Beneficial effect of auto-aggregating Lactobacillus crispatus on experimentally induced colitis in mice

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Abstract

We tested the therapeutic relevance of auto aggregation in lactobacilli by comparing the effect on DSS induced colitis of viable Lactobacillus crispatus M247, isolated from healthy humans, to L. crispatus MU5, an isogenic spontaneous mutants of M247, the latter lacking the auto aggregation phenotype which allows the adhesion to human mucus. Aggregating L. crispatus M247, but not the non-aggregating MU5, was retrievable from mice feces and adherent to the colonic mucosa. Daily administration of L. crispatus M247, but not heat killed L. crispatus M247 or aggregation deficient L. crispatus MU5, dose-dependently reduced the severity of DSS colitis. Indeed, L. crispatus MU5 administered in a 30% sucrose solution, known to restore the aggregation phenotype, had a protective effect comparable to mice receiving L. crispatus M247. These results indicate that a surface-mediated property such as aggregation may play a pivotal role in the protective effects obtained by dietary supplementation with L. crispatus M247 during colitis.

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1. Introduction

Although the pathogenesis of inflammatory bowel disease (IBD) remains elusive, the relevance of intestinal luminal bacteria in the initiation and progression of chronic intestinal inflammatory disorders is gaining crescent supports [1,2]. Thus, enteric bacteria or their products have been detected within the inflamed intestinal mucosa of IBD patients and antibiotic therapy can reduce disease activity [3,4]. Indeed, injection of purified bacterial products in the intestinal wall can directly initiate and perpetuate a chronic inflammation [5]. Furthermore, the spontaneous colitis that develops in transgenic HLA-B27/β 2-microglobulin rats and in mice deficient in IL-2, IL-10 or T-cell receptors requires the presence of luminal bacteria...
However, so far no specific micro-organism has been associated with the pathogenesis of IBD suggesting that qualitative/quantitative differences in the intestinal micro biota may play a major role in the initiation and perpetuation of intestinal inflammation [9].

On the other hand, the intestinal microflora is essential to maintain the homeostasis of host’s colonic mucosa. Germ free animals are highly susceptible to develop colitis following intestinal colonization since normal intestinal micro flora prevent a rapid colonization by intestinal pathogens [10,11]. Furthermore, in humans the colonic anaerobic micro flora manage to degrade non-digested carbohydrates supplying vital energetic substrates to colonicocytes [12,13]. However, the role of the gastrointestinal micro biota is by far more complex as non-pathogenic mucosal associated bacteria can directly modulate the activity of mucosal-associated immune system [14,15].

The intestinal microflora, which contains more than 400 bacterial species, is extremely rich in the colon where the anaerobic bacteria constitute the bulk of the $10^{10–10^{12}}$ bacteria/g of feces [16]. Micro flora considered beneficial to the host include the genera *Bifidobacterium* and *Lactobacillus*, whereas species potentially pathogenic include enterobacteriaceae and clostridia. Interestingly, in patients with active ulcerative colitis (UC) facultative anaerobic Gram+ bacteria and *Lactobacillus* species are decreased, whereas *Bifidobacteria* are decreased in fecal extracts from patients with Crohn’s disease (CD) [9,17,18]. Furthermore, a marked decrease in intestinal *Lactobacillus* spp. precedes the onset of colitis in IL-10 gene deficient mice [19]. These studies offer compelling support to the view that a trigger of intestinal inflammation may be an imbalance in the intestinal micro flora leading to the relative dominance of potentially “aggressive” species. Therefore manipulation of the intestinal micro flora or mucosal-associated bacteria in specific compartments has been proposed as a potential therapy to treat a variety of gastrointestinal disorders including colitis. Although the clinical efficacy of certain probiotic strains is now well documented [20], little is known on the actual characteristics that beneficial bacteria should present in order to rationally select new strains for therapeutic purposes [21]. Therefore, in an effort to identify bacterial characteristics required for the treatment of intestinal inflammatory disorders we tested whether the aggregating properties frequently observed in enteric lactobacilli are required to mediate their therapeutic effects.

To address this question we used a new model of bacterial strains recently characterized in vitro and in vivo, formed by *L. crispatus* M247, isolated from feces collected from an healthy human, and its spontaneous mutant MU5, which suffered the loss of the aggregating trait [22]. Thus we compared the effect of oral supplementation with *L. crispatus* M247 and MU5 on DSS-induced colitis in mice. Survival and persistence into human gut of both strains have been previously established during a randomized, double blind trial [22].

### 2. Methods

#### 2.1. Induction of colitis by dextran sodium sulfate and assessment of colitis severity

Male Balb/c mice, ten weeks old, were randomly divided into groups (6–12 animals each) receiving either water alone (control group) or drinking water containing 5% (wt/vol) dextran sodium sulfate (DSS) (TdB Consultancy, Uppsala, Sweden). Body weight was measured daily and after 5 days of treatment animals were killed, the proximal colon removed, flat opened and washed in PBS. Full-thickness colonic samples were processed to determine myeloperoxidase (MPO) activity [23] or subjected to histological examination [24]. Animals studies were approved by the Institutional Animal Care and Use Committee of the University of Padua.

#### 2.2. Isolation, characterization and culture of *L. crispatus*

*Lactobacillus crispatus* strain M247 was isolated from weaning baby feces and taxonomically identified by a positive hybridization reaction with the *L. crispatus* specific 23S rRNA-targeted probe [22]. Cells grown in MRS (Difco, USA) medium appeared as naked-eye discernible clumps, which sediment at the bottom of the tube, leaving the upper part of the medium clear. A spontaneous non-clumping mutant of M247, named MU5, was isolated from the lower aqueous phase during an hydrophobic assessment test based on water-hexadecane partition assay. Both strains were grown in MRS broth or agar, at 37 °C in anaerobic conditions when required. Cultures are maintained in the bacterial collection of the Instituto di Microbiologia UCSC, Piacenza.

#### 2.3. Scanning electron microscopy

To investigate the ability of *L. crispatus* M247 to adhere to the colonic mucosa, normal full thickness colonic segments (5 × 5 mm) were washed with PBS to remove fecal material, but not adhering mucus, and after 10 min incubation with $10^8$ CFU/ml *L. crispatus* M247 grown for 18 h in MRS (Difco, USA), washed with PBS and fixed with 2.5% glutaraldheyde. After two washings with PBS, samples were dehydrated with ethanol to the critical point, coated with
20 nm of gold in the scanning electron microscopy (SEM) coating unit, and then examined by a SEM XL30 ESEM (Philips).

2.4. Persistence studies

We tested whether _L. crispatus_ M247 and its non-aggregating mutant MU5 administered orally were able to persist in the intestine. We fed normal mice with 10^8 CFU of _L. crispatus_ M247 or MU5 for 3 days and after 48 h fecal and tissue samples were collected from the descending colon. Samples were immediately transferred into Amies medium [25] and stored at 4 °C until they were plated on Rogosa agar (Difco, USA). Plates were incubated under anaerobic conditions (Anaerocult A, Merck, Darmstadt, Germany) for 48 h at 37 °C. Colonies were pre-screened by optical microscopy observation for M247/MU5 morphology and then subjected to strain-specific PCR amplification [22].

2.5. Administration of _L. crispatus_ M247 and MU5

Each treatment (100 μl MRS medium plus 2% skim milk) was administered intragastrically with a polyethylene cannula. To determine the most effective dose, _L. crispatus_ M247 was administered daily at different dosages (10^4, 10^6, 10^8 or 10^10 bacteria). In addition, _B. clausii_ (formerly classified as _B. subtilis_) spores were administered to mice following the same schedule of _L. crispatus_ M247.

2.6. Study design

In order to evaluate a potential therapeutic effect of _L. crispatus_ M247 and MU5 on DSS-induced colitis, mice receiving 5% DSS in drinking water were randomly assigned to receive daily, beginning 24 h after DSS administration was started, the specific treatment for 3 consecutive days and then sacrificed after 24 h (5th day of the experiment).

In order to determine a potential effect on the onset of intestinal inflammation, in a second set of experiments we tested the effect of pre-conditioning the colonic lumen with _L. crispatus_ M247 before the onset of colitis. Mice received 10^8 _L. crispatus_ M247 daily for 5 days prior to the induction of colitis, then 5% DSS was added to drinking water for 5 days. Colitis severity was assessed as described above.

2.7. Statistical analysis

Data are expressed as mean ± standard error. Statistical analysis was performed using ANOVA and Bonferroni's test. Statistical significance was considered for _P_ values < 0.05.

3. Results

3.1. Effect of oral supplementation with _L. crispatus_ M247

On the basis of results previously achieved in human trials [22] showing a greater persistence into the gut of M247, dose finding experiments were performed only with M247. The administration of 10^{10} bacteria for three consecutive days caused weight loss, the appearance of loose stools associated with a macroscopically enlarged caecum, whereas administration of 10^4, 10^6 or 10^8 bacteria had not evident effects on animals. Although colonic mucosal MPO levels in animals receiving 10^{10} _L. crispatus_ M247 were comparable to controls (1.7 ± 0.1 U/mg vs 2.0 ± 0.4 U/mg in tissue of mice receiving no or 10^{10} bacteria, respectively), this dose was not further used.

3.2. Recovery of _L. crispatus_ M247 and MU5 from healthy mice feces and tissues

To determine the involvement of the aggregating phenotype in the persistence of _L. crispatus_ in mouse colon, we fed healthy mice with 10^8 CFU _L. crispatus_ M247 or MU5 for three consecutive days. After 48 h fecal and colonic samples were collected, plated on Rogosa agar and M247/MU5-like colonies were confirmed by strain-specific PCR. While M247 could be recovered from both fecal (5.1 × 10^7 CFU/g) and colonic (7.7 × 10^3 CFU/g) samples, no positive isolates were detected in mice fed with MU5.

3.3. _L. crispatus_ M247 adhesion to colonic mucosa

In order to investigate the ability of _L. crispatus_ M247 to adhere to the colonic mucosa, we incubated colonic explants with 10^8 CFU/ml _L. crispatus_ M247 and then adhering bacteria were detected by SEM. As shown in Fig. 1, _L. crispatus_ M247 efficiently bound to colonic mucus fibers.

3.4. Effect of _L. crispatus_ M247 on DSS-colitis

Mice receiving 5% DSS exhibited within 5 days a wasting syndrome, associated with diarrhea, increased mucosal MPO levels and mucosal ulcerations (Fig. 2). _L. crispatus_ M247 supplementation ameliorated the outcome of DSS colitis in a dose-dependent manner, reducing body weight loss by 78% and 21% in mice treated with 10^8 and 10^6 bacteria, respectively, (Fig. 2). Furthermore, 10^8 and 10^6 _L. crispatus_ M247 reduced by 63% and 35% MPO activity in the colon (Fig. 2). Indeed, administration of 10^8 _L. crispatus_ M247 had no significant effect on colitis outcome (Fig. 2). As shown in Fig. 2, histological examination confirmed that...
administration of L. crispatus M247 reduced the severity of the inflammatory infiltrate and mucosal damage (Fig. 2).

We next evaluated whether live luminal bacteria were required to mediate the protective effects. As shown in Fig. 2, administration of $10^8$ heat killed L. crispatus M247 had no significant effect on DSS colitis. To rule out that the protective effects observed in animals receiving L. crispatus M247 were merely mediated by a competition with other entero-aggressive strains, we investigated the effect of supplementation with B. clausii spores on DSS colitis, previously shown to have a protective effect during colitis triggered by entero-pathogenic E. coli [26]. As shown in Fig. 2, administration of B. clausii spores to mice for 3 days did not modify the onset and the course of colitis.

3.5. Effect of pre-conditioning colonic microflora with L. crispatus M247 on DSS colitis

We next studied whether pre-conditioning of the colonic microflora with L. crispatus M247 before the onset of colitis had any effect on the severity of intestinal inflammation. As shown in Fig. 3, administration of $10^8$ L. crispatus M247 before the onset of colitis had only a partial effect on inflammation reducing the body weight loss and MPO activity by 30% and 50%, respectively.

3.6. Effect of the non-aggregating L. crispatus MU5 on DSS colitis

To investigate the relevance of aggregating properties of L. crispatus M247 on its protective effect on colitis we used a spontaneous isogenic mutant of L. crispatus M247, named MU5 and previously characterized [22]. As shown in Fig. 4 administration of the aggregation deficient L. crispatus MU5 had no significant effect on DSS colitis. Indeed, L. crispatus MU5 administered in a 30% sucrose solution, known to restore the aggregation phenotype, exerted a protective effect on DSS colitis similar to wild type L. crispatus M247 (Fig. 4).
4. Discussion

Recent evidences suggest that an imbalance in the colonic micro flora may predispose to the development of an abnormal response by the host immune system contributing to the development of colitis [1,9]. Indeed, dietary manipulation known to increase the colonic concentration of lactobacilli or the direct administration of *Lactobacillus* spp. significantly reduced the severity of spontaneous or chemically induced colitis in animals [19,27–29], and was effective in the treatment of patients with UC or pouchitis [30–32]. Although many empirical studies demonstrate that selected bacteria may beneficially influence the outcome of gastrointestinal diseases, the work to define the criteria to choose bio-therapeutic agents clearly lags behind [21]. Thus, new strains potentially useful for therapeutic applications are identified on their ability to survive to gastric acid, tolerate bile salts and adhere to gut mucus and epithelial cell monolayers but no attention is paid to identify bacterial characteristics required to exert protective effects [21,33].

We report here that oral supplementation with a single *Lactobacillus* strain recently isolated from healthy humans, *L. crispatus* M247, reduces the severity of DSS induced colitis in mice. *L. crispatus* M247 effects were dose-dependent and required live bacteria suggesting that in order to exert protective effects lactobacilli must reach an adequate level in the intestinal lumen. Indeed discontinuation of *L. crispatus* M247 administration prior to colitis induction was associated with only partial protective effects, that were more evident in the initial phase of the experiments (data not shown).

Recent experimental data suggest that *B. clausii* spores administered to mice may germinate and persist in the gastrointestinal tract and protect experimental animals challenged with enteropathogenic *E. coli* [26,34,35]. However, in agreement with previous reports, administration of *B. clausii* spores was ineffective to modify the outcome of DSS colitis [34], suggesting that *L. crispatus* M247-mediated protective effects were specifically linked to the strain and not simply due to oral supplementation with viable bacteria. Therefore, different microbial species may be beneficial for distinct...
gastrointestinal disorders since they have different effect on the intestinal microbial flora and mucosal immune system [1,14].

This is the first study taking advantage of isogenic variants of lactobacilli to investigate the role of a specific bacterial phenotype on its therapeutic efficacy. We compared the therapeutic effects of wild-type L. crispatus M247 and its spontaneous mutant strain MU5 obtained without any chemical or physical mutagenic treatment [22]. Previous studies by plasmid-profiling, RAPD-PCR and pulsed field gel electrophoresis have demonstrated that M247 and MU5 strains were indistinguishable, supporting their relatedness [22]. The only phenotypic difference between M247 and MU5 was the lack, in the mutant strain, of the receptor for the co-aggregation promoting factor secreted by Lactobacillus M247, a pro-aggregating strategy already described in Lactobacillus 4B2 [22]. This trait was associated with M247 ability to consistently adhere to intestinal epithelial cell lines, human intestinal mucus and to mice colonic explants in vitro [22] and Fig. 1. Indeed, the aggregating phenotype was also required to recover L. crispatus M247 in fecal samples and colonic biopsies from humans or mice following oral supplementation [22]. The different ability of M247 and MU5 to persist in the colon, however, is not simply attributable to the failure of MU5 to survive to the harsh gastrointestinal environment since the two strains showed comparable behaviors regard growth rate and tolerance to acids and bile [22]. Indeed, restoring the aggregating phenotype by administering MU5 in a 30% sucrose solution, we were able to reestablish the protective effects on colitis. Taken together these data strongly suggest that the aggregating phenotype is a required bacterial trait for successful persistence in the mouse colon and a pre-requisite to exert a protective effect during intestinal inflammation.

The mechanism(s) involved in bio-therapeutic agents protective effects during intestinal disorders have not been completely elucidated but probably result from combined effects, some of them probably are in common with the normal commensal microflora [36,37]. Thus, Lactobacillus spp. balance the release of cytokines from the intestinal epithelium preventing exaggerate responses to bacterial lipopolysaccharide for example interfering with IkB ubiquitination occurring in epithelial cells exposed to enteric pathogens [36–39]. An alternative mechanism of action involve the enhancement of the intestinal epithelium barrier function by upregulating mucin gene expression or tight junction protein integrity [36–39]. Oral supplementation with Lactobacillus spp. exert local and systemic immuno-modulatory effects enhancing the release of Th2- over Th1-type cytokines from immune cells [40] and promoting the release of secretory IgA [41,42]. Moreover, live Lactobacillus spp. interact with intestinal epithelial cells to limit adhesion and invasion of pathogens [43]. Finally, the immuno-modulatory activity of probiotics may directly rely on their ability to produce proteins able to inhibit the synthesis or action of host pro-inflammatory cytokines [37].

However, to date researchers have focused on establishing the mechanism(s) behind the beneficial interaction between colonic epithelium and micro biota, paying no attention to probiotics characteristics [30,40]. We identified for the first time a probiotic characteristic that appears relevant to exert protective effects on colitis: the aggregation phenotype. The ability to co-aggregate or aggregate with bacteria belonging to different genera may represent a relevant strategy in L. crispatus M247-mediated protective effects. Indeed, lactobacilli aggregate with E. coli strains or enterococci [22,44]. Aggregation within other bacterial species in the dental plaque, and with yeast is a well known strategy to enhance microorganisms survival in hostile environments [45,46]. In addition, aggregation may favor exchange of genetic material allowing the lactobacilli to acquire new phenotypic characteristics [47]. Since live, aggregating L. crispatus M247 cells are required to obtain beneficial effects in vivo (Fig. 2) we may speculate that the aggregation phenotype, a common feature among enteric Lactobacillus spp., is required to enhance bacterial interaction with intestinal epithelium and eventually to bring in contact with the mucosa different bacterial species co-aggregating with L. crispatus M247.

In conclusion, using a new bacterial model we show for the first time that the protective effects observed during colitis require a specific bacterial trait such as aggregation, which was already shown to determine the persistence into the human gut during in vivo trials. Although it is surprising that a single phenotypic trait could have a so deep impact on the in vivo behavior of a bacterial strain, our efforts should be directed to identify other bacterial factors required to modify intestinal ecology and immune system to rationally design a new generation of probiotics, the so-called “bio-therapeutic agents”.

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