

Limosilactobacillus reuteri DSM-17938 for preventing cough in adults with mild allergic asthma: A double-blind randomized placebo-controlled cross-over study

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Abstract

Background: Cough is a common troublesome symptom in asthma which is neuronally mediated. *Limosilactobacillus reuteri* DSM-17938 (*L. reuteri* DSM-17938) is a probiotic shown to be effective in pre-clinical models at suppressing neuronal responses to capsaicin, a transient receptor potential vanilloid agonist (TRPV1).

Objective: Investigate the effects of DSM-17938 versus matched placebo on capsaicin-evoked coughs in mild allergic asthmatics.

Methods: We performed a 4-visit, randomized, double-blind, placebo-controlled, two-way cross-over study comparing full dose cough responses with inhaled capsaicin in mild allergic asthmatics after 1 month of treatment with DSM-17938 compared with matched placebo. Randomization and allocation to trial group were carried out by a central computer system. Histamine skin prick testing, airway hyper-responsiveness and inflammatory cells in induced sputum were measured at every visit. Blood was collected to extract PBMCs and stimulated with CD3/CD28 to ascertain the effects of DSM-17938 /placebo on T-cell cytokine responses.

Results: Seventeen subjects were recruited and 15 completed the study (8 females, mean age 27.3 years). There was no difference in the change in maximum capsaicin-evoked coughs (Emax) after treatment with *L. reuteri* DSM-17938 compared with placebo [mean difference 2.07 coughs (95% CI -2.77 to 6.91, $p = .38$) or relative changes in geometric mean ratios for the dose evoking at least half the Emax (ED50) [1.05 (95% CI 0.31–3.58, $p = .94$), concentration evoking 2 coughs (C2) [0.63 (0.26–1.53), $p = .28$] and 5 coughs (C5) [0.79 (0.25–2.50), $p = .67$]. There was no effect on histamine skin prick wheal size, intensity of itch sensation, methacholine PC20, airway inflammation or T-cell responses after stimulation with CD3/CD28. There were no serious adverse events. One subject developed a mild upper respiratory tract infection and another mild transient nausea whilst on DSM-17938.

Conclusion: In this small study in adults with mild allergic asthma, we found no evidence that *L. reuteri* DSM-17938 has any systemic effects on airway nerves, smooth muscle, sputum inflammatory cells, skin responses or T-cell responses after oral consumption.

Trial Registration: Clinicaltrials.gov Identifier: NCT03603522.

KEYWORDS

asthma, capsaicin, cough, lactobacillus, probiotic, transient receptor potential vanilloid type 1

1 | INTRODUCTION

Cough is a common, troublesome symptom and a marker of both severity and poor prognosis in subjects with asthma.¹⁻⁴ However, there is a paucity of evidence demonstrating treatment with asthma medications directly improves coughing.⁵ Understanding the mechanisms of cough and developing treatments specifically for coughing has recently gained significant attention with the development of novel therapy targeting ion channels on airway nerves.⁶

Cough is the archetypal airway defensive reflex, which is neurally mediated by the sensory afferents of the parasympathetic vagus nerves and its receptor profile on the nerve terminals. There are two main types of airway nerves.^{7,8} C-fibres are networks of unmyelinated nerves found throughout the airways, sensitive to chemical stimuli including inflammatory mediators (eg bradykinin and prostaglandins), environmental stimuli (eg pollutants and temperature) and characteristically activated by capsaicin via the transient receptor potential vanilloid type 1 (TRPV1) receptor.^{9,10} In contrast, A-delta fibres are faster myelinated nerves which project to the sub-epithelium and respond to punctate mechanical stimuli, changes in osmolarity and acidity but are generally insensitive to capsaicin and inflammatory mediators.¹¹ Hence, capsaicin can be used to study predominantly c-fibre function.

Subjects with mild, moderate and severe asthma demonstrate an exaggerated cough response to capsaicin and they cough at lower doses.¹²⁻¹⁵ Differences in the capsaicin dose-response curves also predict objective hourly cough frequency over a 24-h period.¹² Recent studies have also shown airway nerves become further sensitized after bronchoconstriction and whole lung allergen challenge.^{16,17} Imaging data demonstrate airway nerves to be co-localized with eosinophils with increasing nerve branches.¹⁸ These data suggest airway nerves are an important dimension of asthma pathophysiology, which may be amenable to pharmaceutical intervention.

Limosilactobacillus reuteri DSM-17938 is a probiotic which is currently licensed and recommended in clinical guidelines for the treatment of infantile colic,^{19,20} necrotizing enterocolitis (NEC),²¹ functional constipation, antibiotic-associated diarrhoea and as an adjuvant therapy in *Helicobacter pylori* infection.²² One postulated mechanism is inhibition of visceral pain induced by gastric distension via a local TRPV1 mechanism.^{23,24} Pre-clinical studies in vitro or ex vivo show that mice who were fed DSM-17938 for 2 weeks demonstrated a nearly complete suppression of the thoracic dorsal root ganglion when stimulated with capsaicin, a TRPV1 agonist.^{23,25} Treatment with *L. reuteri* DSM-17938 in animal studies has also shown decreased expression of TRPV1 in the gastric mucosa²⁶ and

Key Messages

- We evaluated whether *L. reuteri* DSM-17938 could suppress cough responses in adults with allergic asthma.
- Compared with placebo, 4 weeks of probiotic treatment resulted in no change in any cough outcomes.
- This specific probiotic strain is unlikely to have short-term systemic effects in mild allergic asthma.

increase in regulatory T cells which was associated with an attenuation of airway hyper-responsiveness (AHR, a characteristic physiologic feature of asthma) and suppression of T-effector cells after antigen challenge.²⁷

The over-arching aim of this study was to assess potential target engagement of TRPV1 in the lung after oral ingestion of *L. reuteri* DSM-17938 (10⁹ CFU per day). The primary objective of this proof of concept study was to investigate the potential systemic effects of oral ingestion of *L. reuteri* DSM-17938 in subjects with mild allergic asthma on capsaicin-evoked cough responses. As TRPV1 is also known to be widely expressed throughout the somatic and autonomic nervous system^{28,29} and on T cells,³⁰ we also sought to investigate effects on histamine-induced itch and wheal responses on skin, differential white cell counts in sputum, T-cell activation responses in blood and airway hyper-reactivity.

2 | METHODS

2.1 | Subjects

Participants with mild steroid naïve allergic asthma were recruited (16th July 2019–18th Jan 2021). All participants had a baseline forced expiratory volumes in 1 s (FEV1) of ≥70% predicted, allergies (based on at least one positive skin prick test), evidence of methacholine AHR (provocative concentration inducing ≥20% fall in FEV1, PC20 < 16 mg/ml), with well-controlled asthma according to Global Initiative for Asthma (GINA) classification and were not taking maintenance treatment, such as inhaled corticosteroids (ICS).³¹ We excluded current smokers, those with a recent exacerbation or uncontrolled symptoms, and use of medication which may have altered cough responses (eg opiates, gabapentin, anti-cholinergics and theophylline). All subjects were asked not to take any concomitant probiotics. To be included in the study, subjects also had to demonstrate a cough response to capsaicin with no significant fall in FEV1

(<10%) post capsaicin requiring salbutamol. The protocol was approved by the local research ethics committees (HiREB:5095) and Health Canada (Control Number: 228322). All subjects provided written informed consent.

2.2 | Study design

This was a 4-visit, single-centre, randomized, double-blind, placebo-controlled, two-way cross-over study comparing cough responses to inhaled capsaicin in subjects with mild steroid naïve allergic asthma before and after treatment with *L. reuteri* DSM-17938 compared with placebo (Figure 1). Full details of all study procedures are found in the Supporting Information.

2.3 | Randomization and masking

Subjects were assigned to one of the two possible treatment sequences using a computer-generated randomization schedule prepared by McMaster University, with a ratio of 1:1 for *L. reuteri* DSM-17938/placebo or placebo/*L. reuteri* DSM-17938. Blinded study product supplies were provided in sequentially numbered identical bottles in accordance with the randomization schedule and dispensed by a pharmacist who was not delegated any other role in the study. The allocation sequence was blinded from the subjects and the research team.

2.4 | Study procedures

Subjects attended for 4 visits. At visit 1, consent, history, examination and baseline spirometry was performed. A capsaicin cough challenge was performed, as previously described,¹² using a dosimeter (KoKo Dosimeter; Ferraris) and a nebulizer pot (Model 646; Devilbiss Healthcare) with an inspiratory flow limiter. Briefly, 4 inhalations of doubling doses of capsaicin (0.48–1000 µmol/L) were administered

30 s apart. After each inhalation, the number of coughs in the first 15 s was counted and later verified by using a cough monitor (VitaloJAK). The challenge was completed when the patient reached the highest capsaicin dose or the maximal tolerated dose. Spirometry was performed before and after each challenge.

All subjects then underwent skin prick testing to common aero-allergens, methacholine challenge (PC₂₀) using the two-minute tidal breathing method,³² sputum induction,³³ incremental histamine skin prick testing (0.25, 0.5, 1.0 mg/ml) and blood sampling. The intensity of itch sensation was assessed 10 min after performing the histamine SPT on a mBorg scale.

Peripheral blood mononuclear cells (PBMC) were isolated from whole blood by density gradient centrifugation. Cell culture plates (96-well) were coated with anti-CD3 antibodies (10 µg/ml) overnight and the PBMC plated at a density 2×10^6 /ml and stimulated with soluble anti-CD28 antibodies (1 µg/ml) for 48 h. Supernatants were collected and stored at -20°C prior to cytokine quantification. Cytokine levels (IFNγ, TNF, IL-10 and GM-CSF) were determined through Multiplex Cytokine/Chemokine Analysis (Eve Technologies).

Subjects who met the inclusion criteria were randomized to a 4-week Treatment Period 1, then returned at visit 2 to repeat all procedures. After a 4- to 5-week washout period, Treatment Period 2 was carried out during visits 3 and 4.

2.5 | Intervention

Limosilactobacillus reuteri DSM-17938 and matched placebo were provided by BioGaia (AB, Eslöv). Both interventions were suspended in oil with identical packaging in a 10ml amber glass bottle and tamper evident screw cap. The active study product *L. reuteri* DSM-17938 was given at a dose of 1×10^9 colony-forming units (CFU) per day corresponding to 2 ml of oil formulation per day administered via a plastic syringe from the 10 ml glass bottle. Eligible subjects started the first day of dosing at the end of visits 1 and 3 in the research facility. The last dose was taken in the morning before visit 2 and 4 study procedures.

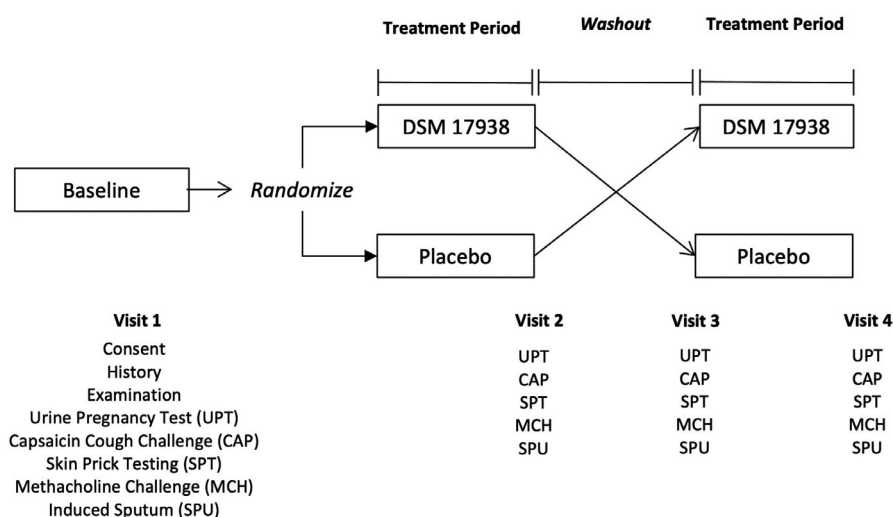


FIGURE 1 Study design—randomized placebo-controlled two-way cross-over design. DSM-17938; *Limosilactobacillus reuteri*, CAP, capsaicin cough challenge; MCH, methacholine challenge; SPT, skin prick testing; SPU, sputum induction; UPT, urine pregnancy test

The intervention was self-administered by the subject whilst at home. Subjects were asked to consume the intervention once per day, at approximately the same time every day for 4 weeks (Treatment Period 1), followed by a washout period for 4–5 weeks, and an additional treatment period for 4 weeks (Treatment Period 2). Subjects were asked to keep the intervention refrigerated between +2 and +8°C.

Dosing compliance was tracked using a diary card, and dispensed study product was returned at the end of each treatment period to confirm adherence.

2.6 | Study end points

The primary end point was the change in maximum number of coughs evoked at any dose of capsaicin ($E_{\max}$) after treatment with *L. reuteri* DSM-17938 compared with placebo after 4 weeks of treatment. Secondary endpoints were the changes in (i) the capsaicin dose evoking half the $E_{\max}$ (ED_{50}), 2 coughs (C2), 5 coughs (C5), and full dose-response curves, (ii) maximum wheal area size (mm^2) and intensity of itch on a mBorg scale (0–10) to graded doubling doses of histamine skin prick testing, (iii) differential white cells in sputum (%), (iv) methacholine PC_{20} , (v) T-cell cytokine responses after stimulation with CD3/CD28. Safety end points included all adverse events for the duration of the study period.

2.7 | Statistical methods and data analysis

Previous data suggest $E_{\max}$ is repeatable with an intraclass correlation of .89 (10). With 15 subjects, this study had approximately 90% power to detect a difference in $E_{\max}$ of 4.6 for DSM-17938 compared with placebo, with a two-sided alpha of 5%.

Analysis on data was performed using SPSS Version 25.0 (IBM Corp.). Demographic data are presented as mean and standard deviation (SD) or as median and interquartile range (IQR). Analyses of the primary and secondary outcomes were performed by comparing the relative change in outcomes after DSM-17938 compared with placebo as a paired *t* test. For $E_{\max}$, histamine wheal size and T-cell responses the absolute mean difference and 95% CI are shown. For other outcomes which were ratio scales (mBorg), doubling doses or % changes (capsaicin, methacholine, % cells in sputum), outcomes are presented as the relative change in the geometric mean ratios and 95% CI.

The effect *L. reuteri* DSM-17938/placebo on capsaicin-evoked coughs across all doses of capsaicin was analysed using general estimating equations (GEE). The GEE model was generated using a linear model for log-transformed capsaicin-evoked coughs (0.1 was added to account for zero values). The exchangeable correlation matrix was selected with co-variables including each doubling dose, intervention (*L. reuteri* DSM-17938/placebo), pre-post treatment, period and sequence. Main effects and interactions were added to the model to generate mean and standard errors at every dose of

capsaicin inhaled. Dose-response curves and all graphs were drawn in GraphPad Prism Version 9.1.

3 | RESULTS

3.1 | Subjects

Seventeen subjects with mild steroid naïve allergic asthma were recruited, of which 15 completed the study (Table 1). One patient developed an upper respiratory tract infection during treatment period 1 and was excluded. One subject screen failed. The eight females and 7 males that completed had a mean age of 27, BMI 25.72 (kg/m^2), and normal FEV1 and forced vital capacity (FVC). The geometric mean methacholine PC_{20} was 3.55 mg/ml.

All subjects coughed during the baseline full dose capsaicin cough challenge; median $E_{\max}$ was 20 coughs (IQR 16–24), ED_{50} 62.5 (15.6–125), C2 7.8 (3.9–31.3) and C5 31.3 (15.6–125) $\mu\text{M}/\text{L}$ of capsaicin. The maximum mean histamine-induced wheal size was 22.30 mm^2 (SD $\pm$ 10.05) and intensity of itch sensation was 4.13 ($\pm$ 2.29) on the mBorg scale.

3.2 | Capsaicin-evoked cough responses

There was no difference in the change in $E_{\max}$ after treatment with *L. reuteri* DSM-17938 compared with placebo [mean difference 2.07 coughs (95% CI -2.77 to 6.91 , $p = .38$, Figure 2A, Table 2)]. There was a significant reduction in the $E_{\max}$ after placebo treatment [mean difference -4.73 coughs (95% CI -8.03 to -1.44), $p = .008$]. There were also no differences in the changes in geometric mean ratios for ED_{50} [1.05 (95% CI 0.31–3.58, $p = .94$, Figure 2B, Table 2), C2 [0.63 (0.26–1.53), $p = .28$, Figure 2C] and C5 [0.79 (0.25–2.50), $p = .67$, Figure 2D] after treatment with *L. reuteri* DSM-17938 compared with placebo. The effect of intervention was not affected by period ($p = .35$) or sequence ($p = .64$). There was no differential effect of DSM-17938 treatment on $E_{\max}$ based on sex (mean difference 5.46 coughs (95% CI -3.55 to 14.46 , $p = .35$, Figure S1)). All subjects tolerated inhaling a capsaicin dose of 31.2 $\mu\text{M}/\text{L}$, after which there was a gradual drop-out rate (Figure S2). Average diary completion rates were above 97% in both treatment periods (Table S1).

Overall, there was no effect after treatment with *L. reuteri* DSM-17938 compared with placebo across the whole dose-response curve [DSM-17938: geometric mean 1.16 coughs (95% CI 0.67–2.00) before vs. 0.86 (0.48–1.57) after, placebo: 1.22 (0.67–2.23) before versus 1.13 (0.61–2.08) after; $p = .33$, Figure 3A/B].

3.3 | Histamine skin prick wheal size and intensity of itch sensation

There was no difference in the change in the maximum histamine wheal size after treatment with *L. reuteri* DSM-17938 compared with

TABLE 1 Baseline demographics of all subjects

Variable	Mean (SD)
Age	27.27 (12.60)
M:F	7:8
BMI (kg/m ²)	25.72 (5.73)
FEV1 (L)	3.65 (1.09)
FEV1%predicted	93.33 (12.79)
FVC (L)	4.49 (1.42)
FVC % predicted	95.40 (14.13)
Methacholine PC ₂₀ (mg/ml)	3.55 (2.49) ^a
Cough endpoints at baseline ^b	
Emax (n)	20 (16–24)
ED50	62.5 (15.6–125)
C2	7.8 (3.9–31.3)
C5	31.3 (15.6–125)
Skin responses at baseline ^b	
Histamine wheal size (mm ²)	19.63 (12.57–28.3)
mBorg itch intensity	4 (2–6)

Note: Data collected at visit 1. Shown as mean (SD).

^aGeometric mean (SD).

^bMedian (IQR). Emax; maximum evoked coughs, ED50; Capsaicin (μmol/L) dose evoking at least half the Emax, C2: 2 coughs, C5: 5 coughs.

placebo [mean difference -0.55 mm² (95% CI -9.09 to 7.99 , $p = .89$, Figure 4A)]. There were also no differences in the geometric mean ratios for the intensity of itch sensation [1.12 (95% CI 0.78 – 1.61), $p = .52$, Figure 4B]. There was also no effect of *L. reuteri* DSM-17938 compared with placebo across the 3 doses of histamine skin prick test ($p = .11$, Figure S3A/B).

3.4 | Changes in sputum inflammatory cells

There was no difference in the change in % eosinophils ($p = .12$), neutrophils ($p = .79$), lymphocytes ($p = .69$) or macrophages ($p = .48$) in sputum after treatment with *L. reuteri* DSM-17938 compared with placebo [Figure S4].

3.5 | Changes in airway hyper-responsiveness

There were no differences in the geometric mean ratios for the methacholine PC₂₀ after *L. reuteri* DSM-17938 compared with placebo [0.99 (95% CI 0.52 – 1.90), $p = .98$, Figure S5].

3.6 | Response to T-cell stimulation

There was no difference in baseline or CD3/CD28-stimulated levels of any of the cytokines assessed before and after DSM-17938 or compared with placebo (Figure 5).

3.7 | Adverse events

There were not serious adverse events (SAEs) throughout the entire study period. Treatment was very well tolerated. One patient developed a mild upper respiratory tract infection during treatment period 1 (on DSM-17938) which required no antibiotics or admission. One subject developed mild transient nausea (on DSM-17938). During the washout period, one subject underwent a pre-planned wisdom tooth extraction (after DSM-17938 period 1) and one subject had fluid accumulate in the inner ear which resolved spontaneously (after placebo).

4 | DISCUSSION

A number of pre-clinical animal models have demonstrated immunomodulatory effects of probiotics on the respiratory immune system; however, whether this translates to clinically meaningful improvement in humans has been a matter of significant debate and controversy.^{34,35} This is the first study to comprehensively assess whether *L. reuteri* DSM-17938 is able to exert its beneficial effects also outside of the gastrointestinal system, utilizing adult subjects with stable mild atopic asthma. Measurements were made on the cough reflex, histamine-induced skin wheal responses, intensity of itch sensation, sputum inflammatory cells, airway smooth muscle hyper-reactivity and T-cell responses. No evidence was found on any of the studied clinical endpoints. This suggests that any beneficial effects observed by this bacterial strain locally in the gastrointestinal tract are not transferred to systemic targets outside of the gastrointestinal system in adult subjects with stable mild atopic asthma.

The motivation for investigating cough responses to inhaled capsaicin and histamine skin wheal area and itch sensation was based on pre-clinical animal experiments in mice showing inhibition of neuronal responses after ingestion of *L. reuteri* DSM-17938 in the thoracic dorsal root ganglion (DRG).²³ There is evidence in animal models in mice to suggest histamine skin prick wheal response is mediated by a TRPV1 reflex in dermal sensory nociceptive neurons in the somatic nervous system.^{36,37} Likewise, the sensation of itch from the forearm would be mediated by the afferent fibres travelling to the thoracic DRG and ascend up the spinal cord to the higher cortex. In contrast, capsaicin inhalation activates vagal sensory afferent which bypass the thoracic DRG and transmit stimuli to the nucleus tractus solitarius and paratrigeminal nuclei in the brainstem before transmitting to the thalamus and higher cortical centres.^{10,38} Thus, the hypothesized mechanism in this study was via the target engagement of TRPV1 systemically after oral ingestion and potential inhibitory effect on the somatic and autonomic nervous system.

The lack of effect on cough responses could simply be due to limited systemic effects of *L. reuteri* DSM-17938 when orally ingested. Furthermore, it is unclear whether mice cough and whether this is a relevant model to translate to study cough in humans. Traditionally, guinea pig models have proved more successful with cough responses to citric acid and capsaicin showing similarities with

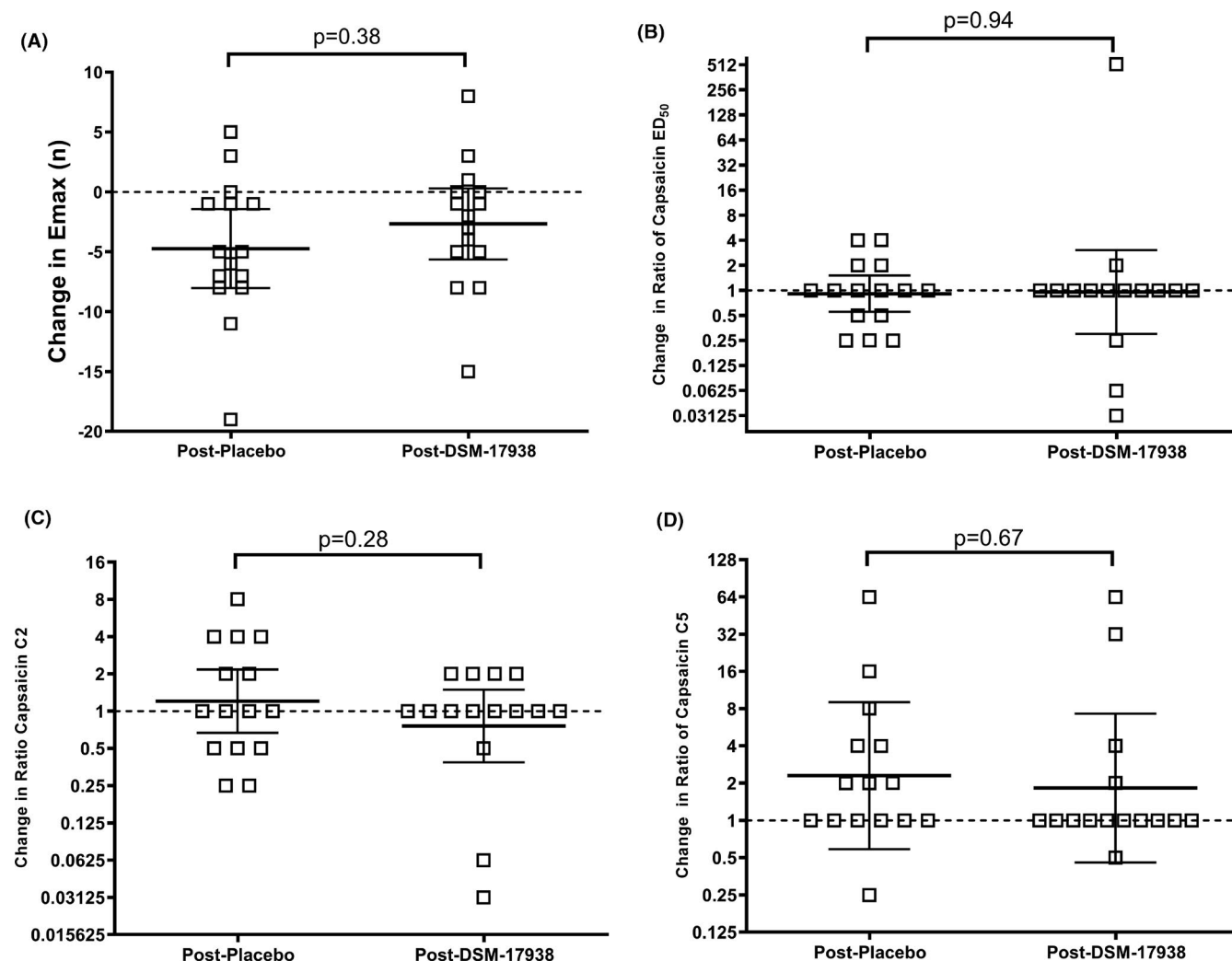


FIGURE 2 The change in cough endpoints after treatment with *Limosilactobacillus reuteri* DSM-17938 compared with placebo, (A) Maximum Cough (Emax), (B) Dose evoking half the Emax (ED₅₀), (C) 2 coughs (C2), (D) 5 coughs (C5). B, C, D shown as the change in geometric mean ratio. Dashed line indicates no change

TABLE 2 Changes in capsaicin-evoked cough responses (Emax and ED₅₀) after placebo compared with after DSM-17938

Variable	Placebo		DSM-17938		Comparison of changes post placebo vs. DSM-17938	
	Change (post-pre) mean difference (95% CI)	p-Value	Change (post-pre) mean (95% CI)	p-Value	Mean difference	p-Value
Emax	-4.73 (-8.03 to -1.44)	.008	-2.67 (-5.63 to 0.30)	.074	2.07 (-2.77 to 6.91)	.38
ED ₅₀	0.91 ^a (0.53 to 1.50)	.70	0.96 ^a (0.30 to 3.04)	.93	1.05 ^a (0.31 to 3.58)	.94

Note: Emax; maximum capsaicin-evoked cough, ED₅₀: dose of capsaicin evoking at least half the Emax.

^aRatio of geometric mean.

humans.^{39,40} Furthermore, pre-clinical allergen and smoke exposure disease models in guinea pigs have shown congruous mechanisms in subjects with allergic asthma and chronic obstructive pulmonary disease (COPD), respectively.^{40,41} Despite these mechanistic similarities, interventional studies targeting TRP channels which have proved to be effective in guinea pigs have not been successful in human trials thus far for refractory chronic cough. There have now

been two TRPV1 antagonist studies, which have both failed to show improvements in spontaneous cough frequency despite reduction in capsaicin-evoked coughs.^{42,43} The major caveat being that such studies were not designed to study effects of cough in allergic asthma where there may be different and distinct neuro-inflammatory mechanisms. Although in this study subjects were all well-controlled with stable mild steroid naïve allergic asthma, it is

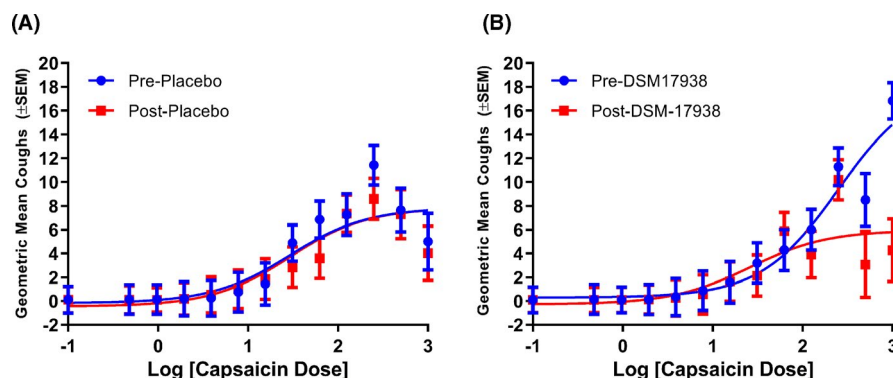


FIGURE 3 The change in capsaicin evoked dose-response after treatment with *Limosilactobacillus reuteri* DSM-17938 compared with placebo, (A) Pre-Post Placebo, (B) Pre-Post DSM-17938. Parameter estimates (geometric mean $\pm$ SEM) derived from a generalized estimating equation (GEE). Overall, there was no effect of intervention compared with placebo ($p = .33$)

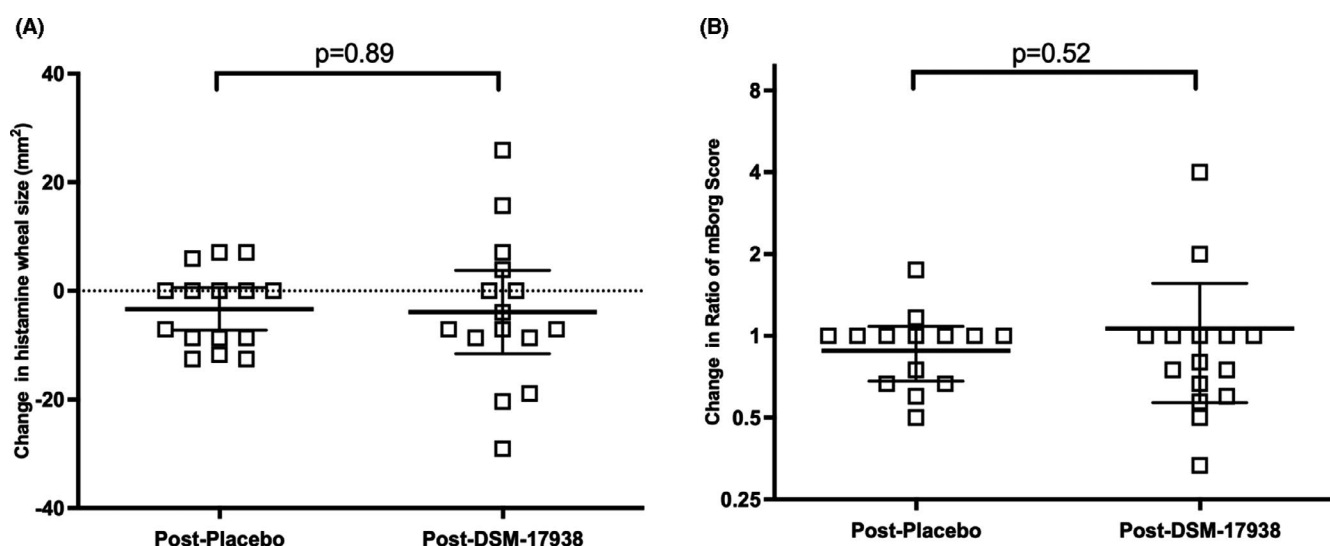


FIGURE 4 The effect of *Limosilactobacillus reuteri* DSM-17938 compared with placebo on the relative changes in (A) Maximum Histamine Skin Prick Wheal Size (mm^2), (B) Intensity of itch sensation on the mBorg scale. Data shown as the change in geometric mean ratio

still uncertain whether during an exacerbation or in more uncontrolled severe asthma whether TRPV1 antagonism has a role to play. A pre-clinical guinea pig model has demonstrated novel TRPV1 expression mediated by neurotrophins after allergen challenge^{41,44}—a process described as phenotypic switching. However, translation of this mechanism with a potent TRPV1 antagonist in an allergen challenge model in humans is yet to be evaluated.

Based on the hypothesis that *L. reuteri* DSM-17938 inhibits TRPV1 systemically, we also assessed the cytokine response of stimulated PBMC following placebo and probiotic treatment. No effect of *L. reuteri* DSM-17938 treatment on baseline or CD3/CD28-stimulated cytokine release was observed. However, there are also TRPV1-independent mechanisms through which *L. reuteri* DSM-17938 might be expected to regulate immune responses. Studies in animal models have demonstrated anti-inflammatory effects of *L. reuteri* strains which are associated with upregulation of Foxp3+ regulatory T cells⁴⁵⁻⁴⁷ and clinically, *L. reuteri* DSM-17938 treatment in infants with colic was shown to increase peripheral blood expression

of FoxP3.^{46,48} While regulatory T cells were not assessed in the current study, it is clear there was no functional effect of these cells on peripheral T-cell response to activation, or on airway immune cell profile as assessed by induced sputum counts. Our results may reflect general poor translation of probiotic effects observed in animal models of asthma to human studies.³⁴ Secondly, it may be that systemic effects of oral probiotic treatment observed in animal models do not occur in human subjects. Thirdly, any inhibitory effects on nerve terminals in the GI tract are only locally on pain fibres and not on any other nerve fibre type. Future clinical studies should focus on the known action of *L. reuteri* DSM-17938 on TRPV1 in the GI tract locally, which may contribute to both analgesic²³ and gastro-protective effects of the bacteria.²⁶

Clinical trials of probiotics have largely focussed on the development of asthma in infants following treatment during the perinatal period,⁴⁹⁻⁵¹ but to date, there is lack of strong evidence to support efficacy of such approaches in treating day to day asthma symptoms in adults. Only three studies using *Lactobacillus reuteri* in children

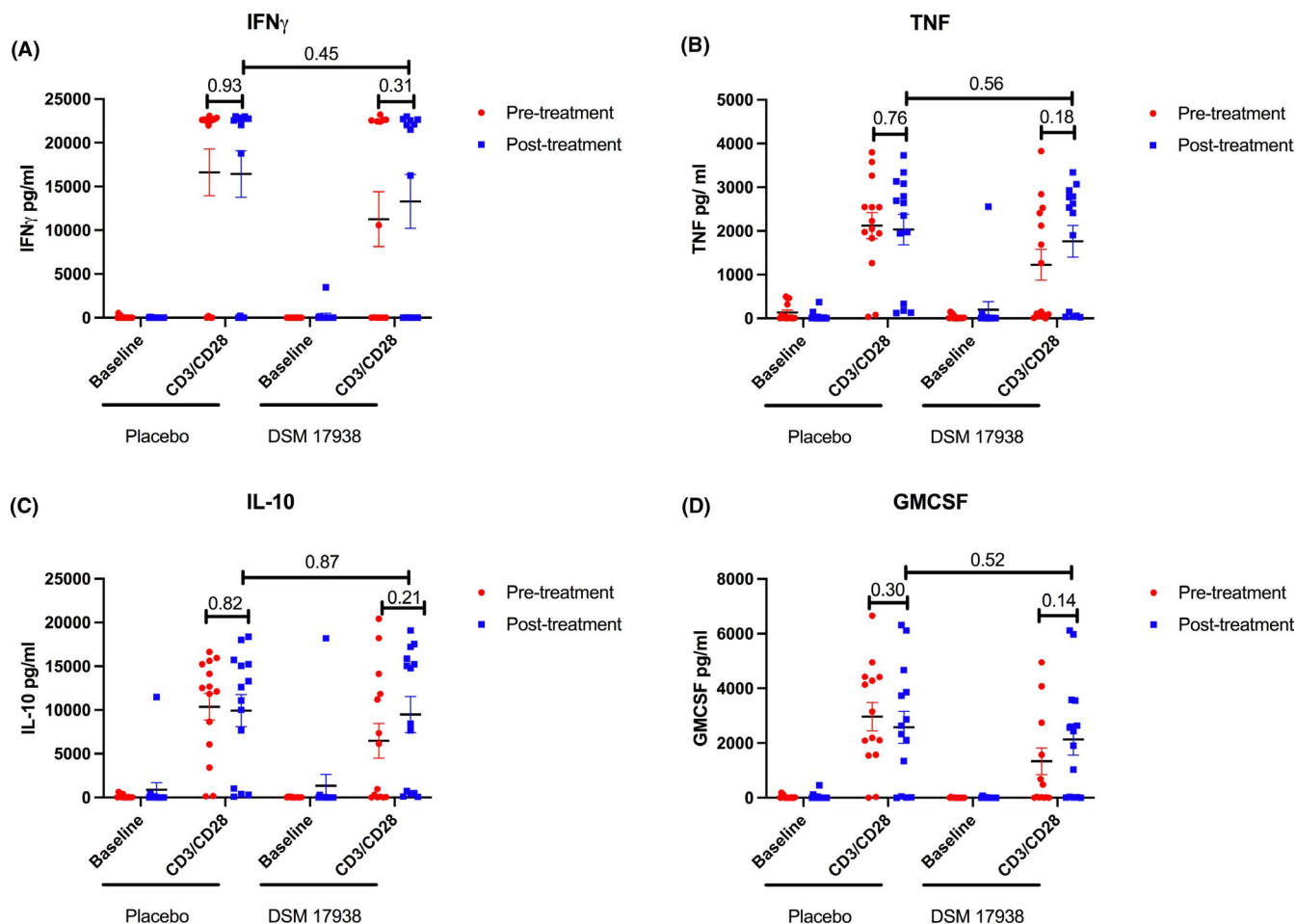


FIGURE 5 (A–D) Baseline and CD3/CD28-stimulated cytokine levels in the supernatants of isolated PBMC before and after treatment with *Limosilactobacillus reuteri* DSM-17938 or placebo. *p* values for specific between-group comparisons are shown

have ever been conducted to evaluate asthma. A non-randomized uncontrolled pilot study over 2 months showed improvement in the Asthma Control Test (ACT) and wheeze in children age 6–17; however, all children also received low-dose inhaled steroids at baseline.⁵² A randomized double-blind placebo-controlled study in children of *Lactobacillus reuteri* plus vitamin D3 compared with placebo found a significant reduction in fraction exhaled nitric oxide (FeNO).⁵³ However, the lack of a *Lactobacillus reuteri* monotherapy arm makes it impossible to conclude whether this was due to vitamin D3 or the probiotic. The third study was a randomized, placebo-controlled trial with oral supplementation with *L. reuteri* ATCC 55730 (the mother strain of DSM-17938) during the last month of gestation and first year of life did not result in a reduction in the prevalence of asthma, allergic rhinitis, eczema at the 7-year follow-up.⁵⁴ Other species such as *Lactobacillus rhamnosus* failed to show improvement in clinical symptoms during and after birch pollen season⁵⁵ and *Lactobacillus acidophilus* also failed to show any improvements in peak flow or spirometry.⁵⁶

There are limitations to this study. First, this study recruited subjects with mild allergic steroid naïve asthma, thus limiting the generalizability of these results to a broader population of more

uncontrolled, unstable or severe asthma. Second, we demonstrated a reduction in capsaicin-evoked coughs after placebo treatment. This could be because (i) cough responses are amenable to voluntary cortical suppression,^{57–60} or (ii) this represents the variability of cough responses repeated over time, particularly after experiencing the cough challenge for the first time. Placebo response rates of up to 30% have recently been reported in phase 2b/3 clinical trials investigating novel anti-tussives.^{61,62} Third, a small number of subjects were investigated, and the study was not powered to detect changes in secondary endpoints. However, the cross-over design would limit some of this variability and no trends were seen in any of the secondary endpoints. Fourth, unlike traditional pharmacokinetic drug studies, it is unclear when the optimum time would be to perform a cough challenge relative to ingestion of the probiotic or the optimum duration of treatment needed to detect systemic effects. The rationale for 4 weeks in this study was based on previous studies showing improvements in abdominal pain and stool frequency after 4 weeks of treatment.^{63–66}

In summary, this is the first double-blind randomized placebo-controlled study in adult patients with allergic asthma testing the potential beneficial effect of *L. reuteri* DSM-17938 outside of the

GI tract. *Limosilactobacillus reuteri* DSM-17938 is known effects in the gastrointestinal tract after oral ingestion. However, our study was unable to demonstrate any systemic effects of *L. reuteri* DSM-17938 on coughs, airway inflammation, smooth muscle, histamine skin prick responses or T-cell activation markers.

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CONFLICTS OF INTEREST

I.S. reports grants from ERS Respire 3 Marie Curie Fellowship, grants and personal fees from Merck Canada, personal fees from GSK and AstraZeneca, outside the submitted work; P.O.B. reports grants and personal fees from AstraZeneca, personal fees from GSK, grants from Novartis, grants and personal fees from Medimmune, personal fees from Chiesi, outside the submitted work. RC, CS, AS, JW, KJK, FM, PF and JB have no disclosures to report. GMG. reports grants and personal fees from AstraZeneca, Genentech, Biohaven and Novartis, and personal fees from Sanofi, and Certior outside of the submitted work.

AUTHOR'S CONTRIBUTIONS

IS, POB, JB, PF and GG involved in concept and design. IS, AS and GG involved in regulatory approvals. IS, RC, CS, JW, FM and PF involved in data generation. IS, KJK, PF and GG involved in statistical analysis and modelling. IS, RC, KJK, POB, JB, PF and GG involved in data interpretation. All authors reviewed the manuscript and approved the final draft.

ETHICAL APPROVAL

The protocol was approved by the local research ethics committees (HiREB:5095, 14th Feb 2019) and Health Canada (Control Number:228322). All subjects provided written informed consent.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request up to 24 months following article publication.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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ONLINE SUPPLEMENT

***Limosilactobacillus reuteri* DSM-17938 for preventing cough in adults with mild allergic asthma: A double-blind randomized placebo-controlled cross-over study**

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STUDY PROCEDURES IN DETAIL:

1. *Full Dose-Response Capsaicin Challenge Testing*

A full dose-response capsaicin challenge was performed at visit 1. Patients inhaled increasing doubling concentrations (0.49-1000 μ M/L) of capsaicin through a nebuliser adapted to control dosage and inspiratory flow rate. Each dose of capsaicin was inhaled in 4 single breaths separated by 30 seconds. Following each inhalation, the total number of coughs evoked (defined as explosive cough sounds) at each concentration within the first 15 seconds were recorded. The total maximum number of coughs evoked by any dose (E_{max}) is documented along with the dose of capsaicin that induced at least 50% of the maximal cough response (ED_{50}). The dose evoking 2 cough and 5 coughs at any dose of capsaicin at the first inhalation was also documented. If subjects did not achieve C2 or C5, then twice the maximum dose of capsaicin 2000 μ M/L was assigned. The lapel microphone of the VitaloJAK cough recorder was attached to the patient throughout the duration of the challenge and used to later verify the number of coughs induced at each dose of capsaicin.

2. *Methacholine Challenge Test (MCT)*

Nebulised methacholine was administered in doubling concentrations from 0.0625mg/mL up to 16 or 32 mg/ml via the 2-minute tidal breathing method. At each concentration, FEV_1 was measured 30s and 90s after the end of each inhalation. The % fall in FEV_1 from baseline was calculated after each dose using the best FEV_1 and the test was terminated once a 20% fall in FEV_1 was documented or the maximum concentration was reached. Salbutamol was withheld for at least 4 hours.

3. *Sputum Induction and Processing:*

Prior to performing the sputum induction, spirometry was repeated to ensure FEV₁ had returned to within 10% of the baseline prior to the MCT. This occasionally required 400mcg of salbutamol in addition to the post-MCT salbutamol. Nebulised hypertonic saline (3/5/7%) was administered at 7 min intervals provided the FEV₁ did not fall by more than 20% from pre-sputum baseline. If there was a drop in FEV₁ between 10-20% then a lower saline concentration was administered.

The total cell count was determined using a Neubauer haemocytometer chamber (Hausser Scientific, Blue Bell, PA) and expressed as the number of cells per gram of sputum. Two slides were stained with Diff Quik (American Scientific Products, McGaw Park, IL) and the differential cell counts (400 cells/slide) of the 2 slides were averaged. Differential cell counts were expressed as the % of the total cell count (400 non-squamous cells).

4. *Skin Prick Testing (SPT) and Histamine Dose Titration*

A single-use plastic lancet was dipped in the common aero-allergen extracts (house dust mite, cat, dog, mixed grass, mixed trees, aspergillus) and gently rotated on the patient's forearm at visit 1 only. A negative control and a positive histamine control were also assessed. A positive result was demonstrated by a wheal of ≥ 2 mm. At least one positive SPT was required to be included in the study. The same method was used for histamine doses of 0.25, 0.5, and 1mg/ml on the opposite arm at the end of each visit (1-4). After 10 mins, the wheal area size (mm²) was measured for each dose of histamine, and the maximum wheal area (mm²) documented. All subjects were asked to rate the intensity of itch sensation on a mBorg Scale after 10 mins.

TABLE S1: Diary Completion Rates (%)

Treatment Period 1		Treatment Period 2	
Treatment	Completion rate	Completion Rate	Treatment
Lactobacillus	94.29	94.44	Placebo
Placebo	100.00	100.00	Lactobacillus
Placebo	100.00	91.18	Lactobacillus
Lactobacillus	100.00	100.00	Placebo
Placebo	100.00	100.00	Lactobacillus
Lactobacillus	100.00	100.00	Placebo
Placebo	97.14	100.00	Lactobacillus
Lactobacillus	93.10	83.87	Placebo
Placebo	96.43	92.86	Lactobacillus
Placebo	100.00	100.00	Lactobacillus
Lactobacillus	100.00	100.00	Placebo
Placebo	100.00	100.00	Lactobacillus
Lactobacillus	96.67	100.00	Placebo
Placebo	100.00	100.00	Lactobacillus
Lactobacillus	100.00	100.00	Placebo

FIGURE S1: The effect of *L. reuteri* DSM-17938 compared with placebo on the relative changes in Emax in males and females. Individual data shown with analysis between groups as ANOVA with Tukey correction.

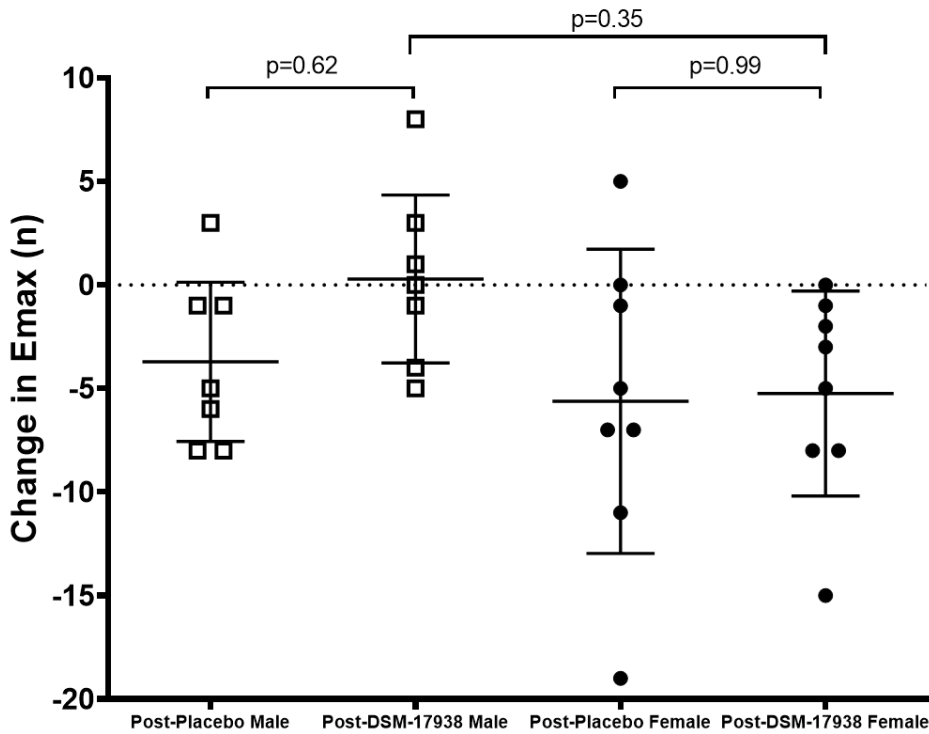


FIGURE S2: The number of subjects dropping out with doubling doses of capsaicin.

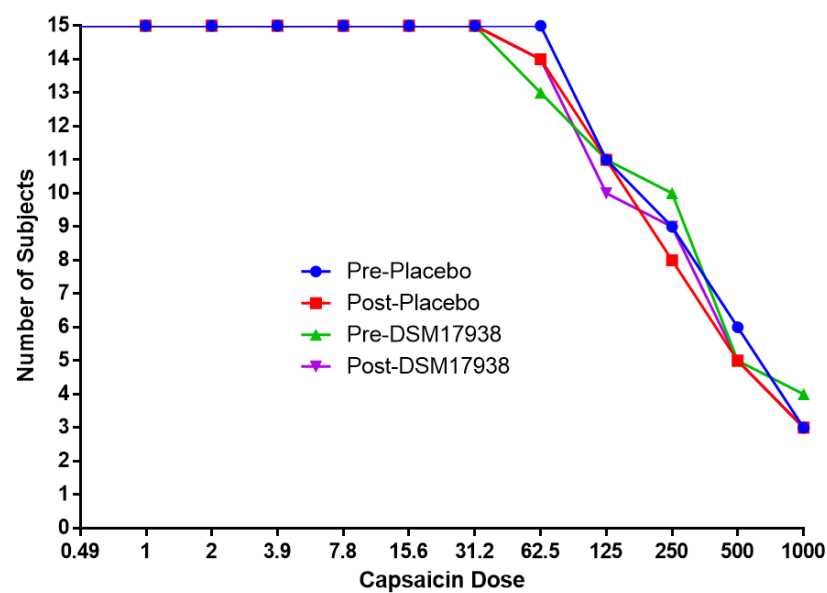


FIGURE S3: The effect of *L. reuteri* DSM-17938 compared with placebo on the relative changes in Histamine Skin Prick Wheal Size (mm²) with incremental doubling doses of histamine A: Pre-Post Placebo, B: Pre-Post DSM-17938. Data shown as mean (± S.E.M).

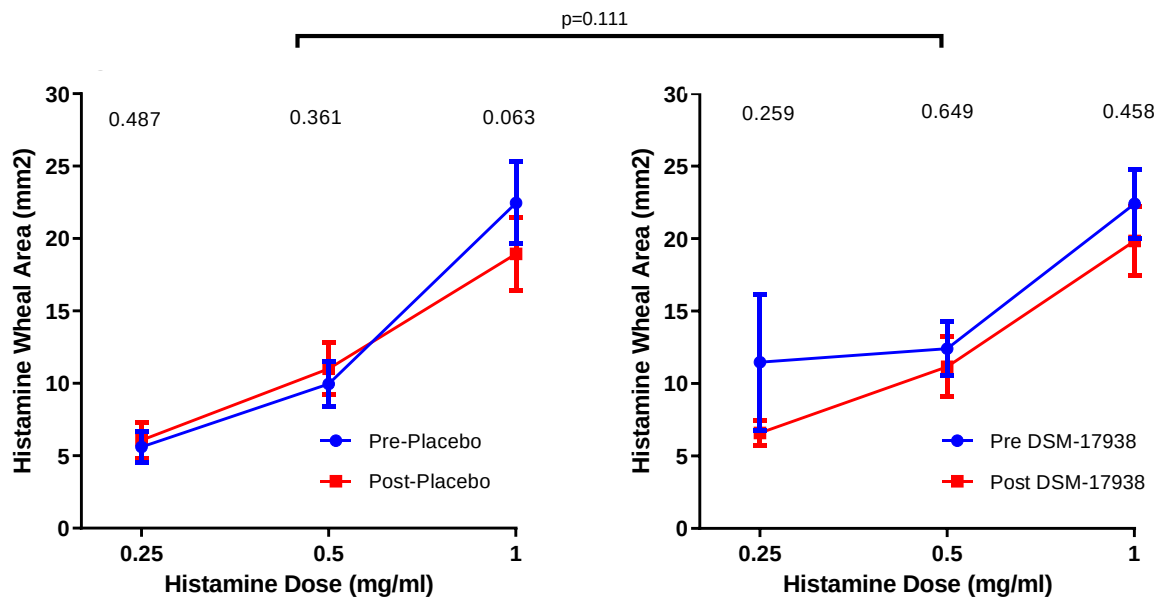


FIGURE S4: The effect of *L. reuteri* DSM-17938 compared with placebo on the relative changes on sputum inflammatory cells (%). Analysis done on the relative % change post-DSM-17938 compared with placebo.

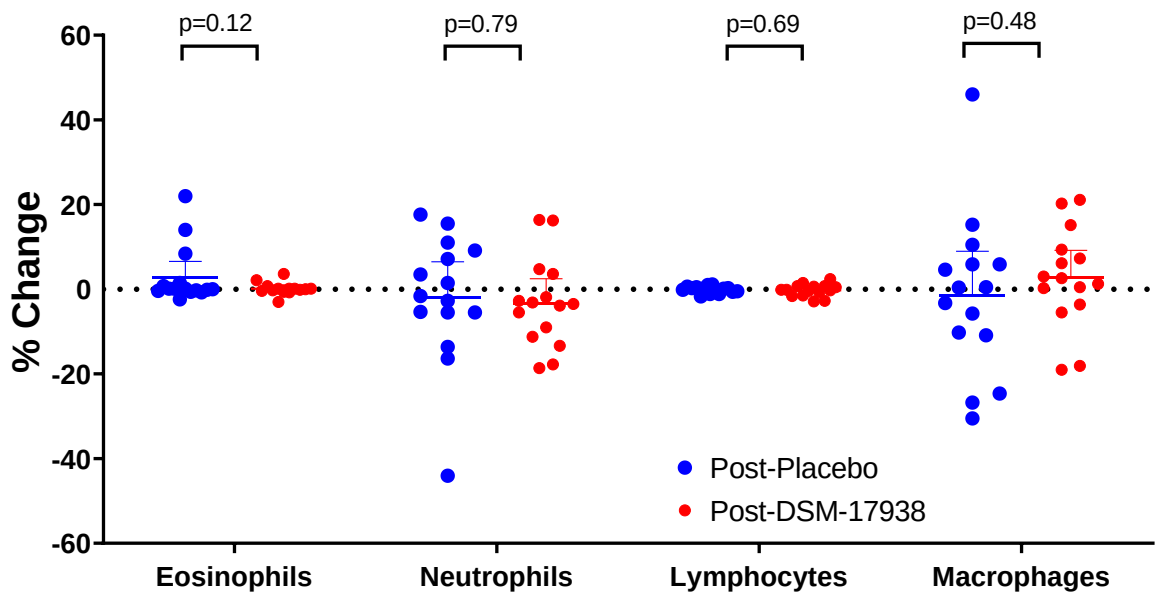


Figure S5: The effect of *L. reuteri* DSM-17938 compared with placebo on the relative change in methacholine PC₂₀. Data shown as the change in geometric mean ratio.

