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Lactulose Feeding Lowers Cecal Densities of Clostridia in Piglets

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Abstract

Background— In order to understand the consequences of persistent enteral feeding in patients with carbohydrate malabsorption, we fed piglets lactulose in sufficient dosage to produce osmotic diarrhea or inulin, using a conventional dose, to determine if this prebiotic can modulate the effects of lactulose. Feeding lactulose increases cecal luminal synthesis of butyrate, with inulin having an intermediate effect. Because clostridia may be a major source of colonic butyrate production, we hypothesized that feeding piglets lactulose or inulin would increase cecal densities of clostridia.

Methods— Piglets were assigned to 3 formula study groups for 6 days: (1) control, fed only sow milk replacer (n = 12); (2) inulin, inulin supplement (3 g/L; n = 11); and (3) lactulose, lactulose supplement (66.7 g/L; n = 6). Cecal fluid for bacteriological studies was sampled intraoperatively.

Results— The wet/dry ratio of the cecal contents (mean ± SEM) was 8.2 ± 0.5, 6.2 ± 0.5, and 18.8 ± 5.5, respectively, in the control, inulin, and lactulose groups (p = .049, Kruskal-Wallis). There were no differences among the diet groups for cecal densities (10^6 colony-forming units [CFU]/g dry wt cecal contents) of total anaerobes, total aerobes, bifidobacteria, or lactobacilli. Densities of clostridia were markedly reduced in the lactulose group (1.14 ± 0.41) *vs* the control (18.39 ± 4.44; p = .001) or inulin groups (8.87 ± 2.20; p = .04).

Conclusions— In piglets, feeding lactulose at a dose known to cause diarrhea reduces cecal densities of clostridia.

Because of our clinical interest in how to approach enteral feeding of patients with osmotic diarrhea secondary to carbohydrate malabsorption (and incomplete fermentation), we developed a piglet model of carbohydrate malabsorption: persistent feeding of lactulose, an indigestible disaccharide of galactose and fructose.[1–3] Our research suggests that osmotic diarrhea caused by lactulose malabsorption has minimal, if any, clinical effects, but lactulose feeding consistently causes decreased cecal cell proliferation.[1–3] Because, *in vivo*, butyrate produced by bacteria and well absorbed from the colonic lumen[4] causes increased cell proliferation,[5–7] we hypothesized that osmotic diarrhea might cause a form of *in vivo* butyrate deficiency, in part characterized by decreased cell proliferation in the colon.[1]

We then sought ways to enhance colonic fermentation so that when challenged with severe carbohydrate malabsorption, fermentation (and butyrate production) might be enhanced. Previously, others[8] had shown that giving adult volunteers a prolonged, non-diarrheogenic

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dose of lactulose attenuated the subsequent diarrhea caused by a "laxative" dose of lactulose, and we theorized that augmenting the fermentation capacity with a fermentable carbohydrate other than lactulose might have a similar effect.[1] Inulin is an indigestible and highly fermentable fructooligosaccharide (FOS), with an average of 35 fructosyl units. FOSs are found naturally in foods such as wheat, onion, and garlic, in an increasing number of processed foods used as low-energy, low-glycemic-index, noncariogenic sweeteners (functional foods), and in various formulas, cereals, and other foods for infants.[4,9] In healthy adults, the threshold for diarrhea with FOS is about 55 g/d (0.8 g/kg/d).[9] Inulin (3 g/L of formula, or about 0.4 g/kg/d) has been used by others to increase colonic cell proliferation in piglets.[10] There is controversy about the extent to which feeding inulin enhances butyrate formation.[3,10,11] In an initial study, we showed a trend in lactulose-treated piglets toward enhanced cecal cell proliferation and reduced diarrhea when inulin was prefed for 7 days and then discontinued before a challenge with lactulose.[2] These results seemed consistent with our original hypothesis that fermentation and butyrate production were inhibited by high purging rates, but enhancing bacterial activity in the colon by prefeeding inulin could reverse these effects.

In a subsequent study, we measured the rate of colonic luminal synthesis of butyric acid in growing, chronically catheterized piglets orally fed sow milk replacement formula, with or without supplements with lactulose, inulin, or inulin plus lactulose.[3] We observed a doubling of the rate of butyrate synthesis in the group fed lactulose (15.2 g/kg/d)[3]; a combination of both prefeeding and then cofeeding inulin (0.4 g/kg/d) with lactulose (15.7 g/kg/d) prevented the decrease in cecal cell proliferation observed with lactulose feeding and was associated with normalization of butyrate synthesis.[3] When compared with the control group, piglets fed inulin without lactulose (0.4 g/kg/d) manifested lower cecal cell proliferation, more diarrhea, and an intermediate rate of butyrate synthesis that was not statistically different from controls or lactulose-fed piglets.[3] One hypothetical explanation for these data is that fructose-containing sugars, lactulose or inulin, exert a "prebiotic" effect,[12] namely, suppression of growth of specific bacterial species, which *via* an independent, unknown mechanism normally stimulate cecal cell proliferation. However, when fed together, they may competitively inhibit the use of either sugar as a substrate for fermentation by this/these bacterial species, resulting in no change in cecal cell proliferation from normal.[2,3] Butyrate synthesis, then, could be a function of the amount of fermentable sugar reaching the colon (much greater for lactulose in our model), rather than related to the prebiotic effect of the sugars. So, in order to gain more insight into the putative effects of lactulose and inulin on the cecal bacterial flora of piglets, we conducted the following piglet study. Because clostridia species have been thought to be major sources for colonic butyrate production,[13,14] we nominally hypothesized that feeding piglets with supplementary lactulose or inulin would increase cecal densities of clostridia.

MATERIALS AND METHODS

Animals, Feedings, and Design

Twenty-nine standard Yorkshire/Hampshire piglets were studied at the University of Vermont, where the Institutional Animal Care and Use Committee approved the research protocol. On approximately day 12 of life, the piglets were transported from the pig farm to the laboratory, where the piglets were housed individually and fed orally a sow milk substitute formula (Control formula, C; SPF Lac; sterile milk replacer; PetAG Inc, Hampshire, IL). According to analysis (Covance Laboratories, Madison, WI), the macronutrient composition was as follows: energy, 3.7 MJ/L; protein, 48.2 g/L; fat, 60.5 g/L; total carbohydrate, 33.9 g/L; and lactose, 28.7 g/L. The formula was further supplemented with lactose to achieve a final concentration of 60 g/L.

The piglets were assigned to 3 treatment groups and fed milk replacer for 6 days. The control group (C; n = 12) received only sow milk replacer. The first experimental group (I; n = 11)

was fed milk replacer with inulin (3 g/L), and the second experimental group (L; n = 6) was fed milk replacer with lactulose (66.7 g/L). Piglets assigned to the C and I groups were randomly assigned to diets as part of a longer study involving cecal infusions of butyrate or phosphate-buffered saline, commencing after the conclusion of the present study. Piglets assigned to the L group were not part of the cecal infusion study. Thus, the number of piglets in the L group was half that of the other 2 groups. During the study, body weight, formula intake, and stool characteristics were monitored. Diarrhea was quantified by computing the fraction of the observation period when diarrhea was observed, as previously described.[1,2]

After 6 days of feeding, the piglets underwent a surgical procedure for collection of cecal luminal fluid for bacteriology studies. Details of the surgical procedures have been described previously.[3,15–18] Anesthesia was induced with a combination of Telazol (Fort Dodge Animal Health, Wyeth, Madison, NJ)/xylazine administered intramuscularly (Telazol, 6 mg/kg; xylazine, 4 mg/kg). General anesthesia was then maintained with isoflurane *via* an endotracheal tube. A laparotomy and cecal incision were performed as previously described.[15] After collection of cecal fluid, piglets in the C and I groups then underwent insertion of a cecal infusion cannula and additional studies, but the piglets in the L group were euthanized with pentobarbital.

Bacteriology Studies

Cecal contents collected *via* the incision were placed in a sterile vial such that the vial was completely filled. The vial was then capped tightly and wrapped with parafilm. The vial was immediately refrigerated until it was mailed on ice the same day to the laboratory of Carol Williams at Mississippi State University (overnight express). Following preparation of dilutions in prereduced, sterile, phosphate-buffered (0.06 M, pH 7.0) gelatin (1% wt/vol) diluent, aliquots were immediately plated to both selective and nonselective agar media for quantification of total anaerobes, total aerobes, *Bifidobacterium* species, *Clostridium* species, and *Lactobacillus* species.[1,19–21] General plate media included Wilkins-Chalgreen blood agar (for both total anaerobes and total aerobes), BIM-25 agar (for *Bifidobacterium* species), MRS agar (for *Lactobacillus*), and MacConkey agar for total enteric count. Selective recovery of *Clostridium* species was accomplished using the ethanol shock treatment method, followed by plating onto anaerobic blood agar. Identification of bacteria was performed to the genus level using standard microbiologic methods. All manipulations for the recovery and identification of anaerobic bacteria were performed in an anaerobic chamber. Bacterial counts were expressed as colony forming units/g dry weight cecal content according to the wet/dry weight ratio of the cecal luminal fluid sample.

Data Analysis and Statistics

During bacteriological examination, the investigators were masked to the identity of the treatment group for each piglet. Because of lack of homogeneity of variance, we generally used the Kruskal-Wallis test initially to determine if there was a significant diet-group effect. Significant results were supplemented with an analysis of variance (ANOVA) performed on the ranks,[22] followed by Scheffe's test to allow comparisons between specific groups (SPSS Base 10.0; SPSS Inc, Chicago, IL). All results are expressed as mean ± SEM.

RESULTS

There was no significant difference among the groups in weight gain and milk replacer intake. There was a significantly higher fraction of days with diarrhea in the lactulose-fed piglets (0.61 ± 0.16) compared with the controls (0.08 ± 0.04; $p = .008$) or to the inulin-fed piglets (0.17 ± 0.08; $p = .029$). The difference between the inulin and control groups would have been statistically significant ($p < .05$) with a power of 0.81, with 43 in each group. The wet/dry ratio

of the cecal contents was 8.2 ± 0.5, 6.2 ± 0.5, and 18.8 ± 5.5, respectively, in the C, I, and L groups (p = .049, Kruskal-Wallis).

Table I shows a lack of differences among the groups for cecal densities of total anerobes, total aerobes, bifidobacteria, or lactobacilli. Figure 1 shows that densities of clostridia (10^6 colony-forming units [CFU]/g dry wt cecal contents) were markedly reduced in the L group (1.14 ± 0.41) compared with the C group (18.39 ± 4.44; p = .001) or the I group (8.87 ± 2.20; p = .04). The 52% reduction in the I group compared with the C group was not statistically significant.

Expressing the bacteriology data per gram dry weight accounts for differences in water content of feces, which may be independently affected by diet group, as noted above. However, changes in total bacterial mass, not quantified in this study, will certainly affect the ratio of bacterial numbers to dry weight.[23] Therefore, because we are interested in how lactulose and inulin alter the relative proportions of the different bacterial groups, we also examined ratios between bacterial classes. The ratio of clostridia/lactobacilli (per dry weight) in the L group (0.0001 ± 0.00002) was significantly lower compared with the C group (0.0042 ± 0.00210; p < .001) or the I group (0.0010 ± 0.00032; p = .004) (rank transformation, Scheffe's test), but there was no significant difference between the C and I groups. There was no effect of treatment group on the ratio of clostridia/bifidobacteria.

DISCUSSION

This study shows that lactulose lowers the cecal density of clostridial species when fed at a dose that produces watery feces (diarrhea) but does not cause nutrition problems or, consistently, cecal inflammation.[1–3] We have been using lactulose as a surrogate for mal-absorbed disaccharides such as lactose. Clostridial species ferment lactose[24] and are cultured from the feces of both bottle-fed and breast-fed infants.[25] However, a literature review did not reveal studies of the effects of dietary lactose on the clostridial colonization of the colon of infant mammals. Kleesen et al[11] showed that lactose supplementation caused a decrease in the density (per dry weight) of clostridia in the feces of elderly subjects. The biologic ("clinical") significance of lowering the normal cecal densities of clostridia is unclear, and we were unable to differentiate among the various species of cecal clostridia, which vary in potential pathogenic effects on the intestine.[14,24] Bacteria of the *Clostridium* genus have been recently implicated in causing necrotizing enterocolitis (NEC)[24,26–28] and enteritis necroticans (pigbel).[29] Moreover, NEC has been linked to feeding and, specifically, the feeding of carbohydrates partially fermented in the colon.[4,30] Cecal, NEC-like lesions were produced frequently in response to lactose feeding and oral inoculation of clostridial strains in alactasic, previously gnotobiotic quails; this pathology was associated with high cecal concentrations of butyric acid.[24] The particular lactulose feeding regimen used in our study has been shown to cause a higher rate of cecal luminal synthesis of butyrate and lower cecal luminal concentration of butyrate.[3] Further studies would be required to determine, for example, if feeding lactulose would lower the risk of NEC or cecitis in a suitable model.[24,28,31] However, apart from relevance to NEC, lactulose-induced changes in the assemblages of various bacteria species in the colon could alter fermentation pathways (eg, the concentrations and relative proportion of organic acids that are produced during fermentation of a given substrate).[32] This, in turn, would affect energy lost as heat during fermentation (eg, lactate *vs* acetate production)[33] or the production of butyrate and other short-chain fatty acids with specific effects on endogenous enzymatic pathways.[34,35]

In the present study, although lactulose feeding (~66 g/L formula) caused diarrhea, it still decreased the cecal density of clostridia expressed either per dry weight cecal contents or as a ratio to another common bacterial species (lactobacilli). Thus, lactulose did not simply purge

the cecum of all bacteria but selectively diminished the density of clostridia. Whether this "prebiotic" effect on clostridia was related to changes in osmolality of the cecal contents cannot be determined from our study, but we have not found evidence in the literature for selective effects of osmolality on the growth of this bacterium. Compared with controls, the inulin group, fed a much lower amount of indigestible carbohydrate (~3 g/L) than the lactulose group, did not exhibit a statistically significant change in either diarrhea frequency (despite a mean 112% increase) or cecal density of clostridia (52% lower). In a previous study, we did find that feeding inulin without lactulose caused more than a doubling of the fraction of days when diarrhea was observed (152% increase), although lactulose had a greater effect (274%).[3] Thus, inulin, at the dose used, may have an intermediate effect on both diarrhea and the cecal density of clostridia, compared with lactulose, given at a much larger daily dose of indigestible carbohydrate.

This study was not intended as a direct comparison of the effects of inulin and lactulose on cecal density of bacteria but rather as a follow-up to our previous studies examining the effects of a very large dose of lactulose on cell proliferation.[1–3] As explained in the introduction, we have been studying a "laxative dose" of lactulose in order to simulate severe sugar malabsorption in infants. Inulin was purposefully not given at such a high dose that it would induce a similarly high purging rate as lactulose, with the realized expectation that, when fed before or with lactulose, it would alter fermentation pathways and prevent the inhibition of cecal cell proliferation.[2,3,8] We had hypothesized that lactulose, more than inulin, would stimulate colonization with clostridia, which ostensibly are important in producing butyrate.[13,14] Because we now have evidence that lactulose (and perhaps inulin as well) inhibits clostridia colonization, it certainly would be reasonable in future studies to compare the effects of these 2 sugars at equal doses of total sugar and at equal doses of fructose. Previous studies have not shown a consistent effect of FOS on clostridia in the colon either in humans[36,37] or nonhuman mammals.[10,38,39] However, in the quail model, FOS supplementation lowered clostridial colonization.[13]

In conclusion, cecal densities of clostridia were markedly decreased by the inclusion of lactulose in a milk replacer fed to 6-day-old piglets at a dose known to cause diarrhea, to lower cecal proliferation, and to cause higher colonic luminal butyrate synthesis. It is not clear whether this prebiotic effect of lactulose would be seen at lower doses or if it could be used to lower abnormally high densities of clostridia under other conditions. These results are consistent with the hypothesis that indigestible carbohydrates including fructose alter the assemblages of bacteria in the cecum.

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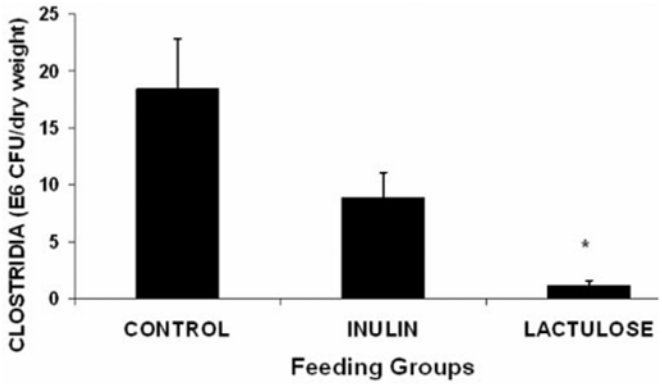

Figure 1.
Cecal clostridial colonization in piglets (10^6 colony-forming units per g dry weight). * Lactulose *vs* control, $p = .001$; lactulose *vs* inulin, $p = .04$ (rank transformation; analysis of variance followed by Scheffe's test).

TABLE I

Densities (10^6 colony-forming units/g dry weight of cecal contents) of bacteria in the cecum of piglets fed milk replacer alone or supplement with inulin or lactulose

Feeding group	Total anaerobes	Total aerobes	Bifidobacteria	Lactobacillus
Control	20,850 ± 6127	4641 ± 1692	2670 ± 684	17,477 ± 5002
Inulin	23,936 ± 5992	6525 ± 2184	1925 ± 623	15,856 ± 4504
Lactulose	100,152 ± 56,442	23,623 ± 13,958	6481 ± 4229	33,605 ± 10,667