Research Article

A single intravesical instillation of *Lactobacillus rhamnosus* GG is safe in children and adults with neuropathic bladder: A phase Ia clinical trial

Catherine S. Forster [1,2], Michael H. Hsieh[2,3], Marcos Pérez-Losada [4,5], Ljubica Caldovic [6], Hans Pohl [3], Inger Ljungberg[7], Bruce Sprague[3], Crystal Stroud[6], Suzanne Groah[7]

[1]Department of Pediatrics, Children's National Health Systems, Washington, DC, USA, [2]Biomedical Research Institute, Rockville, Maryland, USA, [3]Department of Urology, Children's National Health Systems, Washington, DC, USA, [4]Computational Biology Institute, Milken Institute School of Public Health, George Washington University, Ashburn, Virginia, USA, [5]CIBIO-InBIO, Centro de Investigação em Biodiversidade e Recursos Genéticos, Universidade do Porto, Campus Agrário de Vairão, Vairão, Portugal, [6]Children's Research Institute, Children's National Health Systems, Washington, DC, USA, [7]Paralysis Rehabilitation and Recovery Program, Spinal Cord Injury Research, MedStar National Rehabilitation Hospital, Washington, DC, USA

Context/objective: Manipulation of the microbiome is an emerging approach to promote health. We conducted a Phase Ia safety study of a single bladder instillation of probiotics in asymptomatic patients with neuropathic bladder to determine the tolerability and safety of a single *Lactobacillus* instillation.
Design: Phase Ia safety study.
Setting: Outpatient rehabilitation clinic at a rehabilitation hospital (adults) and urology clinic at a free-standing children's hospital (children).
Participants: Ten patients with neuropathic bladder were included: five children with spina bifida and five adults with spinal cord injury.
Interventions: A single *Lactobacillus rhamnosus* GG (Culturelle, 20 billion live organisms) instillation.
Outcome measures: After the instillation, participants self-monitored symptoms using the Urinary Symptoms Questionnaire for People with Neuropathic Bladder using Intermittent Catheterization daily for one week. Repeat urinalysis, urine culture, and 16S bacterial rRNA-based microbiome analyses were performed 7–10 days after instillation.
Results: Probiotic instillation was well-tolerated. One child had upper respiratory tract symptoms during the trial, and two had transient cloudy urine. No adults reported any symptoms following instillation. *Lactobacillus* did not grow on culture post-instillation. There were differences in beta diversity of the urine microbiome in children vs. adults with neuropathic bladder ($P < 0.0156$). *Lactobacillus* was present in the pre-instillation urinary microbiomes all of the adults and 4 out of 5 of the pediatric subjects, and identified in 4 out of 5 of both the adult and pediatric subjects' post-instillation urinary microbiomes.
Conclusion: Intravesical instillation of Culturelle probiotic may be safe and well-tolerated in patients with neuropathic bladder.

Keywords: Microbiome, Urinary tract infection, Neuropathic bladder, Probiotics, Safety study

Correspondence to: Catherine S. Forster, Department of Pediatrics, Children's National Health Systems, 111 Michigan Ave NW, Suite 4800M, Washington, DC 20010, USA. Email: csforster@childrensnational.org

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Supplemental data for this article can be accessed at https://doi.org/10.1080/10790268.2019.1616456

Introduction

The importance of the microbiome, the culture-independent bacterial milieu growing in and on our bodies, is becoming increasingly recognized in human health

DOI 10.1080/10790268.2019.1616456

and disease.[1] The urinary microbiome is hypothesized to be a factor in the pathogenesis of urinary tract infections (UTI), especially in patients with recurrent UTIs, such as those with neuropathic bladders. Indeed, the urinary microbiome in patients with neuropathic bladder is different than non-neuropathic bladders and contains more uropathogens than is seen in those with non-neuropathic bladders.[2,3] Comparatively, the urinary microbiome of individuals with normally functioning bladders have a higher proportion of *Lactobacillus.*[2] Limited data demonstrates that host-detrimental changes in the urinary microbiome, a state referred to as dysbiosis, occur in the setting of UTI. This suggests that microbiome-modulating therapies, such as probiotics, may have utility in correcting dysbiosis and decreasing the number of UTIs in patients with neuropathic bladders. While the use of oral probiotics is increasingly wide-spread, oral probiotics have not been shown to be effective in preventing recurrent UTIs.[4] As UTIs are a local, rather than systemic, infection, instillation of probiotics directly into the bladder may be more likely to correct the dysbiosis and prevent UTIs than oral administration. However, there is no data on the safety or efficacy of intravesical administration of probiotics. Therefore, we conducted an FDA-approved phase 1 safety study of a single intravesical administration of *Lactobacillus* probiotics in healthy adults and children with neuropathic bladder. We sought to determine the tolerability and safety of a single instillation, whether the instilled *Lactobacillus* persists in the urine determined by cultivation and 16S rRNA gene high-throughput sequencing, and if children differ from adults with regards to these endpoints.

Materials and methods

Patients

Pediatric patients with spina bifida (SB) and adults with spinal cord injury (SCI) were enrolled in this study. Patients were eligible for participation if they met the following criteria: neuropathic bladder managed with clean intermittent catheterization, living in the community (i.e. not within a long-term care facility), and presence of SCI for over one year for adults. We excluded patients with: (1) genitourinary pathology beyond neuropathic bladder; (2) instillation of other intravesical agents; (3) psychological or psychiatric conditions influencing the ability to follow instructions; (4) participation in a confounding study; (5) pregnant or breastfeeding women; (6) immunodeficiencies; active or chronic serious infections; (7) cancer/autoimmune disorders; (8) allergy to any component in the probiotic product; (9) a change in neurologic status in the previous 2 weeks; (10) antibiotic use in the previous 2 weeks; (11) sensitivity to ampicillin or tetracycline; and (12) UTI within the previous 2 weeks (as defined by Infectious Diseases Society of America CAUTI Guidelines).[5] Pediatric patients also had to be between 6 and 18 years of age in order to be eligible. This work was conducted with formal approval by the Institutional Review Board at both sites, with informed consent provided by all participants or parents / legal guardians for pediatric patients.

Intervention

At the time of enrollment, subjects completed a 12-question survey on urinary symptoms. Following survey completion, a urine sample was collected at home using a new catheter for urinalysis, urine culture, and bacterial 16S rRNA gene-based analyses. Study personnel then trained subjects on bladder instillation technique, and subjects instilled *Lactobacillus rhamnosus* GG (ATCC 53103, Culturelle GG, 20 billion live organisms) into their bladders with study personnel supervising at the study site. This strain was chosen for use in this work as it is readily available and commercially accessible, allowing for rapid translation into clinical practice. Pediatric patients received smaller volumes of instillate based on their anticipated bladder capacity, which was 10% of their anticipated capacity. Following instillation, subjects were observed in the clinic for 30 min to assess for adverse reactions. Participants then self-monitored symptoms using the Urinary Symptoms Questionnaire for People With Neuropathic Bladder using Intermittent Catheterization (USQNB-IC),[6] a 29-item instrument with face- and content-validity in these populations, to assess patient-reported outcomes regarding urinary symptoms daily for one week. Participants were instructed to contact study personnel for any urinary symptoms. Participants returned to the clinic 7–10 days after instillation for repeat urinalysis, urine culture, and microbiome analyses. Urinalysis and urine culture were performed by Quest Diagnostics (Chantilly, VA). Urinalysis was completed utilizing standard clinical microbiology semiquantitative chemical testing using commercial disposable test strips, and urine culture was performed using standard laboratory techniques.

Sample preparation and DNA isolation

Urinary bacteria were pelleted using low-speed centrifugation, washed with phosphate buffered saline (PBS: 10 mM Na2HPO4, 1.8 mM KH2PO4, 137 mM NaCl and 2.7 mM KCl) and stored at −80°C. Depending on the size of the pelleted material, genomic DNA was isolated either with the DNeasy Kit (Qiagen) using manufacturer's protocol for Gram-negative bacteria or

with the QIAmp DNA Micro Kit (Qiagen) using manufacturer's protocol for DNA isolation from urine. Purified DNA was quantified using NanoDrop spectrophotometer (Thermo Fisher Scientific). Fractions of human and bacterial DNA in each sample were determined using Femto Human and Femto Bacterial DNA quantification kits (Zymo Research) according to the manufacturer's instructions. DNA isolation using PBS as the starting material was used as a negative control.

16S rRNA gene amplification and high-throughput sequencing

V4 regions of 16S rRNA genes were amplified using primers 5'-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGGTGCCAGCMGCCGCGGTAA-3' and 5'-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTWTCTAAT-3' and the following reagent concentrations: 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 1.5 mM MgCl2, 200 µM of each dNTP, 2 µM of each primer, 1% glycerol, 0.3 U AccuPrime Taq polymerase (Thermo Fisher Scientific) and 25 ng of template DNA in 20 µl total volume. Amplification conditions were 2 min at 95°C initial denaturation followed by 30 cycles of 20 s denaturation at 95°C, 15 s annealing at 55°C and a 5-min extension at 72°C, and a 5-min final extension at 72°C. Amplification reaction without template DNA was used as a negative control. Amplification products were purified with the AMPure XP system (Beckman Coulter) and their size was verified with the DNA 1000 Kit (Agilent). Indexing and pooling of amplification products were carried out according to Illumina's 16S Metagenomic Sequencing Library Preparation protocol. Negative control samples were indexed and included in the library preparation. The resulting library was sequenced using Illumina MiSeq Reagent Kit v2 (500 cycles), which produced 250 bases paired-end reads.

Statistical analysis

Raw FASTQ files were processed in Mothur v1.35.1.[7] Default settings were used to minimize sequencing errors.[8] Clean sequences were aligned to the SILVA128-based bacterial reference alignment at http://www.mothur.org. Chimeras were removed using uchime,[9] and non-chimeric sequences were classified using a naïve Bayesian classifier.[10] Sequences were clustered into Operational Taxonomic Units (OTUs) at the 0.03 threshold (species level). OTU sequence representatives and taxonomy were imported (BIOM format) into QIIME for subsequent analyses.[11] Samples were subsampled (rarefaction analysis) to the smallest sample size to remove the effect of sample size bias on community composition. Trees for phylogenetic diversity calculations were constructed using FastTree and midpoint rooting.[12] Taxonomic alpha diversity was estimated as the number of observed OTUs, Chao1 and Shannon indices. Phylogenetic alpha diversity was calculated by Faith's phylogenetic diversity index.[13] Phylogenetic beta diversity Unifrac metrics (unweighted and weighted unifrac) were calculated between pairs of samples. The dissimilarity between samples was explored using principal coordinates analysis (PCoA) and both Unifrac distances. Linear mixed-effects (LME) models analysis was applied to both alpha diversity indices and taxa (genera and phyla) proportions (response) while accounting for non-independence of subjects, subject's baseline levels (random effect), predictors (*e.g.* pyuria) and confounders (*e.g.* age, sex). Beta diversity Unifrac indices were compared using permutational multivariate analysis of variance (adonis) as implemented in the vegan R package.[14] Significance was determined through 10,000 permutations. Sample pairs were compared using the Fisher exact test. Bonferroni or Benjamini-Hochberg FDR multiple test correction methods were applied. All analyses were performed in mothur, QIIME and RStudio.[15]

Results

Patients

Five children with SB and five adults with SCI were included in this study. Adult patients had a mean age of 35 years, were all male, and were, on average, 4.1 years post-SCI. Two patients had a cervical injury, two had a thoracic injury, and one had a lumbar injury, all of which were incomplete. Pediatric patients had a mean age of 8.4 years and were 80% male (Table 1).

Table 1 Patient demographics.

	Adults (*n* = 5)	Pediatrics (*n* = 5)
Age (years)	35.4 (12.7)	8.4 (2.3)
Male (*n* (%))	5 (100)	4 (80)
Level of spinal cord injury		
Cervical (*n* (%))	2 (40)	–
Thoracic (*n* (%))	2 (40)	–
Lumbar (*n* (%))	1 (20)	–
Year post injury	4.1 (3.6)	–
Myelomeningocele level		
L4	–	1 (20)
L5-S1		4 (80)

Note: Data presented as: mean (SD) unless otherwise specified.

Clinical symptoms

Lactobacillus instillations were well-tolerated by all patients, with no immediate adverse events. In the pediatric group, one child developed upper respiratory symptoms following instillation, and two children reported transient cloudy and malodorous urine that self-resolved during the week following instillation. Neither of these children received antibiotics for these symptoms. No adults reported urinary symptoms in the week following probiotic instillation.

Urinalysis and urine culture

Seven of the ten patients had a decrease in urinary pH following probiotic instillation (mean change (95% confidence interval): −0.45 (−0.99, 0.01) and one patient had no change in pH. No children had positive nitrites on their pre-instillation urinalysis whereas four of the five children had positive nitrites following the instillation. Two adults had nitrites present on their pre-instillation urinalysis, and one had nitrites following instillation. One pediatric patient and three adults had pyuria present prior to instillation, and four pediatric patients and two adults had pyuria after the instillation (Table 2).

There were no changes in results from the pre-instillation urine culture to the post-instillation urine culture in any adult patients. Of the five pediatric patients, two had no changes between the pre- and post-instillation urine cultures, two had an increased colony count of the same bacteria in the post-instillation culture compared to the pre-instillation culture, and one patient had a negative pre-instillation urine culture, and a positive urine culture post-instillation (Table 2).

Microbiome

There was a significant difference in beta diversity (inter-sample) of the combined (i.e. both pre- and post-instillation) urinary microbiomes in children versus adults (P = 0.0156) (Fig. 1). There were no differences in any of the alpha diversity (intra-sample) indices (i.e. OTU richness, Shannon diversity, phylogenic diversity, Chao 1 richness) between the pre- and post-instillation microbiomes. *Lactobacillus* was present in the urinary microbiomes of all adult patients, and four out of five pediatric patients pre-instillation. Following instillation, *Lactobacillus* was present in four of the five adult patients, and four of the five pediatric patients. Three of the four pediatric patients had persistent *Lactobacillus* in both their pre- and post-instillation microbiomes, while one pediatric patient only had *Lactobacillus* present following the instillation. In the one pediatric patient that had *Lactobacillus* that was present only in the post-instillation urinary microbiome, it was not the predominant organism: there were higher proportions of *Streptococcus, Prevotella*, *Escherichia*, and *Veillonella* (Fig. 2). The largest mean change in proportion of bacteria in all patients between pre- and post-instillation was *Escherichia,* although there was significant intra-patient variability. In pediatric patients, those patients with changes in their urine culture also had an increase in the proportion of *Escherichia* between their pre- and post- instillation urinary microbiomes. While the majority of bacteria identified were present in both the pre- and post-instillation microbiomes, nine out of ten patients had significant changes in the proportion of specific bacteria between the pre- and post- microbiomes (Table 3).

Table 2 Pre- and post-instillation urinalysis and urine culture results.

	Pre-instillation						Post-instillation					
	pH	Nitrites	Leukocyte esterase	Urine WBCS	Culture result, colony count (CFU/mL)	Culture result, organism	pH	Nitrites	Leukocyte esterase	Urine WBCS	Culture result, colony count (CFU/mL)	Culture result, organism
Children	7.0	Absent	1+	0–5	1,000-10,000	*E. coli*	6.0	Present	2+	20–40	>100,000	*E. coli*
	7.5	Absent	0	0–5	No Growth	–	6.0	Present	1+	10–20	>100,000	*E. coli*
	6.0	Absent	2+	10–20	10,000-50,000	*E. coli*	7.0	Present	1+	6–10	>100,000	*E. coli*
	6.5	Absent	0	0–5	>100,000	*E. coli*	6.0	Present	1+	10–20	>100,000	*E. coli*
	6.5	Absent	0	0–5	No growth	–	7.0	Absent	0	0–5	No growth	–
Adult	6.0	Absent	0	1–2	No growth	–	5.5	Absent	0	0–5	No growth	–
	6.0	Present	1+	10–20	>100,000	*E. coli*	6.0	Present	2+	10–20	>100,000	*E. coli*
	7.5	Absent	2+	0–5	>100,000	*Pseudomonas*	7.0	Absent	2+	0–5	>100,000	*Pseudomonas*
	7.0	Absent	2+	6–10	>100,000	*E. coli*	6.0	Absent	1+	6–10	>100,000	*E. coli*
	7.0	Absent	2+	6–10	>100,000	*E. coli*	6.0	Absent	Trace	0–5	>100,000	*E. coli*

WBCs: white blood cells; CFU: colony-forming units.

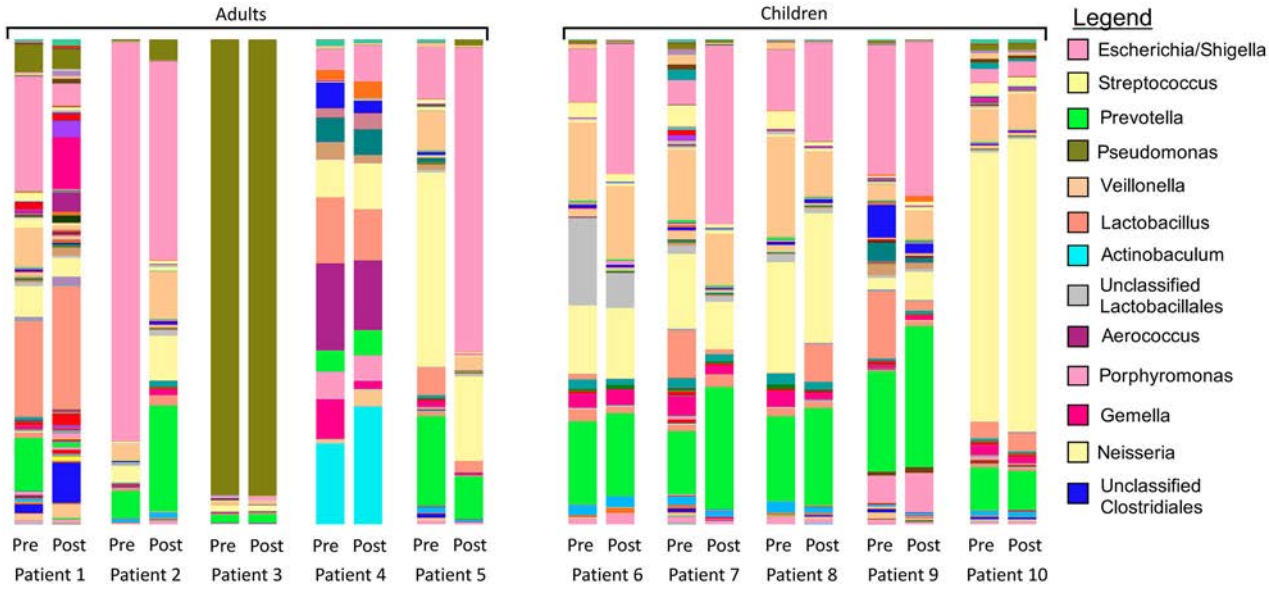

Figure 1 Microbial diversity in each of the 10 patients both pre- and post-instillation of Lactobacillus. Beta diversity is significantly different between all adult samples (both pre- and post-instillation) and all pediatric samples. (P = 0.0156). The legend includes the 13 most common bacteria: the entire list of bacteria identified can be found in the supplementary material.

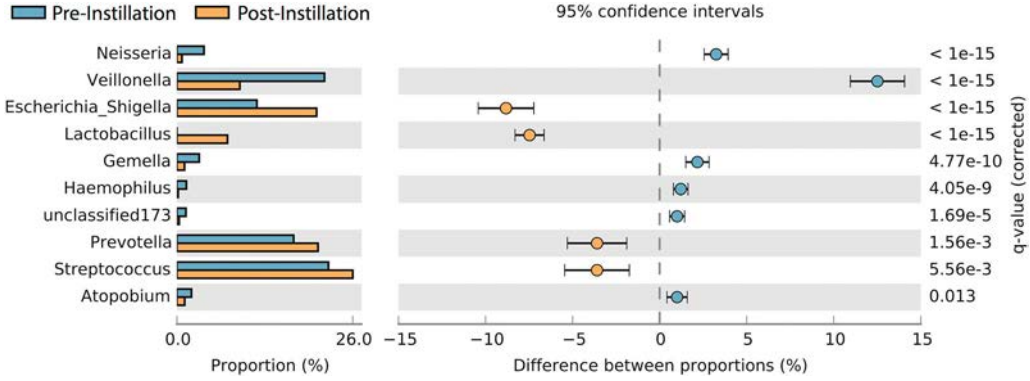

Figure 2 Significant differences (Fisher exact test) in the relative abundance of bacterial genera in the urinary microbiome of a single pediatric patient between pre (first bar)-and post-instillation (second bar). This is the only patient in the cohort to have a new presence of Lactobacillus following the instillation.

Discussion

In this work, we show that one dose of intravesical *L. rhamnosus* GG is well-tolerated, without any adverse events or change in urinary symptoms, suggesting that intravesical instillation is a safe route of administration. The only urinary symptoms reported following probiotic instillation were isolated new onset cloudy and malodorous urine in two pediatric patients, which were not considered to be suggestive of a UTI per the Infectious Disease Society of America's guideline on catheter-associated UTIs. Therefore, no intervention was suggested.[16] The lack of other urinary symptoms suggests that this method of administration of *Lactobacillus* is well-tolerated. Indeed, previous *in vitro* work with *L. rhamnosus* GR-1 demonstrated a lack of urothelial cytotoxicity.[17] Further, 7–10 days following the single instillation, there was no change in alpha diversity, although the majority of patients had significant changes in the proportions of bacteria within their own microbiomes.

No urinary symptoms developed as a result of the intravesical administration of *Lactobacillus.* However, there was a change in asymptomatic bacteriuria in the pediatric patients: Three of the five pediatric patients demonstrated an increase in *E. coli* growth on urine culture at 7–10 days post-instillation. Asymptomatic bacteriuria and pyuria, which are common in these children and frequently persist for weeks in the absence of

Table 3 Bacteria with significant changes in proportions between pre- and post-instillation microbiomes.

Bacteria (*n* = patients with significant change)	Percent of total subjects with significant increase	Percent of total subjects with significant decrease
Escherichia (*n* = 8)	40	40
Prevotella (*n* = 7)	60	10
Lactobacillus (*n* = 7)	30	40
Streptococcus (*n* = 6)	40	20
Veillonella (*n* = 6)	40	20
Acinetobacter (*n* = 3)	20	10
Neisseria (*n* = 3)	0	30
Corynebacterium (*n* = 2)	20	0
Pseudomonas (*n* = 2)	0	20
Campylobacter (*n* = 2)	20	0
Haemophilus (*n* = 2)	0	20
Gemella (*n* = 2)	0	20

Note: Thirteen additional microbes were significantly changed in a single patient. *N* in Bacteria column refers to total number of patients with a significant change in that bacteria, all percentages are out of the total population of *n* = 10.

symptoms, are not associated with either increased risk of developing a UTI or renal scarring.[18,19] As there were not any corresponding clinical symptoms, these positive cultures are not UTIs, but rather asymptomatic bacteriuria that is representative of the underlying bacterial colonization of the urinary tract. Given the clean technique used and the frequency with which these children are catheterized at baseline, it is unlikely that this increase in *E. coli* growth on urine culture is due to inadvertent instillation of *E. coli*. Rather, this relative increase in *E. coli* could potentially be a result of bacterial displacement from urothelial cells. Limited data suggest that various strains of *Lactobacillus* have the ability to displace bacteria adhered to vaginal epithelial cells.[20] *E. coli* colonization of the bladder is mediated though urothelial binding. Therefore, it is possible that the increase in *E. coli* cultured from urine following *Lactobacillus* instillation is a result of bacterial displacement from the uroepithelial cells, a hypothesis that is further supported by the relative increase in *Escherichia* in the post-instillation urinary microbiomes. However, none of the adult patients had a change in urine culture results following probiotic instillation, suggesting that an alternative explanation for the increase in *E. coli* bacteriuria following instillation is likely.

There was an overall decrease in the mean urine pH on the post-instillation urinalysis compared to the pre-instillation urinalysis, with 7 of the 10 patients demonstrating a lower pH following instillation. *L. rhamnosus* GG, like most strains of *Lactobacillus*, produces acids that result in a lowering of environmental pH.[21] Further, the antimicrobial properties of *L. rhamnosus* GG function in a pH-dependent manner.[21] Therefore, changes in urinary pH may serve as a proxy for the increase in such bacteria in the urinary microbiome. However, there are several other bacterial species frequently found within the urinary microbiome that also produce acid, suggesting that a lower pH cannot be solely attributed to the presence of *L. rhamnosus* GG. It is possible that the single instillation of *L. rhamnosus* GG was associated with a microbiome shift, allowing for the growth of other acid-producing bacteria. For example, six of the 10 patients had increased proportions of *Streptococcus*, another lactic-acid producing bacteria. This concept of microbial community shift has been demonstrated to occur in a pH-dependent manner in other settings,[22] providing support for the presence in a community shift in the urinary microbiome concordant with a change in pH. However, our results are preliminary, and further work with recurrent instillation of *Lactobacillus* is needed to fully investigate this hypothesis.

The lack of side effects and the tolerability of intravesical administration of *Lactobacillus* make this route of administration a viable method of UTI prophylaxis. In addition to this favorable safety profile, *Lactobacillus* is a good candidate for UTI prophylaxis given its impact on uropathogens through its ability to regulate uropathogenic growth.[20] However, there are more than 80 species of *Lactobacillus,* each with varying effects on uropathogens.[4] *L. rhamnosus* GG, the strain used in this work, has *in vitro* activity against strains of uropathogenic *E. coli*,[23] suggesting that it is potentially effective in preventing UTIs. Despite this, neither of the two trials examining the utility of *L. rhamnosus* GG in preventing UTIs demonstrated a significant reduction in episodes of UTI.[24,25] However, in these prior studies, the probiotics were administered either orally or vaginally in women with recurrent UTI, both routes of administration that do not lead to bladder colonization. Thus, assuming that *L. rhamnosus* GG can only exert antibacterial properties locally, oral and vaginal administration of this probiotic may preclude potential therapeutic effects. While intravesical *L. casei* has demonstrated efficacy in treating chronic UTIs in mice,[26] to our knowledge, ours is the first human trial of intravesical *Lactobacilli rhamnosus* GG.

There are several limitations to this work, including the small number of patients in this work. Other limitations include the different etiologies of neuropathic bladder in the adults and children, the inability to make comparisons between patients with flaccid and

spastic bladders, and that all patients are from a single geographic region. Future work will focus on a more homogenous patient population. We cannot verify that the *Lactobacillus* which appeared in the urine of some patients post-instillation was *L. rhamnosus* GG due to an inability of our methodologies to detect specific bacterial strains. Although this study was designed to test the safety and tolerability of a single dose of intravesical dose of *Lactobacillus*, and therefore not designed to the study longitudinal changes in the urinary microbiome as a result of intravesical probiotic use, we are unable to comment on the long-term effect of *Lactobacillus* on the urinary microbiome.

Conclusion

In this pilot study, a single dose of intravesical *L. rhamnosus* GG was well-tolerated. Given these results, a larger trial has been initiated to evaluate the safety, tolerability, efficacy and usability of a self-management protocol for pre-infectious urinary symptoms using instilled intravesical *Lactobacillus.*

Acknowledgements

The content of this work are solely the responsibility of the authors and do not necessarily represent the official views of the National Center for Advancing Translational Sciences or the National Institutes of Health.

Disclaimer statements

Contributors None.

Funding This work was funded by Patient-Centered Outcomes Research Institute (PCORI) [Grant Number AD-1310-08215]. This work was supported by Eunice Kennedy Shriver National Institute of Child Health and Human Development [Grant Number K12 HD001399]; National Center for Advancing Translational Sciences [Grant Number UL1TR001876]; National Center for Advancing Translational Sciences [Grant Number KL2TR001877]; National Institutes of Health [Grant Number K12HL119994]; National Institutes of Health (US) [Grant Number RO1: KO113504].

Conflicts of interest Drs Caldovic, Pohl, and Groah have submitted a patent for the intravesical use of *Lactobacillus.*

ORCID

Catherine S. Forster http://orcid.org/0000-0002-6233-1070
Marcos Perez-Losada http://orcid.org/0000-0002-2585-4657
Ljubica Caldovic http://orcid.org/0000-0002-9140-5585
Hans Pohl http://orcid.org/0000-0001-9679-0948

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