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Physiological bioactivity of a postbiotic consisting of heat-treated lactobacilli on mouse small intestine

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ABSTRACT

Lactobacillus LB is a postbiotic generated following fermentation by *Limosilactobacillus fermentum* and *Lactobacillus delbrueckii*. *Lactobacillus* LB alleviates acute diarrhoea and ameliorates the symptoms of irritable bowel syndrome. Here, we investigated whether modulation of intestinal ion transport and motility contributes to these beneficial effects and whether the postbiotic produced with both strains contributes to a unique biophysiological profile. In Ussing chamber studies, low lactose-*Lactobacillus* LB (LL-LB) significantly increased baseline short-circuit current, and this was partially mediated by sodium-D-glucose transporter 1. In organ baths, LL-LB significantly decreased ileal tone and increased carbachol-induced contractility. Relative to LL-LB, preparations produced using a single strain fermentate generated from *L. fermentum* significantly increased baseline short-circuit current and inhibited carbachol-induced contractility. Our data demonstrate a unique biophysiological profile for the dual strain postbiotic and support a direct and immediate effect of LL-LB on host physiology *ex vivo* which could contribute to the clinical efficacy of *Lactobacillus* LB.

1. Introduction

A postbiotic is defined as "a preparation of inanimate microorganisms and/or their components that confers a health benefit on the host" (Salminen et al., 2021). Postbiotics include inactivated microbial cells, cell-free supernatants, and components, commonly inactivated by heat (Mosca et al., 2022). Therefore, postbiotics could act in many ways by interacting directly with the host, by modifying the gut microbiome or both (Mosca et al., 2022; Salminen et al., 2021). *Lactobacillus* LB is defined as a heat-treated fermentate and microbial biomass generated by two *Lactobacillus* strains, *L. fermentum* and *L. delbrueckii* with a ratio of 95:5 respectively. *Lactobacillus* LB is a proprietary product (Adare Biome) and contains the similar active ingredients as Lactéol, an approved anti-diarrheal medication (Liévin-Le Moal, 2016). Moreover, there is strong evidence to support the efficacy of *Lactobacillus* LB in both infectious and non-infectious diarrhoea (Liévin-Le Moal et al., 2007; Salazar-Lindo et al., 2007; Simakachorn et al., 2000; Xiao et al., 2003).

Some of the beneficial properties of *Lactobacillus* LB which might account for its anti-diarrhoeal effects include antibacterial activity against diarrhoea-causing pathogens (Chauvière et al., 1992; Coconnier, Bernet, Chauvière, & Servin, 1993). For example, the heat-killed preparation of *Lactobacillus* LB can adhere to enterocytes and inhibit adhesion of diarrhoeagenic diffusely-adherent *Escherichia coli* (Coconnier, Bernet, Chauvière, & Servin, 1993). Additionally, effects of *Lactobacillus* LB on the gut-brain axis (Warda et al., 2019) could potentially account for the symptom improvement observed in patients with irritable bowel syndrome (IBS) (Halpern et al., 1996). However, modulation of intestinal electrolyte transport, gut barrier function and motility could also

Abbreviations: AEEC, Animal Experimentation Ethics Committee; ANO1, Anoctamin-1 (also known as TMEM16A); CCh, Carbachol; CFTR, Cystic fibrosis transmembrane conductance regulator; CLC-2, Chloride Channel ClC-2; ENaC, Epithelial sodium channel; ENS, Enteric nervous system; I_{sc}, Short-circuit current; LGG, *Lacticaseibacillus rhamnosus* GG; LL-LB, Low lactose-*Lactobacillus* LB; NKCC1, Na-K-Cl cotransporter 1; PPQ, PPQ-102; SGLT1, Sodium-D-glucose transporter 1; TER, Trans-epithelial resistance; TMEM16A, Transmembrane member 16A (also known as ANO1); TTx, Tetrodotoxin.

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contribute to the anti-diarrhoeal effects of *Lactobacillus* LB as well as the beneficial effects in IBS. Several mechanisms have been proposed by which commensal organisms modulate epithelial ion transport, including transcriptional regulation of genes encoding epithelial ion exchangers, increasing ion channel activity, and modulation of intracellular signalling pathways (Lomasney & Hyland, 2013). Moreover, microbial effects on gut motility are often evoked by bioactive molecules from various sources, including microbial breakdown of carbohydrates, fibres, or proteins (Waclawiková et al., 2022).

The small intestine is a major site of nutrient digestion and absorption (Chang & Martinez-Guryn, 2019). There is also an intrinsic relationship in the small intestine between ion transport across the epithelium and motility which may involve sequential activation of the intrinsic nervous system (Greenwood & Davison, 1987). Furthermore, the small intestine also plays an important role in the pathophysiology of infectious diarrhoea (Hodges & Gill, 2010). For example, bacterial enterotoxins stimulate intestinal secretion and propulsive motility via activation of enteric neurones (Lundgren & Svensson, 2003). Moreover, the pathogenesis of Rotavirus-associated diarrhoea involves enterocytes in the upper and mid small intestine (Lundgren & Svensson, 2001). Despite this, little research has focused on host-microbe interactions occurring in this region of the gastrointestinal tract (Chang & Martinez-Guryn, 2019). Therefore, our aim in this study was to increase the mechanistic understanding of the anti-diarrhoeal properties of *Lactobacillus* LB by assessing the effects of a low lactose preparation on small intestinal physiology and to determine whether these are unique to the dual strain fermentate.

2. Materials and methods

2.1. Materials

PPQ-102 (PPQ; CFTR Inhibitor IV) was obtained from Medchemexpress (New Jersey, USA) and tetrodotoxin citrate (TTx) from Tocris (Bristol, United Kingdom). All other chemicals were acquired from Merck Life Science Limited (Arklow, Ireland). Stocks were prepared in water or DMSO as follows, with diluent and working concentration in parenthesis; amiloride (water, 100 μM), carbachol (CCh; water, 100 μM), forskolin (DMSO, 10 μM), furosemide (DMSO, 100 μM), phloridzin (DMSO; 1 mM), PPQ–102 (DMSO, 50 μM), and TTx (water, 3 μM).

2.2. Low lactose-Lactobacillus LB and L. Fermentum preparation for ex vivo studies

Low lactose-*Lactobacillus* LB (LL-LB) was prepared by Adare Biome (Houdan, France) and represents the active ingredient of *Lactobacillus* LB and of their commercial product Lactéol. *Lactobacillus* LB is generated through co-fermentation of *Limosilactobacillus fermentum* CNCM MA65/4E-1b (formerly known as *Lactobacillus fermentum* CNCM MA65/4E-1b) and *Lactobacillus delbrueckii* subsp. *delbrueckii* CNCM MA65/4E-2z (95% and 5% respectively) which are then inactivated through extensive heat treatment and freeze dried using lactose as a carrier (Warda et al., 2019). Given that lactose can influence small intestinal short-circuit current (I_{sc}) in Ussing chambers (Murray et al., 1993), all experiments were conducted with the low lactose version of *Lactobacillus* LB (LL-LB). LL-LB is extracted from the production pipeline prior to freeze drying. 1 ml of LL-LB contains 5–10 $\times$ 10^9 dead cell bodies and metabolites that were generated during the fermentation from the culture medium by the living bacteria prior to inactivation. Unless otherwise stated, LL-LB was filtered (first through a 0.45 μm sterile filter, and then through a 0.2 μm sterile filter) and diluted in Krebs buffer to the final working concentrations (5% LL-LB is equivalent to 2.5–5 $\times$ 10^8 cell bodies).

L. fermentum fermentates were produced at APC Microbiome Ireland. 10 ml MRS broth were inoculated with a single colony of *L. fermentum* from MRS agar plates. The inoculated broth was incubated anaerobically overnight at 37 °C. 1% of this overnight culture was then used to inoculate flasks with MRS broth supplemented with L–cysteine (final concentration 0.6 g/litre) to reproduce the production process of the dual fermentate. Following anaerobic overnight incubation at 37 °C, the culture was distributed into large Petri dishes and stored at –80 °C until freeze-drying. Freeze-dried content was scraped off the plates and resuspended in water (0.34 g/ml) before extensive heat treatment (1 h at 110 °C) which is also applied during the production of *Lactobacillus* LB. Resuspended powder was stored at 4 °C and filtered (first through a 0.45 μm sterile filter, and then through a 0.2 μm sterile filter) before use.

2.3. Lactobacillus LB diet preparation

Lactobacillus LB powder was prepared by Adare Biome. 1 g of the final powder contains 60 $\times$ 10^9 dead cell bodies and metabolites generated during the fermentation process. This powder was incorporated into a low-fat rodent chow (10% fat, Diet 12450 K, Research Diets, Inc, New Jersey, USA) at a concentration of 5% (3 $\times$ 10^9/g food). Control animals were administered a matched diet with equivalent nutrient composition. Animals were kept on the *Lactobacillus* LB or control diet for 4–6 weeks before being euthanised by decapitation for Ussing chamber experiments.

2.4. Ethical approval

All animal experiments were performed in accordance with the European Directive 2010/63/EU and approved by the Animal Experimentation Ethics Committee (AEEC) of University College Cork (AEEC application 21-003).

2.5. Animals used for ex vivo studies

Forty-four 10–18-week-old male and female C57BL/6 mice (Envigo, Bicester, United Kingdom) were housed in enriched (cardboard tubes and shredded paper) open-top cages in groups of 3–4 animals. After delivery, mice were allowed to acclimatise to the animal unit (21 $\pm$ 1 °C, at a humidity of 55 $\pm$ 10%, 12 h light/dark cycle) for 1–2 weeks with ad libitum access to food and water prior to euthanasia.

2.6. Animals used for in vivo feeding study

Twenty-six 6-week-old male and female C57BL/6mice (Envigo) were randomly assigned in groups of 2–3 animals to enriched (cardboard tubes and shredded paper) individually ventilated cages for 2 weeks of acclimatisation. Animals were housed at 21 $\pm$ 1 °C, at a humidity of 55 $\pm$ 10%, with a 12 h light/dark cycle and ad libitum access to control diets and water. After acclimatisation, animals were arbitrarily assigned to treatment groups and fed ad libitum either the *Lactobacillus* LB-containing or control diet 4–6 weeks prior to tissue collection.

2.7. Faecal water content

Faecal pellets were collected from mice fed the *Lactobacillus* LB-containing or control diet. Pellets were then weighed (wet weight, in mg), desiccated in an oven (50 °C, 48 h), and weighed again (dry weight, in mg). Faecal water content was calculated according to the equation: water content (%) = 100 (wet weight - dry weight)/wet weight.

2.8. Ussing chamber studies

Mice were euthanised by decapitation and intestinal or colonic tissue was excised. Distal ileal segments were collected 10 cm proximal to the ileocecal junction and colonic segments were collected proximal to the anus and represent distal to early proximal colon. Seromuscular stripping was carried out by blunt dissection under a stereomicroscope. Both the longitudinal and circular muscle layers were removed. The resulting mucosal-submucosal segments were mounted in Ussing chambers

(NaviCyte Vertical Ussing Chamber System WI-660075, ADInstruments, Oxford, United Kingdom; exposed tissue area of 0.12 cm^2), maintained in 4 mls (serosal and mucosal chambers) Krebs solution (1.2 mM NaH_2PO_4, 117 mM NaCl, 4.8 mM KCl, 1.2 mM $MgCl_2$, 25 mM $NaHCO_3$, 11 mM $CaCl_2$, and either 10 mM glucose (serosal) or 10 mM mannitol (mucosal)) at 37 °C and oxygenated with carbogen gas (95% O_2, 5% CO_2). Tissues were voltage clamped to zero using an automatic voltage clamp (DVC-1000, World Precision Instruments, Florida, USA) and were left to stabilise for approximately 20 min. Once a stable baseline was achieved, basal I_{sc} and trans-epithelial resistance (TER; Ω.cm^2) were recorded. Where a stable baseline was not achieved, tissues were excluded. TER was calculated using Ohm's law after recording the change in I_{sc} evoked by a 2 mV pulse. Tissues with a TER<12 Ω.cm^2 were not used for further analysis. Tissue responses to either increasing concentrations of LL-LB , to a single addition of LL-LB (5%; mucosal), or to the single strain fermentate (*L. fermentum*; 5%; mucosal) were then measured. The resultant peak change in I_{sc} was recorded.

To examine the contribution of the enteric nervous system (ENS) on LL-LB (mucosal)-stimulated changes in I_{sc}, tissues were pre-treated with TTx (3 μM; serosal) for 15 min. For SGLT1 inhibition, phloridzin (1 mM) was added to the mucosal compartment 15 min before the addition of LL-LB (5%). To investigate the contribution of chloride and sodium ion transport to the change in I_{sc} induced by LL-LB (5%), tissues were pre-treated for 15 min with either the cystic fibrosis transmembrane conductance regulator (CFTR) inhibitor, PPQ (50 μM; mucosal), the epithelial sodium channel (ENaC) inhibitor, amiloride (100 μM; mucosal), or the Na-K-Cl cotransporter 1 (NKCC1) inhibitor, furosemide (100 μM; serosal). To examine the effects of LL-LB (5%) or the single strain fermentate on calcium- and cAMP-stimulated changes in I_{sc}, tissues were stimulated with CCh (100 μM; serosal) or forskolin (10 μM; serosal), respectively 15 min after either LL-LB (5%; mucosal) or the single strain fermentate (5%; mucosal).

Mice fed *Lactobacillus* LB were euthanised as described above, and the ileum was removed and placed in chilled Krebs solution. Seromuscular stripping was carried out by blunt dissection and tissues were mounted into Ussing chambers as noted above. Once a stable baseline was achieved, basal I_{sc} and TER were recorded. Tissue responses to CCh and forskolin were then measured.

All measurements were continuously recorded on a computer using LabTrax data acquisition hardware and analysed using LabScribe software (World Precision Instruments). Maximal change in I_{sc} induced by LL-LB, the *L. fermentum* fermentate, CCh and forskolin within 10 min of application was determined using the analysis window and is expressed as Δ I_{sc}.

2.9. Organ bath studies

3 cm of the distal ileum (collected 3 cm proximal to the ileocaecal junction) were flushed with Krebs buffer to remove faecal matter and then mounted in vertical organ bath chambers (20 ml; Panlab Single Chamber Organ Bath, ADInstruments, ML1110). The intact tissue segments were tied to a hook and force transducer (ADInstruments, MLT0210A, Bridge Pod ML301) using surgical thread in oxygenated (95% O_2/5% CO_2) Krebs buffer, maintained at 37 °C. Tissues were stretched to a basal tension of 1 g and were allowed to equilibrate for 15 min. PowerLab 2/25 (ADInstruments) and LabChart8 were used to acquire data. Isometric changes in basal tension were recorded in response to CCh (100 μM), LL-LB (5%) or the *L. fermentum* fermentate (5%) and to subsequent stimulation with CCh (100 μM) in the presence of LL-LB or the *L. fermentum* fermentate. Quantification of the physiologic effect of LL-LB or the *L. fermentum* fermentate on tone was determined relative to basal tone for relaxation as the minimum peak response (Δ force (g) of basal tone). Spontaneous contractility and the response to CCh were pooled and are quoted as area under the curve per second (AUC/sec).

2.10. Statistical analysis

Statistics were performed using GraphPad version 9.0 (GraphPad Software, Inc, California, USA). Statistical significance was set to $p < 0.05$. Outliers were detected using Grubbs' test. Normality was determined by the Shapiro-Wilk test. Groups were compared by two-tailed Student's *t*-test or one-way ANOVA followed by Tukey's multiple comparisons test. Throughout, asterisks denote significance where * represents $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$. Data are presented as mean ± standard error of the mean (SEM) throughout.

3. Results

3.1. Effects of low lactose-Lactobacillus LB on baseline tissue responses in Ussing chambers

Unfiltered 2.5%, 5% and 7.5% LL-LB significantly increased ileal baseline I_{sc} relative to the same volume of Krebs buffer when added to the mucosal compartment of the Ussing chamber (Fig. 1a and b). Moreover, the tissue response to 5% LL-LB was significantly greater in the ileum than in the colon (ileum, 51.5 ± 11.5 μA.cm^{-2}, n = 5 versus colon, 16.3 ± 2.8 μA.cm^{-2}, n = 6, $p < 0.05$).

3.2. Characterisation of the short-circuit current response induced by low lactose-Lactobacillus LB

We compared the effect of filtering LL-LB (5%) on the ileal I_{sc} response to determine whether cellular components of the fermentate contribute to the change in I_{sc} induced by mucosal LL-LB. Filtering LL-LB did not significantly influence the ileal response (Fig. 2a). Concentrations greater than 0.1% LL-LB (filtered) increased ileal baseline I_{sc} when added to the mucosal compartment of the Ussing chamber. A concentration-dependent increase in ΔI_{sc} was observed up to 10% following single additions of filtered LL-LB (0.1–10%; Supplemental Fig. 1). We could not determine whether 10% represented the maximum response as it was not feasible to test concentrations higher than this. To determine whether the change in I_{sc} elicited by 5% LL-LB involved the

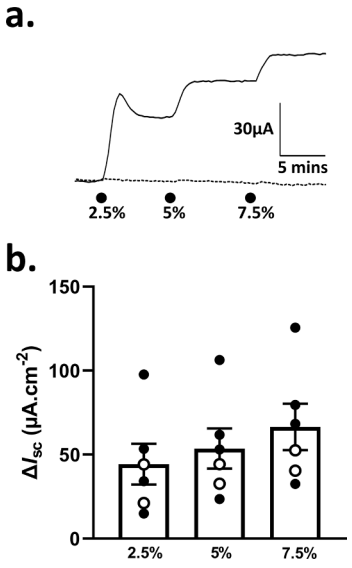

Fig. 1. Effects of low lactose-*Lactobacillus* LB on baseline tissue responses in Ussing chambers. Unfiltered 2.5%, 5% and 7.5% low lactose-*Lactobacillus* LB (LL-LB) significantly increased basal short-circuit current (I_{sc}) when added to the mucosal reservoir of the Ussing chamber. Representative trace (a; filled line, unfiltered LL-LB; dashed line, Krebs buffer) and pooled data (b). Black symbols represent male animals and white symbols represent female animals. n = 6. Data are presented as mean ± SEM.

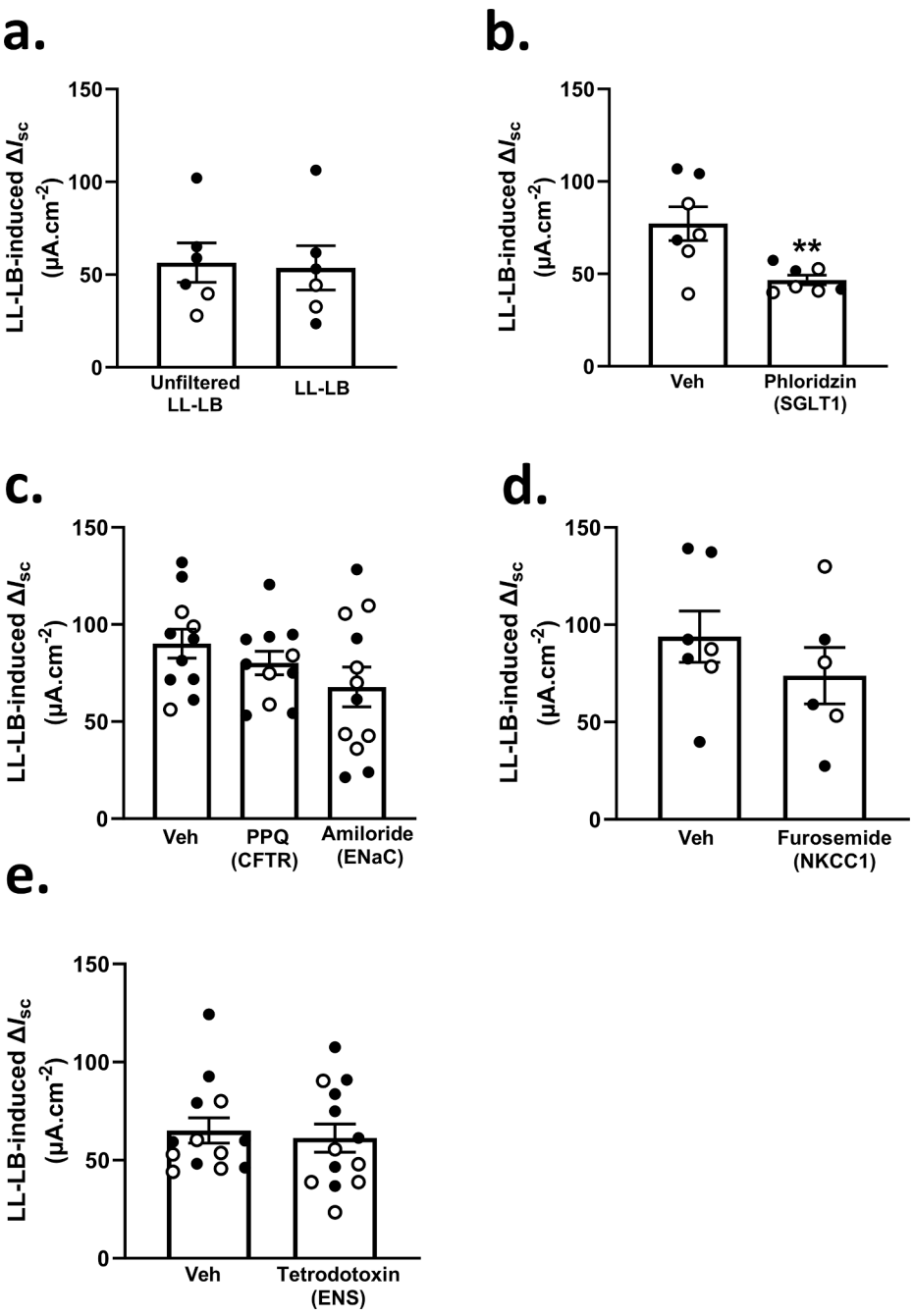

Fig. 2. Characterisation of the effects of low lactose-*Lactobacillus* LB on basal and stimulated short-circuit current. Filtering low lactose-*Lactobacillus* LB (LL-LB; 5%) did not significantly influence the mucosal response to the postbiotic (a). Phloridzin (mucosal) significantly decreased the response to LL-LB (b). Neither preincubation with PPQ (c; mucosal), amiloride (c; mucosal) nor furosemide (d; serosal) significantly affected the response to mucosal 5% LL-LB. Similarly, tetrodotoxin (TTx; serosal) had no significant effect on the response to LL-LB (mucosal; 5%; e). Black symbols represent male animals and white symbols represent female animals. ** $p < 0.01$. n = 6–13. Data are presented as mean ± SEM.

sodium-D-glucose transporter 1 (SGLT1), tissues were pre-treated for 15 min with phloridzin on the mucosal side. This significantly reduced the mucosal response to 5% LL-LB ($p < 0.01$; Fig. 2b). Next, we wanted to determine whether the response to 5% LL-LB could be influenced through inhibition of the CFTR (PPQ), the ENaC (amiloride) or basolateral inhibition of the NKCC1 (furosemide). However, neither preincubation with PPQ, amiloride nor furosemide significantly affected the response to 5% mucosal LL-LB (Fig. 2c and d). Finally, to determine whether the change in I_{sc} elicited by 5% LL-LB involved the ENS, tissues were pre-treated for 15 min with the neurotoxin, TTx on the basolateral side which had no significant effect on the mucosal LL-LB response (Fig. 2e).

3.3. Comparison between the effects of low lactose-Lactobacillus LB and the L. Fermentum fermentate on short-circuit current and trans-epithelial resistance in Ussing chambers

Lactobacillus LB contains a combination of heat-killed lactobacilli of which *L. fermentum* constitutes approximately 95%. Therefore, in the next series of experiments we sought to determine whether a preparation produced using *L. fermentum* alone exerted differential effects on intestinal ion transport when compared to LL-LB. Mucosal addition of the *L. fermentum* preparation (5%) caused an increase in baseline I_{sc} that was significantly greater than that induced by LL-LB (5%; $p < 0.001$; Fig. 3a). Neither LL-LB (5%) nor the *L fermentum* preparation (5%) affected TER 15 min after addition (Fig. 3b). Similarly, neither LL-LB (5%) nor the single strain fermentate (5%) significantly impacted CCh– or forskolin-stimulated responses in the Ussing chamber (Fig. 3c and d).

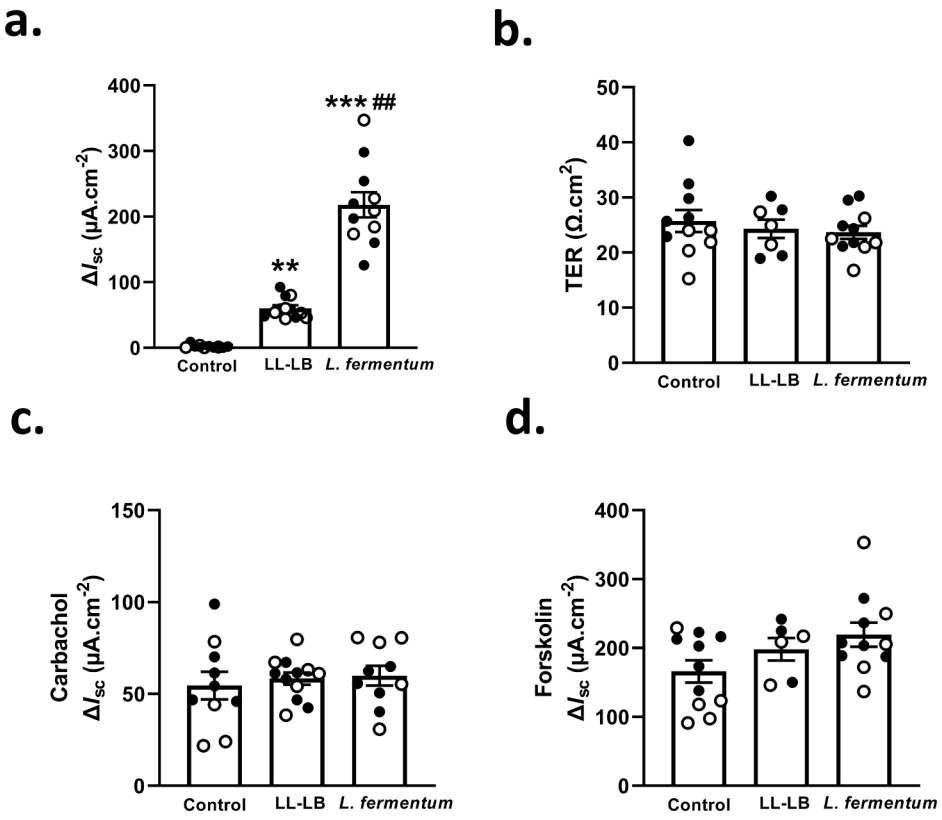

Fig. 3. Comparison between the effects of low lactose-*Lactobacillus* LB to a single strain fermentate produced by *L. fermentum* on intestinal short-circuit current and trans-epithelial resistance. The single strain fermentate (labelled *L. fermentum*; 5%; mucosal) had a significantly greater effect on baseline short-circuit current (I_{sc}) compared to low lactose-*Lactobacillus* LB (LL-LB; 5%; mucosal; a). LL-LB and *L fermentum* had similar effects on trans-epithelial resistance (TER; both 5%; b). Neither 5% LL-LB nor the singe-strain fermentate (5%) significantly impacted carbachol (CCh; serosal)-stimulated (c) or forskolin-stimulated (d; serosal) responses in the Ussing chamber. Black symbols represent male animals and white symbols represent female animals. *, control versus LL-LB or *L. fermentum*. #, LL-LB versus *L. fermentum*. ** $p < 0.01$, *** $p < 0.001$. ##, $p < 0.01$. n = 6–12. Data are presented as mean ± SEM.

3.4. Comparison between the effects of low lactose-Lactobacillus LB to the single strain fermentate produced by L. Fermentum on intestinal motility ex vivo

In the organ bath, the single strain preparation (5%) had the same inhibitory effect as LL-LB (5%) on intestinal tone (Fig. 4a and b). However, the *L. fermentum* fermentate (5%) had an inhibitory effect on CCh-evoked contractions compared to LL-LB (5%) which significantly increased the CCh-induced response ($p < 0.001$; Fig. 4c and d). Neither LL-LB (5%) nor the fermentate produced from *L. fermentum* (5%) significantly influenced spontaneous contractility (Fig. 4e).

3.5. Effects of a Lactobacillus LB-containing diet on intestinal physiology and faecal water content

Given that *Lactobacillus* LB can significantly influence the murine gut microbiome and short-chain fatty acid production (Warda et al., 2020), we wanted to investigate whether *Lactobacillus* LB significantly influenced ileal ion transport and faecal water content after a period of feeding. Ileum collected from mice fed *Lactobacillus* LB did not display any significant difference in basal I_{sc} (control, 18.3 ± 7.8 μA.cm^{-2}, n = 12 versus *Lactobacillus* LB, 3.6 ± 12.3 μA.cm^{-2}, n = 12). CCh– and forskolin-stimulated changes in I_{sc} were similar in tissues collected from animals fed the control and *Lactobacillus* LB-containing diets (Fig. 5a and 5b). No difference in TER was observed between tissues collected from mice fed the control diet or *Lactobacillus* LB-containing diet (Fig. 5c). Moreover, faecal water content was comparable between mice fed *Lactobacillus* LB and the control diet (Fig. 5d).

4. Discussion

Lactobacillus LB, which is the similar active ingredient as Lactéol, is an approved anti-diarrheal medication (Liévin-Le Moal, 2016). We show that the same ingredients modulate I_{sc} in the small intestine (Fig. 1a and b) and that this response displays partial sensitivity to inhibition of SGLT1 (Fig. 2b). A possible explanation for the response to LL-LB may involve rapid upregulation of SGLT1, and subsequent co-transport of sodium. For example, heat-sensitive metabolites produced by probiotic lactobacilli have been shown to increase glucose uptake *in vitro* (Rooj et al., 2010). Supernatants collected from these lactobacilli rapidly increased glucose uptake by Caco-2 colonic epithelial cells. Importantly this effect was not reproduced by the bacterial cell pellets (Rooj et al., 2010). Therefore, a similar secreted factor could account for the effects of LL-LB on SGLT1-mediated glucose transport in our study. Furthermore, as we used a low lactose preparation of *Lactobacillus* LB, any effect of lactose on the immediate change in I_{sc} is unlikely.

However, given that inhibition of sodium absorption (via ENaC) and chloride ion secretion (via CFTR) failed to further inhibit the response to LL-LB (Fig. 2c), we can only speculate on any other ionic mechanism(s) underpinning the ileal increase in I_{sc} we observed. Moreover, the effects of LL-LB on electrogenic ion transport were independent of the ENS (Fig. 2e). We also observed that *Lactobacillus* LB significantly increased baseline I_{sc} in the colon, and this response was approximately two-fold less than that in the ileum. This differential regional sensitivity may reflect different mechanisms of electrogenic ion transport associated with different regions of the gastrointestinal tract. For example, routes for sodium ion entry differ between the ileum and colon (Field, 2003).

We acknowledge, however, that our study focussed only on CFTR and ENaC and that other channels expressed by intestinal epithelia could potentially be influenced by LL-LB. For example, mice lacking ClC-2, a member of the voltage-gated chloride channel family, display defects in intestinal ion transport (Catalán et al., 2012). Although there was no significant effect of ClC-2 knockout observed on either basal I_{sc} or cAMP- and calcium-stimulated responses in mouse colon, in Ussing chamber studies, the absence of ClC-2 increased electrogenic sodium absorption via ENaC (Catalán et al., 2012). As amiloride did not significantly affect

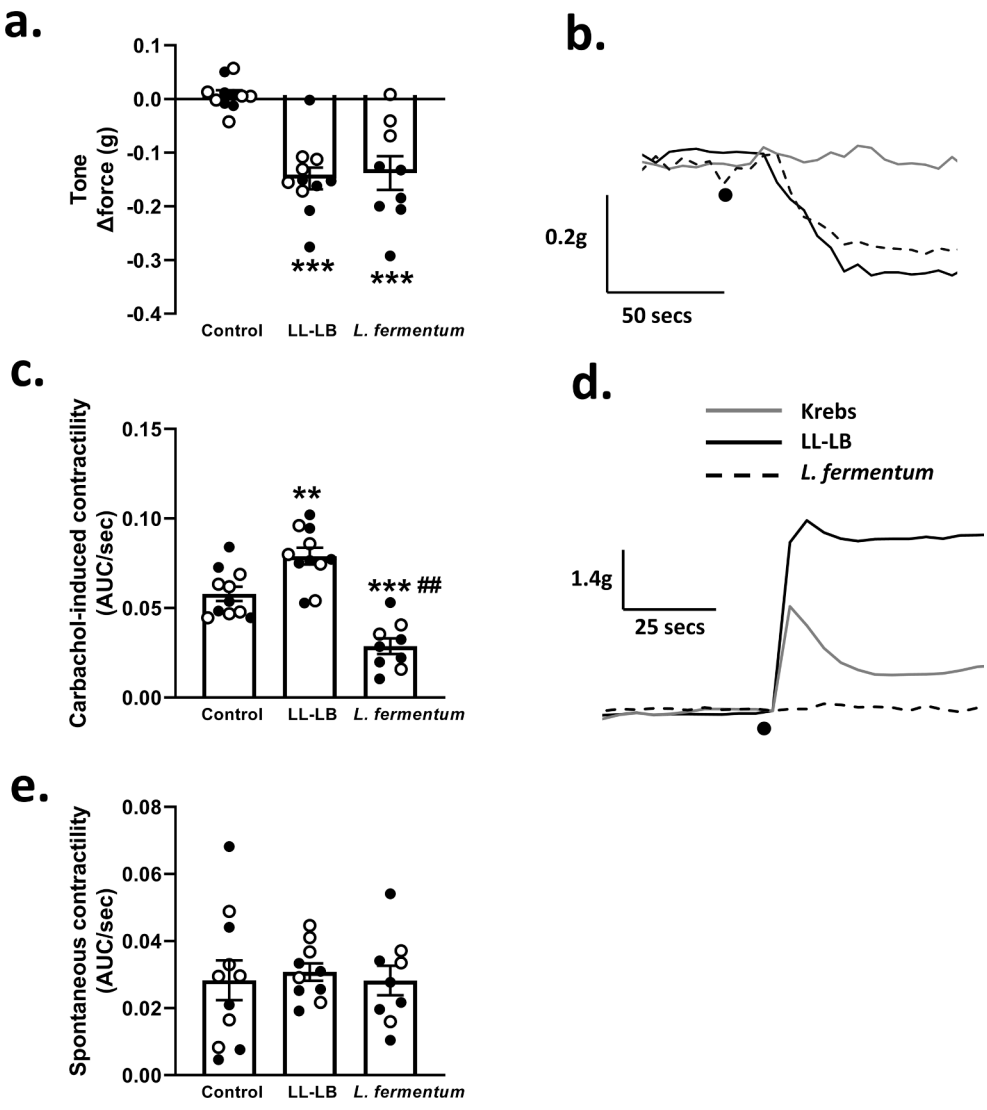

Fig. 4. Comparison between the effects of low lactose-*Lactobacillus* LB to a single strain fermentate produced by *L. fermentum* on intestinal motility *ex vivo*. In the organ bath, both low lactose-*Lactobacillus* LB (LL-LB; 5%) and the single strain fermentate (5%) significantly decreased intestinal tone (a and b). 5% LL-LB significantly increased CCh-evoked contractions whilst the *L. fermentum* fermentate (5%), had an opposing effect (c and d). Neither LL-LB nor the *L. fermentum* fermentate (both 5%) significantly influenced spontaneous contractile activity (e). Black symbols represent male animals and white symbols represent female animals. *, control versus LL-LB or *L. fermentum*. #, LL-LB versus *L. fermentum*. ** $p < 0.01$, *** $p < 0.001$. ##, $p < 0.01$. n = 9–11. Data are presented as mean ± SEM.

the LL-LB response in our study, this might suggest that ClC-2 was not targeted by LL-LB. On the other hand, the absence or inhibition of the calcium-activated chloride channel ANO1 significantly decreased the colonic I_{sc} response to forskolin and significantly increased faecal water content (Lee et al., 2019). However, neither of these parameters were affected by LL-LB. Similarly, other studies have reported that the knockout TEMEM16A significantly inhibited both CCh– and cAMP-mediated responses (Kunzelmann et al., 2019). Again, as neither of these responses were influenced by LL-LB, this could perhaps suggest that TEMEM16A may not be involved in the LL-LB-induced change in I_{sc}. Nonetheless, we acknowledge that there are experimental differences between these studies and ours in terms of intestinal region and experimental protocol, for example the presence of amiloride and indomethacin in the bathing solution.

Given that postbiotics contain inanimate microbial cells or cell fragments as well as metabolites or fermentation products, the observed change in I_{sc} could be elicited by one or more of these entities. For example, short-chain fatty acids can activate electrogenic sodium-coupled monocarboxylate transporter 1 in rat small intestine, resulting in an increase in I_{sc} (Metzler-Zebeli et al., 2022). Likewise, the transport of microbial-derived amino acids and metabolites across the epithelium likely involves the engagement of electrogenic transporters (Chen et al., 2020; Dinges et al., 2018). Overall, whilst we can discount a role for amiloride-sensitive sodium absorption and CFTR in contributing to the change in I_{sc} elicited by LL-LB, based on our data we cannot comment on whether the change in I_{sc} observed in our study reflects a predominantly secretory or absorptive response. However, given the complex nature of a postbiotic it could involve several different mechanisms working in concert.

We also observed a significant change in intestinal tone in response to LL-LB, without a change in spontaneous contractile activity (Fig. 4a and e). Inhibition of intestinal tone can result in the retention of faeces and fluid. For example, the antidiarrheal drug, loperamide significantly reduced mouse ileal tone *ex vivo* and this effect appeared to be mediated by ATP-sensitive K^+ channels and by protein kinase A (Chen et al., 2012). However, loperamide was added to the tissues in the presence of acetylcholine (pre-contracted) whereas the effect of LL-LB on intestinal tone occurred at baseline and was relatively transient and short-lived (Fig. 4a and b), perhaps suggestive of a different mechanism of action. In human studies, the bile acid, glycodeoxycholic acid reduces ileal tone, whilst short-chain fatty acids have an opposing effect (Coffin et al., 1997). Moreover, the diameter of the terminal ileum, reflective of intestinal tone, is significantly reduced (increased tone) in patients with diarrhoea-predominant IBS compared to healthy volunteers which may contribute to decreased small bowel water content and increased transit in these patients (Marciani et al., 2010). Therefore, the immediate effects of LL-LB on intestinal tone could potentially increase time for water absorption and decrease transit, at least in the short term.

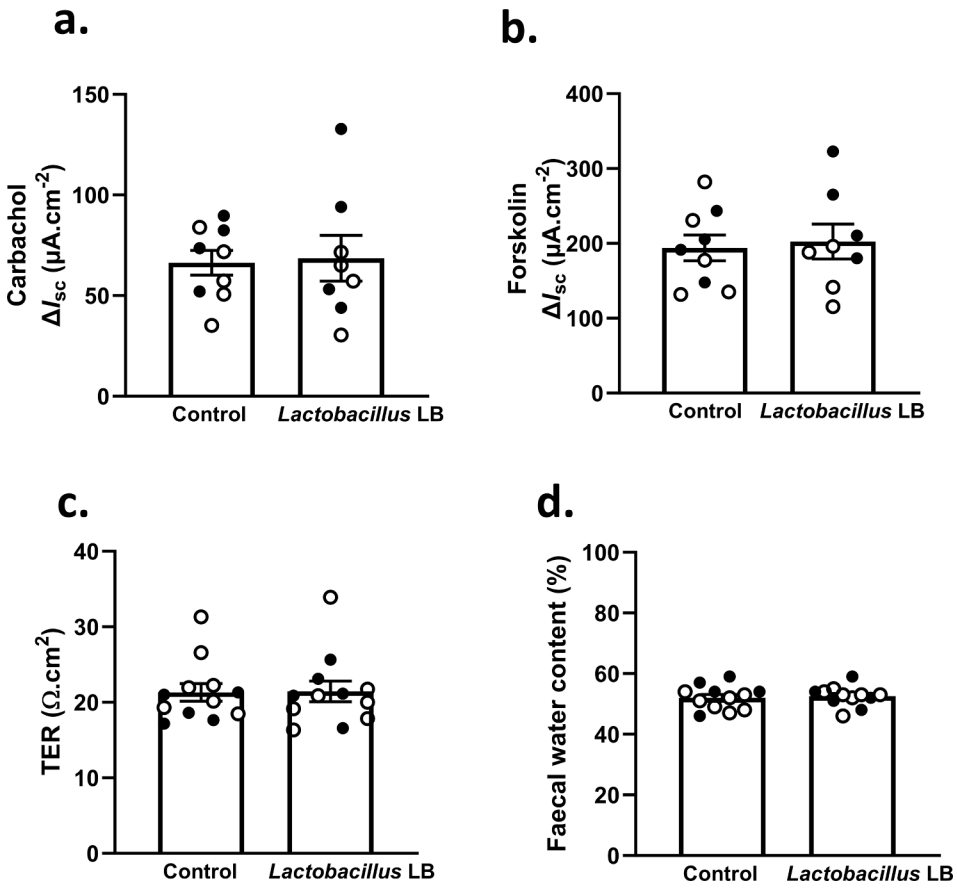

Fig. 5. Effects of four weeks feeding with *Lactobacillus* LB on intestinal physiology and faecal water content. Carbachol-stimulated (a) and forskolin-stimulated (b) responses were comparable between mice fed *Lactobacillus* LB (5% supplemented diet) or the control diet. *Lactobacillus* LB did not significantly affect trans-epithelial resistance (TER; c) relative to mice fed the control diet. *Lactobacillus* LB did not significantly influence faecal water content (e). Black symbols represent male animals and white symbols represent female animals. n = 8–12. Data are presented as mean ± SEM.

Although LL-LB significantly reduced ileal tone, it also significantly enhanced cholinergic-induced contractility (Fig. 4c). Both functions contribute to the role of smooth muscle in modulating the "reservoir" capacity and "propulsion" along the length of the gastrointestinal tract respectively (Bitar, 2003). The small intestine may respond to stimulation by either a change in motility, or tone, or both depending on the stimulus (Lee, 1983). Supernatants derived from *Lacticaseibacillus rhamnosus* GG (LGG) increased cholinergic-induced contraction of human smooth muscle cells *in vitro*, and this effect varied depending on when the supernatants were prepared during the growth of LGG (Cicenia et al., 2016). Moreover, the supernatants derived from LGG were also able to restore lipopolysaccharide-induced inhibition of cholinergic contraction (Cicenia et al., 2016). This enhancement of cholinergic activity by LGG supernatants supports our observations *ex vivo* with LL-LB. Given that we did not use electric field stimulation or pharmacological approaches to examine non-adrenergic, non-cholinergic responses, we cannot comment on whether the effects of LL-LB are specific to cholinergic-induced contractility. *In vivo* studies in the mouse have also demonstrated that lactobacilli were able to increase transit time as well as faecal water content in a model of constipation (Zhang et al., 2018). However, faecal water content was not significantly affected in animals fed *Lactobacillus* LB (Fig. 5d). Therefore, this might suggest that the effects of LL-LB on small intestinal tone and cholinergic-induced contractility may be relatively short-lived, but could be beneficial in the context of acute diarrhoea or constipation respectively.

However, LL-LB and the single strain fermentate differentially influenced cholinergic-induced contractions. Whilst LL-LB increased CCh-induced contractions, the single strain fermentate had an opposing effect (Fig. 4c and d). This is perhaps in line with previous which work has demonstrated that different lactobacilli can have differing effects on motility. For example, *L. rhamnosus* JB-1 significantly increased the velocity of jejunal migrating motor complexes whereas *L. reuteri* DSM 17938 had the opposite effect (Wu et al., 2013). The precise mechanism(s) underpinning this divergence is unclear, but appears to involve the ENS (Wu et al., 2013). The divergent effects on cholinergic responses observed between the single and dual strain fermentates on ileal contractility in our study likely reflects a different repertoire of metabolites or bioactive profile of the single versus the dual strain fermentates. Some of these bioactives could include bacterial cell wall components (toll-like receptor ligands), metabolites or end products of bacterial fermentation, including bile acids, short-chain fatty acids and tryptophan metabolites which have well documented effects on intestinal motility (Zheng et al., 2022).

Epithelial permeability, recorded as TER, was not influenced within 15 min by LL-LB when added to ileal tissue *ex vivo* (Fig. 3b) nor did the TER of tissues collected from animals fed *Lactobacillus* LB differ from that of animals fed a control diet when measured in Ussing chambers (Fig. 5c). Nonetheless, the tissues used in our study were not challenged with a pathogenic or infectious insult. For example, in Ussing chamber studies, incubation of the small intestine with *L. plantarum* 299v did not influence permeability *per se* (Mangell et al., 2002). Similarly, in animals fed *L. plantarum* 299v in their drinking water for one week, no significant effect on baseline permeability was observed. Despite this, however, *L. plantarum* 299v protected against an *E. coli*-induced increase in permeability (Mangell et al., 2002). Therefore, it is possible that *Lactobacillus* LB could protect against pathogen-associated disruption of the intestinal barrier, an effect that could be attributed to the ability of *Lactobacillus* LB to inhibit the adherence of pathogens (Liévin-Le Moal, 2016).

The bioactivity profile of LL-LB was unique to the two strain mix and differed to that of the single strain fermentate (Figs. 3 and 4). This may reflect secretion of different bioactive substances or the importance cell–cell communication between the two strains in co-culture, known as quorum sensing, which may influence the metabolic traits of either of the strains (Kwoji et al., 2021). In other studies which have compared

the bioactivity of different postbiotic mixes derived from two- to six-probiotics, divergent effects were also observed, with a four-mix postbiotic combination having the strongest effect on the expression of tight-junction protein genes in colonic epithelial cells *in vitro* (Lin et al., 2022).

5. Conclusions

Postbiotics represent a complex mixture of metabolic products which may either singularly or synergistically influence either the host, the gut microbiome, or both. In this study, we demonstrate that the effects of a dual strain postbiotic are unique in two *ex vivo* gastrointestinal model systems providing mechanistic insight into the bioactivity of *Lactobacillus* LB on host small intestinal physiology. These studies were informed by clinical observations that the combination of *L. fermentum* and *L. delbrueckii* has proven to be effective in the management of acute diarrhoea and IBS. The physiological significance of our findings may underpin these beneficial effects, but further characterisation is necessary to determine the bioactive mediators involved.

CRediT authorship contribution statement

Friederike Uhlig: Conceptualization, Formal analysis, Funding acquisition, Investigation, Methodology, Writing – original draft, Writing – review & editing. **Alicja K. Warda:** Conceptualization, Formal analysis, Investigation, Methodology, Writing – review & editing. **Cara M. Hueston:** Investigation, Methodology, Writing – review & editing. **Lorraine A. Draper:** Conceptualization, Project administration, Resources, Writing – review & editing. **Gilles Chauvière:** Conceptualization, Funding acquisition, Writing – review & editing. **Erik Eckhardt:** Conceptualization, Funding acquisition, Writing – review & editing. **Colin Hill:** Conceptualization, Funding acquisition, Writing – review & editing. **Niall P. Hyland:** Conceptualization, Funding acquisition, Supervision, Formal analysis, Writing – original draft, Writing – review & editing.

Declaration of Competing Interest

G.C. and E.E. are employees of Adare Biome. Adare Biome was involved in study design and in the decision to submit the article for publication but not in the collection, analysis, and interpretation of data.

Data availability

Data will be made available on request.

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Data statement

The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jff.2023.105730.

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