.05 CFU log<sub>10</sub>/g;  $p = 0.12$ ). Comparing colonization with particular *Lactobacillus* species in *C. difficile* negative and positive cases, we found a significantly higher colonization rate with *L. plantarum* in

negative cases (33.3% vs. 9.4%;  $p = 0.03$ ). A similar trend was found in the case of *L. gasseri*, though not statistically significant (20% vs. 7.8%;  $p = 0.17$ ).

Comparing the presence of *C. difficile* in Estonian and Norwegian samples, no differences were found. Comparing the prevalence of lactobacilli in these samples, we found somewhat higher colonization of Norwegian (64%) as compare with Estonian AAD patients (46%), however this was not a statistically significant difference. The total counts of lactobacilli by culture were higher in Norwegian samples as compared with Estonian ones (medians 4.14 vs. 0 CFU log<sub>10</sub>/g;  $p = 0.018$ ). Using PCR for quantification of lactobacilli, we found the opposite trend: counts in Norwegian samples were lower than in Estonian ones (medians 8 vs. 8.33 CFU log<sub>10</sub>/g;  $p = 0.0002$ ). Comparing colonization with particular lactobacilli species between two countries, we found higher colonization of Norwegians with *L. plantarum* (21% vs. 5%,  $p = 0.053$ ) and Estonians with *L. gasseri* (19% vs. 2%,  $p = 0.023$ ). Presence or absence of *C. difficile* or particular *Lactobacillus* species was not related to AAD patients' age.

Comparing genetic relatedness of *L. plantarum* (Fig. 1a) and *L. gasseri* (Fig. 1b) strains by ERIC-PCR, we found high diversity. Strains isolated from *C. difficile* negative (or positive) cases did not belong to the same clusters.

### 4. Discussion

This is the first published study quantitatively and qualitatively evaluating intestinal lactobacilli in *C. difficile* positive and negative

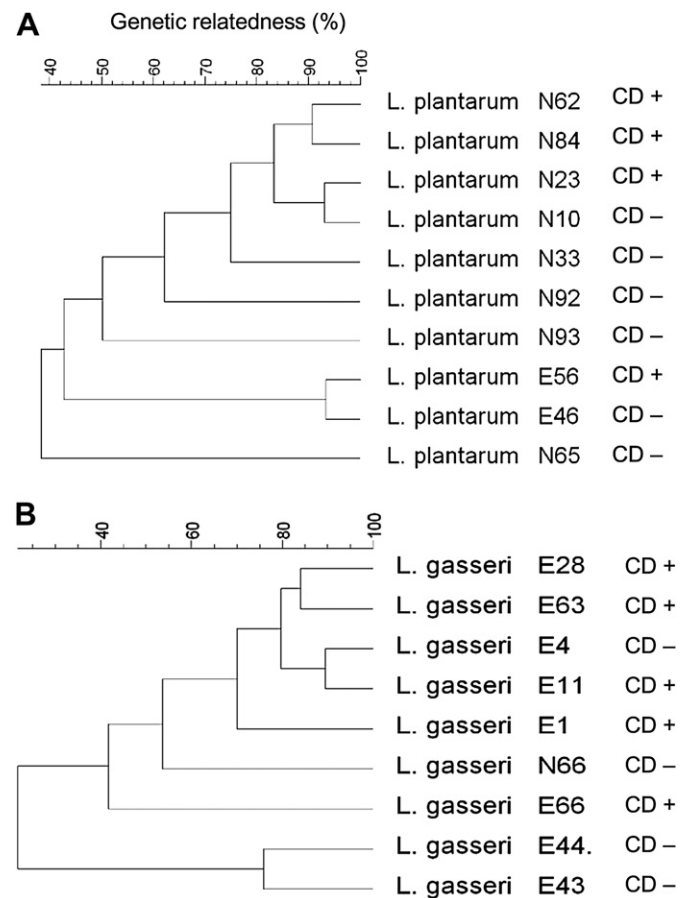


Fig. 1. Genetic relatedness of *L. plantarum* (A) and *L. gasseri* (B) strains. E – Estonian; N – Norwegian patient. CD – *C. difficile* negative and CD + *C. difficile* positive case.

**Table 1**  
Identification of lactobacilli in samples from AAD patients by the culture and PCR methods.

		PCR	
		Positive	Negative
Culture	Positive	40	4
	Negative	28	2

AAD patients and comparing the results of culture and molecular methods. Evaluation of intestinal lactobacilli in connection with *C. difficile* infection could be important on the one hand for assessment of the role of autochthonous lactobacilli in the maintenance of colonization resistance against *C. difficile* and on the other hand for evaluation of the possible isolation of particular *Lactobacillus* strains with potential probiotic activity against *C. difficile*.

We found a borderline trend indicating lower counts of total lactobacilli in *C. difficile* positive AAD patients as compared with negative ones by culture methods. No differences were found counting lactobacilli by PCR. Only a few studies investigating the role of population levels of indigenous lactobacilli in colonization resistance against *C. difficile* are available. Our previous studies indicate that higher counts of intestinal lactobacilli may associate with lower colonization rate of infants and AAD patients with *C. difficile* [8,9]. In one other study, a higher count of intestinal lactobacilli in *C. difficile* diarrhea patients has been shown as compared with healthy elderly persons [10,11]. Since lactobacilli are resistant to several broad-spectrum antibiotics, higher counts of lactobacilli in this study could be related to antibiotic usage rather than connected with *C. difficile* infection.

In identifying members of the dominating *Lactobacillus* population, we found that *L. plantarum* was significantly more common in *C. difficile* negative samples as compared with positive ones. We have previously shown that *L. plantarum* strains usually have the best antagonistic activity against *C. difficile* in vitro as compared to other intestinal lactobacilli [12]. This antagonistic activity was strain – rather than species-specific in this study. Thus, the importance of total counts of intestinal lactobacilli in protection against *C. difficile* remains questionable, and further studies with large patient groups are needed. However, particular species such as *L. plantarum* (or some of its strains) may have a role in supporting colonization resistance against *C. difficile*.

Comparing Norwegian and Estonian AAD patients, we found differences in quantitative counts of lactobacilli as well as in their species composition. In the case of total counts of lactobacilli, conflicting results by culture based and non-culture based methods were found. No correlation was found in counts of lactobacilli between these methods. Thus, it seems that culture and PCR are detecting different groups of lactobacilli that overlap only to some extent. One explanation for this finding is the fact that primers designed for lactobacilli detect also other related bacteria such as *Pediococcus* spp., *Weissella* spp., and *Leuconostoc lactis* [5]. Also, other studies have shown discrepancies between molecular methods and culture in investigation of intestinal microbiota [13]. It has also been supposed that PCR detects high numbers of non-cultivable and nonviable lactobacilli originating from food and proximate parts of the alimentary tract (e.g. oral cavity). If this is true, culture results may represent more autochthonous lactobacilli of the lower part of the intestine and PCR significant numbers of allochthonous micro microbiota [14]. Thus these conflicting results of our study may indicate domination of different lactobacilli groups in Estonian and Norwegian AAD patients. Comparing species

composition of lactoflora, we found the highest difference in prevalence of *L. gasseri* and *L. plantarum*. Reasons for these differences could be in the genetic variations between nations as well as differences in nutritional habits [15]. Thus, due to individual differences in intestinal microbiota (including lactobacilli), different lactobacilli species may have a leading role in the maintenance of colonization resistance against *C. difficile* in different persons or nations.

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