Galactooligosaccharide supplementation provides protection against *Citrobacter rodentium*-induced colitis without limiting pathogen burden

Hatem Kittana,1 Maria I. Quintero-Villegas,1 Laure B. Bindels,1 João Carlos Gomes-Neto,1 Robert J. Schmaltz,1 Rafael R. Segura Munoz,1 Liz A. Cody,1 Rodney A. Moxley,2 Jesse Hostetter,3 Robert W. Hutkins1 and Amanda E. Ramer-Tait1,*

Abstract

Many enteric pathogens, including *Salmonella* and enteropathogenic and enterohemorrhagic *Escherichia coli*, express adhesins that recognize and bind to carbohydrate moieties expressed on epithelial cells. An attractive strategy for inhibiting bacterial adherence employs molecules that mimic these epithelial binding sites. Prebiotic oligosaccharides are non-digestible, fermentable fibres capable of modulating the gut microbiota. Moreover, they may act as molecular decoys that competitively inhibit adherence of pathogens to host cells. In particular, galactooligosaccharides (GOS) and other prebiotic fibres have been shown to inhibit pathogen adherence to epithelial cells *in vitro*. In the present study, we determined the ability of prophylactic GOS administration to reduce enteric pathogen adherence both *in vitro* and *in vivo* as well as protect against intestinal inflammation. GOS supplementation significantly reduced the adherence of the epithelial-adherent murine bacterial pathogen *Citrobacter rodentium* in a dose-dependent manner to the surface of epithelial cells *in vitro*. A 1- to 2-log reduction in bacterial adherence was observed at the lowest and highest doses tested, respectively. However, mouse studies revealed that treatment with GOS neither reduced the adherence of *C. rodentium* to the distal colon nor decreased its dissemination to systemic organs. Despite the absence of adherence inhibition, colonic disease scores for GOS-treated, *C. rodentium*-infected mice were significantly lower than those of untreated *C. rodentium*-infected animals (*P*=0.028). Together, these data suggest that GOS has a direct protective effect in ameliorating disease severity following *C. rodentium* infection through an anti-adherence-independent mechanism.

INTRODUCTION

The definition of prebiotics has been revised several times in the last two decades and was most recently updated as ‘a substrate that is selectively utilized by host micro-organisms conferring a health benefit’ [1]. Among the most studied prebiotics are the galactooligosaccharides (GOS), which are commercially produced from lactose using glycoside hydrolases that catalyse transgalactosylation reactions [2]. Most commercially available GOS products consist of simple or branched polymers of galactose with the degree of polymerizations ranging from two to seven. Many studies have shown that GOS selectively stimulates the growth of beneficial bacteria, including *Bifidobacterium* species in the human gastrointestinal tract [3–6]. Other non-digestible, fermentable carbohydrates including human milk oligosaccharides and other prebiotic oligosaccharides, have similar bifidogenic properties. The latter, however, consists of more than 200 different molecular species, reflecting their significant structural complexity compared to GOS [7]. Regardless of these differences, GOS, human milk oligosaccharides and other prebiotic oligosaccharides enrich for beneficial gut bacteria and suppresses the growth of detrimental bacterial species due to decreased luminal pH in the gut. Subsequent production of short chain fatty acids is also known to improve intestinal barrier function [8–13].

Another mechanism by which prebiotic fibres may improve gut health is via blocking epithelial attachment of intestinal pathogens. Some prebiotic oligosaccharides
possess significant structural similarity to the oligosaccharides that coat host epithelial cells and are recognized by pathogens as receptor sites. These prebiotic carbohydrate moieties may act as molecular decoys that competitively inhibit the adherence of intestinal pathogens to host cells. Specifically, these moieties may serve as binding sites for adhesins expressed by many enteric pathogens such as Salmonella, enteropathogenic Escherichia coli and enterohemorrhagic Escherichia coli [14–18]. By inhibiting bacterial adherence, infection and dissemination could be reduced and ultimately benefit host health [14].

Although several oligosaccharides have been reported to limit the in vitro adherence of gastrointestinal pathogens to various epithelial cell lines [15, 17, 19–21], this phenomenon is currently understudied in animal models. Here, we assessed the ability of GOS treatment to reduce in vivo adherence of the epithelial-adherent murine bacterial pathogen Citrobacter rodentium. This micro-organism is often used to model infections with human-specific enteric pathogens, especially enteropathogenic E. coli. We show that although GOS treatment limited in vitro attachment of C. rodentium to epithelial cells, it did not affect in vivo bacterial colonic adherence or shedding. However, GOS treatment significantly improved pathogen-induced intestinal inflammation compared to untreated C. rodentium-infected mice. Altogether, these findings suggest that GOS supplementation ameliorates host tissue damage following bacterial infection independently of anti-adherence activity.

**METHODS**

**Prebiotics and bacteria**

Galactooligosaccharides (Purimune GOS, formerly GTC Nutrition, Golden, CO) were dissolved in autoclaved distilled water and filter-sterilized through a 0.2 µm membrane filter prior to use in autoclaved dis-tilled water and filter-sterilized through a 0.2 µm membrane. Nutrition, Golden, CO) were dissolved in autoclaved dis-

**LB broth and grown overnight at 37°C** was created by transfer into fresh LB broth and incubated at 37°C for 24 h (late log phase). Bac-

**Type Culture Collection, Manassas, VA** were cultured in

**Citrobacter rodentium** DBS100 (ATCC 51459; American Type Culture Collection) was cultured as previously described [22]. Briefly, C. rodentium was plated on regular Luria–Bertani (LB; Difco, Franklin Lakes, NJ) and grown overnight at 37°C. A single colony was then inoculated into LB broth and grown overnight at 37°C. A 1% inoculum was created by transfer into fresh LB broth and incubated at 37°C with shaking at 200 r.p.m. for 4 h (late log phase). Bacteria were then centrifuged and resuspended to a concentration of 2.5 x 10^8 C. rodentium c.f.u. in 100 µl of PBS (Sigma-Aldrich, St. Louis, MO) for use as an inoculum in mouse experiments.

**Tissue culture**

The HEp-2 cell line is widely used to assess in vitro adherence of enteric pathogens, including C. rodentium, to epithelial cells [23–27]. HEp-2 cells (ATCC CCL-23; American Type Culture Collection, Manassas, VA) were cultured in 75 cm² tissue culture flasks containing 25 ml of minimal essential medium (MEM; Hyclone, Logan, UT) supplemented with 10% fetal bovine serum (FBS; Hyclone) and maintained at 37°C and 5% CO₂. Confluent HEp-2 cells were harvested by removing MEM and washing the cells once with PBS. Subsequently, 0.5 ml of a 0.25% Trypsin-EDTA solution (Hyclone) was added followed by a 10 min incubation at 37°C and 5% CO₂. After incubation, 0.5 ml of FBS was added to inactivate the trypsin. Cells were seeded into 24-well tissue culture plates at approximately 3.6 x 10⁵ cells per well. Then, 500 µl of MEM supplemented with 10% FBS was added to each well. Cells were incubated at 37°C and 5% CO₂ for approximately 20 h prior to the start of each experiment. Before each experiment, cells were viewed using an inverted microscope to verify that they had reached approximately 70% confluency.

For adherence assays, C. rodentium was prepared as described above. Concentrations of GOS ranging from 10 to 50 mg ml⁻¹ were mixed with bacterial cultures (10⁸ c.f.u. ml⁻¹) prior to their addition to tissue culture cells. Auto-

**C. rodentium** infected, (C) GOS-treated (not infected) and (D) GOS-

**control (no treatment and not infected), (B) C. rodentium-

**Mice**

Seven-week-old C57BL/6 conventionally raised female mice (Jackson Laboratory, Bar Harbor, ME) were purchased and maintained under specific pathogen-free conditions at the University of Nebraska-Lincoln. Mice were acclimated to facility conditions for 7 days prior to starting the experiment and then divided into four treatment groups: (A) control (no treatment and not infected), (B) C. rodentium-infected, (C) GOS-treated (not infected) and (D) GOS-treated and C. rodentium-infected. Mice were housed four per cage with two cages per treatment. Groups B and D were inoculated orally with 2.5 x 10⁸ c.f.u. of C. rodentium in 100 µl of PBS as previously reported [28]. GOS was diluted in distilled water at a concentration of 0.026 g ml⁻¹ to give 91 mg of GOS day⁻¹ mouse⁻¹ considering 3.5 ml of daily water consumption per mouse. GOS was provided fresh daily in the drinking water (rather than via oral gavage to avoid repeated handling stress) for 2 weeks prior to inocula-

**C. rodentium** and during the course of infection. All mice were necropsied on day 10 after infection. The Institutional Animal Care and Use Committee at the University of Nebraska-Lincoln approved all procedures involving animals (protocols 817 and 1215).

**Faecal, tissue and serum collection and C. rodentium recovery**

Faeces were collected on day 3 and day 7 after infection to assess shedding of C. rodentium. Specifically, faeces were weighed and homogenized in sterile PBS, serially diluted and plated in triplicate on eosin methylene blue (EMB) agar
(Difco). *C. rodentium* colonies were identified on the basis of their brown phenotype, and bacterial load was calculated as $\log_{10}$ c.f.u. g$^{-1}$ of faeces. At necropsy, a 0.5 cm segment of the distal colon was fixed in 10% neutral buffered formalin (Fisher Scientific, Pittsburgh, PA) for histopathological evaluation. To assess intestinal adherence at the site where *C. rodentium* colonization predominantly occurs [29], a 3 cm segment of distal colon was excised, weighed and washed three times with PBS, resuspended in PBS with 0.1% IGEPAL CA-630 (Sigma-Aldrich) and then homogenized using gentleMACS C tubes and program m_spleen_02 on a gentleMACS dissociator (Miltenyi Biotec; San Diego, CA). Homogenates were serially diluted and plated in triplicate on EMB agar to quantify adherent *C. rodentium* ($\log_{10}$ c.f.u. g$^{-1}$). Livers and spleens were also collected at necropsy, homogenized and plated as described above to assess *C. rodentium* systemic dissemination ($\log_{10}$ c.f.u. g$^{-1}$ of tissue). Portions of liver and spleen were also fixed in 10% neutral buffered formalin for histopathological evaluation. Whole blood was also collected at necropsy and allowed to clot for 15 min at 4°C. Serum was transferred to a new tube and centrifuged at 21,000 g for 3 min before being transferred again to a new tube and subsequently stored at −20°C until use in IL-6 and IL-1β ELISAs (Ready-SET-Go ELISA, eBioscience, San Diego, CA) according to the manufacturer’s instructions.

**Colon explant cultures**

Cultures of colon fragments were prepared as previously described [30]. Briefly, 3 cm segments of colon were washed in sterile PBS to remove colonic contents, cut into 1 cm pieces (but not weighed) and shaken at 280 r.p.m. in washing media for 30 min at 25°C. Washing media consisted of RPMI (RPMI-1640 medium 1X with 2.05 mM of L-Glutamine, Corning Cellgro) supplemented with 2% penicillin/streptomycin (100X penicillin/streptomycin solution, Corning Cellgro) and 0.5% gentamycin (10,000 µg ml$^{-1}$ gentamicin sulfate, Corning Cellgro). Each piece of tissue was placed in an individual well of a 96-well flat bottom well plate (Falcon or Corning) with 200µl of complete culture medium (same formulation as the washing media, but also including 1% L-glutamine, 200 mM L-glutamine solution, Corning Cellgro), 0.1% of a 50 mM 2-mercaptoethanol solution (Thermo Fisher Scientific) and 1% pyruvate (100 mM sodium pyruvate solution, Corning Cellgro). Cultures were incubated at 37°C with 5% CO$_2$ for 24h. Culture supernatants were collected and stored at −20°C until they could be assayed for IL-6 and IL-1β production via ELISA (Ready-SET-Go ELISA, eBioscience, San Diego, CA) according to the manufacturer’s instructions.

**Histopathological analysis**

To assess microscopic lesions, formalin-fixed tissues were processed by standard methods. Specifically, 5 µm paraffin sections were stained with hematoxylin and eosin (H and E) and examined by light microscopy. Tissue sections were evaluated by a board-certified veterinary pathologist who was blinded to the treatments. Colonic tissues were assigned scores based on mucosal height, epithelial injury/ulceration, density of inflammatory cells, edema, stromal collapse, gland hyperplasia and goblet cell depletion as described in [31]. Values for each parameter were added together to generate a cumulative histopathological score. Liver and spleen tissues were evaluated for the severity of inflammatory cell infiltration and degree of hepatic and splenic damage as described in [32].

**Statistical analysis**

An unpaired non-parametric Mann–Whitney test was used to analyse bacterial count data for shedding and for in vivo adherence and systemic translocation. A non-parametric Kruskal–Wallis test followed by a post-hoc pairwise comparison with a Mann–Whitney test was used to analyse all other data sets. All statistical analyses were performed using GraphPad Prism software version 6.0 (GraphPad Software, La Jolla, CA). Differences were considered significant at $P<0.05$.

**RESULTS**

**GOS reduced adherence of *C. rodentium* to HEp-2 cells**

Adherence of *C. rodentium* to HEp-2 cells was assessed in the absence and presence of GOS at concentrations ranging from 10 to 50 mg ml$^{-1}$. Adherence inhibition occurred at all concentrations in a dose-dependent manner. At the lowest dose (10 mg ml$^{-1}$), a 1-log reduction in adherence was observed. As the GOS concentration increased, adherence was reduced, with a nearly 2-log reduction at the highest concentration tested (50 mg ml$^{-1}$) (Fig. 1).

**GOS supplementation did not reduce colonic adherence or faecal shedding of *C. rodentium***

To determine whether GOS supplementation affected *C. rodentium* intestinal adherence and faecal shedding, C57BL/6 mice were provided with GOS for 2 weeks prior to infection with *C. rodentium*. GOS treatment continued until necropsy. GOS supplementation resulted in a modest (0.5 log c.f.u.) yet statistically significant increase ($P=0.032$) in the number of *C. rodentium* adherent to the distal colon compared to the untreated, infected mice (Fig. 2a). We also assessed the effect of GOS treatment on *C. rodentium* faecal shedding. Shedding levels for both GOS-treated and untreated mice were found to be similar at 3 and 7 days post-infection (Fig. 2b, c), indicating that GOS supplementation does not affect the number of *C. rodentium* shed in the faeces. We also collected faecal samples from all mice before infection with *C. rodentium* to confirm the absence of any bacteria (e.g. *E. coli* and *Enterobacter*) that could grow on EMB agar and be counted as *C. rodentium*. However, no colonies were recovered on EMB agar plates (data not shown).
GOS decreased the colonic tissue damage caused by C. rodentium infection

We next evaluated whether the tissue damage caused by C. rodentium was improved by GOS treatment. As expected, marked neutrophilic and monocytic infiltration and extensive crypt hyperplasia were observed in the colonic tissues of untreated, C. rodentium-infected mice (Fig. 3). However, GOS-treated, C. rodentium-infected mice were protected against such tissue damage and presented with scores similar to those of healthy untreated, uninfected mice. Together, our data demonstrate that GOS supplementation provided protection against C. rodentium-mediated tissue damage.

GOS supplementation did not alter local pro-inflammatory cytokine production during C. rodentium infection

A hallmark of C. rodentium infection is increased production of pro-inflammatory cytokines by innate immune cells such as macrophages, dendritic cells and neutrophils [33–36]. Of these cytokines, IL-6 has been reported to protect against the colonic mucosal ulceration caused by C. rodentium infection [37]. Additionally, IL-1β has been shown to contribute to C. rodentium clearance and minimize tissue damage [38]. We therefore examined colonic levels of IL-6 and IL-1β to determine whether GOS treatment would alter their production. Colonic tissues from all mice infected with C. rodentium released significantly higher levels of IL-6 and IL-1β compared to tissues from control mice. Treatment with GOS did not have an impact on the production of these cytokines compared to untreated, C. rodentium-
infected mice (Fig. 4a, b). These results indicate that the protective effects of GOS are not accompanied by altered production of the pro-inflammatory cytokines IL-6 and IL-1β.

GOS treatment did not limit systemic dissemination of *C. rodentium* or affect serum IL-6 and IL-1β levels

Despite being a gut-associated pathogen, *C. rodentium* can disseminate systemically and cause disease in various organs such as the liver, spleen and kidneys [39, 40]. We therefore asked whether GOS supplementation could limit *C. rodentium* dissemination to systemic organs. Portions of liver and spleen were homogenized to enumerate *C. rodentium* burden. Mice treated with GOS had approximately 2-log greater levels of *C. rodentium* in their spleens compared to untreated, *C. rodentium*-infected mice (Fig. 5a). Despite the increased numbers of bacteria detected in the spleens of GOS-treated mice, there were no notable microscopic or pathological findings observed in the red and white pulp in spleen tissues among any treatments (data not shown). GOS supplementation had no effect on the abundance of *C. rodentium* in the liver (Fig. 5b). However, histopathological evaluation described moderate to heavy inflammatory cell infiltration (neutrophils, lymphocytes and macrophages) in the livers of GOS-treated mice infected with *C. rodentium* but only mild to moderate inflammatory cell infiltration in the livers of untreated, infected mice (Fig. 5c–h).

We also examined IL-6 and IL-1β levels in serum as markers of systemic inflammation. Mice infected with *C. rodentium* had significantly higher serum levels of IL-6 and IL-1β compared to uninfected controls; GOS treatment did not alter these levels (Fig. 5i, j). These results show that GOS supplementation did not decrease *C. rodentium* dissemination to systemic organs or alter the associated inflammatory response.

**DISCUSSION**

Previous in vitro research findings have shown that GOS may have a protective effect against enteropathogenic *E. coli* and *Cronobacter sakazakii* through an anti-adherence mechanism [16, 19]. In this study, we showed that although GOS treatment reduced in vitro adherence of *C. rodentium*, it did not affect bacterial colonic adherence or shedding in vivo. However, GOS treatment prevented pathogen-induced intestinal tissue damage. Together, these
findings demonstrate that GOS supplementation provided a protective effect for the host that occurred independently of anti-adherence activity.

Despite the absence of adherence inhibition in vivo, our data showing that GOS treatment prevents colonic tissue damage following C. rodentium infection are consistent with the concept of disease tolerance [41]. This defence strategy occurs when the host is unable to eliminate the invading pathogen. Rather than continue mounting antimicrobial immune responses, the host instead develops tolerance mechanisms that reduce direct tissue damage caused by the pathogen and protect host tissues from indirect immunopathological damage [41–43]. In the present study, GOS may have promoted disease tolerance by diminishing the recruitment of inflammatory cells aimed at pathogen elimination to the site of infection. This outcome could result in decreased local production of pro-inflammatory mediators (other than IL-6 and IL-1β) and ultimately limit the development of immune-mediated pathology. Although we did not assess levels of anti-inflammatory cytokines, it is also possible that GOS may have provided a protective effect by enhancing the production of mediators that limit inflammation such as IL-10 and TGF-β. Future studies assessing cytokine production and other functional responses of various immune cell populations during GOS feeding are certainly warranted.

Our observation that GOS supplementation reduced adherence of C. rodentium in vitro but not in vivo could be explained by differential bacterial gene expression influenced by environment. Although the specific fimbriae and/or adhesins necessary for C. rodentium adherence have not been identified, this organism, like other enteric pathogens, expresses multiple fimbriae-encoding genes. Smith and colleagues recently reported major differences in the transcriptome of C. rodentium when cultured in the laboratory versus when recovered directly from the faeces of infected mice [44]. In particular, they observed that lab cultured C. rodentium did not express 19 fimbrial genes and several other non-fimbrial and non-loci of enterocyte effacement (LEE) encoding genes [44]. In contrast, C. rodentium present in mouse faeces did express the major attaching and effacing virulence genes tir and intimin (cae) as well as other major effector, regulator and structural genes encoded on the LEE island at levels greater than cultured C. rodentium [44]. Together, these findings indicate that C. rodentium adaptation in the host intestinal tract is critical for the expression of the adhesion, effector and structural genes responsible for C. rodentium virulence and fitness. We suggest that GOS only provided an anti-adherence effect in vitro because lab culturing of C. rodentium results in differential gene expression such that only GOS-sensitive adhesins are expressed. In contrast, other adhesins, including those that are GOS-insensitive, are expressed when C. rodentium grows in vivo.

Also in our studies, we observed that GOS supplementation did not reduce the dissemination of C. rodentium to systemic organs. Our findings are consistent with other published reports describing increased systemic pathogen translocation after treatment with prebiotic oligosaccharides. Licht and colleagues reported that treatment with either fructooligosaccharides (FOS) or xylooligosaccharide increased the number of translocated Salmonella Typhimurium to liver, spleen and mesenteric lymph nodes [45]. FOS feeding has also been reported to increase intestinal permeability in rats and enhance dissemination and translocation of Salmonella to extraintestinal tissues [46]. These results subsequently led the authors to speculate that prebiotic treatment promotes bacterial translocation via changes in intestinal permeability. Although we did not evaluate intestinal permeability, there are, however, many reports describing improved intestinal barrier function following prebiotic treatment. For example, Cani and colleagues noted that prebiotic feeding of ob/ob mice improved intestinal barrier permeability and decreased systemic cytokine levels and endotoxemia [47]. Similarly, providing the wheat bran-derived prebiotic arabinoxylan oligosaccharides to high fat
Fig. 5. GOS supplementation did not limit *C. rodentium* dissemination to systemic organs and did not alter systemic IL-6 and IL-1β production. Mice were either left untreated or treated with GOS for 2 weeks prior to inoculation with *C. rodentium*. GOS-treated mice continued to receive GOS during the *C. rodentium* infection. Numbers of *C. rodentium* bacteria translocated to the (a) spleen and (b) liver were assessed by plating. Values were log_{10} transformed, and data were analysed with a Mann–Whitney test. Data are shown as the mean±SEM (*n*=8 mice per treatment). Treatments marked with asterisks (**) are significantly different from one another (*P*=0.008). Representative photomicrographs of hematoxylin and eosin (H and E) stained liver sections taken at x10 (c–e) and x40 (f–h) objective magnification (with bars denoting lengths of 250 and 100 µm, respectively, at the two magnifications) from either uninfected, untreated control mice; untreated *C. rodentium*-infected mice; or GOS-treated *C. rodentium*-infected mice. Note the heavy abundance of hematoxylin (blue)-stained inflammatory cells surrounding the portal tracts (small arrows) in E compared to D as well as the foci of hepatocellular necrosis with accompanying neutrophilic infiltration (large arrows). (f–h) show portions of the same fields as (c–e), respectively, at higher magnification to allow visualization of the inflammatory cell types within the infiltrate. Serum levels of the pro-inflammatory cytokines IL-1β (i) and IL-6 (j) were measured by ELISA. Cytokine data were analysed using a non-parametric Kruskal–Wallis test followed by a Mann–Whitney post-hoc test. Data are shown as mean±SEM (*n*=8 mice per treatment). Treatments with different letters are significantly different from one another (*P*<0.05).
diet-fed mice upregulated intestinal tight junction proteins, lowered blood insulin levels and decreased plasma IL-6 levels and endotoxia [48]. Furthermore, Delzenne and colleagues reported that prebiotic administration in a symbiotic cocktail improved intestinal barrier function in a cancer cachexia mouse model [49]. These contrasting findings regarding intestinal permeability during prebiotic supplementation may be due to differences in prebiotic composition and/or suggest that physiological mechanisms other than changes in barrier function are responsible for the increased systemic bacterial translocation occasionally observed following prebiotic treatment.

Another potential mechanism to explain the protective effects we observed after providing GOS may be shifts in the intestinal microbiota. From our previous studies [50] and those of others [51, 52], GOS supplementation is known to modulate the gut microbiota of rodents. In particular, GOS has been consistently reported to increase abundance of bifidobacteria [3–6]. Although we did not assess microbiota composition in these experiments, we suggest that GOS treatment could have altered the abundance of specific taxa that influenced the experimental outcomes we observed in the present study.

Collectively, our data demonstrate that dietary GOS supplementation provides protection against C. rodentium-induced colonic tissue damage through an anti-adherence-indepen-dent mechanism. These findings suggest that the well-established and highly reported anti-adherence mechanisms observed for GOS in vitro do not explain the protective role of GOS observed in vivo. Future studies to dissect and better understand the exact mechanisms through which GOS provides its protective effects are clearly warranted.

Acknowledgements
We wish to thank Brandon White and the entire staff at the UNL Gnotobi-otic Mouse Facility for outstanding animal husbandry and technical assistance. We would also like to thank Dr. Jennifer Clarke, UNL Departments of Statistics and Food Science and Technology, for helpful discussions regarding statistical analyses.

Conflicts of interest
R.W.H. is a member of the Board of Directors of the International Sci-entific Association for Probiotics and Prebiotics. He has received funding from industry sources involved in the manufacture and marketing of probiotics and prebiotics. He is also a co-owner of Synbiotics Solutions, a developer of synbiotic products.

Ethical statement
The Institutional Animal Care and Use Committee at the University of Nebraska-Lincoln approved all procedures involving animals (Protocols 817 and 1215).

References
19. Shoaf K, Mulvey GL, Armstrong GD, Hutkins RW. Prebiotic galac-toooligosaccharides reduce adherence of enteropathogenic...


