# On Lactobacillus plantarum 299v, bacterial translocation and intestinal permeability.

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# LACTOBACILLUS PLANTARUM 299V INHIBITS ESCHERICHIA COLI-INDUCED INTESTINAL PERMEABILITY

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Running head: *L. plantarum* and intestinal permeability.

**Abstract**

The purpose of this work was to investigate whether a probiotic bacterium, *Lactobacillus plantarum* 299v, could affect *Escherichia coli*-induced passage of mannitol across the intestinal wall. Sprague-Dawley rats were pretreated for one week by either tube-feeding with *L. plantarum* 299v twice daily, free access to *L. plantarum* 299v by adding the bacterium in the drinking water or negative control receiving regular feeding. Intestinal segments were mounted in Ussing chambers and the mucosa was exposed to control medium, *E. coli* and *L. plantarum* 299v (alone or together). $^{14}$C-mannitol was added as a marker of intestinal permeability and samples were taken from the serosal side. *E. coli* exposure induced a 53% increase in mannitol passage across the intestinal wall ($P < 0.05$). One week of pretreatment with *L. plantarum* 299v in the drinking water abolished the *E. coli*-induced increase in permeability. Tube-feeding for one week or short term addition of *L. plantarum* 299v in the Ussing chambers had no effect on the permeability provoked by *E. coli* challenge. Notably, *L. plantarum* 299v itself did not change the intestinal passage of mannitol. These data demonstrate that pretreatment with *L. plantarum* 299v, which is a probiotic bacterium, protects against *E. coli*-induced increase in intestinal permeability, and that *L. plantarum* 299v alone has no influence on the intestinal **permeability**. Thus, this study support the concept that probiotics may exert beneficial effects in the gastrointestinal tract.

**Key words**

*Lactobacillus plantarum*, *Escherichia coli*, intestinal mucosa, permeability, probiotic bacteria, Ussing chamber.

**Introduction**

The intestinal mucosa plays a fundamental role in sustaining a physical barrier against translocation of pathogenic bacteria and noxious substances from the luminal content of the bowel (1). Altered intestinal permeability is an important part of the pathogenesis in several critical conditions, such as major trauma (2), burn injuries (3) and sepsis (4). In addition, an accumulating body of evidence is indicating that chronic inflammatory bowel disease is associated with increased intestinal permeability (5-7). Numerous studies have demonstrated that pathogenic bacteria, such as *Salmonella typhi* (8) and *Escherichia coli (E. coli)* (9), may disrupt the intestinal barrier and enhance permeability in the gastrointestinal tract, although the detailed mechanisms regulating the integrity of the intestinal mucosa remain elusive.

In recent years, an increased effort has been devoted to study the role of potentially beneficial bacteria present in the gastrointestinal tract (10-14). This group of protective microorganisms is referred to as probiotic bacteria and includes several different species, such as lactobacilli and bifidobacteria (15). Interestingly, it has been reported that lactobacilli may reduce bacterial translocation (16) and intestinal inflammation (12,14,17). However, it is not known whether probiotic bacteria, such as lactobacilli may counteract disturbed intestinal integrity induced by pathogenic bacteria.

Based on the above considerations, the objective of the present study was to examine if administration of *Lactobacillus plantarum* 299v *(L. plantarum* 299v) may inhibit *E. coli*-induced increase in permeability in the rat intestine. For this purpose we analyzed the passage of mannitol in the Ussing chamber model.

## Material and Methods

### *Animals*

Male Sprague-Dawley rats (Möllegaard, Skensved, Denmark), weighing 385–456 g, were kept two and two on chopped wood bedding in polycarbonate cages in a 12 hour light/dark cycle at 20 ± 2°C and a relative humidity of 50 ± 10% for at least one week before starting the experiments. The rats had free access to standard rat chow (B & K Universal, Sollentuna, Sweden) and water *ad libitum*. The experiments were approved by the local animal ethics committee at Lund University, Sweden.

### *Bacterial strains and cultivation*

*L. plantarum* 299v (DSM 9843), described by Johansson *et al* (18), was provided by Probi AB (Ideon, Lund, Sweden) and grown stillstanding at 37°C overnight in Lactobacillus Carrying Medium (LCM) (19) supplemented with 1% glucose.

*E. coli* F131was provided by I. Adlerberth (Department of Clinical Immunology, Göteborg University, Göteborg, Sweden) and grown overnight, aerated by vigorous shaking, at 37°C in Brain Heart Infusion (BHI; Difco, Detroit, MI, USA).

The bacteria were harvested by centrifugation, washed twice and resuspended in phosphate-buffered saline (PBS; Difco, Basingstroke, Hampshire, England).

*Pretreatment with L. plantarum* 299v

A total of 25 rats were divided into three groups (Fig. 1) one week before the Ussing chamber experiments: group A (regular feeding only) $n = 8$; group B (regular feeding + tube feeding twice daily with two ml of oatmeal drink), $n = 9$; group C (regular feeding + free access to oatmeal drink which was mixed with the drinking water), $n = 8$. The oat meal drink contained $10^9$ colony-forming units (CFU) of *L. plantarum* 299v/ml. There were no differences in weight gain between the experimental groups.

*Ussing chamber experiments*

Anesthesia was induced and maintained with diethyl-ether. Through a midline incision, 25 cm of the small intestine proximal to the ileo-caecal junction was harvested without its mesentery and divided into twelve segments. Three segments were used for each treatment group in the Ussing chamber experiments. The segments were immediately immersed in room-tempered oxygenated Krebs´ buffer (composition in mM: NaCl 110.0, $CaCl_2$ 3.0, KCl 5.5, $KH_2PO_4$ 1.4, $NaHCO_3$ 29.0, Na-pyruvate 5.7, Na-fumarate 7.0, Na-glutamate 5.7, glucose 13.4) and equilibrated with carbogen (95% $O_2$ and 5% $CO_2$) to maintain physiological pH.

The twelve full thickness intestinal segments were cut along the mesenteric border, rinsed from fecal content in Krebs´ buffer and mounted in iodine decontaminated (Jodopax 5%, Mölnlycke, Sweden) Ussing chambers (Precision Instrument Design, Los Altos, CA, USA) with an exposed area of 1.78 $cm^2$. The Ussing chambers were filled with Krebs´ buffer and continuously bubbled with carbogen until application of the permeability marker. The circulation in the Ussing chambers was maintained by gas lift and temperature kept at 37°C.

All experiments started within 45 min of laparotomy. At t = 0, the Krebs´ buffer in all mucosal reservoirs was substituted with five ml Krebs´ buffer containing the permeability marker $^{14}$C-mannitol (0.031 µCi/ml, MW 182 Da; DuPont, Dreieich, Germany). Intestinal segments from the different pretreatment groups described above were treated with *L. plantarum* 299v and/or *E. coli*, as shown in Fig. 1. Controls received no bacteria in the Ussing chamber. After 20, 40, 60 and 120 min, one ml samples were taken from the serosal chamber and replaced with one ml Krebs´ buffer.

*Passage analysis*

The amount of $^{14}$C-mannitol passage was determined in a beta counter (LKB, Bromma, Sweden) by mixing the serosal sample with five ml liquid scintillation cocktail (Ready Safe™, Beckman, Fullerton, CA, USA).

*Tissue viability*

The transmucosal potential difference (PD, mV) was measured at 0 and 20, 40, 60 and 120 min using Ag/AgCl electrodes embedded in 3 M KCl agar, with one electrode placed in each reservoir and connected to a millivoltmeter (Millicell–ERS, Millipore, Stockholm, Sweden). One rat in group A and two rats in group B was excluded due to insufficient baseline viability, *i.e.* PD was less than 3.0 mV, of intestinal specimens.

*Statistics*

Data are presented as mean ± SEM. Multiple comparisons for unpaired groups were analyzed by use of One Way Repeated Measures Analysis of Variance with Tukey´s test *post hoc*.

**Results**

The transmucosal potential difference (PD) at the start of the experiment, was significantly higher in the untreated group (group A) (Fig. 2 a-c) but decreased similarly in all groups during the course of the experiment and reached similar levels after 120 min. Mannitol permeated across the intestinal segments at a constant rate in all groups during the experiment as indicated by a continuous and linear increase in the amount of mannitol accumulated on the serosal side (data not shown).

Administration of *E. coli* in the Ussing chambers significantly increased the permeability of mannitol by 53 % after 120 min compared to negative controls in group A (Fig. 3, $P < 0.05$, *E. coli* vs. control). Coadministration of *L. plantarum* 299v in the chambers did not reduce the increased permeability to mannitol triggered by *E. coli* in group A (Fig. 3, $P > 0.05$), indicating that acute application of *L. plantarum* 299v in the Ussing chambers had no protective effect against intestinal leakage. Similarly, in group B, which was pretreated by tube-feeding with *L. plantarum* 299v for one week, it was found that *E. coli* enhanced mannitol permeability (Fig 3, $P < 0.05$) and acute addition of *L. plantarum* 299v in the Ussing chambers had no effect in preventing this ($P > 0.05$ vs. *E. coli* alone). In contrast, in group C with free access to *L. plantarum* 299v for one week, it was observed that the *E. coli*-provoked passage of mannitol was abolished, indicating a beneficial impact of long-term pretreatment with *L. plantarum* 299v on pathological permeability changes (Fig. 3, $P < 0.05$). Noteworthy, neither pretreatment nor acute administration of *L. plantarum* 299v *per se* had any effect on mannitol passage (Fig. 3).

The amount of *L. plantarum* 299v consumed by the rats with free access to oatmeal drink containing the bacteria mixed with drinking water (group C) was four times the amount administered to the rats which were tube-fed with the same oat meal drink (group B).

**Discussion**

In the present study, using small intestine from rats mounted in Ussing chambers, we found that presence of *E. coli* increased permeability to mannitol across the intestinal wall. One week of pretreatment with *L. plantarum* 299v in the drinking water abolished this increased permeability induced by *E. coli*. In contrast, pretreatment by tube-feeding twice daily or short term administration of *L. plantarum* 299v in the Ussing chambers had no effect on *E. coli*-induced intestinal permeability. These novel findings provide evidence supporting the concept that *L. plantarum* 299v may exert beneficial effects in the gastrointestinal tract and may be a potential tool in preventing pathological permeability in the intestine.

Changes in intestinal permeability have been suggested to be a pathophysiologic mechanism underlying inflammatory bowel disease (5-7) and sepsis (4) and is thought to play an important role in association with major trauma (2) and burn injuries (3,20) in which the indigenous microflora may ultimately translocate and cause endotoxemia. However, the effect of lactobacilli on pathological permeability in the intestine has yet not been evaluated.

Being one of the most common bacteria of the indigenous microflora of the gut (21), *E. coli* is often cultured from the blood and abscesses in patients suffering from sepsis and critical illness (22), but is also found at extraintestinal sites in the absence of septic events (23). Previous studies have shown that *E. coli* might affect the intestinal barrier and thus cause disturbed integrity (9). Our results demonstrating an increased passage of mannitol across intact intestinal segments after exposure to *E. coli* expand on previous studies reporting an increased mannitol flux across cultured human small intestinal cells exposed to pathogens, such as *Salmonella typhi* (8) and *E.*

*coli* (9). The pathway across the intestinal mucosa for mannitol, a small hydrophilic molecule, is not clearly defined at present (24). However, the increased permeability to mannitol provoked by *E. coli* in this study may be explained by either an increased paracellular mucosal-to-serosal water flux with a concomitant mannitol solvent drag, or an increased transcellular passive diffusion, as this may be of greater importance than solvent drag in the ileum compared to colon (24).

Herein, it was observed that *L. plantarum* 299v given *ad libitum* for one week completely prevented the *E. coli*-induced increase in permeability to mannitol. This potent effect was not seen when the rats were pretreated twice daily with *L. plantarum* 299v by tube-feeding. This discrepancy may be attributable to the fact that rats with free access to *L. plantarum* 299v received a total amount of bacteria four times greater than that of the tube-fed rats, indicating that the quantitative load of lactobacilli may be of importance in order to achieve biological effects in the gastrointestinal tract**. In separate experiments we found that free access to *L. plantarum* 299v caused a clear-cut colonization of this bacterium in the colonic mucosa (data not shown). This colonization** may thus compete with other bacteria, in this case with *E. coli*, and exclude them from binding sites on the mucosa. Previous studies have shown that *L. plantarum* 299v express a mannose-rich adhesin receptor for epithelial cells, similar to a receptor on *E. coli*, constituting a possible basis for a mutually exclusive competition for binding sites on the mucosal surface between *L. plantarum* 299v and *E. coli* (25). **Notably, *L. plantarum* 299v did not decrease baseline passage of mannitol across the intestine, indicating that** *L. plantarum* **299v has no general effect on mannitol permeability but rather may exert a specific effect against pathogen-induced permeability changes.**

Another possible explanation for the effect achieved when the rats where fed *L. planatarum* 299v *ad libitum* may be attributable to the fact that the lactobacilli increase the amount of short chain fatty acids, which are known to be important nutrients for intestinal epithelial cells (26). Thus, *L. plantarum* 299v may on one hand reduce the number of adherent *E. coli* and on the other hand increase the amount of luminal nutrients available for the mucosal epithelial cells.

Besides the above described quantitative effect, we also found a time-dependent factor in the protective effect of *L. plantarum* 299v on *E. coli*-induced permeability. Thus, it was observed that acute (two hours) administration of *L. plantarum* 299v had no effect on mannitol flux provoked by *E. coli*. In most reports on the effect of lactobacilli, the bacterium has been administered for at least five days in order to achieve a biological effect (12,16,25,27). However, it is not presently known how long duration of lactobacilli administration that is required to exert protective effects in the gut. However, based on these considerations, it may be suggested that both the amount and "timing" may be of importance in achieving a protective effect of *L. plantarum* 299v against pathological changes in the gastrointestinal tract.

Although *L. plantarum* 299v was given at a high concentration no adverse effects were observed, *i.e.* there was no difference in weight gain between the fed and unfed groups. Furthermore, exposure of intestinal segments to *L. plantarum* 299v in the Ussing chambers did not change the passage of mannitol. These findings indicate that *L. plantarum* 299v is safe to administer with respect to nutritional interference and intestinal **permeability**.

When studying physiological processes *in vitro* the viability of the specimen studied is of concern. Transmucosal PD, as used in this study, is a simple method and adequately reflects viability of the intestinal segment studied (28). PD is dependent on ATP-dependent $Na^+/K^+$-pumps, which are located in the basolateral membrane of the enterocyte and maintain intracellular electronegativity by lowering intracellular sodium concentration (29). The changes in PD over time as observed here are similar to the results obtained in other studies on rats (30-32).

In summary, our novel data demonstrate that pretreatment by free access to *L. plantarum* 299v protects against *E. coli*-induced increase in permeability in the rat intestine, whereas acute and intermittent treatment had no effect on the pathological leakage. These findings may help explain the beneficial properties of *L. plantarum* 299v in the gastrointestinal tract as reported previously.

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**Figure Legends**

**Fig. 1**. Pretreatment one week before *in vitro* experiment: group A = no pretreatment, group B = tube-feeding twice daily with *L. plantarum* 299v ($10^9$ CFU/ml) in two ml oatmeal and group C = free access to *L. plantarum* 299v ($10^9$ CFU/ml) in oatmeal added to drinking water. Bacteteria added in Ussing chamber at the start of the experiment: L = *L. plantarum* 299v, L + E = *L. plantarum* 299v + *E. coli*, E = *E. coli* and P = phosphat-buffered saline (control). Samples of one ml were taken from the serosal side of each Ussing chamber at 20, 40, 60 and 120 min.

**Fig. 2.** Transmucosal potential difference (PD, mV) in the different pretreatment groups during *in vitro* experiments in the Ussing chambers. Group A = no pretreatment, group B = tube-feeding twice daily with *L. plantarum* 299v ($10^9$ CFU/ml) in two ml oatmeal and group C = free access to *L. plantarum* 299v ($10^9$ CFU/ml) in oatmeal added to drinking water. Data are mean values ± SEM.

Fig. 3. Passage of mannitol expressed as percentage of initially added marker molecule across intestinal segments mounted in Ussing chambers at 120 min. Bacteria added in the Ussing chambers: L = *L. plantarum* 299v, L + E = *L. plantarum* 299v + *E. coli*, E = *E. coli* and P = phosphat-buffered saline (control). Data are mean values ± SEM.

* = $P < 0.05$ L + E and E *vs.* PBS

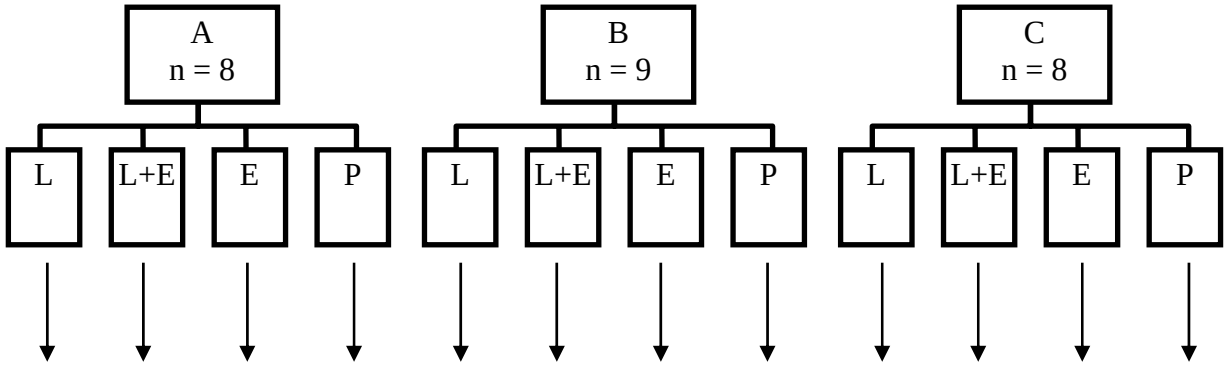
# = $P < 0.05$ L + E in group C *vs.* L + E group A and B

¤ = $P < 0.05$ E in group C *vs.* E in group A and B

# Fig 1

Pretreatment one week before *in vitro* experiment

Bacteria added in Ussing chamber




One ml samples taken at 20, 40, 60 and 120 min

# Fig 2

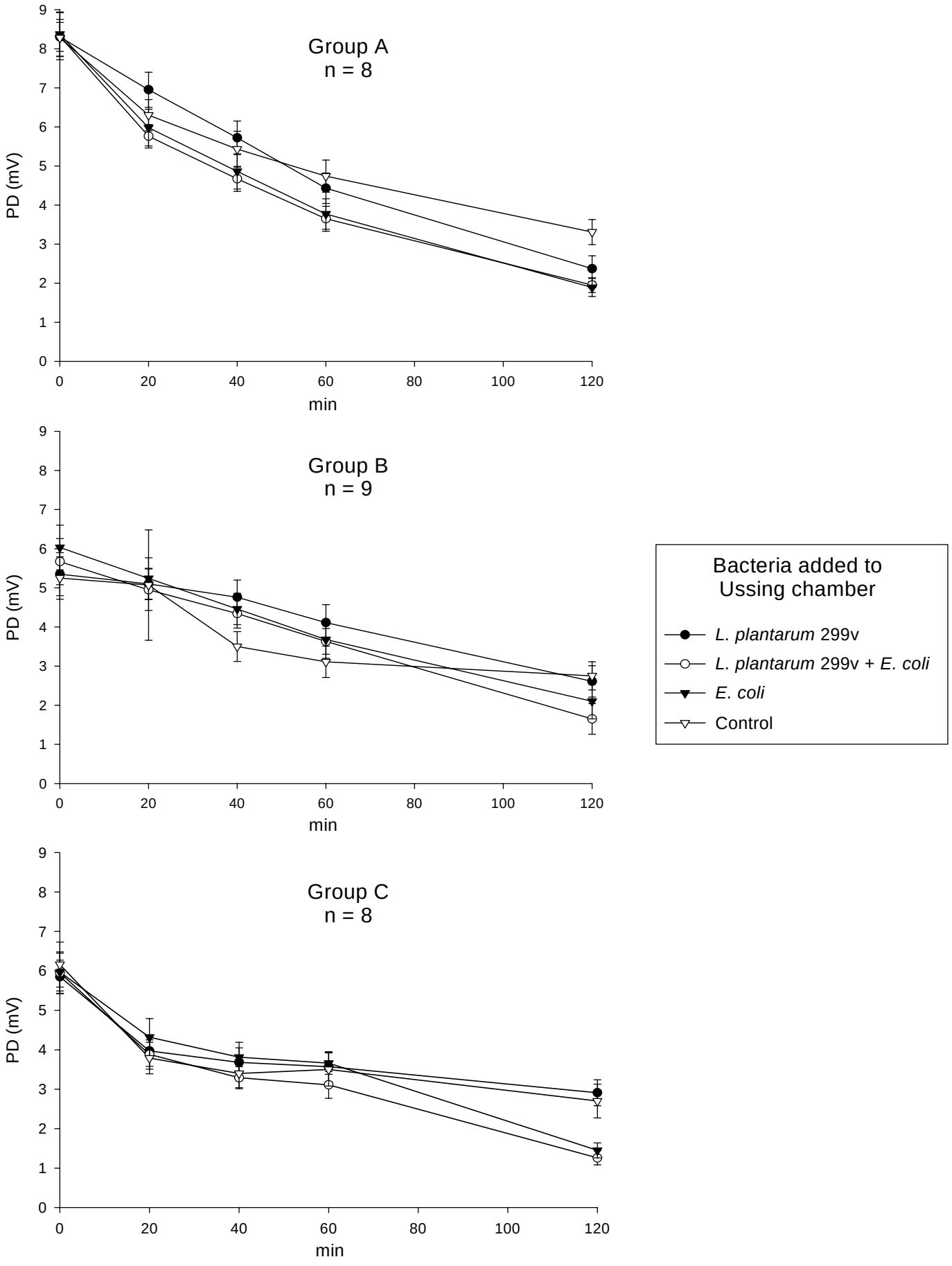
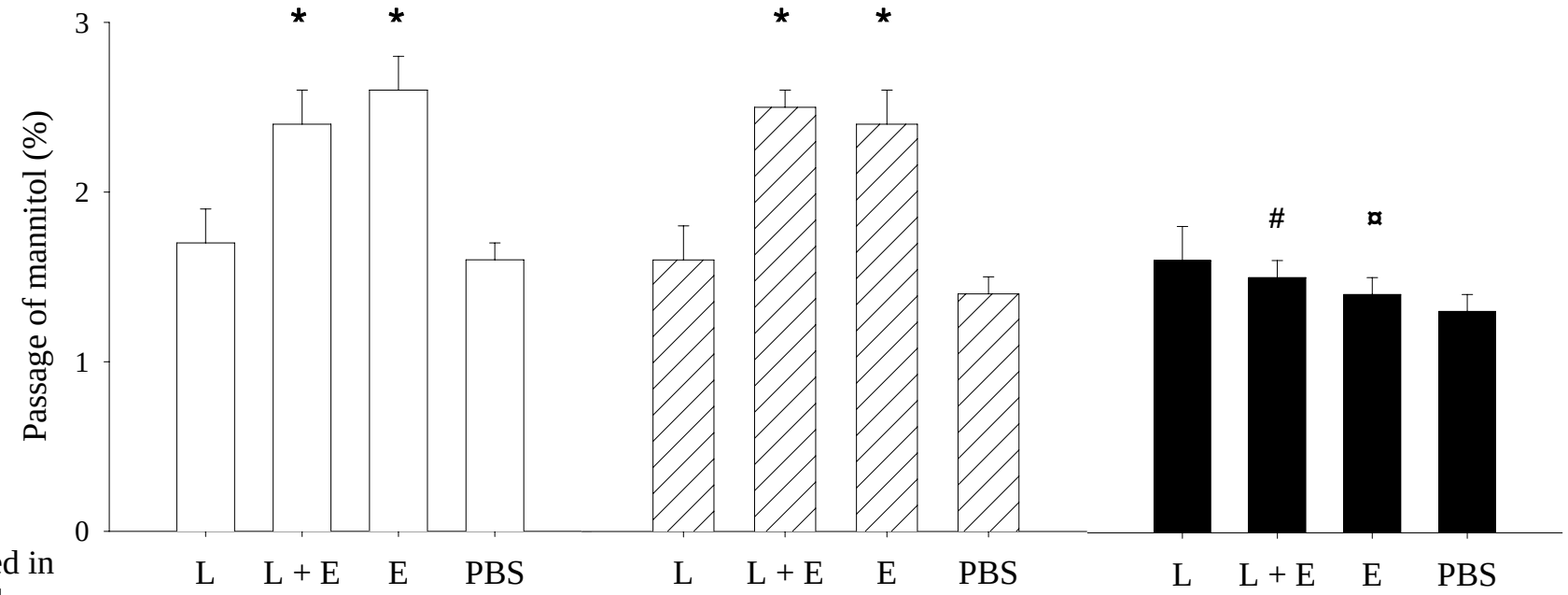

Group A
n = 8
PD (mV)
min
Group B
n = 9
PD (mV)
min
Group C
n = 8
PD (mV)
min
Bacteria added to Ussing chamber
L. plantarum 299v
L. plantarum 299v + E. coli
E. coli
Control

**Fig 3**


Passage of mannitol (%)
3
2
1
0
*
*
*
*
#
¤
L
L + E
E
PBS
L
L + E
E
PBS
L
L + E
E
PBS
Bacteria added in Ussing-chamber
Pretreatment
No pretreatment (group A, n = 8)
Tubefeeding twice daily with *L. plantarum* 299v (group B, n = 9)
Free access to *L. plantarum* 299v (group C, n = 8)